**Klebsiella pneumoniae** subverts the activation of inflammatory responses in a NOD1-dependent manner

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### Summary

*Klebsiella pneumoniae* is an important cause of community-acquired and nosocomial pneumonia. Subversion of inflammation is essential for pathogen survival during infection. Evidence indicates that *K. pneumoniae* infections are characterized by lacking an early inflammatory response although the molecular bases are currently unknown. Here we unveil a novel strategy employed by a pathogen to counteract the activation of inflammatory responses. *K. pneumoniae* attenuates pro-inflammatory mediators-induced IL-8 secretion. *Klebsiella* antagonizes the activation of NF-κB via the deubiquitinase CYLD and blocks the phosphorylation of mitogen-activated protein kinases (MAPKs) via the MAPK phosphatase MKP-1. Our studies demonstrate that *K. pneumoniae* has evolved the capacity to manipulate host systems dedicated to control the immune balance. To exert this anti-inflammatory effect, *Klebsiella* engages NOD1. In NOD1 knock-down cells, *Klebsiella* neither induces the expression of CYLD and MKP-1 nor blocks the activation of NF-κB and MAPKs. *Klebsiella* inhibits Rac1 activation; and inhibition of Rac1 activity triggers a NOD1-mediated CYLD and MKP-1 expression which in turn attenuates IL-1β-induced IL-8 secretion. A capsule (CPS) mutant does not attenuate the inflammatory response. However, purified CPS neither reduces IL-1β-induced IL-8 secretion nor induces the expression of CYLD and MKP-1 thereby indicating that CPS is necessary but not sufficient to attenuate inflammation.

### Introduction

Activation of innate immune responses, particularly inflammation, is a key event in the host defence against pathogens. Innate responses rely on the activation of signalling pathways resulting in the production of antimicrobial molecules, the expression of co-stimulatory molecules and the release of cytokines and chemokines (Mogensen, 2009). These signalling pathways converge on a limited set of transcriptional activators including NF-κB and mitogen-activated protein kinases (MAPKs) (Medzhitov, 2007; Kumar et al., 2009a; Mogensen, 2009). To launch these responses, the host recognizes conserved molecules uniquely expressed by pathogens, the so-called pathogen-associated molecular patterns (PAMPs), through a set of germ line-encoded receptors referred to as pattern-recognition receptors (PRRs) (Medzhitov, 2007; Kumar et al., 2009a). Among TLRs, most of the studies focus on TLR4, mainly involved in the detection of lipopolysaccharide (LPS), and on TLR2 which responds to a variety of Gram-positive PAMPs (Medzhitov, 2007; Kumar et al., 2009a; Mogensen, 2009). Among TLRs, most of the studies focus on TLR4, mainly involved in the detection of lipopolysaccharide (LPS), and on TLR2 which responds to a variety of Gram-positive PAMPs (Medzhitov, 2007; Kumar et al., 2009a; Mogensen, 2009). Among NLRs, NOD1 receives increasing attention. NOD1 is located intracellularly and recognizes a peptidoglycan (PGN) motif, γ-D-glutamyl-meso-diaminopimelic acid from Gram-negative bacteria (Chamaillard et al., 2003; Inohara et al., 2005).

Subversion of the fast-acting inflammatory response is considered important for pathogen survival during the early stages of infection. Such subversion allows pathogens to avoid immediate elimination by the host defence and...
increases the chances of establishing a critical population size. However, little is known about the mechanisms employed by most of the pathogens to subvert the activation of inflammatory responses. At present, the best-characterized strategies are the modification of PAMPs to avoid detection by PRRs and the disruption of those intracellular signalling pathways implicated in host defence (Finlay and McFadden, 2006; Roy and Mocarski, 2007). *Klebsiella pneumoniae* is the most common Gram-negative bacterium causing community-acquired pneumonia and up to 5% of community-acquired urinary tract infections (Sahly and Podschn, 1997). Community-acquired pneumonia is a very severe illness with a rapid onset, and despite the availability of an adequate antibiotic regimen the outcome is often fatal with observed mortality rates of 50%. Importantly, many clinical strains of *K. pneumoniae* are highly resistant to antibiotics, indicating the relative ineffectiveness of current therapy (Timko, 2004). Research over the last 20 years has demonstrated that activation of an inflammatory response is essential to clear *Klebsiella* infections (Greenberger et al., 1996; Standiford et al., 1999; Ye et al., 2001). Furthermore, increasing the response by providing inflammatory mediators decreases morbidity and mortality (Tsai et al., 1998; Standiford et al., 1999). In turn, any exogenous interference with the induction of this response leads to a more severe disease (Laichalk et al., 1996). Strikingly, evidence indicates that *K. pneumoniae* infections are characterized by lacking an early production of cytokines although the molecular bases of these observations are currently unknown (Lawlor et al., 2006).

We have previously tackled the interplay between *K. pneumoniae* and airway epithelial cells by studying the cellular responses activated upon *K. pneumoniae* infection. Epithelial cells play a critical role in the regulation of airway inflammation and immunity, hence acting as the first line of host defence against infections. Our data indicated that wild-type *K. pneumoniae* does not activate a defence response in airway epithelial cells (Regueiro et al., 2006; Moranta et al., 2010). In contrast, infection with a *K. pneumoniae* capsule polysaccharide (CPS) mutant induces the expression of antimicrobial molecules and of IL-8 in a process dependent on the nuclear translocation of NF-κB through TLR2 and TLR4 pathways (Regueiro et al., 2006; Moranta et al., 2010).

In the present study we explored whether wild-type *K. pneumoniae* modulates the activation of inflammatory responses by targeting cellular functions. Here we show that *K. pneumoniae* attenuates IL-8 secretion elicited by pro-inflammatory mediators in human airway epithelial cells. Mechanistically, this anti-inflammatory effect involves inhibition of the NF-κB and MAPKs pathways via upregulation of CYLD and the MAPK phosphatase-1 (MKP-1) in a NOD1-dependent manner.

**Results**

*K. pneumoniae* attenuates pro-inflammatory stimuli-induced IL-8 expression

IL-8 is a multifunctional protein involved in host defence with a prominent role in the recruitment and activation of neutrophils (Hoffmann et al., 2002). Several stimuli including PAMPs and pro-inflammatory cytokines such as IL-1β and TNFα induce the secretion of IL-8 by epithelial cells (Hoffmann et al., 2002). We sought to determine whether wild-type *K. pneumoniae* strain 52145 (hereafter Kp52145) modulates the IL-1β-induced secretion of IL-8 by the human alveolar epithelial cell line A549 and by normal human bronchial epithelial cells (NHBE). As shown in Fig. 1A, Kp52145 reduced IL-1β-induced IL-8 secretion. To generate UV-killed bacteria, a mock infection was carried out, i.e. same infection conditions as Kp52145 but without bacteria. To assess the effect of UV-killed bacteria, we performed the same infection conditions as Kp52145 but with UV-killed bacteria. To assess the effect of UV-killed bacteria, we performed the same infection conditions as Kp52145 but with UV-killed bacteria.

![Fig. 1](image-url) *Klebsiella pneumoniae* attenuates pro-inflammatory stimuli-induced IL-8 expression.

A. ELISA of IL-8 released by A549 or NHBE cells left untreated (CON) or infected for 1 h with Kp52145 (Kp) and then stimulated with IL-1β (50 ng ml⁻¹) for 2 h (n = 5).

