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Intracellular survival of *Burkholderia cepacia* complex in phagocytic cells

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Running title: *Burkholderia* survival in macrophages
Abstract

*Burkholderia cepacia* complex (Bcc) species are a group of Gram-negative opportunistic pathogens that infect the airways of cystic fibrosis patients, and occasionally they infect other immunocompromised patients. Bcc bacteria display high-level multidrug resistance, and chronically persist in the infected host while eliciting robust inflammatory responses. Studies using macrophages, neutrophils and dendritic cells, combined with advances to genetically manipulate these bacteria have increased our understanding of the molecular mechanisms of virulence in these pathogens and the molecular details of cell-host responses triggering inflammation. This article discusses our current view of the intracellular survival of *B. cenocepacia* within macrophages.

Key words: phagocytosis, phagosome, cystic fibrosis, autophagy, inflammasome, cystic fibrosis transmembrane conductance regulator, Pyrin, cepacia syndrome, macrophage, neutrophils, amoebae.
Introduction

The Burkholderia cepacia complex (Bcc) comprises a subset of Burkholderia species that cause respiratory infection in patients with cystic fibrosis (CF) (Coenye and Vandamme 2003; Vandamme and Dawyndt 2011). The Bcc species are opportunistic pathogens acquired from the environment, which can survive intracellularly in human respiratory epithelial cells, neutrophils, macrophages, and amoebae (Loutet and Valvano 2010; Saldías and Valvano 2009). Bcc clinical infections elicit inflammatory responses leading to rapid deterioration of lung function in CF patients. Some species, particularly, B. cenocepacia, cause in CF patients a lethal necrotizing pneumonia associated with sepsis (Cepacia syndrome). Dysregulated inflammatory responses upon B. cenocepacia infection originate from activation of the NLRP3 and pyrin inflammasomes (Gavrilin et al. 2012; Rosales-Reyes et al. 2012a; Xu et al. 2014). In this article, I will focus on our current understanding of the molecular mechanisms of intracellular survival of Bcc bacteria, especially B. cenocepacia, and the inflammatory response of infected macrophages. For additional information, the reader should consult excellent recent reviews (Bazzini et al. 2011; Cullen and McClean 2015; Drevinek and Mahenthiralingam 2010; Ganesan and Sajjan 2011; Leitão et al. 2010; McClean and Callaghan 2009; Parkins and Floto 2015; Vial et al. 2011).

Bcc bacteria - intracellular survivors in professional phagocytes and epithelial cells

Research over the past two decades has demonstrated that at least some species of the Bcc survive intracellularly in free-living amoebae (Anacarso et al. 2010; Lamothe et al. 2004; Landers et al. 2000; Marolda et al. 1999), murine and human macrophages in culture (Lamothe
et al. 2007; Lamothe and Valvano 2008; Martin and Mohr 2000; Saini et al. 1999), and epithelial
cells (Burns et al. 1996; Duff et al. 2006; Keig et al. 2002; Moura et al. 2008; Sajjan et al. 2006;
Schwab et al. 2002; Taylor et al. 2010). The laboratory observations have also been recapitulated
in the human host, as demonstrated in a recent study that investigated resected lungs of Bcc-
infected CF patients undergoing transplant (Schwab et al. 2014). These authors observed that in
contrast to *Pseudomonas aeruginosa*, Bcc bacteria were predominantly identified as single cells
or small clusters within phagocytes and mucus, but not as biofilm-like structures.

