Klebsiella pneumoniae survives within macrophages by avoiding delivery to lysosomes.

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SUMMARY

*K. pneumoniae* is an important cause of community-acquired and nosocomial pneumonia. Evidence indicates that *Klebsiella* might be able to persist intracellularly within a vacuolar compartment. In this work, we demonstrate that *K. pneumoniae* survives killing by macrophages by manipulating phagosome maturation. Engulfment of *K. pneumoniae* was dependent on host cytoskeleton, cell plasma membrane lipid rafts and the activation of PI 3-kinase (PI3K). Microscopy studies revealed that *K. pneumoniae* resides within a vacuolar compartment, the *Klebsiella* containing vacuoles (KCV), which traffics within vacuoles associated with the endocytic pathway. In contrast to UV-killed bacteria, alive bacteria did not colocalize with markers of the lysosomal compartment. Our data suggest that *K. pneumoniae* triggers a programmed cell death in macrophages displaying features of apoptosis. Our efforts to identify the mechanism(s) whereby *K. pneumoniae* prevents the fusion of the lysosomes to the KCV uncovered the central role of the PI3K-Akt-Rab14 axis to control the phagosome maturation. We propose that agents targeting PI3K/Akt might provide selective alternatives to manage *K. pneumoniae* pneumonias. We were keen to identify the *Klebsiella* factors necessary for intracellular survival. Our data revealed that the capsule is dispensable if bacteria were not opsonized. The environment found by *Klebsiella* within the KCV triggered the downregulation of the expression of *cps*. 
INTRODUCTION

In the late nineteenth century, Eli Metchnikoff appreciated phagocytosis as a key process in the battle against pathogens. Phagocytosis can be conceptually divided into phagosome formation and its subsequent evolution into a degradative compartment, a process termed phagosome maturation. This is important because the nascent phagosome is not microbicidal. Maturation not only aids clearing infection, but also generates and routes antigens for presentation on MHC molecules in order to activate the adaptive immune system (Trombeta and Mellman, 2005).

Phagosome maturation involves the sequential acquisition of different proteins, many of them of the endocytic pathway (Vieira et al. 2002, Flannagan et al. 2012). Thus, during and/or immediately after phagosome closure, the phagosome fuses with early endosomes, acquiring Rab5 and early endosome antigen 1 (EEA1). The phagosome rapidly loses the characteristics of early endosome and acquires late endosome features. The late phagosome is positive for Rab7, the mannose-6-phosphate receptor, lysobisphosphatidic acid, lysosome-associated membrane proteins (Lamps) and CD63. Ultimately, the organelle fuses with lysosomes to form the phagolysosome, identified by the presence of hydrolytic proteases, such as processed cathepsin D, cationic peptides and by an extremely acidic luminal pH which is regulated primarily by the vacuolar (V-type) ATP-ase complex. In the course of maturation, an oxidative system formed by the NADPH oxidase and ancillary proteins is also activated.

Many pathogens have developed strategies to counteract the microbicidal action of macrophages (Flannagan et al. 2009, Sarantis and Grinstein. 2012). Some pathogens inhibit phagocytosis. For example, the role of capsule polysaccharides in preventing opsonophagocytosis has been appreciated for many pathogens including Neisseria meningitidis, Staphylococcus aureus and streptococci. Others, such as enteropathogenic Escherichia coli, inhibit engulfment by blocking PI 3-kinase (PI3K) signaling whereas Yersinia species inhibits phagocytosis by injecting type III secretion effectors. Conversely, Salmonella typhimurium induces its own uptake and, once inside a modified phagosome, triggers macrophage death by a caspase-1 dependet process called pyroptosis.
(Fink and Cookson. 2007). *Brucella* spp. resist an initial macrophage killing to replicate in a compartment segregated from the endocytic pathway with endoplasmic reticulum properties (von Bargen *et al*. 2012).

*Klebsiella pneumoniae* is a Gram negative capsulated pathogen which causes a wide range of infections, from urinary tract infections to pneumonia, being particularly devastating among immunocompromised patients with mortality rates between 25% and 60% (Sahly and Podschun. 1997). *K. pneumoniae* is an important cause of community-acquired pneumonia in individuals with impaired pulmonary defences and is a major pathogen for nosocomial pneumonia. Pulmonary infections are often characterized by a rapid clinical course thereby leaving very short time for an effective antibiotic treatment. *K. pneumoniae* isolates are frequently resistant to multiple antibiotics (Munoz-Price *et al*. 2013), which leads to a therapeutic dilemma. In turn, this stresses out the importance of pulmonary innate defense systems to clear *K. pneumoniae* infections.


*K. pneumoniae* has been largely considered as an extracellular pathogen. However, there are reports showing that *K. pneumoniae* is internalized *in vitro* by different cell types being able to persist intracellularly for at least 48 h (Oelschlaeger and Tall. 1997). It has been also reported the presence of intracellular *Klebsiella* spp. within a vacuolar compartment inside human macrophages, mouse alveolar macrophages and lung epithelial cells *in vivo* (Cortes *et al*. 2002b, Fevre *et al*. 2013, Willingham *et al*. 2009, Greco *et al*. 2012). This study was designed to investigate the...
interaction between *K. pneumoniae* and macrophages. We report that *K. pneumoniae* survives within macrophages by deviating from the canonical endocytic pathway and residing in a unique intracellular compartment which does not fuse with lysosomes. Finally, we present evidence indicating that *K. pneumoniae* has the potential to kill and escape from the phagocyte.

**RESULTS**

*K. pneumoniae* survives inside macrophages.

To explore whether *K. pneumoniae* resides inside macrophages *in vivo*, macrophages were isolated from the bronchoalveolar lavage of mice infected intranasally with *K. pneumonia* strain 43816 (hereafter Kp43816R). Microscopy analysis revealed the presence of intracellular bacteria in 10% of the recovered macrophages. Immunofluorescence experiments showed that 99 % ± 7 of the intracellular bacteria did not colocalize with the lysosomal marker cathepsin D (Fig 1A). Z-stack projections confirmed that bacteria were located intracellularly (data not shown).

To assess the intracellular survival of *K. pneumoniae* in macrophages in more detail, we standardized the infection conditions of the mouse macrophage cell line MH-S with *K. pneumoniae* Kp43816R. We optimized the time of bacteria-cell contact (30, 60 and 120 min), the multiplicity of infection (MOI) (100, 50 or 10 bacteria per cell), and the antibiotic treatment necessary to kill the remaining extracellular bacteria after the contact. To synchronize infection, plates were centrifuged at 200 x g during 5 min and intracellular bacteria were enumerated after macrophage lysis with 0.5% saponin in PBS. We found that 90 min treatment with a combination of gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) was necessary to kill 99.9% of the extracellular bacteria. The highest numbers of engulfed bacteria were obtained after 120 min of contact with a MOI of 100:1. However, these conditions also triggered a significant decrease in cell viability as detected by the trypan blue exclusion method. 30 min of contact and a MOI of 50:1 were the conditions in which no decrease in cell viability was observed and, therefore, they were used in the subsequent experiments.
To investigate the molecular mechanisms used by mouse macrophages to engulf Kp43816R, infections were carried out in the presence of drugs which specifically inhibit host cell functions (Fig 1B). Cytochalasin D and nocodazol reduced the engulfment of Kp43816R hence indicating that Kp43816R phagocytosis requires the assembly of F-actin and the host microtubule network. Methyl-β-cyclodextrin (MβCD), which depletes cholesterol from host cell membranes, was employed to analyse the involvement of lipid rafts in Kp43816R phagocytosis. Cholesterol depletion impaired Klebsiella engulfment by MH-S. Similar results were obtained when cells were treated with filipin and nystatin (Fig. 1B). Since the generation of phosphoinositides is linked to phagosome formation (Vieira et al. 2001), we assessed the contribution of the PI3K signalling pathway on Kp43816R phagocytosis. Pre-treatment of MH-S cells with LY294002, a specific inhibitor of PI3K activity, resulted in the blockage of Kp43816R phagocytosis (Fig. 1B). Akt is a downstream effector of PI3K which becomes phosphorylated upon activation of the PI3K signalling cascade. As expected, western blot analysis revealed that Kp43816R induces the phosphorylation of Akt in a PI3K-dependent manner since LY294002 inhibited Klebsiella-induced phosphorylation of Akt (Fig. 1C-D). Similar results were obtained when human macrophages (THP-1 monocytes differentiated to macrophages by phorbol-12-myristate-13-acetate [PMA] treatment; hereafter mTHP-1) were infected (Fig. S1).