B. ELISA of IL-8 released by A549 cells left non-treated (CON) or infected for 1 h and then stimulated for 2 h with TNFα (50 ng ml⁻¹); or Pam3C-SK4 (Pam; 100 pg ml⁻¹) or stimulated for 4 h with non-typable *Haemophilus influenzae* (NTHi; multiplicity of infection 100 bacteria per cell) (n = 4).

C. IL-8 mRNA levels, assessed by RT-qPCR, in A549 cells left untreated (CON) or infected for 2.5 h and then stimulated with IL-1β (1 ng ml⁻¹) for 30 min (n = 3).

D. ELISA of IL-8 released by A549 cells left untreated (CON) or infected for 1 h with Kp52145 (Kp) or UV-killed Kp52145 (UV-Kp) and then stimulated with IL-1β (50 ng ml⁻¹) for 2 h (n = 3). To generate UV-killed bacteria, a mock infection was carried out, i.e. same infection conditions as before, but in a tissue culture well without cells. After 3 h, the bacterial suspension was UV irradiated [1 joule for 3 min in a BIO-LINK BLX cross-linker (Vilber Lourmat)] and used to infect cells.

E. ELISA of IL-8 in the supernatants of A549 cells. Cells were left untreated (CON) or infected for 1 h and then stimulated with IL-1β (50 ng ml⁻¹) for 2 h (n = 3). To obtain bacteria conditioned medium (BCM) a mock infection was carried out and after 3 h, the bacterial suspension was filtered sterilized (0.22 μm; nylon membrane) and used to treat cells. Bacteria–cell conditioned medium (BCCM) was obtained from previously infected cells for 3 h.

F. ELISA of IL-8 released by A549 cells left untreated (CON) or infected for 1 h with Kp52145 (Kp) and then stimulated with IL-1β (50 ng ml⁻¹) for 2 h. Bacteria and cell monolayer were separated by a Transwell insert. IL-1β was added to the upper chamber. Data (A–F) are means and s.e.m. *P* < 0.05 (results are significantly different from the results for untreated cells; one-way ANOVA).
secretion by A549 cells (Fig. 1A, left panel) and NHBE (Fig. 1A, right panel). Next, we explored whether Kp52145 attenuates secretion of IL-8 induced by pro-inflammatory stimuli not signalling via IL-1β. Kp52145 also attenuated TNFα, Pam3CSK4 and non-typable Haemophilus influenzae-induced IL-8 secretion by A549 cells (Fig. 1B). To determine whether the observed effects involved changes in il-8 transcription, real-time quantitative PCR (RT-qPCR) was carried out, and data showed that Kp52145 reduced IL-1β-induced il-8 mRNA levels (Fig. 1C). Analysis by flow cytometry revealed that infected A549 cells expressed amounts of surface molecules and receptors similar to non-infected cells (Fig. S1).

The anti-inflammatory effect triggered by Kp52145 could be abrogated by UV-killing bacteria (Fig. 1D) and was not exerted by either bacteria conditioned medium (BCM) or by bacteria–cell conditioned medium (BCCM) (Fig. 1E). Furthermore, removal of bacteria by washing, followed by 1 h gentamicin treatment, rendered cells responsive to IL-1β (50 ng ml⁻¹) challenge [1140 ± 90 pg ml⁻¹ IL-8 secreted by non-infected cells versus 990 ± 75 pg ml⁻¹ IL-8 secreted by control cells; \( P > 0.05 \); one-way analysis of variance (ANOVA)]. We verified that Kp52145 itself did not inactivate IL-1β. To this end, IL-1β (50 ng ml⁻¹) was incubated with Kp52145 (10⁷ cfu ml⁻¹) for 3 h in tissue culture medium. Bacteria were pelleted (12 000 g, 5 min) and the supernatant used as a source of IL-1β. This bacteria-incubated IL-1β performed in an identical way to non-pre-incubated IL-1β hence inducing secretion of IL-8 (1300 ± 80 and 1100 ± 100 pg ml⁻¹ IL-8 respectively; \( P > 0.05 \) one-way ANOVA). Collectively, these data suggest that Kp52145 anti-inflammatory effect is mediated by bacteria–cell contact. To further explore this effect, Kp52145 and A549 cells were separated by a Transwell insert (0.4 μm pore size, polycarbonate membrane) and IL-1β (50 ng ml⁻¹) was added to the upper compartment containing bacteria. As shown in Fig. 1F, the Kp52145 anti-inflammatory effect was abrogated.

Taken together, these findings demonstrate that Kp52145 attenuates the induction of IL-8 elicited by pro-inflammatory stimuli in a process requiring alive bacteria and bacteria–cell contact.

K. pneumoniae affects NF-κB activation by upregulating the expression of CYLD

We next aimed to identify the cellular functions targeted by Kp52145 to exert the observed anti-inflammatory effect. Given that it is known that NF-κB activation is associated with IL-8 expression (Hoffmann et al., 2002), we analysed the effect of Kp52145 on the NF-κB activation pathway, by studying the activation of a reporter construct controlled by synthetic NF-κB response elements. Klebsiella inhibited the IL-1β and TNFα-dependent activation of the reporter construct (Fig. 2A). Immunolocalization of the NF-κB p65 subunit in A549 cells revealed that Kp52145 prevented IL-1β- and TNFα-induced nuclear translocation of the p65 subunit (Fig. 2B). In the canonical NF-κB activation pathway, nuclear translocation of NF-κB is preceded by phosphorylation and subsequent degradation of IκBα (Hayden and Ghosh, 2008). We analysed the levels of IκBα in cell extracts by immunoblot. Infection of A549 cells with Kp52145 before IL-1β challenge resulted in IκBα stabilization in contrast to its nearly complete degradation induced by IL-1β (Fig. 2C). In summary, these data indicate that Kp52145 inhibits cytokine-dependent activation of NF-κB by altering IκBα degradation.

Having established that Kp52145 blocks the activation of the NF-κB pathway we sought to identify the underlying molecular mechanisms. Ubiquitination is a key event in the regulation of NF-κB signalling (Sun, 2008; Skaug et al., 2009). In fact, ubiquitination of proteins belonging to the TNF receptor-associated factor family (TRAF) is essential for signal transduction leading to phosphorylation of IκBα (Sun, 2008; Skaug et al., 2009). Given that ubiquitination of TRAF6 is a necessary event for IL-1β-dependent activation of NF-κB (Hacker and Karin, 2006), we asked whether Kp52145 affects IL-1β-induced ubiquitination of TRAF6. Figure 3A shows that IL-1β-induced ubiquitination of TRAF6 was reduced in cells infected with Kp52145.

Recent evidence indicates that deubiquitinating enzymes regulate the activation of innate immune responses (Sun, 2008). CYLD, originally identified as a tumour suppressor, is a deubiquitinating enzyme that targets members of the TRAF family and acts as a negative regulator of NF-κB (Trompouki et al., 2003). We explored whether Kp52145 downregulates the NF-κB pathway by inducing the expression of CYLD. RT-qPCR analysis showed that Kp52145 induced the expression of cyld in both A549 and NHBE cells (Fig. 3B). Western blot analysis demonstrated that Kp52145 increased the expression of CYLD in both cell types (Fig. 3C). Next, we investigated whether CYLD could play a role in Kp52145-triggered block of NF-κB activation by using small interfering RNA (siRNA) targeting CYLD. In CYLD knock-down cells, Kp52145 did not inhibit IL-1β-dependent activation of the NF-κB reporter construct (Fig. 3D). Finally, we asked whether Kp52145 inhibits the ubiquitination of TRAF6 via CYLD. As shown in Fig. 3E, Kp52145 did not affect IL-1β-induced ubiquitination of TRAF6 in CYLD knock-down cells.

