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Role of Bacterial Surface Structures on the Interaction of *Klebsiella pneumoniae* with Phagocytes

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**Abstract**

Phagocytosis is a key process of the immune system. The human pathogen *Klebsiella pneumoniae* is a well known example of a pathogen highly resistant to phagocytosis. A wealth of evidence demonstrates that the capsule polysaccharide (CPS) plays a crucial role in resistance to phagocytosis. The amoeba *Dictyostelium discoideum* shares with mammalian macrophages the ability to phagocytose and kill bacteria. The fact that *K. pneumoniae* is ubiquitous in nature and, therefore, should avoid predation by amoebae, poses the question whether *K. pneumoniae* employs similar means to counteract amoebal and mammalian phagocytes. Here we developed an assay to evaluate *K. pneumoniae*-D. discoideum interaction. The richness of the growth medium affected the threshold at which the cps mutant was susceptible to predation by amoebae. Given the critical role of bacterial surface elements on host-pathogen interactions, we explored the possible contribution of the lipopolysaccharide (LPS) and outer membrane proteins (OMPs) to combat phagocytosis by *D. discoideum*. We uncover that, in addition to the CPS, the LPS O-polysaccharide and the first core sugar participate in *Klebsiella* resistance to predation by *D. discoideum*. *K. pneumoniae* LPS lipid A decorations are also necessary to avoid predation by amoebae although PagP-dependent palmitoylation plays a more important role than the lipid A modification with aminoarabinose. Mutants lacking OMPs OmpA or OmpK36 were also permissive for *D. discoideum* growth. Except the LPS O-polysaccharide mutants, all mutants were more susceptible to phagocytosis by mouse alveolar macrophages. Finally, we found a correlation between virulence, using the pneumonia mouse model, and resistance to phagocytosis. Altogether, this work reveals novel *K. pneumoniae* determinants involved in resistance to phagocytosis and supports the notion that *Dictyostelium* amoebae might be useful as host model to measure *K. pneumoniae* virulence and not only phagocytosis.

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**Introduction**

Phagocytosis is the process by which particles are recognized, bound to the surface of cells and internalized into a plasma membrane-derived intracellular vacuole, or phagosome. In mammals, phagocytosis is a special feature of the so-called professional phagocytes, i.e. polymorphonuclear leukocytes (also known as neutrophils), dendritic cells, monocytes and macrophages. When a microorganism enters the sterile sections of the body, professional phagocytes are chemotactically attracted, bind the microorganism, ingest and kill it. In the case of macrophages and dendritic cells, the invader’s antigenic molecules are presented to other immune cells hence initiating adaptive immune responses.

The social amoeba *Dictyostelium discoideum* lives in soil, where it feeds on both Gram-negative and positive bacteria [1]. Typically, upon starvation, *Dictyostelium* cells initiate a multicellular development stage leading to the formation of a fruit body. However, there are *D. discoideum* axenic strains that can feed not only by phagocytosis but also by macrophagocytosis of liquid nutrients [2]. Of special interest is the fact that *Dictyostelium* cytoskeleton architecture is similar to that found in mammalian cells. Furthermore, the process of particle uptake in *Dictyostelium* is similar to macrophage phagocytosis [1]. The fact that the strategies evolved to counteract mammalian professional phagocytes are considered essential to establish an infection has led to the notion that *Dictyostelium* amoebae could be used as host model to measure virulence [3,4]. The similarities between *Dictyostelium* and mammalian cells also extent to membrane trafficking, endocytic transport and sorting events [5]. There are genetic tools available to manipulate *D. discoideum* cells thereby facilitating
the study of cellular mechanisms at the molecular level. Moreover, the genome of *D. discoideum* strain AX4 has been sequenced [5].

*Klebsiella pneumoniae* is a Gram negative pathogen common cause of nosocomial infections that include urinary tract, respiratory, and wound infections [6]. *K. pneumoniae* isolates are frequently resistant to multiple antibiotics [7], which leads to a therapeutic dilemma. In contrast to many bacterial pathogens, *K. pneumoniae* is ubiquitous in nature. The non-clinical habitats include the mucosal surfaces of animals and environmental sources such as vegetation, soil and surface waters [8]. Notably, it has been shown that environmental *Klebsiella* isolates are nearly identical to clinical ones with respect to the expression of virulence factors and ability to infect animal models [9]. The factors mediating *Klebsiella* survival in the environment are poorly characterized but predominance in the environment is likely to correlate with the ability of *Klebsiella* to avoid predation by protozoa, including amoebae.

Macrophages and neutrophils play a critical role in the clearance of bacteria from the lung and other organs by their capacity for phagocytosis and killing. In this regard, it has been shown that depletion of either neutrophils or alveolar macrophages results in reduced killing of *K. pneumoniae* in vivo [10,11]. Conversely, this suggests that *Klebsiella* countermeasures against phagocytosis should be important virulence factors. Supporting this notion, *K. pneumoniae* capsule (CPS) reduces phagocytosis by neutrophils and macrophages [12–14] and CPS mutant strains are avirulent being not able to cause pneumonia and urinary tract infections [13,15,16]. Notably, CPS is also important to prevent phagocytosis by *D. discoideum* [17–19]. Therefore, a tantalizing hypothesis could be that *K. pneumoniae* may employ the same determinants for resistance to phagocytosis by neutrophils, macrophages and amoebae. Moreover, given the critical role of bacterial surface elements on host-pathogen interactions, we speculated that the lipopolysaccharide (LPS) and outer membrane proteins (OMPs), major components of the outer membrane (OM) of Gram negative bacteria, could be also involved in the resistance to phagocytosis by *K. pneumoniae*.

LPS consists of a hydrophobic membrane anchor, lipid A, substituted with an oligosaccharide core region that can be extended in some bacteria, including *Klebsiella*, by a repeating oligosaccharide, the O-poly saccharide (OPS). The LPS contains a molecular pattern recognized by the innate immune system thereby arousing several host defence responses. The lipid A could be decorated with aminoarabinose, palmitate or phosphoethanolamine [20]. Several studies have demonstrated that these modifications are involved in the resistance to antimicrobial peptides, key weapons of the innate immune system against infections [21–25]. In a recent study we have shown that *K. pneumoniae* lipid A is decorated with palmitate and aminoarabinose which contribute to *K. pneumoniae* resistance to antimicrobial peptides [26]. OMPs are important for membrane integrity and transport of molecules across (for a review see [27]). OmpA is one of the best characterized OM protein and data support the notion that plays an important role in the interaction of bacteria with the innate immune system (for a review see [28]). OmpA and OmpK36 are the most abundant OMPs on *K. pneumoniae* OM [29]. Mounting evidence indicates that *K. pneumoniae* OmpA is important for immune evasion in vitro and in vivo [30,31].

