Klebsiella pneumoniae NdpA suppresses ERK pathway-mediated host early inflammatory responses and is degraded through the ubiquitin-proteasome pathway

Dear Editor,

Klebsiella pneumoniae (KP) is an important opportunistic pathogen causing community-acquired and nosocomial infections. When the host is immunocompromised, the pathogen would infect the host and cause diseases, such as pneumoniae, sepsis, liver abscess, meningitis, urinary tract inflammation and wound infection (Karaiskos et al., 2016; Park et al., 2015). The phenomenon that K. pneumoniae has a preference to infect immunocompromised populations, especially seniors suggests that the outcomes of K. pneumoniae infection depend on the pathogen-host interactions, but up to now, the molecular mechanisms underlying K. pneumoniae-host interactions remain largely unknown.

Previous studies reported that K. pneumoniae suppresses inflammatory cytokine production during early period of infection (Lawlor et al., 2006), and this bacteria can block the activation of inflammatory responses by antagonizing NF-κB and MAPK signaling pathways (Frank et al., 2013; Regueiro et al., 2011). We discovered that the K. pneumoniae nucleoid-associated protein (NdpA) is highly conserved among gram-negative bacteria. Transient expression of NdpA in human embryonic kidney HEK239T cells inhibited the Elk activation induced by RasV12, as well as V-Raf (constitutive active Raf) and MEK1-ED (constitutive active MEK1), respectively (Fig. 1A–C). K. pneumoniae NdpA also promoted tumor necrosis factor (TNF) α-stimulated NF-κB activation (Fig. S1A), and had little, if any, inhibitory effect on JNK and p38 signaling pathways (Fig. S1B). Given the lack of inflammation at the early stage of K. pneumoniae infection (Lawlor et al., 2006), we thus focused on the elucidation of the suppressive effects of K. pneumoniae NdpA on ERK signaling pathway-mediated host early inflammatory responses. Because many pathogenic bacteria have secretion systems to inject their virulence factors into host cells to interfere their functions. Thus, we sought to examine whether K. pneumoniae NdpA could be secreted into host cells during infection. Immunoblot analysis showed that NdpA entered into the cytosol of the human alveolar epithelial cells A549 during K. pneumoniae infection (Fig. S2A). In addition, we found that the phosphorylation of ERK1/2 activated by MEK1-ED was largely reduced by NdpA (Fig. 1D). Consistently, NdpA also abolished extracellular stimuli epidermal growth factor (EGF)-activated ERK1/2 phosphorylation (Fig. 1E). To further confirm the role of NdpA in the suppression of ERK signaling during K. pneumoniae infection, we tried to knockout the gene encoding NdpA with several methods available, but after many attempts we failed to obtain the expected mutant strain. We thus adopted the alternative strategy to investigate the host immune-regulatory function of NdpA by overexpressing it in K. pneumoniae. We found that overexpression of NdpA in K. pneumoniae and E. coli resulted in down-regulation of phospho-ERK1/2 (p-ERK1/2) in A549 cells during K. pneumoniae infection (Figs. 1F and S2B).

With a central role in recruiting and infiltrating neutrophils into inflammatory sites, IL8 is known as a main inflammatory molecule involved in host defense against K. pneumoniae infection (Harada et al., 1994). We thus next explored whether NdpA regulates the expression of IL8 in human alveolar epithelial cells during K. pneumoniae infection. The data from quantitative real-time PCR showed that compared with the wild-type (WT) K. pneumoniae strain, the NdpA-overexpressing K. pneumoniae strain significantly down-regulated the mRNA of IL8 in A549 cells (Fig. 1G). Accordingly, the secretion of IL8 was apparently attenuated by the overexpression of NdpA in K. pneumoniae as analyzed by enzyme-linked immunosorbent assay (ELISA) (Fig. 1H). To determine whether the inhibitory effects of NdpA on IL8 production is dependent on ERK signaling pathway, we pretreated A549 cells with U0126, a specific inhibitor of ERK pathway, before the infection assay, and we found that the WT K. pneumoniae strain and the NdpA-overexpressing K. pneumoniae strain showed similar amount of IL8 production during infection of A549 cells (Fig. S3A and S3B).

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We further sought to investigate the underlying mechanisms by which NdpA suppresses host inflammatory responses. Based on the observation that NdpA inhibited ERK1/2 phosphorylation stimulated by MEK1-ED, the activator of ERK pathway next to ERKs (Fig. 1D), MEK1 and ERKs were chosen to verify their interactions with NdpA. HEK293T cells expressing Flag-tagged ERK2 or Myc-tagged MEK1 were used for co-immunoprecipitation analysis with His-tagged NdpA purified from *E. coli* BL21, and the data indicated that NdpA could specifically bind to ERK2, but not MEK1 (Fig. S4A). Furthermore, we found that overexpression of NdpA in HEK293T cells completely abolished the binding of MEK1 to ERK2 (Fig. S4B).