Collectively, our data demonstrate that Kp52145 blocks the activation of NF-κB signalling pathway by upregulating the expression of CYLD.

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K. pneumoniae affects MAPKs phosphorylation via activation of MKP-1, a MAPK phosphatase

Pro-inflammatory stimuli also activate MAPKs which are considered important regulators of gene expression including IL-8 (Hoffmann et al., 2002). The activation of the three MAPKs p38, JNK and p44/42 occurs through phosphorylation of serine and threonine residues. IL-1β is known to be a potent inducer of MAPKs’ phosphorylation (O’Neill, 2000). We asked whether Kp52145 affects IL-1β-induced phosphorylation of p38, p44/42 and JNK. Western blot analysis showed that infection with Kp52145 reduced IL-1β-induced phosphorylation of the three MAPKs in both A549 and NHBE cells (Fig. 4A and B respectively). These findings led us to explore whether Kp52145 upregulates the expression of inhibitors of MAPKs’ phosphorylation. Evidence indicates that MAPK phosphatases are pivotal in the regulation of immune responses by deactivating MAPKs (Liu et al., 2007). Several studies highlight the importance of the phosphatase MKP-1 in the attenuation of MAPKs activities (Liu et al., 2007). Therefore, we hypothesized that Kp52145-triggered reduced phosphorylation of MAPKs could be mediated by MKP-1. Consistent with this, we observed that Kp52145 increased the expression of m kp-1 and MKP-1 levels in both A549 and NHBE cells (Fig. 4C and D). To study the role of MKP-1 in Kp52145-mediated reduced phosphorylation of MAPKs, mkp-1 was knocked down using siRNA. In MKP-1 knock-down cells; Kp52145 did not affect IL-1β-induced phosphorylation of MAPKs (Fig. 4E).

In summary, our findings indicate that Kp52145 inhibits IL-1β-dependent activation of MAPKs by upregulating the expression of MKP-1, which in turn leads to dephosphorylation of p38, p44/42 and JNK MAPKs.

K. pneumoniae attenuates IL-1β-induced IL-8 secretion by upregulating the expression of CYLD and MKP-1

Having established that Kp52145 blocks the activation of NF-κB and MAPKs pathways by upregulating the
Fig. 3. *Klebsiella pneumoniae* affects NF-κB activation via CYLD.

A. Immunoblot analysis of ubiquitinated TRAF6 and TRAF6 in lysates of A549 cells left untreated, stimulated with IL-1β (20 ng ml⁻¹) for the indicated times, infected for 3 h, or infected for 3 h and then stimulated with IL-1β, assessed after immunoprecipitation of TRAF6. Data are representative of four independent experiments.

B. *cyld* mRNA levels, assessed by RT-qPCR, in A549 or NHBE cells left untreated (CON) or infected for the indicated times (data are means and s.e.m.; n = 3). *P < 0.05 (results are significantly different from the results for untreated cells; one-way ANOVA).

C. Immunoblot analysis of CYLD levels in A549 or NHBE cells left uninfected (CON) or infected for the indicated times. Data are representative of three independent experiments.

D. Activation of a NF-κB luciferase reporter plasmid in A549 cells transfected with either control or CYLD siRNA, which were left untreated or infected for 1 h and then stimulated for 4 h with IL-1β (20 ng ml⁻¹). Activity is normalized by correction of Renilla expression and is presented relative to the untreated cells (data are means and s.e.m.; n = 3). *P < 0.05 (one-way ANOVA).

E. Immunoblot analysis of ubiquitinated TRAF6 and TRAF6 in lysates of A549 cells transfected with either control or CYLD siRNA, which were left untreated, stimulated with IL-1β (20 ng ml⁻¹) for 30 min, infected for 3 h, or infected for 3 h and then stimulated with IL-1β, assessed after immunoprecipitation of TRAF6. Data are representative of five independent experiments.

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Fig. 4. *Klebsiella pneumoniae* affects MAPKs’ phosphorylation via activation of MKP-1.

A and B. Immunoblot analysis of phospho p38 (P-p38), phospho p44-42 (P-p44-42), phospho JNK (P-JNK) and tubulin levels in lysates of A549 or NHBE cells left untreated (CON), stimulated with IL-1β (1 ng ml⁻¹) for the indicated times, infected for 3 h (Kp), or infected for 3 h and then stimulated with IL-1β as before. Data are representative of three independent experiments.

C. *mkp-1* mRNA levels, assessed by RT-qPCR, in A549 or NHBE cells left untreated (CON) or infected for the indicated times (data are means and s.e.m.; n = 3). *P < 0.05 (results are significantly different from the results for untreated cells; one-way ANOVA).

D. Immunoblot analysis of MKP-1 levels in A549 or NHBE cells left uninfected (CON) or infected for the indicated times. Data are representative of three independent experiments.

E. Immunoblot analysis of phospho MAPKs and tubulin levels in lysates of A549 cells transfected with either control or MKP-1 siRNA, which were left untreated (CON), stimulated with IL-1β (1 ng ml⁻¹) for the indicated times, infected for 3 h (Kp), or infected for 3 h and then stimulated with IL-1β as before. Data are representative of three independent experiments.
expression of CYLD and MKP-1, we sought to determine the involvement of both proteins in Kp52145-induced anti-inflammatory effect. In MKP-1 knock-down cells by siRNA, Kp52145 attenuated partially IL-1β-dependent induction of IL-8 (Fig. 5). Similarly, knock-down of CYLD by siRNA also reduced Kp52145-triggered attenuation of IL-1β-induced IL-8 secretion (Fig. 5). In cells knock-down for both CYLD and MKP-1, Kp52145 did not reduce IL-1β-induced IL-8 levels (Fig. 5).

K. pneumoniae induces CYLD and MKP-1 in vivo

We asked next whether Kp52145 induces the expression of CYLD and MKP-1 in the lungs of infected mice. To this end, mice were infected intranasally and mRNA expression of cyld and mkp-1 was measured by RT-qPCR at 6, 12 and 24 h post infection. Results displayed in Fig. 6 show that both genes were upregulated in the lungs of infected mice. Furthermore, the expression of both genes...
increased over time, being significantly higher at 24 h than at 6 h post infection (Fig. 6A and B). We also measured the expression of the CXC chemokine KC in the lungs of infected mice. It has been shown that high KC lung levels result in increased *K. pneumoniae* clearance and improved survival (Tsai *et al*., 1998). Analysis by RT-qPCR revealed that Kp52145 induced the expression of *kc* in the lungs of infected mice; however, the levels of *kc* at 24 h post infection were 10-fold lower than at 6 h post infection (Fig. 6C). Of note, the reduction of *kc* expression correlated with the increased expression of *cyld* and *mkp-1*.

**K. pneumoniae-induced anti-inflammatory effect relies on the activation of NOD1**

We investigated the potential contribution of TLRs-dependent pathways to Kp52145-induced anti-inflammation. Almost all TLRs activate cellular signalling pathways through TIR domain-mediated interactions with the adaptor molecule MyD88 (Kenny and O’Neill, 2008). Therefore, the involvement of TLRs in Kp52145-induced anti-inflammatory effect can be examined by reducing the expression of the MyD88 adaptor molecule using siRNA.

TNFα was used as a pro-inflammatory stimulus because MyD88 is essential for IL-1β-dependent induction of IL-8 expression (O’Neill, 2000). After siRNA knock-down of MyD88, Kp52145 was still able to attenuate TNFα-induced IL-8 expression (Fig. 7A). These data suggested that there is no role for MyD88-dependent TLR signalling in Kp52145 anti-inflammatory effect. However, considering that TLR4 is also able to signal through the protein adaptor TRIF (Kenny and O’Neill, 2008), we asked whether TLR4 participates in Kp52145-elicited anti-inflammation. Kp52145 still inhibited TNFα-induced IL-8 expression in TLR4 knock-down cells by siRNA (Fig. 7A).