In this study, we report that *K. pneumoniae* employs the same determinants to counteract phagocytosis by *D. discoideum* and alveolar macrophages, the resident defenders of the lung against infections. We uncover that the LPS, the first LPS core sugar, the lipid A decorations with palmitate and aminoarabinose, and the OMPs OmpA and OmpK36 contribute to the resistance to phagocytosis by *D. discoideum* and alveolar macrophages. Finally, we report a correlation between virulence, using the pneumonia mouse model, and resistance to phagocytosis.

### Materials and Methods

#### Ethics statement

Mice were treated in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/EEC) 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.

#### Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in lysogeny broth (LB) at 37°C on an orbital shaker (180 rpm). When appropriate, antibiotics were added to the growth medium at the following concentrations: rifampicin (Rif) 25 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 *K. pneumoniae* mutants**

To construct a *pmrF* mutant, *pGEMTApmrF* was amplified by inverse PCR to delete internal coding regions of *pmrF* using primers KpmrFpnmrFF and KpmrFpnmrFR (Table 2). The PCR product was digested with DpnI, gel purified and ligated to obtain *pGEMTApmrF*. Δ*pmrF* allele was PCR-amplified using Vent polymerase and primers KpmrFpnmrFF and KpmrFpnmrFR (Table 2), and cloned into SmaI-digested pKOV [32] to obtain pKOVΔ*pmrF*. This vector was electroporated into 52145-*ΔpagP*GB and 52145-*ΔwcaG*GB-*ΔpagP*GB and clones were selected after growth on LB agar plates supplemented with Cm at 30°C. Bacteria from 10 individual colonies were pooled in 500 μl PBS, serially diluted in PBS, and spread on LB agar plates with Cm which were incubated at 42°C in order to select merodiploids in which the suicide vector was integrated into the chromosome by homologous recombination. 5–10 merodiploids were serially diluted in PBS and dilutions spread in LB agar plates containing 10% sucrose and without NaCl which were incubated at 30°C. The recombinants that survived 10% sucrose were checked for their antibiotic resistance. The replacement of the wild-type alleles by the mutant ones was confirmed by PCR (data not shown). Recombinants selected were named 52145-*ΔpagP*GB-*ΔpmrF* and 52145-*ΔwcaG*GB-*ΔpagP*GB-*ΔpmrF*.

To confirm that *pmrF* mutation does not have polar effects, the expression of the downstream gene, *pmrI*, was analyzed by real time quantitative PCR (RT-qPCR). Bacteria were grown in 5 ml of LB on an orbital incubator shaker (180 r.p.m.) until an OD₆₀₀ of 0.3. 0.5 ml of ice-cold solution ÉTOH/phenol (19:1 v/v [pH 4.3]) were added to the culture and the mixture was incubated on ice for 30 min to prevent RNA degradation. Total RNA was extracted using a commercial NucleoSpin RNA II kit as recommended by the manufacturer (Macherey-Nagel). cDNA was obtained by retrotranscription of 2 μg of total RNA using a commercial M-MLV Reverse Transcriptase (Sigma), and random primers mixture (SABiosciences, Qiagen). 200 ng cDNA were used as a template in a 25 μl reaction mixture containing 1x SYBR green RT® qPCR Master Mix (Superarray Bioscience Corporation) and primer mix (KpnmybGF1 and KpnmybGR1). *tpoD* was amplified as control using primers KpnpoDLEFT and KpnpoDRIGHT (Table 2). RT-qPCR analyses were performed...
### Table 1. Strains and plasmids used in this study.

| Bacterial strains and plasmids | Genotype or comments | Source or references |
|--------------------------------|----------------------|----------------------|
| *Escherichia coli*             |                      |                      |
| C600                           | *Thr, thr, leuB, tonA, lacY, supE* | [61]                 |
| CC118-λpir                     | Δara-λpir ΔaraΔ139 ΔaraΔX4 galE galK ΔpsaA20 thi-1 rpsE rpoB argE Amr recA1 |                      |
| *Klebsiella pneumoniae*        |                      |                      |
| Kp52145                        | clinical isolate (serotype O1:K2), RifR | [13,62]              |
| 52145-ΔwcaK2; wcaK2 gene inactivated, no CPS expression; RifR | [63]     |
| 52145-ΔpmrF; the pmrF gene inactivated; nonpolar mutant; RifR | [26]     |
| 52145-ΔpagPGB; the pagP gene inactivated; nonpolar mutant; RifR, KmR | [26]     |
| 52145-ΔwcaK2-ΔpmrF             | 52145-ΔwcaK2; the pmrF gene inactivated in CPS mutant background; RifR, KmR | [26]     |
| 52145-ΔwcaK2; the pagP gene inactivated in CPS mutant background; RifR, KmR | [26]     |
| 52145-ΔwcaK2-ΔpmrF-ΔpG1         | 52145-ΔwcaK2-ΔpG1; the pagP gene inactivated in pG1 mutant background; RifR, KmR | This work |
| 52145-ΔwcaK2-ΔpmrF-ΔpG1-ΔpmrF   | 52145-ΔwcaK2-ΔpmrF-ΔpG1; the pmrF gene inactivated in pG1 mutant background; RifR, KmR | This work |
| 520ompA2                       | Kp52145, ompA gene inactivated by insertion of pKNOCKIntKpnOmpA; RifR, CmR | [30]     |
| 52145-ΔwcaK2-ompA              | 52145-ΔwcaK2-ompA; the ompA gene inactivated by insertion of pKNOCKIntKpnOmpA; RifR, CmR | [30]     |
| 520ompA2Com                    | Kp52145 ompA mutant harbouring mini-Tn7KpnOmpA; OmpA levels restored; RifR, CmR, KmR | [30]     |
| 52145-ΔwcaK2-ompACom           | 52145-ΔwcaK2-ompACom; the OmpA gene inactivated by insertion of pKNOCKIntKpnOmpA; OmpA levels restored; RifR, CmR, KmR | [30]     |
| 520ompK36                      | Kp52145, ompK36 gene inactivated by insertion of pKNOCKIntKpnOmpK36; RifR, CmR, KmR | [30]     |
| 52145-ΔwcaK2-ompK36            | 52145-ΔwcaK2-ompK36; the ompK36 gene inactivated by insertion of pKNOCKIntKpnOmpK36; RifR, CmR, KmR | This work |
| 52145-ΔwcaK2-ompK36Com         | Kp52145 ompK36 mutant harbouring mini-Tn7KpnOmpK36; OmpK36 levels restored; RifR, CmR, KmR | This work |
| 52201                           | Kp52145, wbbM gene inactivated; RifR, KmR | [13]     |
| 52145-ΔwaaL                     | Kp52145, ΔwaaL; the waaL gene inactivated; nonpolar mutant; RifR | [34]     |
| 52145-ΔwaaL-ΔwaaL              | 52145-ΔwaaL-ΔwaaL; the waaL gene inactivated; nonpolar mutant; RifR | This work |
| 52145-ΔwabM                     | Kp52145, ΔwabM; the wabM gene inactivated; nonpolar mutant; RifR | [35]     |
| 52145-ΔwabH                     | Kp52145, ΔwabH; the wabH gene inactivated; nonpolar mutant; RifR | [35]     |
| 52145-ΔwabK                     | Kp52145, ΔwabK; the wabK gene inactivated; nonpolar mutant; RifR | [35]     |
| 52145-ΔwabG                     | Kp52145, ΔwabG; the wabG gene inactivated; nonpolar mutant; RifR | [34]     |
| 52145-ΔwaaQ                     | Kp52145, ΔwaaQ; the waaQ gene inactivated; nonpolar mutant; RifR | [52]     |
| 52145-ΔwaaL-ΔwaaQ               | 52145-ΔwaaL-ΔwaaQ; the waaL gene inactivated; nonpolar mutant; RifR | [52]     |
| 52145-ΔwcaK2-ΔwabM              | 52145-ΔwcaK2-ΔwabM; the wabM gene inactivated; nonpolar mutant; RifR | This work |
| 52145-ΔwcaH-ΔwabH               | 52145-ΔwcaH-ΔwabH; the wabH gene inactivated; nonpolar mutant; RifR | This work |
| 52145-ΔwcaK-ΔwabK               | 52145-ΔwcaK-ΔwabK; the wabK gene inactivated; nonpolar mutant; RifR | This work |

