ERJ Express. Published on April 8, 2010 as doi: 10.1183/09031936.00002710

OSTEOPONTIN PROMOTES HOST DEFENSE DURING *KLEBSIELLA PNEUMONIAE*-INDUCED PNEUMONIA

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ABSTRACT

Klebsiella (*K.*) *pneumoniae* is a common cause of nosocomial pneumonia. Osteopontin (OPN) is a phosphorylated glycoprotein involved in inflammatory processes, some of which mediated by CD44. Aim of this study was to determine the role of OPN during *K.pneumoniae*-induced pneumonia.

Wild-type (WT) and OPN knockout (KO) mice were intranasally infected with 10^4 colony forming units of *K.pneumoniae*, or administered *Klebsiella* LPS. In addition, rOPN was intranasally administered to WT and CD44 KO mice.

During *Klebsiella* pneumonia, WT mice displayed elevated pulmonary and plasma OPN levels. OPN KO and WT mice showed similar pulmonary bacterial loads at 6 h post infection; thereafter *Klebsiella* loads were higher in lungs of OPN KO mice and the mortality rate in this group was higher than in WT mice. Early neutrophil recruitment into the bronchoalveolar space was impaired in the absence of OPN after intrapulmonary delivery of either *Klebsiella* bacteria or *Klebsiella* LPS. Moreover, rOPN induced neutrophil migration into the bronchoalveolar space, independent from CD44. *In vitro*, OPN did not affect *K.pneumoniae* growth or neutrophil function.

In conclusion, OPN levels are rapidly increased in the bronchoalveolar space during *K.pneumoniae* pneumonia, where OPN serves a chemotactic function towards neutrophils, thereby facilitating an effective innate immune response.

Keywords

Infection, *Klebsiella pneumoniae*, mice, lung, osteopontin

INTRODUCTION

Pneumonia is a common and serious illness that is a major cause of morbidity and mortality in humans and *Klebsiella* (*K.*) *pneumoniae* is a frequently isolated causative pathogen in nosocomial pneumonia [1].

Osteopontin (OPN) is a phosphorylated glycoprotein, expressed by a broad range of tissues and cells, which is involved in a number of physiological and pathological processes. OPN has been implicated as an important regulator of inflammation, occupying a central role in both innate and adaptive immunity by mediating inflammatory cell differentiation, maturation and migration, and cytokine production. Some of these processes are mediated through one of its receptors, the transmembrane molecule CD44 that is known to affect cellular migration and chemokine responses [2]. Animal studies have especially pointed to a role for OPN in lung inflammation, such as seen in allergy and asthma [3 , 4], acute respiratory distress syndrome [5], fibrosis [6] and parasitic infection [7]. Furthermore, patients suffering from diverse pulmonary diseases, including tuberculosis, silicosis and sarcoidosis, displayed enhanced OPN expression in their lungs [5, 7], and patients with tuberculosis or interstitial pneumonia had dramatically elevated plasma OPN levels as compared to healthy controls [8, 9]. Recently, plasma OPN concentrations were found to be increased in patients with sepsis, the majority of whom suffered from pneumonia as the primary source of infection [10].

Considering the association between OPN expression and pulmonary disease and sepsis, we here sought to determine the potential role of OPN in the host response to lower respiratory tract infection caused by *K. pneumoniae*. We show for the first time that OPN is important for early neutrophil recruitment to the bronchoalveolar space and

thereby for an effective immune response during *K. pneumoniae* induced pneumonia.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type (WT) mice were purchased from Harlan Sprague Dawley Inc. (Horst, The Netherlands). OPN knockout (KO) mice (Jackson Laboratories, Bar Harbor, ME) and CD44 KO mice (kindly provided by Dr. A. Berns, Netherlands Cancer Institute, Amsterdam, The Netherlands [11]), both on a C57BL/6 genetic background, were bred in the animal facility of the Academic Medical Center (Amsterdam, The Netherlands).