Another PRR constitutively expressed by airway epithelial cells is NOD1 (Girardin *et al*., 2001; Hisamatsu *et al*., 2003; Inohara and Nunez, 2003; Strober *et al*., 2006). To determine whether NOD1 is involved in Kp52145-induced anti-inflammatory effect, siRNA was used to knock-down its expression. Kp52145 did not reduce IL-1β-dependent IL-8 expression in NOD1 knock-down cells (Fig. 7B). Similar results were obtained using two different siRNA (Fig. 7B). Kp52145 increased IL-8 expression in NOD1 knock-down cells in contrast to cells transfected with control siRNA (Fig. 7B). Furthermore, Kp52145 did not inhibit IL-1β-induced phosphorylation of MAPKs in cells in which nod1 was knocked-down by using siRNA (Fig. 7C). In addition, Kp52145 did not block IL-1β-dependent activation of the NF-κB reporter construct in NOD1 knock-down cells (Fig. 7D). The efficiency of siRNA-mediated downregulation of target gene mRNA levels was confirmed by RT-qPCR (Fig. S2).

Having established the involvement of CYLD-, MKP-1- and NOD1-dependent signalling on Kp52145-triggered anti-inflammatory effect, we questioned whether Kp52145 upregulates the expression of CYLD and MKP-1 through NOD1. Kp52145 did not increase the expression of both CYLD and MKP-1 in NOD1 knock-down cells in contrast to cells transfected with control siRNA (Fig. 7E).

Altogether, these data show that Kp52145-triggered anti-inflammatory effect relies on NOD1-dependent expression of both CYLD and MKP-1.

**Rac1 inhibition triggers NOD1-mediated CYLD and MKP-1 expression**

Considering that NOD1 recognizes PGN (Chamaillard *et al*., 2003; Inohara *et al*., 2005), one could speculate that *K. pneumoniae* PGN mediates the anti-inflammatory effect. Yet, *K. pneumoniae* PGN does not differ from PGNs expressed by other *Enterobacteriaceae* (Botta and Buffa, 1981) known to activate inflammatory responses in a NOD1-dependent manner. On the other hand, evidence suggests that actin cytoskeletal reorganization may contribute to the activation of members of the NLR family (Legrand-Poels *et al*., 2007; Eitel *et al*., 2008; Kufer *et al*., 2008). Furthermore, inhibition of the Rho family GTPase Rac1 is associated to activation of NOD2, a close homologue of NOD1 (Eitel *et al*., 2008). Therefore, we hypothesized that *K. pneumoniae* may negatively modulate Rac1 activity to contribute to NOD1-mediated induction of CYLD and MKP-1.

To pursue this notion, we explored whether *K. pneumoniae* inhibits Rac1 activation by analysing the amount of activated protein bound to the GST-PAK Rac1 interaction binding site. Pull-down assays of activated Rac1 revealed that the levels of Rac1-GTP were lower in Kp52145 infected cells than in control cells (Fig. 8A). Moreover, Kp52145 also inhibited PMA-dependent Rac1 activation (Fig. 8A). To further sustain the notion that *K. pneumoniae* inhibits Rac1 activation, we questioned whether Kp52145 affects *Yersinia enterocolitica* invasion of epithelial cells. It is firmly established that *Y. enterocolitica* invasion is dependent on Rac1 activation (Wong and Isberg, 2005). Indeed, a 99% reduction in *Y. enterocolitica* invasion was observed in Kp52145 infected cells (Fig. S3). Control experiments showed that *Y. enterocolitica* adhesion was not significantly different between control cells or *Klebsiella* infected cells (Fig. S3).

Rac1 inhibition perturbs membrane recruitment of NOD2, a close homologue of NOD1 (Eitel *et al*., 2008). To test whether this is also the case for NOD1, HEK293T cells were transiently transfected with a plasmid containing HA-tag NOD1 and the subcellular location of NOD1 was assessed by Western blot. NSC23766 inhibits the generation of Rac1-GTP (Gao *et al*., 2004). This inhibitor...
Fig. 7. *Klebsiella pneumoniae*-induced anti-inflammatory effect relies on the activation of NOD1.

A and B. *il-8* mRNA levels, assessed by RT-qPCR, in A549 cells transfected with control, MyD88, TLR4 or NOD1 (two different ones) siRNAs which were left untreated, stimulated with cytokines (IL-1β, 1 ng ml⁻¹; TNFα, 20 ng ml⁻¹) for 30 min or infected for 2.5 h and then stimulated with the indicated cytokines (*n* = 3) (data are means and s.e.m.).

C. Immunoblot analysis of phospho MAPKs and tubulin levels in lysates of A549 cells transfected with either control or NOD1 siRNA (NOD1#1), which were left untreated (CON), stimulated with IL-1β (1 ng ml⁻¹) for the indicated times, infected for 3 h (Kp), or infected for 3 h and then stimulated with IL-1β. Data are representative of three independent experiments.

D. Activation of a NF-κB luciferase reporter plasmid in A549 cells transfected with either control or NOD1 siRNA (NOD1#1), which were left untreated or infected for 1 h and then stimulated for 4 h with IL-1β (20 ng ml⁻¹). Activity is normalized by correction of Renilla expression and is presented relative to the cells untreated (*data are means and s.e.m.; *n* = 3).

E. Immunoblot analysis of CYLD, MKP-1 and tubulin levels in A549 cells transfected with either control or NOD1 siRNA (NOD1#1), which were left uninfected (CON) or infected for the indicated times. Data are representative of three independent experiments.
Fig. 8. Rac1 inhibition triggers NOD1-mediated CYLD and MKP-1 expressions.

A. Rac1 activation, detected by the amount bound to the GST-PAK Rac1 interaction binding site, was determined in A549 cells left uninfected (CON), infected for 90 or 120 min, stimulated with PMA (100 nM) for 40 min, or infected for 90 or 120 min and then stimulated with PMA. Tubulin Western blot was used as a loading control before the pull-down ($n = 3$).

B. Western blot analysis of cytosol (C) and membrane (M) fractions from HEK293T cells transfected with HA-NOD1 plasmid which were left untreated (CON), treated with Rac1 inhibitor NSC23766 (4 h, 200 μM final concentration), NOD1 agonist M-Tri-DAP (40 min, 10 μg ml$^{-1}$) or infected with Kp52145 (Kp) for 3 h. NOD1 was revealed using anti-HA antibody (HA-NOD1); flotillinE 2 and tubulin are used as markers of the fractions.

C. Immunoblot analysis of CYLD, MKP-1 and tubulin levels in A549 cells which were left uninfected (CON), treated with Rac1 inhibitor NSC23766 (200 μM) for the indicated hours or infected with Kp52145 (Kp). Data are representative of three independent experiments.

D. Immunoblot analysis of CYLD, MKP-1 and tubulin levels in A549 cells transfected with either control or NOD1 siRNA (NOD1#1), which were left uninfected (CON), treated with Rac1 inhibitor NSC23766 (200 μM) for the indicated hours or infected with Kp52145 for 3 h (Kp). Data are representative of three independent experiments.

E. ELISA of IL-8 secreted by A549 cells which were left untreated (CON) or treated with Rac1 inhibitor NSC23766 (200 μM) for the indicated hours and then stimulated with IL-1β (50 ng ml$^{-1}$) for 2 h (data are means and s.e.m.; $n = 3$). *P < 0.05 (one-way ANOVA).