### Plasmids

| Plasmids                        | Genotype or comments | Source or references |
|--------------------------------|----------------------|----------------------|
| pGEM-T Easy                    | Cloning plasmid, AmpR | Promega              |
| pK03                           | Suicide vector, Pic101 replication origin, sacB gene, CmR | [32]     |
| pKOV                           | pK03 with the addition of a 3 kb stuffer sequence in the multiple cloning site; CmR | Addgene plasmid 25769 |
| pGEM-T ΔpmrF                   | pGEM-T Easy containing ΔpmrF; AmpR | [26]     |
| pKOV ΔpmrF                     | pKOV containing ΔpmrF; CmR | This study |

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as previously described [33]. The expression of pmrI was not significantly different between strains (data not shown).

To obtain *K. pneumoniae* mutant strains with defects in LPS core, chromosomal in-frame nonpolar *waa* deletions were generated [34,35]. pKO3_DwabM, pKO3_DwabH, and pKO3_DwabK suicide vectors were used to introduce each mutation into the *cps* mutant, strain 52145-DwcaK2, by double homologous recombination, as previously described [34,35]. Likewise, a double mutant lacking *cps* and *ops* was constructed by mobilizing the suicide vector pKO3_DwaaL into 52145-DwcaK2.

An *ompK36* mutant in the genetic background of the *cps* mutant, strain 52145-DwcaK2, was obtained by insertion-duplication mutagenesis using the suicide vector pKNOCKIntKpnOmpK36. Correct insertion was verified by Southern blot (data not shown). OMPs were purified and analyzed by SDS-PAGE using 12% polyacrylamide gels as previously described [30,36]. Proteins were visualized by Coomassie brilliant blue staining. 52145-DwcaK2-ΔompK36 did not express OmpK36 whereas the expression of other OMPs was not affected (Figure S1).

### Table 1. Primers used in this study

| Purpose/target gene | Name             | Sequence (5’ to 3’) |
|---------------------|------------------|---------------------|
| Mutagenesis         |                  |                     |
| pmrF                | KpnpmrFF         | CGGATCCACCTGCGCAGCTGGCGGAC |
|                     | KpnpmrFR         | CGGATCCGGGCGTCATCCGCGCCAATC |
|                     | KpnpmrFinvF      | TCTCCTCCGGCAGGGTTTTGC |
|                     | KpnpmrFinvR      | CAAATACAGCTTTATGCGCCTG |
| Complementation     |                  |                     |
| ompK36              | ComKpnOmpK36F    | GAGTGGTAGACTGCTTACGAC |
|                     | ComKpnOmpK36R    | AGGGAAATCCATTAGCCGAC |
| RT-qPCR             |                  |                     |
| pmrI                | KpnyfbGF1        | CGCTGGATCTACTCAGTTCC |
|                     | KpnyfbGR1        | TCTTTGTCTCGGCTGAGTT |
| rpoD                | KpnporpDLEFT     | CCGAAGAACAATCGCGGA |
|                     | KpnporpDRIGHT    | CGGGTAACGCTGAACTG |
| Tn7 insertion       |                  |                     |
| glmS                | KpnpglmSup       | GCGACAAGTGTTGAGCCGAG |
|                     | KpnpglmSdown     | TGCCATACGCTGGCCGCT |
| Tn7                 | Ptn7L            | ATTAGCTGACGCTGACCC |
|                     | Ptn7R            | CAGCGATACGCTGACTT |

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Complementation *ompK36* mutants

A 1.7 kb fragment encompassing *ompK36* and its promoter was PCR-amplified (using primers ComKpnOmpK36F and ComKpnOmpK36R [Table 2], Vent polymerase [New England Biolabs]) and cloned into SmaI-digested pUC18R6KT-mini-Tn7TKm to give pUC18R6KT-mini-Tn7TKmKpnOmpK36. Tn7 delivery to 520ompK36 and 52145-DwcaK2-ΔompK36 was performed as described [37] and insertion was verified by colony-PCR with primer pairs: KpnglmSup/Ptn7L; and KpnglmSdown/Ptn7R [37]. Tn7 transposon integrates at the site-specific attTn7, located