Engulfed *B. cenocepacia* in murine macrophages reside on a membrane-bound compartment
with a maturation defect (Fig. 1) (Andrade and Valvano 2014; Flannagan et al. 2012; Huynh et al.
2010; Keith et al. 2009; Lamothe et al. 2007; Maloney and Valvano 2006; Rosales-Reyes et al.
2012b). Phagosomes containing *B. cenocepacia* transiently recruit early endosome autoantigen
(EEA1) and Rab5, and synthesize phosphatidylinositol-3-phosphate, indicating that the bacterial
containing vacuoles progress normally to the early phagosomal stage (Huynh et al. 2010;
Lamothe et al. 2007). However, these vacuoles exhibit a significant delay in the accumulation of
the late endosome/lysosome marker LAMP-1 (Lamothe et al. 2007) while acquiring CD63 and
Rab7 (Huynh et al. 2010). During their arrested maturation, the bacterial containing vacuole
remain in contact with newly formed endosomes and maintain a luminal pH around 6.4
(Lamothe et al. 2007). Delay in acidification correlates with delayed recruitment of the 16-kDa
subunit of the phagosomal vATPase onto the membrane of the bacterial containing vacuole
(Rosales-Reyes et al. 2012b). Further, the arrested phagosome has a delay to assemble the
NADPH oxidase complex (Keith et al. 2009; Rosales-Reyes et al. 2012b). Fluorescence recovery
after photobleaching and use of a probe that detects Rab7-guanosine triphosphate revealed that
the Rab7 recruited to the bacterial containing vacuole is inactive, suggesting a potential
mechanism for the inability of these vacuoles to fuse with lysosomes as a consequence of Rab7 defect (Huynh et al. 2010). The B. cenocepacia-containing phagosome also acquires LC3 (Abdulrahman et al. 2012; Abdulrahman et al. 2011; Al-Khodor et al. 2014)(Kevin, Torres and Valvano, unpublished), a marker for autophagy, and the intracellular infection downregulates the expression of other components involved in autophagy (Abdulrahman et al. 2012; Abdulrahman et al. 2011) suggesting the possibility that B. cenocepacia survives in an arrested autophagosome (Fig. 1). A recent study reported that B. cenocepacia also escape the bacteria-containing vacuoles, as determined by the apparent recruitment of galectin-3 (Al-Khodor et al. 2014), a marker for vacuole disruption. Attempts to repeat this work in our laboratory have failed to clearly demonstrate galectin-3 recruitment and colocalization with intracellular B. cenocepacia (Torres-Bustos and Valvano, unpublished).

B. cenocepacia and B. multivorans are the Bcc species most commonly isolated from CF patients (Drevinek and Mahenthiralingam 2010). Despite that most research groups have focused on the study of B. cenocepacia, the incidence of B. multivorans infections has steadily increased and B. multivorans is now the most prevalent Bcc strain infecting CF patients in North America and also the most common species infecting patients with chronic granulomatous disease (Zelazny et al. 2009). B. multivorans infects human lung epithelial cells (Duff et al. 2006; Moura et al. 2008), monocytes (Zelazny et al. 2009), and dendritic cells (Macdonald and Speert 2008). B. multivorans isolates survive and slowly replicate within murine macrophages in a manner similar to B. cenocepacia (Schmerk and Valvano 2013). In contrast, the B. multivorans and B. cenocepacia strains do not replicate within human THP-1 macrophages and their numbers diminished marginally after 48 hours of infection (Schmerk and Valvano 2013).
Similar survival and intracellular replication of *B. cenocepacia* and *B. multivorans* in macrophages does not preclude differences in their trafficking properties after engulfment. Indeed, whereas *B. cenocepacia* delays phagosomal maturation in murine macrophages (Huynh et al. 2010; Lamothe et al. 2007) the endocytic trafficking pattern of *B. multivorans* does not show a maturation arrest of the bacteria-containing vacuole (Schmerk and Valvano 2013). Therefore, *B. cenocepacia* and *B. multivorans* appear to survive within immune cells using different strategies. These differences have become useful to investigate factors associated to phagosomal arrest using *B. multivorans* recombinant strains expressing *B. cenocepacia* genes (Andrade and Valvano 2014).

**To replicate...or not replicate - *B. cenocepacia* and the gentamicin protection assay**

Intracellular Bcc in amoebae and macrophages are viable and metabolically active (Marolda et al. 1999; Saini et al. 1999). However, reliable quantification of the intracellular infection is difficult due to the extreme resistance of Bcc bacteria to aminoglycoside antibiotics (Saini et al. 1999). This precludes using the classical gentamicin protection assay traditionally employed to quantify intracellular bacteria (Elsinghorst 1994; Isberg and Falkow 1985). Antibiotic treatment of Bcc-infected macrophages requires large doses of kanamycin in combination with ceftazidime, which at best do not efficiently kill all extracellular bacteria (Burns et al. 1996; Saini et al. 1999), and may cross the cell membrane reaching the intracellular bacteria. There are no reports systematically assessing whether very high antibiotic concentrations used in some studies (Al-Khodor et al. 2014) directly affect the macrophages. Preliminary experiments in our laboratory indicate that high concentrations of ceftazidime dramatically alter the bacterial uptake and
trafficking (Torres-Bustos and Valvano, unpublished). An efficient protocol for constructing markerless gene deletions in *B. cenocepacia* (Flannagan et al. 2008) allowed the creation of gentamicin-sensitive strains of *B. cenocepacia* in which the genes encoding an AmrAB-OprA-like efflux pump were deleted (Hamad et al. 2010). *B. cenocepacia* strains carrying this deletion are hypersensitive to gentamicin, allowing the use of low concentrations of gentamicin to effectively kill extracellular bacteria and demonstrating that engulfed bacteria can replicate slowly over 24-48 hours post infection (Hamad et al. 2010).