The bacterial intracellular location in MH-S cells was assessed 3 and 6 h post infection by transmission electron microscopy (TEM). In good agreement with other published in vivo observations (Cortes et al. 2002b, Fevre et al. 2013, Willingham et al. 2009, Greco et al. 2012), bacteria were located in a vacuolar compartment (Fig. 1E). To determine the fate of intracellular Kp43816R, MH-S cells were infected with GFP-expressing Kp43816R and the number of intracellular bacteria was assessed microscopically using differential (inside/outside) staining and by plating after different incubation times. Bacteria could be clearly observed through the entire course of infection. To elucidate whether those intracellular bacteria were indeed viable, fluorescent in situ hybridisation (FISH) was carried out by using the oligonucleotide probes EUB338 and
GAM42a (see Experimental procedures). The detection of bacteria by these oligonucleotide probes is dependent on the presence of sufficient ribosomes per cell, hence providing qualitative information on the physiological state of the bacteria on the basis of the number of ribosomes per cell (Christensen et al. 1999, Morey et al. 2011). Microscopy analysis revealed that the number of bacteria metabolically active (FISH positive) versus the total number of intracellular bacteria (GFP positive) was maintained through the infection (Fig. 1F-G). We did not observe any change of host cell morphology (data not shown). Time course experiments showed that the number of intracellular bacteria in MH-S cells decreased during the first 2.5 h of infection but then it remained constant until 8 h post infection (Fig 2A). Immunofluorescence analysis revealed that the phagocytic index (number of phagocytosed bacteria per 100 macrophages) decreased during the first 2 h and then it did not change until the end of the experiment (Fig 2B). Similar results were obtained when mTHP-1 cells were infected (Fig 2C-D).

Collectively, these results showed that Kp43816R phagocytosis by macrophages is an event dependent on host cytoskeleton and cell plasma membrane lipid rafts. Moreover, the PI3K/Akt host signalling pathway is activated by Kp43816R infection and it is required for bacterial phagocytosis. Our data demonstrate that Kp43816R survives within macrophages through the course of infection and the TEM experiments suggest that Kp43816R may reside in a specific compartment that we named the *Klebsiella* containing vacuole (KCV).

**K. pneumoniae elicits a cytotoxic effect on macrophages.**

Examination of the infected monolayers by immunofluorescence at different time points revealed a decrease in the overall monolayer density at 10 h post infection which was more evident 20 h post infection (Fig S2A). This observation prompted us to study whether Kp43816R exerts a cytotoxic effect on macrophages. We assessed the viability of infected MH-S cells by measuring the levels of LDH release. Kp43816R infection was associated with a 35% decrease in cell viability.
Cellular Microbiology

after 20 h of infection. Kp43816R-triggered cytotoxic effect on macrophages was also evident when cell viability was estimated by the neutral red uptake assay (Fig S2B).

The induction of host cell apoptosis is one mechanism used by some pathogens to augment infection (Navarre and Zychlinsky. 2000). To test whether Kp43816R causes apoptosis of MH-S cells, apoptosis was measured with annexin V, to analyze phosphatidylserine translocation to the outer leaflet of the plasma membrane, and 7-actinomycin D (AAD) to evaluate plasma membrane integrity. Flow cytometry analysis of infected cells showed a significant increased in annexin V\(^+\)AAD\(^-\) cells over time (Fig. 3). The amount of double-positive annexinV\(^+\)AAD\(^+\) cells, which corresponds to a necrotic-like phenotype, was markedly lower at all times analyzed. These results indicate phosphatidylserine translocation and intact membrane integrity, a classical apoptotic phenotype, hence suggesting that Kp43816R triggers apoptosis in macrophages.

*K. pneumoniae* prevents phagosome fusion with lysosomes.

Because Kp43816R is able to survive within macrophages, we hypothesized that *Klebsiella* must either divert the normal process of phagosome maturation or withstand the hostile environment of the mature phagolysosome. Therefore, we analyzed the maturation of the KCV during the course of an infection. We analysed the association of the KCV with compartments of the exocytic pathway. Bacteria did not colocalize with either markers of the endoplasmic reticulum (calnexin) or markers of the Golgi network (GM 130) at any time analyzed (Fig S3). Next, we examined the presence on the KCV of markers specific of the successive stations of the endocytic pathway. EEA1 is an early endosome-specific peripheral membrane protein which colocalizes with the small GTP binding protein Rab5 (Vieira et al. 2002, Flannagan et al. 2012). As shown in Figure 4, by 15 min post infection we could detect the presence of EEA1 on 22 ± 4% of KCVs. EEA1 was lost from KCVs as the percentage of vacuoles positive for this marker dropped to 15% ± 9% and 5% ± 1% at 60 and 90 min post infection, respectively (Fig 4). We next sought to determine whether the KCV acquires the late endosomal markers Lamp1 and Rab7 (Vieira et al.
KCVs were positive for Lamp1 already at 30 min post infection and the percentage of positive KCVs increased over time. KCVs remained positive for Lamp1 until 8 h post infection. Rab7 is a small G-protein that controls vesicular transport to late endosomes and lysosomes in the endocytic pathway (Rink et al. 2005). To assess the presence of Rab7 on KCVs, macrophages were transfected with GFP-Rab7 and then infected with Kp43816R. The majority of the vacuoles containing Kp43816R were positive for Rab7. To determine the activation status of Rab7 we asked whether RILP, a Rab7 effector protein that exclusively recognizes the active (GTP bound) conformation of the GTPase (Cantalupo et al. 2001, Jordens et al. 2001), labels the KCV. Before infection, cells were transfected with a plasmid containing GFP fused to the C-terminal Rab7-binding domain of RILP, called “RILP-C33”, which can be used as a reliable index of the presence and distribution of active Rab7 (Cantalupo et al. 2001, Jordens et al. 2001). As shown in Figure 4 RILP-C33-EGFP colocalized with the majority of KCVs.

Since the interaction of Rab7 with RILP drives fusion with lysosomes (Cantalupo et al. 2001, Jordens et al. 2001), we sought to determine whether KCV colocalizes with lysosomal markers. Although there are not markers that unambiguously distinguish late endosomes from lysosomes, mounting evidence indicates that an acidic luminal pH and the presence of hydrolytic proteases, such as processed cathepsin D, are characteristics of the phagolysosomal fusion (Vieira et al. 2002, Flannagan et al. 2012). We used the fixable acidotropic probe LysoTracker to monitor acidic organelles in infected macrophages. We found a major overlap between the dye and the KCVs (Fig 5), hence indicating that the KCV is acidic. We next examined the presence in the vacuole of cathepsin D as a marker for the lysosomal soluble content. However, the majority of the KCVs did not colocalize with cathepsin D (Fig 5), thereby suggesting that the KCV does not fuse with lysosomes. To further sustain this notion, we assessed colocalization of the KCV with tetramethylrhodamine-labelled dextran (TR-dextran). Prior to bacterial infection macrophages were pulsed with TR-dextran for 2 h followed by a 1 h chase in dye-free medium to ensure that the probe is delivered from early and recycling endosomes to phagolysosomes (Morey et al. 2011, Eissenberg...
et al. 1988, Hmama et al. 2004, Lamothe et al. 2007). Confocal immunofluorescence experiments showed that the majority of the KCVs did not colocalize with TR-dextran (Fig 5B). In contrast, when macrophages were infected with UV-killed Kp43816R the majority of the KCVs did colocalize with cathepsin D and TR-dextran (Fig S4). Collectively, these results strongly support the notion that KCVs containing alive bacteria prevent the fusion of the vacuole with lysosomes.

Similar findings were obtained when mTHP-1 cells were infected. KCV was not associated with compartments of the exocytic pathway, either Golgi network or endoplasmic reticulum, but acquired markers of the endocytic pathway, EEA1 and Lamp1 (Fig S5A). The majority of KCVs colocalized with LysoTracker (Fig S5A) but they were negative for cathepsin D (Fig S5B). In contrast, nearly 70% of UV-killed Kp43816R colocalized with cathepsin D after 2 h post infection (Fig S5B). Altogether, these results indicate that only phagosomes containing UV-killed Klebsiella bacteria fuse with lysosomes in human macrophages.