### Table 2. Primers used in this study

| Purpose/target gene | Name             | Sequence (5’ to 3’) |
|---------------------|------------------|---------------------|
| Mutagenesis         |                  |                     |
| pmrF                | KpnpmrFF         | CGGATCCACCTGCGCAGCTGGCGGAC |
|                     | KpnpmrFR         | CGGATCCGGGCGTCATCCGCGCCAATC |
|                     | KpnpmrFinvF      | TCTCCTCCGGCAGGGTTTTGC |
|                     | KpnpmrFinvR      | CAAATACAGCTTTATGCGCCTG |
| Complementation     |                  |                     |
| ompK36              | ComKpnOmpK36F    | GAGTGGTAGACTGCTTACGAC |
|                     | ComKpnOmpK36R    | AGGGAAATCCATTAGCCGAC |
| RT-qPCR             |                  |                     |
| pmrI                | KpnyfbGF1        | CGCTGGATCTACTCAGTTCC |
|                     | KpnyfbGR1        | TCTTTGTCTCGGCTGAGTT |
| rpoD                | KpnporpDLEFT     | CCGAAGAACAATCGCGGA |
|                     | KpnporpDRIGHT    | CGGGTAACGCTGAACTG |
| Tn7 insertion       |                  |                     |
| glmS                | KpnpglmSup       | GCGACAAGTGTTGAGCCGAG |
|                     | KpnpglmSdown     | TGCCATACGCTGGCCGCT |
| Tn7                 | Ptn7L            | ATTAGCTGACGCTGACCC |
|                     | Ptn7R            | CAGCGATACGCTGACTT |

doi:10.1371/journal.pone.0056847.t002
Dictyostelium was prepared in 10 mM PBS (pH 6.5). To test the ability of the wild-type strain (Figure S1).

Growth of Dictyostelium on bacteria

Procedures to test growth of Dictyostelium on bacteria have been described previously [42]. Briefly, bacteria were grown overnight in 5-mL LB, harvested (2500 × g, 20 min, 24°C), washed once with PBS and a suspension containing approximately 1 × 10^6 cfu/ml was prepared in 10 mM PBS (pH 6.5). 300 µl from this suspension was spread onto standard medium (SM)-agar plates (10 g/l glucose, 10 g/l peptone, 1 g/l yeast extract, 1 g/l MgSO_4·7H_2O, 1.9 g/l KH_2PO_4, 0.6 g/l K_2HPO_4, 20 g/l agar; pH 6.3) or dilution series of HL5-agar plates. The plates were dried in a greenhouse and allowed to grow at 21°C for 4–5 days, i.e. until Dictyostelium growth became visible.

Eukaryotic cells culture

D. discoideum AX2 cells were grown at 21°C in HL5 medium (pH 6.5) supplemented with 1.12 mg/ml glucose, 20 µg/ml streptomycin and 10 µg/ml tetracycline, and subcultured twice a week to maintain a density <10^6 cells/ml [42,43].

Marine alveolar macrophages

Marine alveolar macrophages MH-S (ATCC, CRL-2019) were grown on RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and Hepes 10 mM at 37°C in an humidified 5% CO_2 atmosphere.

Construction of pFPV25.1Cm plasmid

A cat cassette, obtained by Smal digestion of p34S-Cm [38], was cloned into EcoRV-digested pFPV25.1 [39,40] to obtain pFPV25.1Cm. This plasmid expresses gfpmut3 under the control of Salmonella rpsM promoter region. This fusion has been reported to be expressed at similar levels in various environments, including growth media and mammalian cells [39,40]. pFPV25.1Cm was cloned into EcoRV-digested pFPV25.1 to obtain pFPV25.1Cm. This plasmid expresses gfpmut3 under the control of Salmonella rpsM promoter region.

LPS analysis

Small scale LPS extraction using hot phenol was performed following the procedure described by Marolda et al. [41], with the exception that ethyl ether was replaced by ethanol for the washing of the LPS pellet. The LPS was run on a 12% SDS-PAGE and visualized using Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen).

Growth of Dictyostelium on bacteria

Procedures to test growth of Dictyostelium on bacteria have been described previously [42]. Briefly, bacteria were grown overnight in 5-mL LB, harvested (2500 × g, 20 min, 24°C), washed once with PBS and a suspension containing approximately 1 × 10^6 cfu/ml was prepared in 10 mM PBS (pH 6.5). 300 µl from this suspension was spread onto standard medium (SM)-agar plates (10 g/l glucose, 10 g/l peptone, 1 g/l yeast extract, 1 g/l MgSO_4·7H_2O, 1.9 g/l KH_2PO_4, 0.6 g/l K_2HPO_4, 20 g/l agar; pH 6.3) or dilution series of HL5-agar plates. The plates were dried in a laminar hood for 30 min. Variable numbers of Dictyostelium amoebac (10 000, 1000, 100, 10) were deposited on the bacterial lawn, and allowed to grow at 21°C for 4–5 days, i.e. until Dictyostelium growth became visible.

Phagocytosis and killing of bacteria by Dictyostelium

Experiments were performed as previously described [44]. Briefly, bacteria were grown in 5-mL LB, harvested in the exponential phase (2500 × g, 20 min, 24°C), washed once with PBS and a suspension containing approximately 1 × 10^6 cfu/ml was prepared in 10 mM PBS (pH 6.5). To test the ability of Dictyostelium to ingest and kill live bacteria, 10^7 cfu from the indicated suspension were mixed with 10^7 Dictyostelium in 500 µl of KK2 buffer (16.5 mM KH_2PO_4, 3.9 mM K_2HPO_4; pH 6.3) and incubated at 21°C with shaking. After 90 or 180 min of incubation, a 10 µl aliquot of the suspension was collected and diluted in 40 µl of ice-cold sucrose (400 g/l). 200 µl of 0.5% saponin in KK2 were added, before plating on a LB agar plate and incubating at 37°C. Control experiments showed that this procedure does not affect bacterial viability (this work and [44]). When indicated, the number of viable bacteria associated with Dictyostelium cells (intracellular fraction) was determined by washing the cells twice with ice-cold HL5 medium before diluting in sucrose [44]. Results are expressed as percentage of the colony count of bacteria not exposed to Dictyostelium. All experiments were done with triplicate samples on at least four independent occasions.

Immunofluorescence analysis was performed as described previously [45] by infecting AX2/RPF, a D. discoideum strain constitutively expressing the red fluorescent protein [46]. 2.5 × 10^7 cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates and, after 2 h, infected with GFP-expressing K. pneumoniae at a ratio of 100 bacteria per 1 cell in a final volume of 500 µl of HL5. To synchronize infection, plates were centrifuged at 200 × g during 5 min. Plates were incubated at 21°C for 30 min. Cells were washed twice with KK2 buffer and fixed with 3.7% paraformaldehyde in PBS pH 7.4 for 20 min at room temperature. Coverslips were washed two times in KK2 buffer before mounting onto glass slides using Aqua poly/Mount (Polysciences). Confocal microscopy was carried out with a Leica TCS SP5 confocal microscope. Experiments were carried out by duplicate in three independent occasions. The number of infected cells and the number of intracellular bacteria per cell was quantified within 300 cells.