**Bacterial entry and survival in macrophages**

Very little is known about bacterial factors involved in entry and intramacrophage bacterial survival of *B. cenocepacia*. The O-antigen moiety of the lipopolysaccharide molecule is associated with reduced bacterial engulfment by macrophages (Saldías et al. 2009; Schmerk and Valvano 2013). However, the macrophage receptor involved with bacterial recognition remains unknown. Recently, Eierhoff et al. (2014) uncovered a novel mechanism for the entry of *P. aeruginosa* into epithelial cells that occurs independently of actin polymerization and involves the interaction of a bacterial surface lectin LecA with a membrane glycolipid receptor. *B. cenocepacia* produced several LecA-like surface lectins (Šulák et al. 2011), suggesting that a similar mechanism may operate in these bacteria for entry in epithelial cells and perhaps also macrophages.

Using the rat agar bead model of chronic lung infection revealed that a *B. cenocepacia* transposon mutant carrying an insertion in the *mgtC* gene was dramatically attenuated (Hunt et al. 2004), and a subsequent study demonstrated that this mutant fails to delay phagosomal maturation resulting in rapid targeting of the bacteria-containing vacuole to the lysosomal
compartment and a compromise in bacterial survival (Maloney and Valvano 2006). MgtC is essential for the intramacrophage survival of several intracellular bacteria (Alix and Blanc-Potard 2007), a function that was recently attributed to its ability to regulate the bacterial physiological ATP levels and cytosolic pH (Lee et al. 2013). Furthermore, two sigma factors controlling the regulation of multiple genes, RpoN and RpoE, are required for the intracellular survival of *B. cenocepacia* (Flannagan and Valvano 2008; Saldías et al. 2008), but the specific gene determinants involved in survival have not been elucidated.

Factors involved in resistance to oxidative damage play a role in intracellular survival of *B. cenocepacia*. Indeed, vacuoles containing a *B. cenocepacia* mutant unable to produce a melanin-like pigment rapidly traffic to the lysosome, but this process can be halted in the presence of the NADPH oxidase and nitric oxide inhibitors (Keith et al. 2007). Therefore, melanin-like pigment production by *B. cenocepacia* protects intracellular bacteria from oxidative damage. Indeed, the delay of the bacteria-containing vacuole to incorporate NADPH oxidase components (Keith et al. 2009; Rosales-Reyes et al. 2012b) suggests that *B. cenocepacia* must escape oxidative damage to overcome the host cell defensive mechanisms. Bylund *et al.* (2006) showed that many Bcc isolates produce large amounts of exopolysaccharide that interfere with the function of human neutrophils *in vitro*. These interactions lead to inhibition of neutrophil chemotaxis and production of reactive oxygen species that are essential components of innate neutrophil-mediated host defences. These authors also provided direct evidence that the exopolysaccharide inhibits enzymatic generation of ROS in a cell-free system (Bylund et al. 2006).

*B. cenocepacia* also produces two Zn$^{2+}$-dependent metalloproteases, ZmpA and ZmpB, whose expression is controlled by quorum sensing and the AtsR hybrid sensor kinase (Aubert et al. 2013; Corbett et al. 2003; Gingues et al. 2005; O'Grady et al. 2012). These proteases can
cleave and inactivate *in vitro* antimicrobial peptides involved in innate immunity (Kooi and Sokol 2009), and are required *in vivo* for the maturation arrest of the bacteria-containing vacuoles (Rosales-Reyes et al. 2012a).