Through protein sequence alignment analysis, we found that NdpA harbors a canonical D motif (15-KRDEQNLEL-23) for ERKs docking. The D motif contains the consensus...
sequence \((K/R)_{1,2}(X)_{2,4,6}(\Omega_A \cdot \Omega_B)\) \((\Omega_A\) and \(\Omega_B\) are hydrophobic residues) (Supplementary Fig. 4C) (Zhou et al., 2006; Zhu et al., 2007). To identify which site plays the most critical role in the binding to ERK1/2, four mutants of NdpA were constructed, including NdpA (K15A), NdpA (R16A), NdpA (L21E) and NdpA (L23E). Through dual-luciferase assay, we found that NdpA (L21E) evidently impaired the inhibitory effects of NdpA on ERK pathway (Fig. S4D). Hist-

![Diagram of protein interactions](image_url)
ubiquitin-conjugated proteins (Polo et al., 2003). Therefore, for interacting with free Ub, poly-ubiquitin chains, as well as free eukaryotic cells instead of prokaryotic cells and it is specific for the bacteria might gain the upper hand and cause diseases.

The UIM motif has been reported to occur more frequently in unknown regulatory functions in hosts.

Since the suppressive effects of *K. pneumoniae* are usually attenuated at about 4 hours post infection of host cells, we thus further questioned whether the host could counteract the regulatory effects of NdpA during the course of *K. pneumoniae* infection by regulating the protein stability of NdpA. Interesting, by online bioinformatics analysis (http://smart.embl-heidelberg.de/), we identified two tandem ubiquitin (Ub)-interacting Motifs (UIMs) in *K. pneumoniae* NdpA. The UIM motif has been reported to occur more frequently in eukaryotic cells instead of prokaryotic cells and it is specific for interacting with free Ub, poly-ubiquitin chains, as well as ubiquitin-conjugated proteins (Polo et al., 2003). Therefore, we hypothesized that NdpA might be degraded by the host through an ubiquitin-dependent pathway. When the vectors encoding NdpA and ubiquitin were co-transfected in HEK293T cells, we thus further questioned whether the host could counteract the regulatory effects of NdpA during the course of *K. pneumoniae* infection by regulating the protein stability of NdpA. Increasing the expression of ubiquitin in HEK293T cells caused the reduction of NdpA protein (Fig. 2B). Tag pull-down analysis further confirmed that NdpA could directly bind to ERK2 in vitro, but the NdpA (L21E) mutant had little binding affinity (Supplementary Fig. 4E). Consistently, NdpA (L21E) lost the ability to block the interactions between ERK2 with MEK1 (Fig. 5A). Unlike WT NdpA, NdpA (L21E) could not efficiently inhibit MEK1-ED-stimulated ERK1/2 phosphorylation in HEK293T (Supplementary Fig. 5A), and also failed to inhibit ERK1/2 phosphorylation in A549 cells during *K. pneumoniae* infection (Fig. S5B). Consistently, quantitative real-time PCR and ELISA analysis also showed that NdpA (L21E) couldn’t effectively downregulate IL8 expression in A549 cells during *K. pneumoniae* infection (Fig. SSC and SSD). Together, these data indicate that NdpA inhibited ERK signaling pathway-mediated inflammation in a D motif-dependent manner.

The suppression of ERK1/2 phosphorylation is attenuated during aging process (Tonoki et al., 2009), such as the seniors. Since the suppressive effects of *K. pneumoniae* are usually attenuated at about 4 hours post infection of host cells, we thus further questioned whether the host could counteract the regulatory effects of NdpA during the course of *K. pneumoniae* infection by regulating the protein stability of NdpA. Increasing the expression of ubiquitin in HEK293T cells caused the reduction of NdpA protein (Fig. 2B). Tag pull-down analysis further confirmed that NdpA could directly bind to ERK2 in vitro, but the NdpA (L21E) mutant had little binding affinity (Supplementary Fig. 4E). Consistently, NdpA (L21E) lost the ability to block the interactions between ERK2 with MEK1 (Fig. 5A). Unlike WT NdpA, NdpA (L21E) could not efficiently inhibit MEK1-ED-stimulated ERK1/2 phosphorylation in HEK293T (Supplementary Fig. 5A), and also failed to inhibit ERK1/2 phosphorylation in A549 cells during *K. pneumoniae* infection (Fig. S5B). Consistently, quantitative real-time PCR and ELISA analysis also showed that NdpA (L21E) couldn’t effectively downregulate IL8 expression in A549 cells during *K. pneumoniae* infection (Fig. SSSC and SSD). Together, these data indicate that NdpA inhibited ERK signaling pathway-mediated inflammation in a D motif-dependent manner.

Therefore, the D motif might provide selectivity for the development of novel pan-anti-gram-negative pathogen therapies.

While in immunocompromised hosts (such as the aged patients) whose innate immunity such as proteasome function is attenuated during aging process (Tonoki et al., 2009), the bacteria might gain the upper hand and cause diseases. Thus our findings help to explain, at least partially, that why *K. pneumoniae* tends to infect immunocompromised hosts such as the seniors.

**FOOTNOTES**

This work was supported by research funding from the National Basic Research Program (973 Program) (Nos. 2014CB744400 and 2012CB518700), the National Natural Science Foundation of China (Grant Nos. 81371769, 81571954 and 81571536), the Beijing Natural Science Foundation (Grant No. 5162021), and the Youth Innovation Promotion Association CAS (Grant No. Y12A027BB2). We thank Feng Shao (National Institute of Biological Sciences, Beijing, China), Lingqiang Zhang (Beijing Institute of Radiation Medicine) and Youjun Feng (Zhejiang University) for providing our plasmids.

Guanghua Xu, Jing Wang, and Cui Hua Liu declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by the any of the authors.

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Electronic supplementary material The online version of this article (doi:10.1007/s13238-016-0341-y) contains supplementary material, which is available to authorized users.

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