Killing of Dictyostelium by bacteria

Bacteria were grown in 5-mL LB, harvested in the exponential phase (2500 × g, 20 min, 24°C), washed once with PBS and a suspension containing approximately 1 × 10^7 cfu/ml was prepared in 10 mM PBS (pH 6.5). To test the ability of Klebsiella to kill Dictyostelium, 10^7 cfu were mixed with 10^7 Dictyostelium cells in 500 µl of KK2 buffer and incubated at 21°C with shaking (180 rpm). After 3 h, serial dilutions of the mixture were plated on a K. aerogenes lawn on SM agar plates, which were incubated at 21°C for 4–5 days, i.e. until individual colonies of Dictyostelium became visible. All experiments were done with triplicate samples on three independent occasions.

Phagocytosis of bacteria by alveolar macrophages

MH-S cells were seeded in 24-well tissue culture plates at a density of 7 × 10^4 cells per well 15 h before the experiment. Bacteria were grown in 5-mL LB, harvested in the exponential phase (2500 × g, 20 min, 24°C), washed once with PBS and a suspension containing approximately 1 × 10^7 cfu/ml was prepared in 10 mM PBS (pH 6.5). Cells were infected with 33 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 × g during 5 min. Plates were incubated at 37°C in an humidified 5% CO_2 atmosphere. After 30 min of contact, cells were washed twice with PBS and incubated for additional 90 min with 500 µl of 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). Cells were then 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. Phagocytosis data are represented as cfu per well. All
experiments were done with triplicate samples on at least three independent occasions. Immunofluorescence was performed as previously described [47]. 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. After 90 min, cells were washed three times with PBS, and fixed with 3.7% paraformaldehyde in PBS pH 7.4. The actin cytoskeleton was stained with Rhodamine-Phalloidin (Invitrogen) diluted 1:100, DNA was stained with Hoescht 33342 (Invitrogen) diluted 1:2500. 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. The number of infected cells and the number of intracellular bacteria per cell was quantified within 300 cells. Experiments were carried out by triplicate in three independent occasions.

**Intranasal infection model**

Five- to 7-week-old female C57BL/6JOlalHsd 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, 24°C), resuspended in PBS and adjusted to 5 x 10⁷ cfu/ml. 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. At indicated times after infection, mice were euthanized by cervical dislocation and lungs were rapidly dissected for bacterial loads determination. Dissected lungs were homogenized in 500 μl of PBS using an Ultra-Turrax T10 basic (IKA) on ice. Serially diluted bacteria from the homogenates were recovered in LB agar plates containing Rif for wild-type *K. pneumoniae* strain 52145-ΔwacKZ, but not Kp52145, was susceptible to predation even by 10 amoebae at 5% HL5 (Figure 1C). Kp52145 and 52145-ΔwacKZ exhibited similar growth rates in 5% HL5 (data not shown). Control experiments indicated that Kp52145 was not cytotoxic for *Dictyostelium* because the number of amoebae after incubation with Kp52145 was similar to that after incubation with *K. aerogenes* (1.6±0.5 x 10⁴, versus 1.7±0.6 x 10⁴, respectively, *P>0.05*).

Survival experiments were carried out to determine the total number of remaining bacteria, as well as the number of live cell-associated bacteria. *Dictyostelium* ingested and killed 52145-ΔwacKZ as fast as the *K. aerogenes* control strain (Figure 2A). The number of intracellular 52145-ΔwacKZ was higher than that of intracellular Kp52145 after co-culture of bacteria and *Dictyostelium* with an average of 2 bacteria per infected amoeba (Figure 2B). The percentage of infected *Dictyostelium* with Kp52145 was 7±3% whereas it reached 63±9% when the challenging strain was 52145-ΔwacKZ. Aligned with this, experiments indicated that 52145-ΔwacKZ is more easily engulfed by *Dictyostelium* than Kp52145. Intracellular killing was so fast that viable intracellular bacteria were hardly detectable already at 60 min post infection (Figure 2C), suggesting that under these experimental conditions the limiting factor for killing was the rate of phagocytosis.

Gentamicin protection assays showed that 52145-ΔwacKZ was ingested by MH-S alveolar macrophages in higher numbers than the wild-type strain (Figure 2D). This correlated with the microscopic observation of higher numbers of intracellular 52145-ΔwacKZ than Kp52145 after co-culture with alveolar macrophages with an average of 4 bacteria per infected macrophage (Figure 2E). The percentage of macrophages infected with 52145-ΔwacKZ was significantly higher than that of macrophages infected with Kp52145 (43±9% and 7±3%, respectively; *P<0.05*).

Collectively, these results highlight the role of *K. pneumoniae* CPS of K2 serotype in resistance to phagocytosis by *D. discoideum* and alveolar macrophages.

**Role of *K. pneumoniae* CPS on phagocytosis resistance**

We evaluated the resistance of the highly virulent clinical isolate *K. pneumoniae* strain 52145 (hereafter Kp52145) to predation by *D. discoideum* in comparison to the previously analyzed *K. aerogenes* susceptible strain, by using SM medium. Further confirming previous results [42], amoebae feed only upon *K. aerogenes*, creating phagocytic plaques (Figure 1A). Since CPS reduces phagocytosis, the lack of growth of *Dictyostelium* on Kp52145 may simply reflect that these bacteria are not ingested by the amoebae. Unexpectedly, the isogenic cps mutant, strain 52145-ΔwacKZ, was also not permissive for *Dictyostelium* growth (Figure 1A). This might be due to the fact that the CPS of this *Klebsiella* strain, of the K2 serotype, is not required for phagocytosis resistance. However, there are studies showing that indeed CPS of the K2 serotype mediate resistance to phagocytosis [18,48,49]. Another possibility could be that the assay conditions were too favourable for Kp52145. In fact, a similar scenario was reported when the virulence of *Aeromonas* spp was analyzed using *D. discoideum* model [50], i.e. *Dictyostelium* was incapable of growing on wild-type bacteria or on any of the nonvirulent mutants of *Aeromonas* tested when the assays were performed on SM medium. Since the richness of the growth medium affects the threshold at which a bacteria is permissive for *Dictyostelium* [42], we tested a range of dilutions of HL5 to determine the medium where only Kp52145 remains non-permissive (Figure 1B). We observed that 52145-ΔwacKZ, but not Kp52145, was susceptible to predation even by 10 amoebae at 5% HL5 (Figure 1C). Kp52145 and 52145-ΔwacKZ exhibited similar growth rates in 5% HL5 (data not shown). Control experiments indicated that Kp52145 was not cytotoxic for *Dictyostelium* because the number of amoebae after incubation with Kp52145 was similar to that after incubation with *K. aerogenes* (1.6±0.5 x 10⁴, versus 1.7±0.6 x 10⁴, respectively, *P>0.05*).
cantly lower than that of the *cps* mutant, 52145-*ΔwcaK2* already after 90 min of co-culture with the amoebae (Figure 3B).