In summary, these findings suggest that K. pneumoniae trafficks inside macrophages within vacuoles associated to the endocytic pathway, and that living K. pneumoniae seem to perturb the fusion of the KCV with the hydrolases-rich lysosomal compartment.

**Inhibition of compartment acidification affects K. pneumoniae intracellular survival.**

Phagosome acidification has been shown to be essential for the intracellular survival of different pathogens (Morey et al. 2011, Ghigo et al. 2002, Porte et al. 1999). Therefore, we investigated the effect of inhibiting KCV acidification on K. pneumoniae survival. Bafilomycin A₁ is a specific inhibitor of the vacuolar type H⁺-ATPase in cells, and inhibits the acidification of organelles containing this enzyme, such as lysosomes and endosomes. As expected, phagolysosomal acidification was sensitive to bafilomycin A₁ treatment (Fig 6A), hence confirming dependence on the vacuolar H⁺-ATPase. Moreover, bafilomycin A₁ treatment also abrogated the overlap between Kp43816R and the probe LysoTracker (Fig 6A). To assess the effect of vacuolar acidification on Kp43816R survival, cells were treated with bafilomycin A₁ at the onset of the
gentamicin treatment and bacteria were enumerated by plating at different time points. Data shown in Figure 6C revealed that the number of intracellular Kp43816R decreased in bafilomycin A₁ treated cells over time compared to infected untreated cells. Control experiments revealed that bafilomycin A₁ has no toxic effect on K. pneumoniae (our control experiments [data not shown]) or on other Gram-negative bacteria (Morey et al. 2011, Porte et al. 1999). Altogether, these observations suggest that Kp43816R intracellular survival is dependent on KCV acidification.

PI3K-AKT and Rab14 aid intracellular survival of K. pneumoniae.

S. enterica serovar typhimurium perturbs the fusion of the phagosomes with lysosomes by activating the host kinase Akt (Kuijl et al. 2007). In turn, inhibition of Akt activation reduces the intracellular survival of Salmonella (Kuijl et al. 2007, Chiu et al. 2009). Several pathogens also target the PI3K-Akt axis to manipulate cell biology for their own benefit (Krachler et al. 2011). Since Kp43816R induced the activation of Akt in a PI3K-dependent manner we sought to determine the contribution of the PI3K-Akt axis to the intracellular survival of K. pneumoniae. Treatment of cells with the PI3K inhibitor LY294002 or the Akt inhibitor AKT X at the onset of the gentamicin treatment reduced the number of intracellular bacteria in MH-S cells (Fig 7A). Moreover, pulse-chase experiments revealed that more than 70% bacteria colocalized with TR-dextran in cells treated with the Akt inhibitor (Fig 7B). Collectively, these results support the notion that Kp43816R targets the PI3K-Akt axis to survive intracellularly.

At least 18 Rab GTPases are implicated in phagosomal maturation (Smith et al. 2007). Interestingly, Salmonella targets Rab14 to prevent phagosomal maturation in an Akt dependent manner (Kuijl et al. 2007). We speculated that Kp43816R may also target Rab14 to control the maturation of the phagosome. Immunofluorescence experiments revealed that GFP-Rab14 colocalized with the majority of the KCVs (Fig 7C). To determine whether recruitment of Rab14 is required for intracellular survival cells were transfected with a Rab14 dominant-negative construct (DN-Rab14) or control vector and then infected with Kp43816R. As shown in figure 7D, we found...
a 60% decrease in the number of intracellular bacteria in cells transfected with DN-Rab14.

Altogether, these results support the notion that Kp43816R recruits Rab14 to the KCV to control
the maturation of the phagosome.

In summary, our results are consistent with a model where Kp43816R targets the PI3K-Akt-
Rab14 axis to control the phagosome maturation to survive inside macrophages.

**K. pneumoniae capsule polysaccharide is dispensable for intracellular survival.**

We were keen to identify *K. pneumoniae* factors necessary for intracellular survival. Given
the importance of *K. pneumoniae* CPS on host-pathogen interactions, we explored whether CPS is
also necessary for *K. pneumoniae* intracellular survival. As anticipated, a CPS mutant was engulfed
by MH-S and mTHP1 macrophages in higher numbers than Kp43816R (data not shown). For the
sake of comparison with the wild-type strain in time-course experiments, we adjusted the MOI of
the CPS mutant to get comparable numbers of intracellular bacteria at the beginning of the
infection. Time course experiments showed no differences between the number of intracellular
bacteria of both strains in MH-S and mTHP1 cells (Fig 8A).

Given the critical role of CPS in preventing complement-mediated opsonophagocytosis
(Alvarez *et al.* 2000, de Astorza *et al.* 2004, Cortes *et al.* 2002a), we evaluated whether the
intracellular fate of the CPS mutant could be modified by bacterial opsonization with human serum.
In agreement with previous reports (de Astorza *et al.* 2004, Cortes *et al.* 2002a), opsonization of the
CPS mutant resulted in an increase in the number of ingested bacteria by mTHP1 cells compared to
nonopsonized bacteria (Fig 8B). For the sake of comparison, the MOI was adjusted to get
comparable numbers of intracellular bacteria at the beginning of the infection. The number of CFU
recovered from cells infected with the opsonized CPS mutant was significantly lower than the
number of CFU recovered from cells infected with non-opsonized bacteria (100 fold lower at 8 h
post infection; Fig 8C). These data indicate that internalization via the C3 receptor results in a
significant loss of intracellular viability, presumably because these bacteria are ultimately delivered to lysosomes.

The lack of contribution of CPS to intracellular survival prompted us to analyze the expression of *cps* in the KCV. To monitor *cps* expression over time, we constructed a transcriptional fusion in which the *cps* promoter region was cloned upstream a promoterless *gfp* that encodes a protein tagged at the C terminus with the (LVA) peptide. The GFP(LVA) protein is targeted for Tsp protease degradation within the bacteria and has been reported to have 40-min half-life, while untagged GFP is very stable (estimated *in vivo* half-life, 24 h) (Miller et al. 2000). Kp43816R containing the unstable GFP reporter was used to infect MH-S. Control experiments showed that there were no differences in the number of intracellular bacteria recovered over time from cells infected with bacteria containing the reporter plasmid, the empty vector or no plasmid (data not shown). Cells were lysed, processed as described in Experimental procedures, and analyzed for fluorescence by FACS at different time points post infection. As shown in Figure 8D, GFP fluorescence decreased over time reaching the levels of the control strain carrying the empty vector. To explore whether the acidic pH of the KCV might be responsible for the downregulation of *cps* expression, bacteria were grown in M9 minimal medium, with different magnesium concentrations, buffered to different pHs. The expression of the *cps::gfp* fusion was 5-fold lower when bacteria were grown at pH 5.5 (Fig 8E).

Collectively, these findings show that *K. pneumoniae* CPS is dispensable for intracellular survival. Furthermore, the environment found by *Klebsiella* within the KCV triggers the downregulation of the expression of *cps*. The fact that opsonization affects the intracellular survival of the CPS mutant indicates that the mechanism of bacteria entry into macrophages has a major impact in the ability of *K. pneumoniae* to survive intracellularly.
In this work, we present compelling evidence demonstrating that *K. pneumoniae* survives killing by macrophages by manipulating phagosome maturation. Our data sustain that *K. pneumoniae* traffics within vacuoles associated with the endocytic pathway in mouse and human macrophages. In contrast to UV-killed bacteria, which colocalize with lysosomal markers, alive bacteria modify the vacuole biogenesis preventing the fusion of the KCV with the hydrolases-rich lysosomal compartment. *K. pneumoniae* thus increases the list of pathogens able to alter phagosome maturation.