A low molecular protein tyrosine phosphatase, referred to as Dpm (delayed phagosome maturation), is also required for the phagosome maturation arrest upon macrophage infection (Andrade and Valvano 2014). Heterologous expression of Dpm in *B. multivorans* confers to this bacterium a similar phagosomal maturation delay as found with *B. cenocepacia*. A mycobacterial low molecular protein tyrosine phosphatase prevents incorporation of the host vacuolar ATPase (vATPase) to the vacuole containing *Mycobacterium tuberculosis* and blocks acidification (Wong et al. 2011). Therefore, it is conceivable that Dpm could be responsible for the delayed acidification of the bacteria-containing vacuole by impairing the recruitment of the 16-kDa subunit of the phagosomal vATPase (Rosales-Reyes et al. 2012b). However, the Dpm mechanism is unclear since the protein appears to be biochemically inactive *in vitro*, suggesting that its contribution to phagosomal maturation arrest must be unrelated to tyrosine phosphatase activity (Andrade and Valvano 2014). Also, efforts to detect the host cell target of Dpm by co-immunoprecipitation experiments did not afford any reproducible candidates (Andrade and Valvano, unpublished).

The role of the *B. cenocepacia* specialized secretion system in macrophage survival and inflammation

Bacterial intracellular survival requires in most cases one or more specialized secretory systems responsible for injecting bacterial molecules into the cytoplasm of eukaryotic cells (Costa et al. 2015; Galán 2009). These molecules target host-signalling pathways, allowing bacteria to
establish an intracellular niche (Records 2011; van der Heijden and Finlay 2012; Voth et al. 2012). *B. cenocepacia* expresses one Type 3 (T3SS), two Type 4 (T4SS-1 and T4SS-2), and one Type 6 (T6SS) secretion systems. A report by Sajjan *et al.* (2008) concluded that the plasmid encoded T4SS-1 was required for intracellular survival and replication in macrophages. Using markers of the classical endocytic pathway in colocalization experiments, these authors showed that mutant and parental strains reside transiently in early endosomes, but a greater proportion of the mutant bacteria are targeted for lysosomal degradation. In our laboratory, we have constructed deletion mutants defective in each of the secretion systems singly and in combination, including a mutant defective in all the secretion systems (Tolman and Valvano, unpublished data). Using these mutants, we could not find differences in intracellular survival in murine macrophages measured as recovered bacteria at 24 h post-infection (Tolman and Valvano, unpublished data). Therefore, the role of the T4SS and the other secretion systems in the biology of *B. cenocepacia* intracellular infection is controversial and requires further investigation. It is possible that one or more secretion system in *B. cenocepacia* will be needed for specific conditions such as survival in different tissues or establishment of infection in non-mammalian hosts, experiments that are currently underway in our laboratory.

**T6SS affects the normal function of small Rho GTPases**

The T6SS is a recently recognized secretion system widely spread in Gram-negative pathogenic (Cascales 2008; Pukatzki *et al.* 2006; Zoued *et al.* 2014) and non-pathogenic bacteria (Jani and Cotter 2010). A distinct feature of the T6SS in comparison with the other secretion systems is its predominant role for bacterial competition, as most of the identified Type 6 secreted effectors are directed to inhibit growth of neighbouring bacteria in mixed populations (Russell *et al.* 2011;
Russell et al. 2014). In some cases, the same effector targets other bacteria and the eukaryotic host (Jiang et al. 2014). The *B. cenocepacia* T6SS is essential for virulence *in vivo*, as a library of mutants attenuated for virulence in the rat agar bead model of lung infection (Hunt et al. 2004) contained three independent transposon mutants in a region that was subsequently identified as a T6SS gene cluster (Aubert et al. 2008). The T6SS gene expression is negatively regulated by a global regulator designated AtsR (Aubert et al. 2013). Macrophage infection assays showed that an *atsR* mutant induces the formation of actin-mediated cell projections in a process that is T6SS dependent (Aubert et al. 2008). Surprisingly, the T6SS is not required for the maturation arrest of the bacteria-containing vacuole (Rosales-Reyes et al. 2012b). Further research established that intracellular *B. cenocepacia* expressing the T6SS alter the activation of Rac1 and Cdc42 GTPases by reducing the cellular pool of GTP-bound Rac1 and Cdc42 (Fig. 2) (Flannagan et al. 2012; Rosales-Reyes et al. 2012b). GTPase inactivation requires internalization of viable bacteria and also inhibits macropinocytosis and phagocytosis (Flannagan et al. 2012). These effects lead to abnormal actin polymerization causing collapse of lamellipodia and failure to retract the uropod. The T6SS also prevents the recruitment of soluble subunits of the NADPH oxidase complex including Rac1 to the membrane of bacteria-containing vacuole membrane, but is not involved in causing the phagosome maturation arrest (Rosales-Reyes et al. 2012b). We have recently identified a novel T6SS effector protein, designated as TecA (*T*6SS effector protein affecting the cytoskeleton architecture of macrophages), which is required for actin rearrangements (Aubert and Valvano, unpublished data). Macrophage infection by *B. cenocepacia* can result in the T6SS-dependent deamidation of a critical asparagine-41 residue in the RhoA GTPase (Xu et al. 2014), suggesting that TecA may be effector responsible for the modification of Rho-family GTPases.
T6SS mediates damage of the bacteria-containing vacuole leading to increased proinflammatory responses