Study design

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. Pneumonia was induced by intranasal inoculation of 10^4 colony forming units (CFU) of *K. pneumoniae* serotype 2 (ATCC 43816; American Type Culture Collection, Rockville, MD), as described [12]. Sample harvesting and processing, and determinations of bacterial loads and cell counts were done as described [12]. In some experiments *K. pneumoniae* LPS (100 μg; L1519, Sigma, St. Louis, MO) or recombinant mouse OPN (rOPN, 1 or 10 μg; < 1.0 EU endotoxin per 1 μg as determined by the Limulus amoebocyte lysate assay; R&D Systems, Minneapolis, MN) was administered intranasally. For neutralization of keratinocyte-derived cytokine (KC), 50 μg of monoclonal anti-murine KC antibody (R&D) was injected intraperitoneally 2 h before rOPN, as described [13]; control animals received isotype control antibody (R&D).

Assays

Myeloperoxidase (MPO), OPN, KC, LPS-induced CXC chemokine (LIX) and macrophage inflammatory protein (MIP)-2 levels were determined by ELISA (MPO; Hycult, Uden, the Netherlands, others; R&D Sytstems, Abingdon, United Kingdom). Tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA). Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), urea and creatinin were determined with commercially available kits (Sigma-Aldrich), using a Hitachi analyzer (Roche) according to the manufacturer's instructions.

Immunohistochemistry

Paraffin lung sections were stained with hematoxilin and eosin or Ly-6G monoclonal antibody (BD Pharmingen, San Diego, CA) and semi-quantitatively scored as described [14].

Bactericidal assay, phagocytosis and phago-lysosomal fusion

K. pneumoniae was cultured in the presence of 800-0.8 ng/ml rOPN or in TSB only. At indicated time points the number of bacteria was determined by plating on blood-agar plates and counting colonies after 16 h of incubation at 37°C. Neutrophil phagocytosis of *K. pneumoniae* was determined as described [15]. To determine phago-lysosomal fusion in neutrophils after phagocytosis of *K. pneumoniae*, the procedure was similar except for *K. pneumoniae* being labeled with pHrodo (Invitrogen, Breda, The Netherlands) and neutrophils being labeled using anti-Gr-1-FITC (BD Pharmingen) [16]. Phagocytosis and

phago-lysosomal fusion index of each sample was calculated: mean fluorescence of positive cells x % positive cells.

Statistical analysis

All values are expressed as mean ± SEM. Comparisons for two groups were done with Mann-Whitney U tests, comparisons for more than two groups were done with Kruskall Wallis followed by Dunn's Multiple Comparison tests. Numbers of surviving animals at the end of observation period were compared by Chi-square test. These analysis were performed using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Osteopontin levels are increased during K. pneumoniae *infection*

To obtain a first insight into a potential role for OPN during gram-negative pneumonia, we measured OPN concentrations in bronchoalveolar lavage fluid (BALF), lung and plasma from WT mice before, 6, 24 and 48 h after infection mice with 10^4 CFU *K. pneumoniae*. OPN concentrations in BALF increased rapidly and peaked already at 6 h after infection (Figure 1A). Lung OPN levels increased more slowly and were significantly elevated compared to baseline from 24 h onward (Figure 1B). Plasma levels were increased already at 6 h after *K. pneumoniae* infection as compared to baseline and continued to rise up to 48 h (Figure 1C).

Osteopontin limits pulmonary bacterial growth

To determine whether OPN affected mortality due to *Klebsiella* pneumonia, we followed WT and OPN KO mice for 14 days after *K. pneumoniae* infection. Although median survival times did not differ, a significant part of WT mice (27%) survived the observation period whereas none of the WT mice did ($P = 0.03$, Figure 2). Next we questioned whether OPN affects the antibacterial response against *K. pneumoniae*. Therefore we determined bacterial outgrowth in BALF and lung homogenates of WT and OPN KO mice 6, 24 and 48 h after infection. At 6 h after infection bacterial loads were similar in BALF and lungs from both mouse strains (Figure 3A-B). At 24 and 48 h after infection, however, bacterial loads were 10-100 fold higher in BALF and lungs of OPN KO as compared to WT mice (BALF: $P < 0.01$ and lung: $P < 0.05$ for both time points,