Engulfment of *K. pneumoniae* by mouse and human macrophages was dependent on host cytoskeleton, cell plasma membrane lipid rafts and the activation of PI3K which are all commonly needed to engulf pathogens and inert particles such as latex beads (Vieira et al. 2002, Flannagan et al. 2012). TEM analysis suggested that *K. pneumoniae* resides inside a vacuolar compartment and FISH experiments revealed that intracellular *Klebsiella* were metabolically active for a long period of time. Several lines of evidence indicate that *K. pneumoniae* infections are associated with cell death (Willingham et al. 2009, Cano et al. 2009, Cai et al. 2012). In good agreement, in this study we show that *K. pneumoniae* triggers a programmed cell death in macrophages displaying features of apoptosis. Of note, kinase activity profiling in whole lungs during *K. pneumoniae* infection showed the activation of kinases associated to induction of apoptosis (Hoogendijk et al. 2011). However, Willingham and co-workers have reported recently that *K. pneumoniae* activates the NLRP3-dependent cell death programme termed pyroptosis (Willingham et al. 2009). Similar contradictory findings have been reported for *Shigella flexneri* infections. *Shigella* triggers apoptotic and pyroptotic cell death in macrophages depending on the bacterial dosage and time of infection (Willingham et al. 2007, Hilbi et al. 1998). In the case of *Shigella*, shorter time of contact and low MOI are associated to induction of apoptosis (Willingham et al. 2007, Hilbi et al. 1998). Notably, the infection conditions in our study are different to those used by Willingham and co-
workers who used a MOI four times higher than ours (Willingham et al. 2009). Future studies are warranted to carefully assess the influence of infection conditions on Klebsiella-induced cell death.

The vacuole of K. pneumoniae and its biogenesis was studied by immunofluorescence. The presence of EEA1 on the KCV indicates that internalized bacteria are initially present in a vacuole related to the endocytic pathway. However, K. pneumoniae does not remain in early endosomes as demonstrated by the acquisition of Lamp1 and Rab7. A hallmark of the maturation is the exclusion of lysosomal hydrolases in the majority of KCVs containing live bacteria. In contrast, more than 50% of the KCVs containing UV-killed bacteria were positive for lysosomal markers already 90 min post infection. The KCV is acidic most likely due to the activity of vacuolar proton-ATPases. Notably, inhibition of these pumps by bafilomycin A1 resulted in a decrease in intracellular bacterial numbers. Similar findings have been reported for non typable H. influenzae, Tropheryma whippiei, and Brucella suis (Morey et al. 2011, Ghigo et al. 2002, Porte et al. 1999). The reduction of intracellular viability may have several explanations. Bafilomycin A1 might affect other cellular functions necessary for K. pneumoniae survival. An alternative hypothesis, and more appealing to us, is that K. pneumoniae requires a low pH environment for survival within the KCV. For example, the acidic environment may facilitate the uptake of nutrients by Klebsiella. Acidic pH is required for the transport of glucose in Coxiella burnetii (Howe and Mallavia. 2000) and localization in an acidic environment facilitates the availability of iron for the growth of Francisella tularensis (Fortier et al. 1995). In addition, low pH may regulate the expression of factors essential for intracellular survival. This has been shown to be true for virulence gene transcription in S. typhimurium (Yu et al. 2010). In this context, our data have revealed that Klebsiella downregulates the expression of cps when residing within the KCV. Interestingly, when Klebsiella was cultured in low magnesium and acidic pH we also found a downregulation of cps expression. It is tempting to speculate that these signals could trigger cps downregulation within the KCV. In fact, in this work we have shown that the KCV is acidic and there are reports suggesting that the magnesium concentration in pathogen-containing vacuoles is in the micromolar range (Garcia-del Portillo et al.
1992). Future efforts will be devoted to characterize the chemical composition of the KCV as well
as the transcriptional landscape of intracellular *K. pneumoniae*.

It was interesting to consider the mechanism(s) whereby *K. pneumoniae* prevents the fusion
of the lysosomes to the KCV. The overall resemblance between the KCV and the *Salmonella*
containing vacuole (acidic Lamp-1-positive cathepsin-negative vacuole) prompted us to explore
whether *K. pneumoniae* employs similar strategies as *Salmonella* to subvert phagosome maturation.
Kuijl and coworkers (Kuijl et al. 2007) demonstrated that *S. typhimurium* activates Akt to prevent
phagosome-lysosome fusion. Since *K. pneumoniae* activates Akt in vitro (this work and (Frank et
al. 2013)) and in vivo (Hoogendijk et al. 2011) we speculated that activated Akt may also promote
*Klebsiella* intracellular survival. Indeed this was the case. Akt inhibition resulted in a significant
decrease in bacterial intracellular survival associated with an increased colocalization of the KCV
with lysosomal markers. The fact that Akt is implicated in the intracellular survival of other
pathogens, including *M. tuberculosis* (Kuijl et al. 2007), strongly suggests that this kinase is a
central host node targeted by pathogens to take control over cellular functions.

PI3K/Akt governs phagosome maturation by controlling, at least, the activation of Rab
GTPases (Thi and Reiner. 2012), although Rab14 is emerging as a central Rab in this process.
Recent data indicate that pathogens hijack Rab14 to manipulate phagosome maturation. The *M.
tuberculosis* vacuole recruits and retains Rab14 to maintain early endosomal characteristics (Kyei et
al. 2006) whereas *S. typhimurium* containing vacuole retains Rab14 in an Akt-dependent manner to
arrest phagosome maturation (Kuijl et al. 2007). Immunofluorescence experiments confirmed that
the KCV is positive for Rab14 whereas transient transfection of the dominant-negative Rab14
resulted in a decrease in bacteria intracellular survival. In aggregate, this evidence supports a
scenario in which *K. pneumoniae* manipulates phagosome maturation by targeting a PI3K-Akt-
Rab14 pathway. Nevertheless, we do not rule out that there are additional pathways necessary for
*Klebsiella* intracellular survival.
We were keen to identify the bacterial factors interfering with the phagosomal maturation pathway. Given the critical role of *K. pneumoniae* CPS in preventing host defense responses (March *et al.* 2013, Regueiro *et al.* 2006, Lawlor *et al.* 2005, Frank *et al.* 2013, Moranta *et al.* 2010, Campos *et al.* 2004, Lawlor *et al.* 2006), we hypothesized that CPS is necessary for intracellular survival. To our initial surprise, CPS does not play a large role, if any, in intracellular survival of *Klebsiella* since a *cps* mutant did not display any loss of viability upon phagocytosis. Furthermore, the *cps* mutant also triggered a programmed cell death in macrophages (data not shown). Altogether, these findings may seem contradictory with the well establish role of CPS in *K. pneumoniae* virulence. However, considering the presence of complement in the bronchoalveolar fluid (Wu *et al.* 2005), the fact that opsonization results in more efficient internalization of pathogens and maturation of phagosomes (Aderem and Underhill. 1999), and the well-known role of CPS in preventing complement opsonization (de Astorza *et al.* 2004, Cortes *et al.* 2002a), we hypothesized that opsonization of the mutant is deleterious to its intracellular fate. Indeed, this was the case hence revealing the critical role of CPS on *Klebsiella*-macrophage interplay. These results also illustrate how the mode of entry of a pathogen influences its intracellular outcome. Similar findings have been reported for other pathogens (Geier and Celli. 2011, Gordon *et al.* 2000, Drevets *et al.* 1993) but it cannot be considered a general feature since complement opsonization does not affect the intracellular fate of *Salmonella* and *M. tuberculosis* (Drecktrah *et al.* 2006, Zimmerli *et al.* 1996).

Finally, it is worthwhile commenting on the clinical implications of this study. The antibiotics commonly used to treat *Klebsiella* infections are not very efficient against intracellular bacteria. In turn, our findings provide rationale for the use of inhibitors targeting the PI3K-Akt signaling cascade to eliminate intracellular *K. pneumoniae*. The concept of eradicating pathogens through targeting host factors modulated by pathogens has received wide attention in the infectious disease arena. Several promising drugs have been developed or are being developed to antagonize PI3K/Akt due to its relevance for many human cancers. Of note, there are *in vitro* and *in vivo* studies supporting the use of Akt inhibitors to eliminate intracellular *Salmonella* and *M.*
tuberculosis (Kuijl et al. 2007; Chiu et al. 2009). Therefore, we propose that agents targeting PI3K/Akt might provide selective alternatives to manage *K. pneumoniae* pneumonias. Careful designed preclinical trials using the well establish mouse pneumonia model are warranted to test this hypothesis.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions.**

Kp43816R is a rifampicin-resistant derivative of *K. pneumoniae* pneumonia clinical isolate expressing a type 1 O-polysaccharide and a type 2 capsule (ATCC 43816). Bacteria were grown in lysogeny broth (LB) at 37°C on an orbital shaker (180 rpm). To UV kill bacteria, samples were UV irradiated (1 joule for 15 min) in a BIO-LINK BLX crosslinker (Vilber Lourmat). When appropriate, antibiotics were added to the growth medium at the following concentrations: rifampicin (Rif) 50 µg/ml, ampicillin (Amp), 100 µg/ml for *K. pneumoniae* and 50 µg/ml for *E. coli*; kanamycin (Km) 100 µg/ml; chloramphenicol (Cm) 12.5 µg/ml.