F. ELISA of IL-8 secreted by A549 cells transfected with either control, MKP-1 or CYLD siRNAs, which were left untreated (CON) or treated with Rac1 inhibitor NSC23766 (200 μM) for 2 h and then stimulated with IL-1β (50 ng ml$^{-1}$) for 2 h (data are means and s.e.m.; $n = 3$). *P < 0.05 (one-way ANOVA).
does not affect the activity of endogenous Cdc42 or RhoA (Gao et al., 2004). NSC23766 decreased membrane association of NOD1 compared with untreated cells (Fig. 8B). Consistent with Rac1 inhibition by Kp52145, infection also decreased NOD1 membrane association. M-Tri-DAP, a NOD1 agonist, did not affect membrane association of NOD1 (Fig. 8B).

We next examined whether Rac1 inhibition could be sufficient to induce the expression of CYLD and MKP-1. Western blot analysis showed that NSC23766 induced the expression of CYLD and MKP-1 in A549 cells (Fig. 8C). Furthermore, NSC23766-induced CYLD and MKP-1 expression is dependent on NOD1 because this inhibitor did not increase the expression of CYLD and MKP-1 in NOD1 knock-down cells (Fig. 8D). These findings prompted us to evaluate whether Rac1 inhibition reduces IL-1β-dependent induction of IL-8 secretion. Indeed, NSC23766 attenuated IL-1β-induced IL-8 levels (Fig. 8E). Moreover, knock-down of MKP-1 or CYLD by siRNA reduced NSC23766-triggered attenuation of IL-1β-induced IL-8 (Fig. 8F).

In summary, our findings suggest that Kp52145-triggered anti-inflammatory effect is dependent on Rac1 inhibition which in turn induces NOD1-mediated CYLD and MKP-1 expression.

K. pneumoniae capsule polysaccharide is necessary but not sufficient to attenuate pro-inflammatory stimuli-induced IL-8 expression

K. pneumoniae CPS is recognized as an important virulence factor. We have previously shown that 52K10, an isogenic capsule polysaccharide mutant from Kp52145 (Cortes et al., 2002), activates an inflammatory programme in airway epithelial cells (Regueiro et al., 2006). To explore whether CPS could account for Kp52145 anti-inflammatory effect we determined the effect of 52K10 on IL-1β-induced IL-8 expression by A549 cells. Data obtained by RT-qPCR showed that 52K10 did not significantly reduce IL-1β-induced IL-8 mRNA levels (Fig. 9A). Furthermore, 52K10 did not significantly attenuate IL-1β-induced IL-8 secretion by A549 cells (Fig. 9B). These results might indicate that CPS could mediate the observed Kp52145 anti-inflammatory effect. However, purified CPS (up to 600 μg ml⁻¹) did not reduce IL-1β-induced IL-8 secretion (Fig. 9C). Furthermore, purified CPS did not induce the expression of either CYLD or MKP-1 (Fig. 9D) and it did not activate NF-κB-dependent reporter activity in NOD1-expressing HEK293T cells (Fig. 9E). Addition of Fugene HD, a transfection reagent, together with CPS did not lead to the activation of NF-κB-dependent reporter activity (data not shown).

Taken together it can be concluded that CPS is necessary but not sufficient for Kp52145 anti-inflammatory effect.

Discussion

In the present study, we provide evidence that K. pneumoniiae dampens the activation of inflammatory responses by targeting NF-κB and MAPKs pathways, via upregulation of CYLD and MKP-1. Our data show that, in order to exert this anti-inflammatory effect, Kp52145 engages a NOD1-dependent pathway by inhibiting Rac1 activation. Collectively, these findings unveil a previously unknown strategy employed by a human pathogen to counteract host defence mechanisms.

Inflammation is a key element of the infected host response against pathogens (Barton, 2008). In turn, pathogens try to counteract this fast-acting response to survive during the early stages of infection. One of the best-characterized strategies is the avoidance of detection by PRRs (Finlay and McFadden, 2006; Roy and Mocarski, 2007). For example, pathogens modify their LPS lipid A to evade detection by TLR4 (Miller and Mocarski, 2007). Another strategy is the disruption of cellular communications by targeting NF-κB and MAPKs signalling pathways (Finlay and McFadden, 2006; Roy and Mocarski, 2007), which in most cases is achieved by injecting protein effectors via a type III secretion system.

The evidence provided in this study describes a novel strategy utilized by bacteria to subvert the activation of inflammatory responses. K. pneumoniiae upregulates the expression of two proteins, CYLD and MKP-1, which cells normally activate to return to homeostasis after an inflammatory response (Liu et al., 2007; Sun, 2008). Our data support the notion that Klebsiella increases the transcription of both genes; however, it cannot be ruled out that post-transcriptional mechanisms could be operating as well. In vitro and in vivo studies suggest that CYLD and MKP-1 may protect the host from an overwhelming inflammatory response by terminating the activation of NF-κB and MAPKs-dependent signalling pathways respectively (Chi et al., 2006; Zhao et al., 2006; Lim et al., 2007; 2008; Liu et al., 2007). Then, K. pneumoniiae-induced expression of CYLD and MKP-1 could be explained as part of a normal cell response to avoid a Klebsiella-dependent overactive inflammatory response. However, this explanation seems unlikely given the fact that wild-type K. pneumoniiae does not induce the secretion of inflammatory mediators by airway cells (this work and Regueiro et al., 2006). Therefore, based on the presented data, we propose that K. pneumoniiae hijacks two host systems used by the cells to return to homeostasis in order to actively block the activation of inflammatory responses. We put forward the notion that manipulation of systems...
dedicated to control the host immune balance represents an example of a novel theme employed by pathogens to subvert cell innate immunity.

Another important finding of our study is that *K. pneumoniae* engages a PRR to exert its anti-inflammatory effect. This strategy is unusual and only one example has been reported. *Yersinia* species secrete a protein, LcrV, which signals in a CD14-TLR2-dependent manner to induce IL-10 secretion and hence immunosupression (Sing et al., 2002). To the best of our knowledge *K. pneumoniae* is the first pathogen hijacking NOD1 to block the activation of inflammatory responses. Moreover, available evidence implicates only TLRs in the expression of CYLD and MKP-1 (Yoshida et al., 2005; Chi et al., 2006), being this the first study showing a role for a NLR receptor in the expression of both proteins.

Evidence indicates that extracellular bacteria, like *K. pneumoniae*, may engage NOD1 by delivering PGN to the

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**Fig. 9. Klebsiella pneumoniae** capsule mutant does not attenuate IL-1β-induced IL-8 expression.