Figure 3A-B). To obtain insight into the dissemination of the infection we determined bacterial loads in blood and spleen. At 6 h bacteria were not detectable yet in these organs, whereas after 24 and 48 h bacterial loads were similar in blood and spleen from WT and OPN KO mice (Figure 3C-D). In line, neither plasma cytokine levels (TNF-α and IL-6) nor liver injury (as indicated by ASAT and ALAT plasma levels) and kidney injury (as indicated by urea and creatinin plasma levels) were different in OPN KO mice as compared to WT mice (Table I). Together these data suggest that OPN contributes to local host defense, within the pulmonary compartment, during *Klebsiella* pneumonia.

Osteopontin deficiency does not influence pulmonary pathology

To obtain insight into the role of OPN in pulmonary inflammation in response to *K. pneumoniae* we analyzed lung histology slides obtained 6, 24 or 48 h after infection using a semi-quantitative scoring system. Already at 6 h after infection mild interstitial inflammation and pleuritis were found in all mice. These parameters were dramatically increased at 24 and 48 h, and bronchitis, endothelialitis and edema were also present from 24 h onward. Importantly, the extent of lung inflammation was similar in both groups at all time points (Figure 4). In addition, lung weights did not differ at 24 h, however, lungs from OPN KO mice were significantly heavier as compared to lungs from WT mice at 48 h after induction of pneumonia (24h: 115 ± 2 versus 109 ± 3; 48h: 109 ± 5 versus 134 ± 9, for WT versus OPN KO mice, $P = 0.13$ and $P < 0.01$ respectively).

Osteopontin deficiency results in impaired early neutrophil recruitment into the bronchoalveolar space upon K. pneumoniae *infection*

As pulmonary bacterial loads were similar at 6 h after infection but enhanced in the absence of OPN from 24 h onward, we next questioned whether OPN affects the early neutrophil recruitment to the lungs upon *K. pneumoniae* infection. At 6 h neutrophil numbers in BALF were significantly reduced in OPN KO mice as compared to WT mice (Figure 5A, $P < 0.05$). In lung tissue, however, neutrophil infiltration was similar in WT and OPN KO mice, as determined by MPO measurements in lung homogenates and quantification of Ly6+ cells in lung tissue slides (Figure 5B-C). Of interest, at this early time point OPN levels were strongly increased in BALF but not in lung homogenates as compared to uninfected mice (see Figure 1). As chemokines play an important role in the recruitment of inflammatory cells, we measured CXC chemokine levels in BALF at 6 h after infection. Whereas KC levels were significantly decreased in OPN KO mice as compared to WT mice, MIP-2 and LIX concentrations were similar (Figure 5D-F). Of note, at 24 and 48 h after infection neutrophil numbers in BALF were significantly enhanced in OPN KO mice as compared to WT mice (24 h: 6.39 ± 0.7 versus $15.3 \pm 1.7 \times 10^5$ cells/ml and 48 h: 5.1 ± 0.6 versus $13.2 \pm 3.3 \times 10^5$ cells/ml BALF, $P < 0.01$ and $P < 0.05$ respectively); similarly, chemokine levels were higher in BALF from OPN KO mice than in BALF from WT mice at these later time points (data not shown). Importantly, pulmonary bacterial loads were 10-100 times higher in OPN KO mice from 24 h on (see Figure 3), probably causing the enhanced proinflammatory response in these mice at these later time points. Together these data suggest that OPN present in BALF contributes to the influx of neutrophils into the bronchoalveolar space early after pulmonary infection by *K. pneumoniae*.

Osteopontin deficiency results in reduced early neutrophil influx into the bronchoalveolar space after intrapulmonary delivery of Klebsiella *LPS.*

To obtain further proof for a role for OPN in early neutrophil recruitment during gram-negative lung inflammation without the possible influence of different bacterial loads, we instilled *Klebsiella* LPS via the airways of OPN KO and WT mice and determined the numbers of neutrophils in BALF harvested 6 and 24 h later. In WT mice LPS administration caused a significant rise in the BALF levels of OPN at 6 h after LPS ($P < 0.05$), whereas at 24 h OPN levels were back to baseline again (Figure 6A). Like in mice infected with viable *Klebsiella*, OPN KO mice administered with *Klebsiella* LPS displayed fewer neutrophils in their BALF as compared to WT mice at 6 but not at 24 h after LPS (Figure 6B). Importantly, OPN concentrations were elevated in WT mice at 6 but not at 24 h after LPS; thus these data further indicate that OPN present in BALF contributes to the influx of neutrophils into the bronchoalveolar space.