**Construction of a *K. pneumoniae* cps mutant.**

Primers for *manC* mutant construction were designed from the known *K. pneumoniae* K2 gene cluster sequence (Arakawa et al. 1995). Primer pairs ManCUPF (5’-CGCTTAAGACCAGCGGTGTCG-3’), ManCUPR (5’-CGGATCCGATCAGCGGGTCGCCGTG-3’) and ManCDOWNF (5’-CGGATCCGCAGCGAGGAGCTGGTGG-3’ BamHI site underlined), ManCDOWNR (5’-GGATATCCCGAGGCGGC-3’) were used in two sets of asymmetric PCRs to obtain DNA fragments ManCUP and ManCDown, respectively. DNA fragments ManCUP and ManCDOWN were annealed at their overlapping region and amplified by PCR as a single fragment using primers ManCUPF and ManCDOWNR. This PCR fragment was cloned into pGEM-T Easy to obtain pGEMTΔmanC. A kanamycin cassette, obtained as a 1.5 kb PCR fragment from pKD4 (Datsenko and Wanner. 2000) using primers cassette-F1 (5’-CGGATCCGCAGCGAGGAGCTGGTGG-3’) and cassette-R1 (5’-GGATATCCCGAGGCGGC-3’).
CGCGGATCCGTAGGCTGGAGCTGCTTCG-3’ BamHI site underlined) and cassette-R1 (5’-
CGCGGATCCCATGGGAATTAGCCATGGTCC -3’ BamHI site underlined), was BamHI-
digested and cloned into BamHI-digested pGEMTΔmanC to obtain pGEMTΔmanCKm. Primers
ManCUPF and ManCDOWNR were used to amplify a 3.5 kb fragment which was electroprated
into Kp43816R containing pKOBE-G-sacB plasmid (Derbise et al. 2003). The vector pKOBE-G-
sacB contains the Red operon expressed under the control of the arabinose inducible pBAD
promoter and the sacB gene that is necessary to cure the plasmid. A recombinant in which the wild-
type allele was replaced by Δman::Km was verified by PCR and named 43ΔmanCKm. The mutant
was resistant to the CPS-specific phage φ2.

Eukaryotic cells culture.

Murine alveolar macrophages MH-S (ATCC, CRL-2019) and human monocytes THP-1
(ATCC, TIB-202) were grown in RPMI 1640 tissue culture medium supplemented with 10% heat-
inactivated fetal calf serum (FCS) and 10 mM Hepes at 37ºC in an humidified 5% CO2 atmosphere.
THP-1 cells were differentiated to macrophages by PMA-treatment (10 ng/ml for 12 h).

Infection of macrophages.

Macrophages were seeded in 24-well tissue culture plates at a density of 7 x 10^5 cells per
well 15 h before the experiment. Bacteria were grown in 5-ml LB, harvested in the exponential
phase (2500 x g, 20 min, 24ºC), washed once with PBS and a suspension containing approximately
1x10^9 cfu/ml was prepared in 10 mM PBS (pH 6.5). Cells were infected with 35 µl of this
suspension to get a multiplicity of infection of 50:1 in a final volume of 500 µl RPMI 1640 tissue
culture medium supplemented with 10% heat-inactivated FCS and 10 mM Hepes. To synchronize
infection, plates were centrifuged at 200 x g during 5 min. Plates were incubated at 37ºC in a
humidified 5% CO2 atmosphere. After 30 min of contact, cells were washed twice with PBS and
incubated for additional 90 min with 500 µl RPMI 1640 containing 10% FCS, 10 mM Hepes,
gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) to eliminate extracellular bacteria. This
treatment did not induce any cytotoxic effect which was verified measuring the release of lactate
dehydrogenase (LDH) and by immunofluorescence microscopy (data not shown). For time course experiments, after the 90 min treatment period, cells were washed three times with PBS and incubated with 500 µl RPMI 1640 containing 10% FCS, 10 mM Hepes, gentamicin (100 µg/ml). To determine intracellular bacterial load, cells were washed three times with PBS and lysed with 300 µl of 0.5% saponin in PBS for 10 min at room temperature. Serial dilutions were plated on LB to quantify the number of intracellular bacteria. Intracellular bacterial load is represented as cfu per well. All experiments were done with triplicate samples on at least three independent occasions.

When indicated, cells were pre-incubated for 1 h with nocodazole (50 µg/ml), filipin (5 µg/ml), nystatin (25 µg/ml), LY294002 hydrochloride (75µM), or for 30 min with cytochalasin D (5 µg/ml) before carrying out infections as described above. Cells were also pre-incubated for 1 h with 1 mM methyl-β-cyclodextrin (MβCD), washed twice with PBS to remove cholesterol and infected. In other experiments, LY294002 hydrochloride (75µM), AKT X (10 µM), or 100 nM bafilomycin A₁ were added to the cells during the gentamicin treatment and kept until the end of experiment. All drugs were purchased from Sigma.

**Immunofluorescence and transmission electron microscopy.**

Cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates. Infections were carried out as described before with *K. pneumoniae* strains harbouring pFPV25.1Cm (March et al. 2013). Control experiments showed that there were no differences in the number of intracellular bacteria recovered over time from cells infected with bacteria containing pFPV25.1Cm or no plasmid (data not shown). When indicated, cells were washed three times with PBS and fixed with 3% paraformaldehyde (PFA) in PBS pH 7.4 for 15 min at room temperature. For EEA1 staining, cells were fixed with 2.5% PFA for 10 min at room temperature followed by 5% PFA + methanol (1:4 v/v) at -20°C for 5 min. Methanol fixation (3% PFA for 20 min at room temperature followed by 1 min cold methanol) was used for cathepsin D whereas periodate-lysine-paraformaldehyde fixation (0.01 M NaIO₄, 0.075 M L-lysine, 0.0375 M NaPO₄ buffer pH 7.4, 2% paraformaldehyde: 20 min room temperature) was used for calnexin. The actin cytoskeleton was
stained with Rhodamine-Phalloidin (Invitrogen) diluted 1:100, DNA was stained with Hoechst 3342 (Invitrogen) diluted 1:2500. *Klebsiella* was stained with rabbit anti-*Klebsiella* serum diluted 1:5000. Early endosomes were stained with goat anti-EEA1 (N-19) antibody (Santa Cruz Biotechnology) diluted 1:50. Late endosomes were stained with rat anti-Lamp-1 (1D4B) antibody (Developmental Studies Hybridoma Bank). Lysosomes were labelled with goat anti-human cathepsin D (G19) or rabbit anti-human cathepsin D (H-75) antibodies (Santa Cruz Biotechnology) diluted 1:100. Golgi network was stained with mouse anti-GM130 (BD Laboratories) diluted 1:400. Endoplasmic reticulum was stained with rabbit anti-calnexin (SPA-860; Enzo Life Sciences) diluted 1:400. Donkey anti-rabbit, donkey anti-mouse, donkey anti-rat and donkey anti-goat conjugated to Rhodamine, Cy5 or Cy2 secondary antibodies were purchased from Jackson Immunological and diluted 1:200.

Fixable dextran 70,000 (molecular weight) labelled with Texas red (TR-dextran) (Molecular Probes) was used to label lysosomes in a pulse-chase assay. Briefly, macrophages seeded on glass coverslips were labelled by pulsing with 250 µg/ml of TR-dextran for 2 h at 37°C in 5% CO₂ in RPMI 1640 medium. To allow TR-dextran to accumulate in lysosomes, medium was removed; cells were washed three times with PBS, and incubated for 1 h in dye-free medium (chase). After the chase period, cells were infected. Acidic compartments were loaded with 0.5 µM Lysotracker RedDN99 (Invitrogen), 45 min before fixation. At the end of the infection period, the residual fluid marker was removed by washing the cells three times with PBS, followed by fixation.