A. *il-8* mRNA levels, assessed by RT-qPCR, in A549 cells left untreated (CON) or infected for 2.5 h and then stimulated with IL-1β (1 ng ml⁻¹) for 30 min (n = 3).
B. ELISA of IL-8 released by A549 cells left non-treated (CON) or infected with strain 52K10 for 1 h and then stimulated for 2 h with IL-1β (50 ng ml⁻¹).
C. ELISA of IL-8 released by A549 cells left non-treated (CON) or treated with different amounts of purified Kp52145 capsule for 1 h and then stimulated for 2 h with IL-1β (50 ng ml⁻¹). Data are means and s.e.m. (n = 3). *P < 0.05 (results are significantly different from the results for untreated cells; one-way ANOVA).
D. Immunoblot analysis of CYLD, MKP-1 and tubulin levels in A549 cells which were left uninfected (CON), treated with purified Kp52145 capsule (CPS, 300 µg ml⁻¹) for the indicated hours or infected for 3 h (Kp). Data are representative of three independent experiments.
E. Activation of a NF-κB luciferase reporter plasmid in HEK293T cells transfected with HA-NOD1 plasmid, which were left untreated (white bar), or treated with M-Tri-DAP (grey bars; 5 or 10 µg ml⁻¹) for 3 h or treated with different amounts of purified Kp52145 capsule (black bars; from 10 to 150 µg ml⁻¹) for 3 h. Activity is normalized by correction of *Renilla* expression and is presented relative to untreated cells (data are means and s.e.m.; n = 3). *P < 0.05 (results are significantly different from the results for untreated cells; one-way ANOVA).
cytoplasm using either type IV or type III secretion systems (Viala et al., 2004; Travassos et al., 2005). However, the fact that K. pneumoniae PGN does not differ from other PGNs activating inflammatory responses and that in silico analysis of the available K. pneumoniae genomes does not support that this pathogen encodes type III or IV secretion systems makes unlikely that K. pneumoniae engagement of NOD1 would be PGN-dependent. As an alternative possibility, we examined the role of Rac1 in K. pneumoniae induced NOD1 signalling. This was based on three considerations: (i) several pathogens manipulate the activation state of Rac1, (ii) Rac1 has been implicated in different inflammatory pathways, and (iii) Rac1 mediates actin cytoskeletal reorganization which in turn modulates the activation of NOD2 and NOD1 (Legrand-Poels et al., 2007; Eitel et al., 2008; Kufer et al., 2008). By pull-down assays detecting the activated form of Rac1 and by functional assays based on testing the effect of K. pneumoniae infection on the Rac1-dependent Y. enterocolitica invasion, we show that Kp52145 inhibits the activation of Rac1. To connect Rac1 inhibition and the NOD1-dependent anti-inflammatory phenotype, we took advantage of the well-characterized Rac1 chemical inhibitor NSC23766 and explored whether the inhibitor may recapitulate the features of Klebsiella-triggered anti-inflammation. Indeed, NSC23766 attenuated IL-1β-induced IL-8 levels via upregulation of CYLD and MKP-1 in a NOD1-dependent manner. On the whole, our findings are consistent with a model in which K. pneumoniae, by negatively modulating Rac1, engages NOD1 to activate the expression of CYLD and MKP-1.

We further confirmed previous findings showing the membrane association of NOD1 (Kufer et al., 2008). Moreover, and similar to NOD2 (Eitel et al., 2008), Rac1 inhibition decreases membrane location of NOD1 (this work). In agreement with Kp52145 inhibition of Rac1 activation, Kp52145 infection was also associated with a decreased membrane association of NOD1. These findings do not necessarily contradict recent reports showing that membrane recruitment of NOD1 is crucial for NOD-mediated signalling as shown for Shigella flexneri (Kufer et al., 2008). It should be noted that, in contrast to wild-type K. pneumoniae, Shigella activates Rac1 and invades tissue culture cells (Tran Van Nhu et al., 1999). On the other hand, Rac1 inhibition induces NOD1- and NOD2-mediated signalling (Eitel et al., 2008 and this work) and for NOD2 it has been postulated that release of the protein from cytoskeletal structures may allow more efficient signalling due to a reduction of NOD2 interaction with its negative regulator Erbin (Kufer et al., 2006; Eitel et al., 2008). Therefore, a tantalizing hypothesis could be that membrane location is not essential for NOD1 activation but only crucial for recognition of invading bacteria. Additional work is needed to further clarify the impact of NOD1’s membrane recruitment on its activation level and the type of responses under different situations of Rac1 activation induced by different bacterial infections.

Having deciphered intracellular signalling mechanisms responsible for the anti-inflammatory phenotype we were keen to identify the bacterial system(s) involved. We first focused on the possible involvement of CPS because we and others have shown that K. pneumoniae CPS mutants activate an inflammatory program in contrast to wild-type strains (Lawlor et al., 2006; Regueiro et al., 2006; Moranta et al., 2010). Here we have shown that strain 52K10 did not attenuate IL-1β-induced IL-8 secretion by A549 cells. However, purified CPS did not reduce IL-1β-induced IL-8 secretion, it did not induce the expression of CYLD and MKP-1 and it did not activate NOD1 using a standard activation assay. Taken together, these results argue against a direct role of CPS in the anti-inflammatory phenotype. In turn, recently we have shown that presence of the CPS on the bacterial surface interferes with TLR signalling (Regueiro et al., 2006; Moranta et al., 2010). Interestingly, this seems to be a feature of bacterial CPSs because Salmonella typhi and Neisseria meningitidis CPSs also inhibit TLR-dependent signalling (Kocabas et al., 2007; Wilson et al., 2008). Therefore, we propose that the reduced inflammatory response characteristic of K. pneumoniae infections is the sum of the CPS-mediated inhibition of TLR signalling and the NOD1-dependent anti-inflammatory effect described in this study. This model implies that TLR-dependent inflammatory signal overcome the NOD1-dependent anti-inflammatory signal. Supporting this, IL-8 secretion induced by 52K10 is TLR-dependent (Regueiro et al., 2006). Future studies will aim to identify K. pneumoniae factor(s) required to attenuate inflammatory responses in a NOD1-dependent manner.

It should be pointed out that the findings reported in this study do not contradict two studies from our group (Regueiro et al., 2008; Moranta et al., 2010). In a first study, we showed that LPS-induced IL-8 secretion is increased in A549 cells infected for 5 h with Kp52145 compared with non-infected cells (Regueiro et al., 2008). Note that in the experimental set-up, bacteria were washed off because otherwise cells would not respond to LPS (Regueiro et al., 2008; V. Regueiro and J.A. Bengoechea; unpubl. data). This is in agreement with the findings reported here demonstrating, on one hand, that Kp52145 blocking effect was dependent on bacteria–cell contact and, on the other hand, that removal of bacteria by washing followed by gentamicin treatment rendered cells responsive to IL-1β. In a second study, we showed that strain 52145ΔwcaC2, a cps mutant derived from Kp52145, does induce the expression of CYLD and MKP-1 which in turn acted as negative regulators for 52145ΔwcaC2-induced...
expression of human β-defensins (Moranta et al., 2010). Interestingly, 52145-ΔwcaO engaged TLR-dependent pathways to activate the expression of human β-defensins, CYLD and MKP-1 (Moranta et al., 2010). As discussed before, these results are consistent with the concept that activation of an inflammatory programme also triggers the induction of negative regulatory proteins to return to homeostasis. The fact that Kp52145 did induce the expression of CYLD and MKP-1 led us to postulate that wild-type bacteria could target cellular functions controlling the expression of CYLD and MKP-1 to block host defence responses (Moranta et al., 2010). Findings reported here give experimental support to this notion.