Osteopontin is chemotactic in the bronchoalveolar space

To determine whether OPN is indeed chemotactic in the bronchoalveolar space we intranasally instilled PBS, 1 or 10 μg of rOPN in WT mice. At 6 h after instillation we determined the numbers of infiltrated cells in BALF. Although 1 μg of rOPN was not associated with influx of cells, instillation of 10 μg rOPN resulted in the recruitment of neutrophils to the bronchoalveolar space (Figure 6C, $P < 0.001$ as compared to PBS). Since the reduced neutrophil numbers in OPN KO mice early after *K. pneumoniae* infection were accompanied by reduced KC concentrations, we determined whether the chemotactic effect of rOPN was KC dependent. Therefore, we pretreated mice with an a

neutralizing anti-KC or control antibody (as described [13, 17]) 2 h prior to intrapulmonary delivery of rOPN (10 μg). Anti-KC treatment did not affect neutrophil recruitment into BALF induced by rOPN (Figure 6D). CD44 is a transmembrane molecule mediating cellular migration and chemokine responses [18], and an important receptor for OPN [19]. Therefore, we determined whether neutrophil recruitment induced by OPN was CD44 dependent. Upon 10 μg of rOPN administration WT and CD44 KO mice demonstrated similar neutrophil recruitment after 6 h (Figure 6E). Together, these data suggest that OPN is chemotactic in the lung by a mechanism that is independent from KC and CD44.

Osteopontin does not affect K. pneumoniae *growth and neutrophil phagocytosis or phago-lysosomal fusion*

The experiments described above established that in the absence of OPN mice display diminished early recruitment of neutrophils to the bronchoalveolar space, accompanied by increased bacterial loads. Apart from impaired early neutrophil influx, the enhanced bacterial outgrowth in the absence of OPN might additionally be explained by direct effects of OPN on bacterial growth or by altered phagocytosis capacity or phago-lysosomal fusion in OPN KO neutrophils. Therefore, we added OPN to *K. pneumoniae* and monitored whether bacterial growth was affected. OPN did not alter the growth of this pathogen *in vitro* (Figure 7A). In addition, phagocytosis capacity and phago-lysosomal fusion were not altered in OPN KO as compared to WT neutrophils (Figure 7B-C). Taken together, the observed impairment of bacterial clearance in OPN KO mice

cannot be explained by the absence of a bactericidal effect of OPN or by diminished phagocytosis or fusion of phagosomes with lysosomes in OPN KO neutrophils.

DISCUSSION

The present study is the first to investigate the functional role of OPN during bacterial infection. In accordance with a recent report revealing elevated circulating levels of OPN in patients with bacterial sepsis predominantly suffering from pneumonia [10], we here demonstrate elevated local and systemic levels of OPN during *Klebsiella* pneumonia in mice. OPN was released rapidly into the bronchoalveolar space upon infection of the airways with *K. pneumoniae*, where it contributed significantly to the early recruitment of neutrophils. Subsequently, OPN KO mice displayed a reduced antibacterial defense at the primary site of infection, as reflected by higher bacterial loads in their lungs, which resulted in a higher mortality rate. The chemotactic function of OPN in the bronchalveolar space towards neutrophils was confirmed in studies using LPS derived from *Klebsiella* and experiments using rOPN. OPN did not influence antibacterial effector functions of neutrophils. Our data suggest that OPN improves local host defense during *Klebsiella* pneumonia at least in part by facilitating early neutrophil recruitment.