To delineate the possible contribution of the LPS core to phagocytosis resistance, we first analyzed strains 52145-*ΔwaaQ*, lacking one heptose and its attached variable residue, and 52145-*ΔwabG*, lacking the first four sugars of the LPS core (Figure 3A). Whereas 52145-*ΔwaaQ* expresses OPS and similar levels of CPS than the wild-type strain [51,52], 52145-*ΔwabG* is devoid of cell-surface attached CPS and OPS [51]. Results shown in Figure 3C revealed that 52145-*ΔwabG* was more susceptible to predation by

**Figure 1. Virulence of *K. pneumoniae* against *D. discoideum* can be modulated.** (A) The ability of *Dictyostelium* to grow on a bacterial lawn was assessed by depositing amoebae (from 10 to 10,000) on a lawn of bacteria grown on SM agar medium. A phagocytosis plaque was observed 5 days later when bacteria were permissive. Bacteria tested were: *K. pneumoniae* (Kp52145), *cps* mutant (52145-*ΔwcaK2*; *ΔwcaK2*), or control strain (*K. aerogenes*). (B) The ability of wild-type *K. pneumoniae* (Kp52145), *cps* mutant (52145-*ΔwcaK2*), or control strain (*K. aerogenes*) to resist predation by *D. discoideum* was tested on HL5-agar, pure or diluted. 1,000 amoebae were deposited on the bacterial lawns and plaques were recorded 5 days later. (C) The ability of *Dictyostelium* to grow on a bacterial lawn was assessed by depositing amoebae (from 10 to 10,000) on a lawn of bacteria grown on HL5–5% agar medium. A phagocytosis plaque was observed 5 days later when bacteria were permissive (K *aerogenes* and 52145-*ΔwcaK2*; *ΔwcaK2*). doi:10.1371/journal.pone.0056847.g001
D. discoideum than 52145-ΔwcaK2, which, in turn, was as susceptible as the OPS mutants. We did not observe significant differences between 52145-ΔwcaK2 and 52145-ΔwcaK2 (Figure 3C). Since 52145-ΔwcbG lacks the cell-surface attached CPS, the OPS and the sugars of the outer core region we analyzed other core mutants expressing CPS and a less truncated core. 52145-ΔwabM, 52145-ΔwabK and 52145-ΔwabH, lack, in addition to the OPS, the first, second and third sugar of the LPS core, respectively (Figure 3A), but expressed similar levels of CPS than the wild-type strain [35]. Results shown in Figure 3C revealed that 52145-ΔwabM; 52145-ΔwabK and 52145-ΔwabH were susceptible to predation by D. discoideum already after 90 min of co-culture with amoebae (Figure 3C). No significant differences were observed between these mutants and 52145-ΔwabG (Figure 3C). In the genetic background of the cps mutant, the three core mutants were more susceptible to predation by the amoebae than the LPS OPS mutant but only after 90 min of co-culture (Figure 3D).

Next, we analyzed the susceptibility of this set of mutant strains to phagocytosis by alveolar macrophages. No differences were found between the phagocytosis of the LPS OPS mutants, 52O21 and 52145-ΔwcaK2, and that of the wild-type strain (Figure 3E). In contrast, all the LPS core mutants tested were ingested in higher numbers by the macrophages (Figure 3E).
numbers than the LPS OPS mutants and the wild-type strain (Figure 3E). In the genetic background of the cps mutant, only wabk and wabH mutants were internalized in higher numbers than the LPS OPS mutant by the alveolar macrophages (Figure 3F).

In summary, our findings indicate that, in addition to the CPS, _K. pneumoniae_ LPS OPS and the core sugars participate in the resistance to predation by _D. discoideum_. In the case of alveolar macrophages, the LPS core plays a more prominent role than the LPS OPS in _K. pneumoniae_ avoidance of phagocytes.

**Role of _K. pneumoniae_ LPS lipid A decorations on phagocytosis resistance**

52145-ΔpmrF, 52145-ΔpagFBG and 52145-ΔpmrF-ΔpagFBG are mutant strains lacking lipid A species containing aminoorabinose, palmitate or both [26]. These mutants express the same levels of CPS than the wild type [26]. LPS analysis showed that these mutants expressed OPS (Figure S2).

We asked whether these modifications contribute to phagocytosis resistance by _D. discoideum_. After 90 min of co-culture, pagP mutant was more susceptible to predation by the amoebae than the wild type and the pmrF mutant whereas the pagP-pmrF double mutant was the most susceptible strain (Figure 4A). After 180 min, the three lipid A mutants were more susceptible to predation than the wild type and no significant differences between the mutat strains were observed (Figure 4A). In the background of the cps mutant, after 90 min of co-culture, the pagP and pmrF single mutants were more susceptible to predation than 52145-ΔwacK2 whereas 52145-ΔwacK2-ΔpmrF-ΔpagFBG triple mutant was the most susceptible strain (Figure 4B). After 180 min, the three lipid mutants were more susceptible than 52145-ΔwacK2 (Figure 4B).

Next, we assessed the contribution of lipid A decorations to phagocytosis resistance by alveolar macrophages. The pagP mutants, strains 52145-ΔpagFBG, 52145-ΔpmrF-ΔpagFBG, 52145-ΔwacK2-ΔpmrF-ΔpagFBG, and 52145-ΔwacK2-ΔpagFBG, were internalized in higher numbers by alveolar macrophages than 52145 and 52145-ΔwacK2, being the numbers of 52145-ΔwacK2-ΔpmrF-ΔpagFBG the highest (Figure 4C-D).

Altogether, these data support the notion that the PagP-dependent lipid A modification with palmitate plays a more important role to reduce _K. pneumoniae_ phagocytosis by _D. discoideum_ and alveolar macrophages than the lipid A modification with aminoorabinoise. The impact of the latter is more evident in the pagP mutant background.

**Role of _K. pneumoniae_ OMPs on phagocytosis resistance**

Previously, we have shown that ompA and ompK36 mutants express similar levels of CPS than Kp52145 [30,31]. We aimed to establish whether OmpA and OmpK36 are involved in the resistance to phagocytosis by _D. discoideum_ and alveolar macrophages.

Results displayed in Figure 5A indicate that ompA and ompK36 mutants were susceptible to predation by amoebae. The contribution of both OMPs to resist predation by _D. discoideum_ was also observed in the _cps_ mutant background (Figure 5B). Likewise, OMPs mutants were phagocytosed by alveolar macrophages in higher numbers than Kp52145 and 52145-ΔwacK2 (Figure 5C–D). OMPs mutants could be complemented (Figure 5).