Finally, it is worthwhile commenting on the clinical implications of our findings. A wealth of evidence clearly indicates the importance of the inflammatory response in clearing K. pneumoniae infections (Greenberger et al., 1996; Standiford et al., 1999; Ye et al., 2001). Conversely, these data suggest that K. pneumoniae limits the induction of these defence responses. Our in vivo data give experimental support to this hypothesis. We found a reduction in the levels of the pro-inflammatory cytokine kc in the lungs of infected mice associated with an increased expression of cyld and mkp-1. Therefore, we reason that in the onset of pneumonia K. pneumoniae does induce the expression of CYLD and MKP-1 to dampen host defence inflammatory responses. It is tempting to postulate that treatments designed to interfere with this Klebsiella-induced response could be the basis of new therapeutic strategies to treat K. pneumoniae pneumonias. It is important to note that glucocorticoids, widely used anti-inflammatory drugs to treat respiratory diseases including pneumonia, induce the expression of MKP-1, which is one of the molecular mechanisms underlying their anti-inflammatory effect (O’Neill, 2008). Therefore in the context of K. pneumoniae infections, setting of pneumonia would be facilitated by the treatment with glucocorticoids because, on one hand, they activate the expression of the same mechanism hijacked by Klebsiella and, on the other hand, they reduce the inflammatory response necessary to clear the infection. In agreement with this notion, a number of clinical studies already point out that glucocorticoid therapy is associated with a higher risk of pneumonia (Drummond et al., 2008), thereby casting doubts on the use of these drugs to treat pneumonias, particularly those caused by Klebsiella.

**Experimental procedures**

**Bacterial strains, growth conditions and reagents**

*Klebsiella pneumoniae* 52145 is a clinical isolate (serotype O1:K2) previously described (Nassif et al., 1989). *K. pneumoniae* 52K10 is an isogenic capsule mutant from strain 52145 (Cortes et al., 2002). *Y. enterocolitica* strain 8081-c R-M (serotype O:8) is a restriction minus mutant derived from 8081-c which lacks the pYV virulence plasmid (Zhang and Skurnik, 1994). Bacteria were grown in Luria–Bertani medium at 37°C (*Klebsiella* strains) or at 21°C (*Yersinia*). Recombinant human IL-1β and TNFα were purchased from Peprotech; NSC23766 was purchased from Calbiochem; Pam3CSK4 and M-TriDAP were purchased from InvivoGen.

**Cell culture and infection**

Monolayers of A549 (ATCC CCL185) and NHBE (Lonza) cells were grown as previously described (Regueiro et al., 2006). HEK293T (ATCC CRL-11268) cells were grown in DMEM tissue culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics (penicillin, streptomycin and amphotericin B) in 25 cm² tissue culture flasks at 37°C in a humidified 5% CO₂ atmosphere. For infections, A549 cells were seeded to 90% confluence (2 × 10⁶ cells well⁻¹) in 24-well tissue culture plates. Cells were serum starved 16 h before infection. For NHBE cells, cells were seeded to 80% confluence (8 × 10⁴ cells well⁻¹) in collagen coated 24-well tissue culture plates using 1 ml of Bronchial Epithelial cell Growth Medium (Lonza) per well. Bacteria were prepared as described (Regueiro et al., 2006; 2008) and infections were performed using a multiplicity of infection of 150 bacteria per cell, unless otherwise indicated. To synchronize infection, plates were centrifuged at 200 g during 5 min. Cell viability was assessed by trypan blue dye exclusion and it was >95% even after 4 h of infection.

**IL-8 stimulation assay**

IL-8 in the supernatants from stimulated cells was determined by ELISA using a commercial kit (Endogen) with a sensitivity <2 pg ml⁻¹.

**RT-qPCR**

Cells seeded into 60 mm tissue culture dishes were infected as described above. After infection, cells were washed with PBS and total RNA was purified using a Nucleospin RNAII kit (Macherey-Nagel) as recommended by the manufacturer. cDNA was obtained by retrotranscription of 2 μg of total RNA using a commercial RT² First Strand kit as recommended by the manufacturer (Superarray Bioscience Corporation). The reaction included one step to eliminate traces of genomic DNA. Real-time PCR (RT-qPCR) analyses were performed with a Smart Cycler real-time PCR instrument (Cepheid, Sunnyvale, CA). To amplify human *il-8*, *cyld* and *mkp-1* 200 ng of cDNA was used as template in a 25 μl reaction mixture containing 1× SYBR green RT² qPCR Master Mix (Superarray Bioscience Corporation) and primer mix. Human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin were amplified as controls using 100 ng of cDNA as template. The following intron-spanning primers were used: [GAPDH] sense (5’-GAAGATGGTGATGGGATTTC-3’) and antisense (5’-GAAGTATGGTGATGGGATTCC-3’); [Actin] sense (5’-AGAAATCTGGCCACACCAC-3’) and antisense (5’-GGGTTGTGAAGGTCTCCAAA-3’); [CYLD] sense (5’-TATGGGAAGGA CGATTCTGC-3’) and antisense (5’-CTGCCTTTCAGTTTCTTGCC-3’); [MKP-1] sense (5’-GCTTGCAGCAAAACAGTCCA-3’) and antisense (5’-CGATTTAGTCCCTCATAAGGTA-3’). To amplify...
*il-8* the Hs_IL8-1_SG QuantiTect Primer Assay (Qiagen; catalogue number QT00000322) were used. To amplify mouse *kc*, *cyld* and *mkp-1* 400 ng of cDNA was used whereas 200 ng of cDNA was used to amplify mouse actin and mouse *gadph*. The following primers were used: [mouse actin] sense (5′-TGT TACCAACTGGGACGACA-3′) and antisense (5′-CTGGGGCACT TCTTTCAAGG-3′); [mouse GAPDH] sense (5′-CCCAT ACTACAAATGGGG-3′) and antisense (5′-CTTCCCAAAATG CCAAAGTT-3′); [KC] sense (5′-GACAGACTGCTGATGGGCA-3′) and antisense (5′-TGCACTTTCTTTGCGCAAC-3′); [mouse CYLD] (5′-CTGGGTGTCTTTGAGGACGACA-3′) and antisense (5′-CTGGTGGAGCGAGGTTTCTGC-3′) and antisense (5′-CCCAT ACTACAAATGGGG-3′) and antisense (5′-CTTCCCAAAATG CCAAAGTT-3′). The thermocycling protocol was as follows: 95°C for 15 min for hot-start polymerase activation, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. SYBR Green dye fluorescence was measured at 521 nm during the annealing phase. Relative quantities of mRNAs were obtained using the comparative threshold cycle (ΔΔCt) method by normalizing to GADPH and actin.

**Immunoprecipitation**

A549 cells grown in six-well plates were transfected with 1 μg of pcDNA3-hTRAF6 (donated by M. Kracht). After infection, cells were lysed in RIPA buffer (sc-24948 Santa Cruz Biotechnology). Five hundred micrograms of cell lysates were pre-cleared with protein A/G PLUS agarose beads (sc-200, Santa Cruz Biotechnology) for 2 h at 4°C. After centrifugation (1000 g, 13 000 rpm, 4°C), the supernatants were incubated with 2 μg of anti-TRAF6 antibody (Santa Cruz Biotechnology) for 2 h at 4°C. Protein A/G PLUS agarose beads were added and then incubated overnight at 4°C. Immunoprecipitates were washed four times with RIPA buffer and suspended in 2x electrophoresis sample buffer (Santa Cruz Biotechnology).

**Plasmids, transfections and luciferase assays**

PathDetect® NF-κB cis-Reporting plasmid and pRL-TK Renilla luciferase control reporter vector were purchased from Stratagene and Promega respectively. A549 cells seeded in 24-well plates were transiently transfected when they reached 40–60% confluence. Transfections were carried out in 500 μl of Opti-MEM Reduced-Serum Medium (Invitrogen) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen). Two hundred and fifty nanograms of the NF-κB reporter plasmid were co-transfected with 20 ng of pRL-TK Renilla Luciferase plasmid. Forty-eight hours post transfection cells were infected as described. Cell extracts were prepared using Passive Lysis Buffer (Promega) and luciferase activities were measured with the Dual Luciferase Assay kit (Promega). Firefly luciferase values were normalized to Renilla control values. Results were plotted as relative luciferase activity compared with activity measured in non-stimulated control cells.