Several studies have demonstrated enhanced OPN expression during subacute and chronic pulmonary inflammation [4, 5, 7, 20]. However, knowledge of the production of OPN during acute inflammatory diseases is limited. So far, only one study on experimental gram-negative infection, induced by *Francisella novicida* administered intratracheally, has shown induction of OPN mRNA in lungs of infected mice [21]. We here show that intranasal instillation of viable *K. pneumoniae* results in a rapid release of

OPN into the bronchoalveolar space, whereas OPN levels in lung tissue increased more gradually. Of note, OPN was already detectable in BALF of uninfected mice. Respiratory epithelial cells are a possible source of constitutive OPN levels in BALF: we observed significant spontaneous OPN release upon culturing two distinct murine epithelial cell lines (MLE-12 and MLE-15) without any stimulus added (data not shown). In addition, alveolar macrophages are known to secrete OPN during inflammation [2]. In line, we found profound OPN release by murine alveolar macrophages (MH-S cells) upon stimulation with either *Klebsiella* LPS or intact *K. pneumoniae* (data not shown).

In this study we demonstrate that the increase in OPN levels induced by *K. pneumoniae* pneumonia serves a functional role in host defense against this infection. Although pulmonary bacterial growth was still similar in OPN KO and WT mice at 6 h post infection, enhanced bacterial growth at the primary site of infection was found in the absence of OPN from 24 h onward. In line, OPN KO mice demonstrated a higher mortality rate. Dissemination of bacteria and organ failure was similar in both groups, suggesting that the difference in pulmonary bacterial loads dominantly determined outcome of the infection. As rapid neutrophil recruitment to the lungs is essential for effective clearance of *K. pneumoniae* [22], we investigated whether OPN affects this process early during infection. Indeed, we found impaired early neutrophil recruitment into the bronchoalveolar space, but not into lung tissue, in the absence of OPN at 6 h after infection. As at this early time point OPN levels were increased in BALF, but not in lung homogenates, as compared to uninfected mice, these data suggest that the rapid release of OPN into the bronchoalveolar space upon respiratory tract infection by *Klebsiella* acts to attract neutrophils into the airways. This suggestion is supported by our experiments in

which *Klebsiella* LPS was used as inciting stimulus (revealing reduced neutrophil influx into BALF of OPN KO mice) and by studies in which the intrapulmonary delivery of rOPN induced neutrophil recruitment into the bronchoalveolar space. Our data confirm and extend earlier data on the role of OPN as a chemoattractant for neutrophils in the liver during alcoholic and Con A induced hepatitis [23, 24]. Moreover, OPN has been implicated as a chemoattractant for neutrophils in the peritoneal cavity by studies showing an impaired intraperitoneal neutrophil recruitment in OPN KO mice in response to sodium periodate and a robust increase in neutrophil numbers in peritoneal fluid upon local injection of rOPN [25]. In accordance, OPN KO neutrophils were reported to exhibit reduced chemokinesis and chemotaxis towards fMLP, whereas rOPN exerted direct chemotactic effects on neutrophils *in vitro* [25, 26]. A recent study has indicated that polymerization of OPN results in an interaction with the α9β1 integrin on neutrophils, which is essential for the chemotactic function of OPN [27]. Whether this interaction also drives OPN induced neutrophil migration into the bronchoalveolar space *in vivo* remains to be established. Our current results argue against an important indirect role for CXC chemokines in the effect of OPN on neutrophil recruitment into the bronchoalveolar space. KC, LIX and MIP-2 have been shown to mediate neutrophil recruitment during gram-negative lung inflammation [28]. Of these, only KC levels were reduced in BALF of OPN KO mice early after infection with *Klebsiella*, which might have contributed to a diminished neutrophil influx in these animals. However, anti-KC did not impact on influx of these inflammatory cells upon intranasal administration of rOPN, arguing against an intermediate role for KC in OPN mediated neutrophil recruitment. Of note, the human and mouse chemokine systems differ and therefore the

current results on the role of OPN in a mouse model of pneumonia cannot be extrapolated directly to human respiratory tract infection. In addition, *Klebsiella* predominantly causes infections in immunocompromised patients; although enhanced OPN expression has been described in patients with immunodeficiency [29], further research is warranted to establish the functional role of OPN in bacterial infection in the immunocompromised host.