Staining was carried out in 10% horse serum, 0.1% saponin in PBS. Coverslips were washed twice in PBS containing 0.1% saponin, once in PBS, and incubated for 30 minutes with primary antibodies. Coverslips were then washed twice in 0.1% saponin in PBS and once in PBS and incubated for 30 minutes with secondary antibodies. Finally, coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H₂O, mounted on Aqua Poly/Mount (Polysciences) and analysed with a Leica TCS SP5 confocal microscope. Depending of the marker, a KCV was considered positive when it fulfilled these criteria: (i) the marker was detected throughout the area
occupied by the bacterium; (ii) the marker was detected around/enclosing the bacterium, (iii) the marker was concentrated in this area, compared to the immediate surroundings. To determine the percentage of bacteria that colocalized with each marker, all bacteria located inside a minimum of 100 infected cells were analysed in each experiment. Experiments were carried out by triplicate in three independent occasions.

For extra-/intracellular bacteria differential staining, PFA fixed cells were incubated with PBS containing 10% horse serum, Hoechst 33342 and rabbit anti-
\textit{Klebsiella} for 20 min. Coverslips were washed three times with PBS and stained as described above with donkey anti-rabbit conjugated to Rhodamine secondary antibody. Coverslips were washed three times in PBS and once in distilled water before mounting onto glass slides using Prolong Gold antifade mounting gel (Invitrogen).

Immunofluorescence was analysed with a Leica CTR6000 fluorescence microscope. Images were taken with a Leica DFC350FX monochrome camera. Confocal microscopy was carried out with a Leica TCS SP5 confocal microscope.

For transmission electron microscopy (TEM), cells were seeded in 24-well tissue culture plates. Infections were carried out as described before, fixed with glutaraldehyde and processed for TEM as described previously (Kruskal \textit{et al}. 1992).

\textbf{Fluorescent in situ hybridisation}

We carried out hybridization of PFA fixed infected cells with fluorescently labelled oligonucleotides as described before (Morey \textit{et al}. 2011). Alexa488 conjugated DNA probes EUB338 (5’-GCTGCTCCGTAGGAGT-3’) and GAM42a (5’-GCCTCCACATCGTTT-3’) were designed for specific labelling of rRNA of eubacteria and gamma subclass of Proteobacteria, respectively (Manz \textit{et al}. 1993). A DNA probe non-EUB338, complementary to EUB338 was used as a negative control. The detectability of bacteria by such oligonucleotide probes is dependent on the presence of sufficient ribosomes per cell, hence providing qualitative information on the physiological state of the bacteria on the basis of the number of ribosomes per cell. These probes
were used together to obtain a stronger signal, added to a final concentration of 5 nM each in the hybridization buffer. The hybridization buffer contained 0.9M NaCl, 20mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS) and 35% formamide. Coverslips were first washed with deionized water. Hybridization was carried out for 1.5 h at 46°C in a humid chamber; followed by a 30 min wash at 48°C. Washing buffer contained 80 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS) and 5 mM EDTA (pH 8). After washing, DNA staining for total bacteria was carried out by incubating the coverslips in PBS containing Hoechst 33342 for 20 min. Coverslips were then washed three times in PBS and once in distilled water before mounting onto glass slides using Prolong Gold antifade mounting gel.

**Isolation of in vivo infected macrophages**

Mice were treated in accordance with the Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU) and in agreement with the Bioethical Committee of the University of the Balearic Islands. This study was approved by the Bioethical Committee of the University of the Balearic Islands with the authorisation number 1748.

Infections were performed as previously described (Insua et al. 2013). Briefly, five- to seven-week-old male CD-1 mice (Harlan) were anesthetized by intraperitoneal injection with a mixture containing ketamine (50 mg/kg) and xylazine (5 mg/kg). Overnight bacterial cultures were centrifuged (2500 x g, 20 min, 22°C), resuspended in PBS and adjusted to 5 x 10^4 CFU/ml for determination of bacterial loads. 20 µl of the bacterial suspension were inoculated intranasally in four 5 µl aliquots. To facilitate consistent inoculations, mice were held vertically during inoculation and placed on a 45° incline while recovering from anaesthesia. 24 h post infection, mice were euthanized by cervical dislocation and bronchoalveolar lavage was performed as previously described (Cai et al. 2012). The lavage fluid was spun at 300 x g for 10 min to pellet alveolar macrophages. Cells were cultured on 12 mm circular coverslips in 24-well tissue culture plates at a concentration of 0.5 x 10^6 cells/well in 1 ml RPMI 1640 tissue culture medium supplemented with...
10% heat-inactivated FCS and 10 mM Hepes and gentamicin (100 µg/ml). After 2 h of incubation, nonadherent cells were washed off with PBS, and cells were fixed. Klebsiella and cathepsin D staining was performed as previously described. Immunofluorescence was analysed with a with a Leica TCS SP5 confocal microscope.

**Neutral red uptake assay for the estimation of cell viability.**

Cell viability was determined by assessing the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The protocol described by Repetto and coworkers (Repetto et al. 2008) was followed with minor modifications. Macrophages were seeded on 96-well tissue culture plates at 5 x 10^5 cells/well 18 h before the experiment. Cells were infected to get a multiplicity of infection of 50:1 in a final volume of 200 µl RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated FCS and 10 mM Hepes. To synchronize infection, plates were centrifuged at 200 x g during 5 min. Plates were incubated at 37°C in a humified 5% CO_2 atmosphere. After 90 min of contact, cells were washed twice with PBS and incubated overnight with 200 µl RPMI 1640 containing 10% FCS, 10 mM Hepes, gentamicin (100 µg/ml). Cells were washed twice with PBS and incubated with 100 µl of freshly prepared neutral red medium (final concentration 40 µg/ml neutral red [Sigma] in tissue culture medium) for 2 h. Wells were washed once with PBS and the remaining biomass-adsorbed neutral red was solubilized with 150 µl neutral red destaining solution (50% ethanol 96%; 49% deionised water; 1% glacial acetic acid). Staining was then quantified by determining the OD_540 in a 96-well microplate reader, and used to compare relative neutral red staining of uninfected cells and cells that were lysed completely with 1% Triton X-100. Experiments were carried out by triplicate in six independent occasions.

**Detection of Akt phosphorylation by Western blotting**

Macrophages were seeded on 6-well tissue culture plates at 10^6 cells/well. Cells were infected with Kp43816R, washed 3 times with cold PBS, scraped and lysed with 100 µl lysis buffer (1x SDS Sample Buffer, 62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) on ice. Samples were sonicated, boiled at 100°C for 10 min and
cooled on ice before polyacrylamide gel electrophoresis and Western Blotting. Akt phosphorylation was detected with primary rabbit anti-phospho Ser473 Akt (Cell Signaling Technology) antibody diluted 1:1,000 and secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Thermo Scientific) diluted 1:10,000. Tubulin was detected with primary mouse anti-tubulin antibody (Sigma) diluted 1:3,000 and secondary goat anti-mouse antibody (Pierce) conjugated to horseradish peroxidase diluted 1:1,000. When necessary, the membrane was washed twice for 15 min with PBS-0.5% Tween-20, incubated for 30 min in Restore™ Western Blot Stripping Buffer (Thermo Scientific) at 37ºC, and washed twice for 15 min with PBS-0.5% Tween-20. Images were recorded with a GeneGnome HR imaging system (Syngene).

Apoptosis analysis in vitro.

Apoptosis of macrophages was analysed as previously described (Aguilo et al. 2013). Briefly, phosphatidylserine exposure and membrane integrity were analyzed by using Annexin-V and 7-AAD (BD Biosciences) and FACS according to manufacturer instructions. Cells were washed with PBS and incubated with APC-conjugated Annexin-V and 7-AAD in Annexin-binding buffer for 15 min. After that, cells were washed twice with PBS, fixed with 4% PFA during 30 min and washed again with PBS. Both PBS and PFA contained 2.5 mM CaCl₂.

Bacterial opsonisation.

Normal human serum (NHS), kindly donated by the Balearics Blood Centre, was obtained from five different donors (blood type O negative) and kept frozen at -80°C. 35 µl from a suspension containing approximately 1x10⁹ cfu/ml in 10 mM PBS (pH 6.5) were added to 500 µl RPMI 1640 tissue culture medium supplemented with 10 mM Hepes and 1% NHS. The suspension was incubated at 37ºC shaking (180 rpm) for 45 min. The suspension was used to infect mTHP1 cells as previously described.