**Virulence of _K. pneumoniae_ ompK36 mutant**

We and others have assessed the contribution of CPS, LPS polysaccharides, lipid A decorations and _ompK_ to _K. pneumoniae_ virulence [13,18,26,31,34,35,35]. Notably, there is a strong correlation between resistance to phagocytosis (this work) and attenuation in _in vivo_. Therefore, the fact that OmpK36 contributed to phagocytosis resistance prompted us to determine the ability of the _ompK36_ mutant to cause pneumonia. C57BL/6J mice were infected intranasally and bacterial loads in trachea and lung homogenates were determined at 24 and 72 h post-infection (Figure 6). Kp52145 and _ompK36_ mutant colonized trachea although bacterial loads of the mutant were lower than those of the wild type at 24 and 72 h post-infection (Figure 6A). _ompK36_ mutant also colonized the lungs and at 72 h post infection bacterial loads of the mutant were lower than those of the wild type (Figure 6B).

To evaluate the ability of the mutant to disseminate to other organs, bacterial loads in spleen and liver were determined. _ompK36_ mutant reached both organs (Figure 6C–D) and at 72 h post-infection the bacterial loads in spleen and liver were significantly lower than those of the wild type (Figure 6C–D).
Figure 4. Role of *K. pneumoniae* lipid A decorations on phagocytosis resistance. (A) Dictyostelium cells were incubated with *Klebsiella* strains (wild type [Kp52145], pagP mutant (ΔpagP, 52145-ΔpagPGB), pmrF mutant (ΔpmrF, 52145-ΔpmrF), or pagP-pmrF double mutant (ΔpagP-ΔpmrF, 52145-ΔpagPGB-ΔpmrF). The number of surviving bacteria (total or cell-associated) was determined at different times by killing the *Dictyostelium* and plating the bacteria on LB plates. Bars represent mean ± s.e.m (n = 4). *; P<0.05 (results are significantly different from the results for the wild-type strain [Kp52145]; two tailed t test). n; P>0.05 (results are significantly different from the results for 52145-ΔpagP GB; two tailed t test). (B) Dictyostelium cells were incubated with *Klebsiella* lipid A mutants constructed in the background of the cps mutant (52145-ΔwcaK2 [ΔwcaK2]). The number of surviving bacteria was determined at different times by killing the *Dictyostelium* and plating the bacteria on LB plates. Bars represent mean ± s.e.m (n = 4). *; P<0.05 (results are significantly different from the results for the cps mutant [52145-ΔwcaK2]; two tailed t test). Δ; P<0.05 (results are significantly different from the results for 52145-ΔwcaK2-ΔpagP GB; two tailed t test). (C) Phagocytosis of lipid A mutants by MH-S cells. Wild type [Kp52145], pagP mutant (ΔpagP, 52145-ΔpagPGB), pmrF mutant (ΔpmrF, 52145-ΔpmrF), or double pagP-pmrF mutant (ΔpagP-ΔpmrF, 52145-ΔpagPGB-ΔpmrF). Bars represent mean ± s.e.m (n = 4); *; P<0.05 (results are significantly different from the results for the wild-type strain [Kp52145]; two tailed t test). Δ; P<0.05 (results are significantly different from the results for 52145-ΔpagPGB; two tailed t test). (D) Phagocytosis of lipid A mutants constructed in the background of the cps mutant (52145-ΔwcaK2 [ΔwcaK2]) by MH-S cells. Bars represent mean ± s.e.m (n = 4); *; P<0.05 (results are significantly different from the results for the cps mutant [52145-ΔwcaK2]; two tailed t test). Δ; P<0.05 (results are significantly different from the results for 52145-ΔwcaK2-ΔpagP GB; two tailed t test).

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Discussion

Phagocytosis is one of the key processes of the immune system. Although most bacteria are successfully internalized and eliminated by phagocytes, several pathogens have developed survival strategies that interfere with the internalization and/or maturation processes. *K. pneumoniae* is a well known example of one pathogen displaying resistance to phagocytosis. Prevention and management of the infections caused by such pathogens would obviously benefit from understanding the manner in which they circumvent and often co-opt the immune response. The fact that *K. pneumoniae* is ubiquitous in nature and, therefore, should avoid predation by protozoa, including amoebae, poses the question whether *K. pneumoniae* employs similar means to counteract predation by amoebae and engulfment by mammalian phagocytes. In this study, we provide evidence for this notion. Furthermore, our data reveal novel information about the implication of *K. pneumoniae*.
LPS polysaccharide and lipid A sections, and of the OMPs OmpA and OmpK36 to *K. pneumoniae* avoidance of phagocytosis.

In this study, we found a correlation between the findings obtained testing alveolar macrophages, key cells responsible for lung defence against infections, and those found challenging *D. discoideum*. Therefore, our results add further evidence to the notion that *D. discoideum* model is useful for investigating phagocytosis. Our data further support the idea that the limiting factor for killing *Klebsiella* by the amoebae is the rate of phagocytosis [17,44] since bacterial survival was not affected in those strains not engulfed by *D. discoideum*. Nevertheless, our results also highlight the importance of adjusting the assay conditions in order to set the threshold of the assay for supposedly permissive bacteria, in our case the cps mutant [17–19]. This was so even for a bacterial species previously tested, *K. pneumoniae*, thereby suggesting that the interplay between *D. discoideum* and bacterial pathogens is strain specific. Likewise, not all strains of *Vibrio cholerae* are able to avoid predation by *D. discoideum* [54]. The assay established and used in this study allowed a comprehensive analysis of *Klebsiella* surface determinants mediating resistance to phagocytosis with a high degree of reproducibility.

While this work was in progress, Pan and co-workers [19] reported the results of a screening to identify *K. pneumoniae* NHTU-K2044 determinants preventing predation by *D. discoideum*. Seventy two of the mutants permissive for *D. discoideum* growth had transposon insertions in the cps operon [19], which is in good agreement with our findings showing the importance of CPS to resist phagocytosis. Of note, Kp52145 and NHTU-K2044 express CPS of different *K* serotypes, K2 and K1 respectively, thereby suggesting that the CPS serotype may not be the determinant factor mediating CPS-dependent reduction of phagocytosis. In fact, evidence points out that the critical factor is the amount of CPS expressed [19,55]. Like most clinical isolates associated to

Figure 6. Virulence of *K. pneumoniae ompK36* mutant. Bacterial counts in mouse organs at 24 h post infection or 72 h post infection. Mice were infected intranasally with a bacterial mixture containing 5 × 10^8* bacteria of wild type (Kp52145, ◦) or ompK36 mutant (∆ompK36, ○) Results were reported as log CFU per gram of tissue (Log CFU/g). *, results are significantly different (P<0.05; two-tailed t test) from the results for Kp52145. (A) Trachea; (B) Lung; (C) Spleen, (D) Liver.