CD44 is an important receptor for OPN [2, 19] and involved in polarization and chemotaxis of neutrophils [30]. In contrast to macrophages, the expression and localization of CD44 in neutrophils is independent of OPN expression [25]. We here demonstrate that rOPN induced neutrophil recruitment to the bronchoalveolar space was CD44 independent as this response was indistinguishable between CD44 KO and WT mice. Notably, the accumulation of macrophages induced by OPN *in vivo* was previously found to be dependent on CD44, indicating that OPN exerts chemotactic effects on neutrophils and macrophages by different mechanisms [31]. As Toll-like receptor (TLR) signaling significantly contributes to host defense during *Klebsiella pneumoniae* infection [32-35], a potential interaction between OPN signaling and TLR responses might be of interest. Although the knowledge on such an interaction is very limited, one study showed that OPN expression by cementoblasts, induced by *Porphyromonas gingivalis*-derived LPS which is known to interact with TLR2 and TLR4 [36], was partially inhibited by antibodies against the TLR4/MD2 complex [37].

The enhanced bacterial growth in the absence of OPN could, apart from impaired early neutrophil influx into the bronchoalveolar space, additionally be explained by an altered phagocytosis capacity or phago-lysosomal fusion in OPN KO neutrophils. We

found no alterations in the absence of OPN in either of these processes. This result is in accordance with earlier data showing that several antibacterial effector functions of neutrophils are not affected by OPN deficiency [25].

This current model of *Klebsiella* pneumonia bears resemblance with human pneumonia and sepsis, as it is associated with invasive respiratory tract infection leading to dissemination of infection and distant organ injury, caused by a clinically relevant gram-negative respiratory pathogen. The findings generated in this model of bacterial pneumonia have been considered of relevance for understanding the pathogenesis of pneumonia [38, 39]. Nonetheless, extrapolation to human disease should be made with caution.

In conclusion, we show here for the first time that OPN levels are rapidly elevated in the bronchoalveolar space during *K. pneumoniae* pneumonia, serving a chemotactic function towards neutrophils and thereby an effective innate immune response.

ACKNOWLEDGEMENTS

We thank Joost Daalhuisen, Marieke ten Brink, and Regina de Beer for expert technical assistance.

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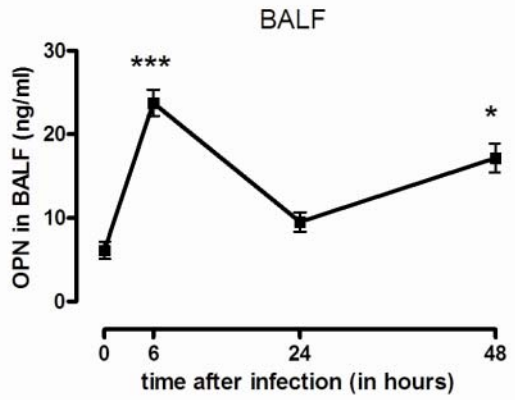
FIGURE LEGENDS

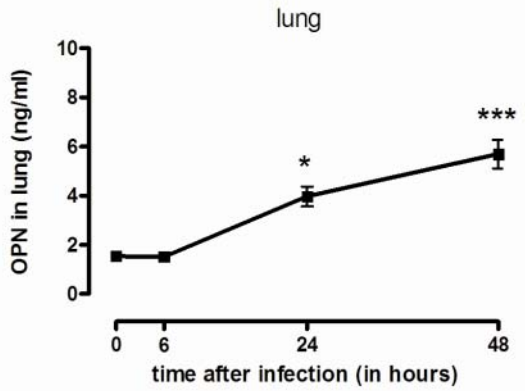
Figure 1: Pulmonary and plasma OPN concentrations are elevated during *K. pneumoniae* infection. OPN concentrations in (A) BALF, (B) lung and (C) plasma before, 6, 24 and 48 h after infection with 10^4 CFU of *K. pneumoniae*. Data are expressed as means ± SEM; n = 8 mice/group, * $P < 0.05$, *** $P < 0.001$ as compared to t=0.