Plasmids and transient transfections

For transient transfections with GFP-Rab7 (Cantalupo et al. 2001), GFP-Rab14 (Kuijl et al. 2007), and RILP-C33-EGFP (Cantalupo et al. 2001), macrophages were seeded in 24-well tissue
culture plates at a density of 3 x 10^5 cells per well 24 h before transfection. Cells were transfected with 750 ng DNA using FuGENE (Promega) according to manufacturer’s instructions (reagent/DNA ratio 3.5:1). In all cases, samples were fixed, stained and analysed by immunofluorescence microscopy. pcDNA3 and DN-Rab14 (Seto et al. 2011) were transfected using jetPEI-macrophage (Polyplus) following manufacturer’s instructions. After 24 h, cells were washed twice with PBS, infected, and intracellular bacterial load determined as previously described.

**Construction of cps reporter strain**

DNA fragment containing the promoter region of the Kp43816R capsule operon was amplified by PCR using *Vent* polymerase (NewEngland Biolabs) and primers K2ProcpsF (5’-gaattcTGCTGGGACAAATTGCCACC-3’) and K2ProcpsR (5’-AGATGGATGACCCCGATC-3’). To construct a green fluorescent protein (GFP) reporter, the PCR product was EcoRI-digested and cloned into the EcoRI-SmaI digested low-copy-number vector pPROBE’-gfp[LVA] (Miller et al. 2000) to obtain pPROBE’43Procps. The plasmid was introduced into Kp43816R by electroporation.

**Analysis of cps expression**

The reporter strains were grown at 37°C on an orbital incubator shaker (180 r.p.m.) until OD_{540} 1.2. The cultures were harvested (2500 x g, 20 min, 24°C) and resuspended to an OD_{540} of 0.6 in PBS. 0.8-ml aliquot of this suspension was transferred to 1-cm fluorimetric cuvette and fluorescence was measured with a spectrofluorophotometer (Perkin Elmer LS55) set as follows: excitation, 485 nm; emission, 528 nm; slit width 5 nm; integration time 5 seconds. Results were expressed as relative fluorescence units (RFU). All measurements were carried out in quintuplicate on at least three separate occasions.

For analysis of cps expression from intracellular bacteria, macrophages were seeded in 6-well plates and infected with Kp43816R containing pPROBE’43Procps or pPROBE’-gfp[LVA] control vector at a MOI of 150:1. After 40 min, cells were washed twice with PBS and incubated
with 500 µl RPMI 1640 containing 10% FCS, 10 mM Hepes, gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) to eliminate extracellular bacteria. At the indicated time points, cells were lysed with 900 µl 0.5 % saponin in PBS. The samples from two wells were combined and serial dilutions were plated on LB to quantify the number of intracellular bacteria. Control experiments showed that there were no differences in the number of intracellular bacteria recovered over time from cells infected with bacteria containing pPROBE’-gfp[LVA] derivatives or no plasmid (data not shown). By replica plating on plates containing kanamycin, it was determined that 85-100% of the bacteria contained the reporter plasmid at any time point analysed. The rest of the lysate was centrifuged (13 000 rpm, 1 min, room temperature) and resuspended in 1 ml 1 % BSA in PBS for staining. Bacteria were stained with rabbit anti-Klebsiella serum diluted 1:5000 for 20 min, washed twice with PBS, and incubated for 20 min with a 1:200 dilution of Rhodamine-conjugated donkey anti-rabbit secondary antibody. Flow cytometry analyses were performed using a Cultek Epics XL flow cytometer. Samples were gated for bacteria-like particles by using the rhodamine fluorescence of the anti-Klebsiella labelling to identify bacterial cells and to exclude mammalian cell debris and background noise. Lysed and stained uninfected macrophages were not rhodamine positive, indicating that there was no cross-reactivity of the primary or secondary antibodies with MH-S cells. Fluorescence compensation settings were determined in parallel under identical conditions by using the constitutively GFP-expressing Kp43816R strain or the non-expressing strain, with and without anti-Klebsiella antibody labelling. Approximately 10,000 events identified as Klebsiella cells were collected per sample. A histogram of GFP fluorescence for the negative-control sample (bacteria containing pPROBE’-gfp[LVA] ) was created, and the area of the histogram containing the bacterial population was considered to be negative for GFP fluorescence. All experiments were done with triplicate samples on at least three independent occasions.

Statistical analysis.
Statistical analyses were performed using the one-tailed $t$ test or, when the requirements were not met, by the Mann-Whitney U test. $P < 0.05$ was considered statistically significant. The analyses were performed using Prism4 for PC (GraphPad Software).

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REFERENCES


FIGURE LEGENDS

FIGURE 1. Phagocytosis of *K. pneumoniae* by macrophages.

(A) Immunofluorescence confocal microscopy showing the lack of colocalisation between *K. pneumoniae* and the lysosome marker cathepsin D in macrophages isolated from the BALF of infected mice with *K. pneumoniae* harbouring pFPV25.1Cm. Methanol fixation was used for cathepsin D staining. (B) Involvement of PI3K, cytoskeleton and lipid rafts on Kp43816R phagocytosis by MH-S cells. (C) Immunoblot analysis of Akt phosphorylation (P-Akt) in lysates of MH-S cells infected with Kp43816R for the indicated times. Membranes were probed for tubulin as a loading control. Data are representative of three independent experiments. (D) Immunoblot analysis of Akt phosphorylation (P-Akt) in lysates of PI3K inhibitor (LY294002) or DMSO (vehicle solution)-treated MH-S cells infected with Kp43816R for 20 min. Membranes were probed for tubulin as a loading control. Data are representative of three independent experiments. (E) TEM analysis of MH-S macrophages infected with Kp43816R for the indicated time points. Data are representative of two independent experiments. (F) Detection of FISH positive Kp43816R inside MH-S cells 5.5 h post infection. Kp43816R harbored pFPV25.1Cm (green), host cell nuclei were stained with Hoechst (blue), and metabolically active bacteria were labelled with probes EUB338-Cy3 and GAM42a-Cy3 (red). (G) Quantification of the number of metabolically active (FISH positive) intracellular bacteria in MH-S infected cells. Results are represented as percentage of FISH positive bacteria versus total number of intracellular bacteria (green). At least 300 infected cells belonging to three independent experiments were counted per time point.

FIGURE 2. Dynamics of *K. pneumoniae* survival in macrophages.

(A) MH-S cells were infected with Kp43816R for 30 min (MOI 50:1). Wells were washed and incubated with medium containing gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) for 90 min to eliminate extracellular bacteria, and then with medium containing gentamicin 100 µg/ml for up to 12.5 h. Intracellular bacteria were quantified by lysis, serial dilution and viable counting on LB agar plates. (B) MH-S cells were infected with Kp43816R harboring pFPV25.1Cm and the phagocytic
index calculated as the number of intracellular bacteria (determined by extra-/intracellular differential staining) per the percentage of infected cells. (C) Quantification of intracellular bacteria in mTHP-1 cells infected with Kp43816R. (D) Phagocytic index of infected mTHP-1 cells.

In panels A and C, data, shown as Log_{10}CFU/well, are the average of three independent experiments. In panels B and D, at least 500 infected cells belonging to three independent experiments were counted per time point.

**FIGURE 3. Apoptosis of MH-S cells.**

(A) MH-S cells were mock-treated or infected with Kp43816R harboring pFPV25.1Cm. 6 h post infection, cells were stained with Annexin V and 7-AAD and analysed by flow cytometry. A representative experiment of three is shown. (B) Data from three independent experiments are represented as mean ± SD.

**FIGURE 4. Phagosome maturation during *K. pneumoniae* infection of MH-S cells.**

(A) Upper and middle panels show the colocalization of Kp43816R harboring pFPV25.1Cm and EEA1 (images were taken 30 min post infection) and Lamp1 (images were taken 4 h post infection) using goat anti-EEA1 and donkey anti-goat conjugated to Rhodamine, and rat anti-Lamp-1 and donkey anti-rat conjugated to Rhodamine antibodies, respectively. Lower panels show the colocalization of Kp431816R and EGFP-Rab7 and RILP-C33-EGFP (images were taken 4 h post infection). Bacteria were stained using rabbit anti-*Klebsiella* and donkey anti-rabbit conjugated to Rhodamine antibodies. Images are representative of triplicate coverslips in three independent experiments.

(B) Percentage of Kp43816R colocalization with EEA1, Lamp1, and EGFP-Rab7 and RILP-C33-EGFP over a time course. Cells were infected, coverslips were fixed and stained at the indicated times. Values are given as mean percentage of Kp43816R colocalizing with the marker ± SE. At least 300 infected cells belonging to three independent experiments were counted per time point.