**Small interfering RNA (siRNA)**

RNA-mediated interference for downregulating MyD88, CYLD, MKP-1 was carried out by transfection of MyD88 siRNA (5′-AAGCGAAACAGACAAACTATC-3′), CYLD siRNA (5′-CTGTTGAGCGAGGTTTCTGC-3′) and antisense (5′-CCCAT ACTACAAATGGGG-3′) and antisense (5′-CTTCCCAAAATG CCAAAGTT-3′); [mouse MKP-1] sense (5′-CTTCCCAAAATG CCAAAGTT-3′) and antisense (5′-CCCAT ACTACAAATGGGG-3′) and antisense (5′-CTTCCCAAAATG CCAAAGTT-3′). The thermocycling protocol was as follows: 95°C for 15 min for hot-start polymerase activation, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. SYBR Green dye fluorescence was measured at 521 nm during the annealing phase. Relative quantities of mRNAs were obtained using the comparative threshold cycle (ΔΔCt) method by normalizing to GADPH and actin.

**Immunofluorescence microscopy**

Cells were seeded on 13 mm circular coverslips in 24-well tissue culture plates. After infection, cells were washed three times with PBS and fixed with 3.7% paraformaldehyde in PBS pH 7.4 for 20 min.
15 min at room temperature, p65 NF-κB subunit was stained with anti-p65 antibody (1:200, Santa Cruz Biotechnology). Cy2-conjugated donkey anti-rabbit antibody (Jackson Immunological) was diluted 1:200. Staining was carried out as previously described (Martí-Lliteras et al., 2009). Immunofluorescence was analysed with a Leica CTR6000 fluorescence microscope.

Rac1 activation assay

Rac1 activation was determined with the Rac1 Activation assay kit (Millipore) according to the manufacturer’s instructions. Briefly, A549 cells were grown to ~10^5 cells per sample (80% confluence) in 25 cm² tissue culture flasks. After treatments, cells were washed twice with ice-cold Tris-Buffered Saline (TBS) and resuspended in the assay buffer provided by the kit. Protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific). Two hundred micrograms of lysates were mixed with PAK-1 PBD agarose slurry (10 μg) and incubated for 1 h at 4°C with gentle rocking. Beads were collected by centrifugation (14 000 x g, 5 s), washed three times with the assay buffer provided by the kit, and resuspended in SDS sample buffer. Activated Rac1 in the cell lysates was visualized by Western blot using mouse anti-Rac1 (clone 23A8, provided by the kit) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (1:1000, Pierce). Proteins were detected using the SuperSignal West-dura system (Pierce). To confirm that equal amounts of protein from each sample were pulled down, tubulin levels of the lysates were also analysed by immunoblotting.

Yersinia adhesion and invasion assays

Yersinia enterocolitica strain was grown aerobically for 4 h at 21°C, pelleted and resuspended to an OD_{600} of 1.0 in PBS. Bacteria suspensions were added to subconfluent A549 cells grown in 24-well plates (multiplicity of infection of ~150:1) which, when indicated, were pre-infected for 3 h with Kp52145 as described. To determine Yersinia adhesion, cells were infected during 30 min, monolayers were washed five times with PBS and then lysed with PBS-0.5% saponin. Bacteria were plated in CIN agar plates, a selective medium for Yersinia. To determine Yersinia invasion, cells were infected during 30 min, monolayers were washed twice with PBS and incubated during 60 min in medium containing gentamicin (100 μg ml⁻¹) to kill extracellular bacteria. Gentamicin treatment was long enough to kill all extracellular bacteria. After this period, cells were washed three times with PBS, lysed with PBS-0.5% saponin and bacteria plated on CIN agar plates. Experiments were carried out in triplicate on three independent occasions. Invasion was calculated as per cent invasion = 100 x (No. of bacteria recovered after gentamicin treatment/No. of bacteria initially added) whereas adhesion was calculated as per cent adhesion = 100 x (No. of bacteria recovered after washes/No. of bacteria initially added).

NOD1 NF-κB activation assay

HEK293T cells, seeded on six-well plates, were transfected with 50 ng of pCI-NOD1 plasmid and 450 ng of pcDNA3 to balance the transfected DNA concentration in a final volume of 2500 μl using 1 μl of Lipofectamine 2000 following manufacturer’s instructions. Twenty-four hours post transfection, cells were washed once with PBS and M-Tri-DAP, or CPS were added for 3 h before performing luciferase measurements.

Cell fractionation (membrane/cytosol) experiments

HEK293T cells, seeded on six-well plates, were transfected with 50 ng of pCI-NOD1 plasmid and 450 ng of pcDNA3 to balance the transfected DNA concentration in a final volume of 2500 μl using 1 μl of Lipofectamine 2000 following manufacturer’s instructions. 21°C, pelleted and resuspended to an OD 600 of 1.0 in PBS. Gentamicin treatment was long enough to kill all extracellular bacteria. After this period, cells were washed twice with ice-cold Tris-Buffered Saline (TBS) and resuspended in the assay buffer provided by the kit. Protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific). Two hundred micrograms of lysates were mixed with PAK-1 PBD agarose slurry (10 μg) and incubated for 1 h at 4°C with gentle rocking. Beads were collected by centrifugation (14 000 x g, 5 s), washed three times with the assay buffer provided by the kit, and resuspended in SDS sample buffer. Activated Rac1 in the cell lysates was visualized by Western blot using mouse anti-Rac1 (clone 23A8, provided by the kit) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (1:1000, Pierce). Proteins were detected using the SuperSignal West-dura system (Pierce). To confirm that equal amounts of protein from each sample were pulled down, tubulin levels of the lysates were also analysed by immunoblotting.

Statistical methods

Statistical analyses were performed using one-way ANOVA with Bonferroni contrasts or the Mann–Whitney U-test. P < 0.05 was considered statistically significant.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** *Klebsiella pneumoniae* infection does not affect the levels of surface molecules in A549 cells. Flow cytometry analy- sis of TLR2, TLR4, TNFR1, TNFR2, CD54 and HLA-I expression by A549 cells left uninfected (white bars) or infected with Kp52145 for 3 h (grey bars). Cells were detached by incubation with trypsin-EDTA and washed with 0.1% sodium-azide in PBS. Non-permeabilized cells were incubated with PE-conjugated antibo- dies (10 μg ml-1) or IgG2a, κ isotype labelled antibodies for 15 min at room temperature (22–25°C) for 15 min. Analysis were performed using a CuluiTeck Epics XL flow cytometer. At least 9000 cells were acquired in every experiment. The levels of proteins were expressed as relative mean fluorescence intensity (rMFI) measured in arbitrary units and the non-specific binding was corrected by subtraction of MFI values corresponding to isotype- matched antibodies. Data are means and s.e.m. (n = 3).

**Fig. S2.** siRNA knock-down efficiency. siRNA efficiency in A549 cells was quantified by RT-qPCR in samples from the same experiments shown in Fig. 7A and B. mRNA level was normalized to GADPH and actin and then relative mRNA levels in cells transfected with control siRNA or specific siRNAs were compared. mRNA levels in control siRNA-transfected cells were set to 100%. Data are means and s.e.m. of RT-qPCR duplicates.

**Fig. S3.** *Klebsiella pneumoniae* reduces *Yersinia enterocolitica* invasion of A549 cells. Invasion and adhesion assays were per- formed without centrifugation. Values for invasion and adhesion were calculated as described in *Experimental procedures*. Data are means and s.e.m. (n = 3).

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