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severe infections, Kp52145 and NHTU-K2044 are heavily capsulated strains.

Twenty one of the NHTU-K2044 mutants supporting the growth of the amoebae had transposon insertions affecting the biosynthesis of the LPS OPS [19]. However, those mutants were less capsulated than the wild type [19] making difficult to delineate the relative contribution of the OPS and CPS to resist predation by D. discoideum. The OPS mutants tested in this work express wild-type levels of CPS hence allowing us to study the role of OPS on resistance to phagocytosis by D. discoideum. Indeed, our data highlight that the OPS limits predation by D. discoideum. Unexpectedly, the OPS mutants were phagocytosized by alveolar macrophages in similar numbers than the wild-type strain. Likewise, it has been reported no role for Klebsiella OPS on the resistance to phagocytosis by human dendritic cells [56]. In contrast, it has been shown that the OPS does play a role in the interaction with mouse and human neutrophils [19,57]. Therefore, it is tempting to postulate that the contribution of Klebsiella OPS to prevent phagocytosis is not uniform to all professional phagocytes.

The contribution of LPS core to virulence is poorly characterized in most Gram negative pathogens and it has been only conclusively established for Yersinia enterocolitica and Kp52145 [35,38]. To the best of our knowledge, our study is the first one highlighting the contribution of LPS core residues to phagocytosis resistance. Our findings showed that the heptose branch linked to the core by WaaQ is implicated in resistance to phagocytosis by D. discoideum and alveolar macrophages even in the presence of OPS. To investigate the contribution of other core residues in an OPS-bearing strain, we have used defined mutants that lack the OPS in addition to core residues which, in turn, suggest that the core residues are never exposed in a wild-type strain. However, it should be noted that epidemiological data indicate that nearly 10% of Klebsiella clinical isolates do not express the LPS OPS [59] and, therefore, core residues will not be masked by the OPS. Our results revealed that the first glucose residue of the LPS core is necessary to avoid engulfment by D. discoideum and alveolar macrophages since the relative survival of vwhAM mutant, lacking also OPS, was lower than those of the OPS mutants. Elimination of additional core residues did not further decrease the observed phagocytosis resistance.

Perusal of the literature clearly shows the importance of lipid A decorations with aminoarabinose and palmitate to counteract the microbial action of antimicrobial peptides. However, the role of these lipid A decorations, if any, to resist phagocytosis was unknown. Our data indicated that PagP-dependent lipid A palmitoylation plays an important role to reduce Klebsiella engulfment by D. discoideum and alveolar macrophages. Intriguing, a pagP-like gene also confers Legionella pneumophila resistance to antimicrobial peptides and contributes to the intracellular life of the pathogen in Hartmannella vermiformis amoebae and human macrophages [60]. It is tempting to formulate that PagP-dependent lipid A modification is a major bacterial determinant against the soluble and the cellular arms of the innate immune system. As a consequence, pagP mutants should be attenuated as indeed it has been indeed shown for K. pneumoniae and Legionella [26,60]. Studies in other bacterial models are required to further validate our hypothesis.

Finally, we showed that Klebsiella OMPs also contribute to phagocytosis resistance in K. pneumoniae. Mounting evidence indicates that an essential attribute of K. pneumoniae OmpA is to thwart the innate immune system [30,31]. The findings reported in this work further corroborate this notion and add new features to the previously described panoply of OmpA-dependent anti-immune strategies. In turn, the possible role of OmpK36 on K. pneumoniae evasion of innate immunity is poorly characterized. Our results revealed that OmpK36 also contributes to phagocytosis resistance by Klebsiella. However, and in contrast to OmpA, OmpK36 does not play any role in the resistance to antimicrobial peptides [30] and therefore it seems that both OMPs are not functionally redundant in terms of immune evasion. We are currently assessing whether OmpK36 modulates the cellular responses upon Klebsiella infection.

Previous reports suggested that alveolar macrophages play a major role in host defence against K. pneumoniae since the depletion of these cells results in reduced killing of the pathogen in vivo [10,11]. Conversely, this suggests that Klebsiella countermeasures against phagocytosis could be important virulence factors. Data reported in this work give experimental support to this hypothesis since we have found a nearly perfect correlation between virulence using the pneumonia mouse model and resistance to phagocytosis by D. discoideum and alveolar macrophages. Thus, cps, pagP, LPS core, ompA and ompK36 mutants are attenuated in vivo (this work and [13,18,26,31,34,35,53]) and in this study we have demonstrated that these loci mediate phagocytosis resistance. A tantalizing observation is that Dictyostelium amoebae might be useful as host model to measure K. pneumoniae virulence and not only phagocytosis. Given the relatively simplicity and low cost to create banks of D. discoideum mutants in comparison to mouse or human macrophages, it can be envisaged a more systematic analysis of the complex interactions between Klebsiella and the host, aiming to identify host resistance genes. First attempts challenging a non-saturating D. discoideum library of mutants with a laboratory adapted K. pneumoniae strain led to the identification of a type V P-ATPase as an essential element for killing of Klebsiella [44]. Interestingly, this P-ATPase was dispensable for the elimination of other bacteria [44] hence suggesting that Klebsiella may mobilize a specific set of host gene products only necessary for resistance to K. pneumoniae infection.

Supporting Information

Figure S1 Analysis of OMPs from Klebsiella strains. SDS-PAGE (the acrylamide concentration was 4% in the stacking gel and 12% in the separation one) followed by Coomasie brilliant blue staining of OMPs from (A) Kp52145, 520ompK36 and 520ompK36Com; and (B) 52145-ΔwcaK3, 52145-ΔwcaK2-ompK36 and 52145-ΔwcaK2-ompK36Com. MW, molecular weight marker. (TIF)

Figure S2 Analysis of LPSs from Klebsiella strains. SDS-PAGE (the acrylamide concentration was 4% in the stacking gel and 12% in the separation one) followed by staining using Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen) of LPSs from Kp52145, 52145-ΔpmrF (ΔpmrF), 52145-ΔpagP/GB (ΔpagP) and 52145-ΔpagP/GB-ΔpmrF/ΔpagP-ΔpmrF. (TIF)

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Author Contributions

Conceived and designed the experiments: JAB TS JG. Performed the experiments: CM VC EL DM CPG. Analyzed the data: CM VC JAB TS JG. Contributed reagents/materials/analysis tools: JMT. Wrote the paper: JAB TS JG.
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