**FIGURE 5. Colocalization of *K. pneumoniae* with phagolysosomal markers.**
(A) Upper panels show the colocalization of Kp43816R harboring pFPV25.1Cm and the dye LysoTracker at 4 h post infection. Middle panels show the colocalization of Kp43816R harboring pFPV25.1Cm and cathepsin D at 2 h post infection. Cathepsin D was stained using goat anti-human cathepsin D (G19) and donkey anti-goat conjugated to Rhodamine antibodies. Lower panels display the colocalization of Kp43816R harboring pFPV25.1Cm and TR-dextran at 2 h post infection. Images are representative of three independent experiments. (B) Percentage of Kp43816R colocalization with LysoTracker, cathepsin D and TR-dextran over a time course. Cells were infected, coverslips were fixed and stained at the indicated times. Values are given as mean percentage of Kp43816R colocalizing with the marker ± SE. At least 300 infected cells belonging to three independent experiments were counted per time point.


(A) Microscopy analysis showing that bafilomycin A₁ (100 nM) treatment abrogates LysoTracker staining of the KCV (images were taken at 4 h post infection). MH-S cells were infected with Kp43816R harboring pFPV25.1Cm. Images are representative of triplicate coverslips in two independent experiments. (B) Experimental outline to investigate the effect of vacuolar acidification on the intracellular survival of Kp43816R. (C) Intracellular bacteria in MH-S cells, treated (white symbols) or not (black symbols) with bafilomycin A₁, were quantified by lysis, serial dilution and viable counting on LB agar plates. Data, shown as CFU/well, are the average of three independent experiments. Significance testing performed by Log Rank test. *, P < 0.05.

FIGURE 7. PI3K-AKT and Rab14 aid intracellular survival of K. pneumoniae.

(A) Quantification of intracellular bacteria in MH-S cells infected with Kp43816R which were mock-treated (black bar) or treated with LY294002 hydrochloride (75 µM) or with AKT X (10 µM). Treatments were added after the time of contact and kept until cells were lysed for bacterial enumeration. Data, shown as CFU/well, are the average of three independent experiments. *, P < 0.05 (results are significantly different from the results for untreated cells; Mann-Whitney U test).

(B) Percentage of Kp43816R colocalization with TR-dextran in cells mock-treated or treated with
the Akt inhibitor AKT X over a time course. Cells were infected, coverslips were fixed and stained at the indicated times. Treatments were added after the time of contact and kept until cells were fixed. Values are given as mean percentage of Kp43816R colocalizing with the marker ± SE. At least 300 infected cells belonging to three independent experiments were counted per time point. *, $P < 0.05$ (results are significantly different from the results for untreated cells; Mann-Whitney U test). (C) Colocalization of Kp43816 and EGFP-Rab14 (image was taken 3.5 h post infection) in MH-S cells. Bacteria were stained using rabbit anti-*Klebsiella* and donkey anti-rabbit conjugated to Rhodamine antibodies. Image is representative of three independent experiments. Left graph shows the percentage of Kp43816R colocalization with EGFP-Rab14 over a time course. Cells were infected, coverslips were fixed and stained at the indicated times. Values are given as mean percentage of Kp43816R colocalizing with the marker ± SE. At least 300 infected cells belonging to three independent experiments were counted per time point. (D) Quantification of intracellular bacteria in transfected MH-S cells with plasmid pcDNA3 or with Rab14 dominant-negative construct (DN-Rab14) at 3.5 h post infection. Data, shown as CFU/well, are the average of three independent experiments. *, $P < 0.05$ (results are significantly different from the results for cells transfected with control plasmid pcDNA3; Mann-Whitney U test).

**FIGURE 8. Role of CPS in *K. pneumoniae* intracellular survival.**

(A) MH-S or mTHP-1 cells were infected with Kp43816R (black symbols) or the capsule mutant (Kp43816des; white symbols) for 30 min. Wells were washed and incubated with medium containing gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) for 90 min to eliminate extracellular bacteria, and then with medium containing gentamicin 100 µg/ml for up to 10 h. Intracellular bacteria were quantified by lysis, serial dilution and viable counting on LB agar plates. Data, shown as Log$_{10}$CFU/well, are the average of three independent experiments. (B) Opsonization with 1% normal human sera (NHS) increased the phagocytosis of the capsule mutant (Kp43816Rdes) by mTHP-1 cells. Data, shown as CFU/well, are the average of three independent experiments. *, $P < 0.05$ (results are significantly different from the results for cells infected with
the non-opsonized capsule mutant; Mann-Whitney U test; n.s., no significant difference. (C) mTHP-1 cells were infected for 30 min with Kp43816R or the capsule mutant which were either opsonized or not. Wells were washed and incubated with medium containing gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) for 90 min to eliminate extracellular bacteria, and then with medium containing gentamicin 100 µg/ml for up to 7.5 h. Intracellular bacteria were quantified by lysis, serial dilution and viable counting on LB agar plates. Data, shown as Log_{10} CFU/well, are the average of three independent experiments. Significance testing performed by Log Rank test. *, P < 0.05. (D) Analysis of cps::gfp expression over time by flow cytometry. Analysis was performed after lysing MH-S cells and staining the bacteria using rabbit anti-Klebsiella and donkey anti-rabbit conjugated to Rhodamine antibodies (red histogram). In these populations, GFP fluorescence was analyzed (green histogram). Gray histogram represents GFP fluorescence for the negative-control sample, and the area of the histogram is considered negative for GFP fluorescence. Results are representative of three independent experiments. (E) Fluorescence levels of Kp43816R containing pPROBE'43Procps. Data, shown as relative fluorescence units (RFUs), are the average of three independent experiments. *, P < 0.05 (results are significantly different from the results for cells grown in medium buffered to pH 7.5; Mann-Whitney U test).
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CFU per well

Lipid rafts

P13K

Cytochalasin

Nocodazol

MβCD

Filipin

Nystatin

Lipid rafts

Cytoskeleton

PI3K

* 

* 

* 

* 

* 

* 

Time (min) infection

CON 5 15 25 35 45 55 65 75

P-Akt

Tubulin

3 h

6 h

Kp   -    +    +

LY294002 - - +

P-Akt

Tubulin

3 h

6 h

Merge

GFP

EUB388

GAM42a

Hoechst

% FISH positive cells

Time (h)
A

Non infected cells

Infected cells

GFP

SSC

7-AAD

AnnexinV

Ann+AAD-

Ann+AAD+

B

% cells

0 25 50 75 100

0h 2h 6h 8h 24h

Ann+AAD-

Ann+AAD+
A

Kp43816R

LysoTracker

Merge

Kp43816R

Cathepsin D

Merge

Kp43816R

TR-dextran

Merge

B

LysoTracker

Cathepsin D

TR-dextran
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**A**

- LysoTracker
- Phase contrast

**B**

- Gentamicin
- Bafilomycin A1

<table>
<thead>
<tr>
<th>CFU determinations</th>
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<tbody>
<tr>
<td>Contact</td>
</tr>
<tr>
<td>1.5 h</td>
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<tr>
<td>3.5 h</td>
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<tr>
<td>5.5 h</td>
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**C**

- Log_{10} CFU per well

- **Kp43816R**
- **Kp43816R + bafilomycin**

**Time (h)**

0.0 1.5 3.0 4.5 6.0

*
A) Bar graph showing CFU per well for LY294002 and AKT inhibitors at 3.5 h and 5.5 h. * indicates significant difference.

B) Bar graph showing % colocalization at 3.5 h and 5.5 h for LY294002 and AKT inhibitors. * indicates significant difference.

C) Fluorescence image of EGFP-Rab14 showing % colocalization at 1.5 h, 3.5 h, and 5.5 h. Scale bar = 10 μm.

D) Bar graph showing CFU per well for pcDNA3 and DN-Rab14 at 5.5 h. * indicates significant difference.
A

MH-S

mTHP1

Time (h)

Log_{10} CFU per well

0.0 2.5 5.0 7.5 10.0

0 2 4 6

B

Kp43816R Kp43816Rdes

1% NHS

n.s.

C

Kp43816R Kp43816Rdes Kp43816R opsonized Kp43816Rdes opsonized

1% NHS

D

Inoculum 1 h 2 h

4 h 6 h

E

RFU

pH 7.5 8 \mu M Mg pH 5.5 8 \mu M Mg

* n.s.