Bcc infections are often persistent and lead to exacerbated inflammatory responses (Downey et al. 2007; Moura et al. 2008; Ventura et al. 2009). The *B. cenocepacia* T6SS induces a proinflammatory response in macrophages, which depends on the activation of the NLRP3 inflammasome and results in the stimulation of IL-1β secretion in a caspase-1-dependent manner that ultimately leads to cell death by pyroptosis (Rosales-Reyes et al. 2012a). Furthermore, the T6SS-mediated disruption of the phagosomal membrane allows the escape of proteins secreted by the T2SS into the macrophage cytoplasm (Rosales-Reyes et al. 2012a), namely the proteases ZmpA and ZmpB, which may also contribute to proinflammatory signaling (Kooi and Sokol 2009).

Murine macrophages use the NLRP3 and pyrin inflammasomes together with the host adapter molecule ASC to detect intracellular *B. cenocepacia* (Rosales-Reyes et al. 2012a; Xu et al. 2014). In contrast, detection of intracellular *B. cenocepacia* in human macrophages requires pyrin (Gavrilin et al. 2012). The activation of the NLRP3/ASC and pyrin/ASC inflammasomes also required a functional T6SS (Gavrilin et al. 2012; Rosales-Reyes et al. 2012a; Xu et al. 2014). Therefore, a model is emerging from all of these studies in murine and human macrophages indicating the expression of T6SS plays a proinflammatory role, resulting in damage of the phagosomal membrane with the concomitant release of bacterial mediators of inflammation accumulated in its lumen. Also, the T6SS causes the modification of RhoA and possibly other Rho-family GTPases (Xu et al. 2014). The activation of both NLRP3 and pyrin inflammasomes is not completely elucidated and may not require direct sensing of pathogen molecules (Elliott
and Sutterwala 2015; Yang et al. 2014), but rather cellular modifications due to the infecting pathogen, which ultimately contributes to inflammasome activation and host cell death. This notion correlates well with clinical observations documenting the proinflammatory nature of the respiratory infection by *B. cenocepacia* in CF patients.

**Role of the CF transmembrane conductance regulator (CFTR) in *B. cenocepacia* intracellular survival**

A remarkable observation in the biology of the *B. cenocepacia* infection in macrophages was the finding that the maturation arrest of the bacteria-containing vacuoles is greatly exaggerated in CFTR-defective macrophages than in CFTR-normal macrophages (Lamothe and Valvano 2008). This suggests that not only defective CFTR, but also viable *B. cenocepacia* are required for the altered trafficking phenotype. Therefore, CFTR may play a role in the mechanism of clearance of the intracellular infection, as *B. cenocepacia* bacterial cells that localized to the lysosome lost cell envelope integrity (Lamothe et al. 2007). The suggestion that CFTR is the Cl⁻ ion channel essential for phagosomal acidification (Di et al. 2006) has been disproved by several groups (Barriere et al. 2009; Haggie and Verkman 2007; Lamothe and Valvano 2008; Painter et al. 2008; Painter et al. 2010) and therefore cannot explain the delayed maturation arrest of bacteria-containing vacuoles in CFTR-defective macrophages. Additional possibilities to explain how the CFTR defect enhances the *B. cenocepacia* intracellular survival have been discussed elsewhere (Valvano et al. 2012). However, current evidence indicates that defective CFTR leads to defective autophagy and decreases the clearance of protein aggregates, which in turns leads to inflammation (Luciani et al. 2010; Luciani et al. 2011; Luciani et al. 2012). Conceivably, defective autophagy might alter the ability of macrophages to clear the intracellular infection.
Indeed, several intracellular pathogens reside in vacuoles with autophagosome features (Deretic and Levine 2009). In this scenario, the CFTR defect and Bcc infection could have a synergistic effect down regulating autophagy, which would be consistent with the pronounced delay in the bacteria-containing vacuole maturation under a functionally impaired CFTR.

An autophagosome is a compartment that engulfs non-functional organelles and parts of the cytoplasm, and subsequently delivers these components to the lysosome for degradation and recovery of essential nutrients during periods of cell starvation or stress. Recent results have shown that in CFTR-defective murine macrophages, *B. cenocepacia* persists in vacuoles that rarely fuse with lysosomes. These infected cells produce higher levels IL-1β than infected CFTR-normal macrophages (Abdulrahman et al. 2011). It was previously suggested that intracellular *B. cenocepacia* reside in autophagosomes that fuse with lysosomes at later stages of infection (Sajjan et al. 2008). A comparison between infected CFTR-defective and CFTR-normal macrophages revealed that the autophagy genes are downregulated. However, the CFTR-defective cells contain more bacteria than the CFTR-normal macrophages. Treatment of CFTR-defective macrophages with the autophagy stimulating agent rapamycin markedly decreases *B. cenocepacia* infection *in vitro* by enhancing the clearance of *B. cenocepacia* via induced autophagy (Abdulrahman et al. 2011). More importantly rapamycin decreased bacterial burden and inflammation in the lungs of CF mice, revealing that autophagy can control the fate of the *B. cepacia* intracellular infection and the ensuing inflammatory response.

The mechanism for the link between *B. cenocepacia* and autophagy in CFTR-defective macrophages involves the adaptor protein SQSTM1/p62, an ubiquitin binding adaptor protein that is required for the delivery of ubiquitinated cargo to the autophagosome (Abdulrahman et al. 2012). In CFTR-normal macrophages, p62 depletion and overexpression cause increased and
decreased bacterial intracellular survival, respectively. However, depletion of p62 in CFTR-defective macrophages results in decreased bacterial survival, whereas overexpression of p62 leads to increased *B. cenocepacia* intracellular multiplication. Therefore, these studies expose a strong link between *B. cenocepacia* infection and the CFTR defect.

**Conclusions**

The survival of Bcc bacteria, in particular *B. cenocepacia* and *B. multivorans* in macrophages provides a model system to investigate the interactions of opportunistic pathogens with host cells responsible for innate immune recognition and beneficial or pathological inflammatory responses at the molecular level. The elucidation of the details of the survival, especially the ability of *B. cenocepacia* to synergize with the CFTR defect and its consequences on the mechanism of autophagy, provides new avenues to explore novel therapeutic approaches to ameliorate and control the profuse inflammatory responses triggered by infection and eradicate the bacterial load.

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Legend to Figures

Fig. 1. Delayed maturation of the B. cenocepacia-containing phagosome. Shortly after engulfment the B. cenocepacia-containing vacuole acquires LC3 and EEA1 (early endosome marker). EEA1 is lost upon maturation but instead of rapid acidification the B. cenocepacia-containing vacuole remains with high pH and delays the incorporation of LAMP1 for up to 6 h. A defective Rab7 is also incorporated to the vacuole. The possibility that during this process the bacteria escape the vacuole (red dashed lines) and live in the cytosol is not clearly established.

Fig. 2. Model for the central role of the T6SS in the B. cenocepacia intracellular lifestyle in macrophages. T6SS effectors are delivered into the cytosol where affect the function of Rho-family GTPases which in turns leads with a defect in the recruitment of soluble components of the NADPH oxidase to the membrane of the phagosome and defects in actin remodelling (affecting lamellipodia formation and compromising phagocytosis). These functional defects are somehow detected by an unknown mechanism and result in the activation of the NLRP3/Pyrin inflammasomes and subsequent cell death by pyroptosis and sustained inflammation.
Fig. 1

Fig. 2