Figure 1

A


BALF
OPN in BALF (ng/ml)
30
20
10
0
***
*
0
6
24
48
time after infection (in hours)

B


lung
OPN in lung (ng/ml)
10
8
6
4
2
0
*
***
0
6
24
48
time after infection (in hours)

C

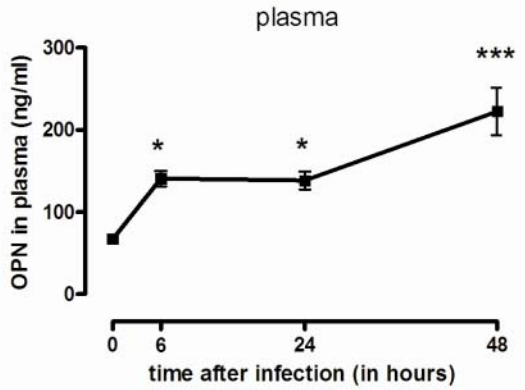

plasma
OPN in plasma (ng/ml)
300
200
100
0
*
*
***
0
6
24
48
time after infection (in hours)

Figure 2: OPN KO mice show enhanced mortality. Percentage survival of WT (closed symbols) and OPN KO (open symbols) mice until 14 days after intranasal infection with 10^4 CFU of *K. pneumoniae*. n = 14-15 per group. *P* value indicates the difference between groups.

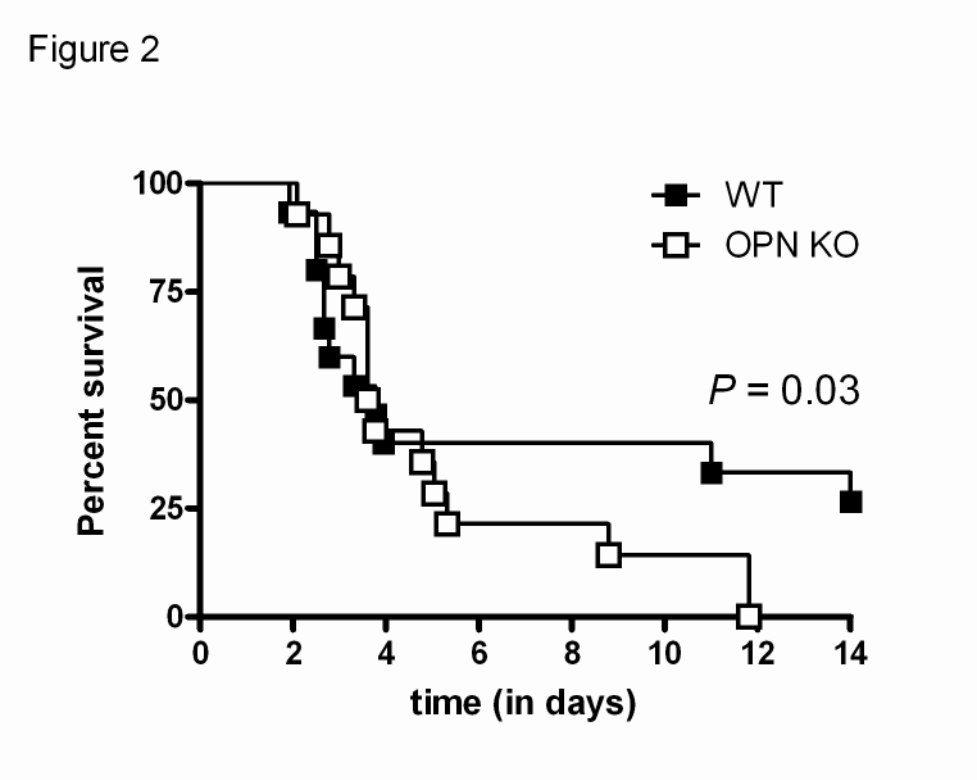


Figure 3: Enhanced bacterial outgrowth in OPN KO mice. WT (black bars) and OPN KO (white bars) mice were infected with 10^4 CFU of *K. pneumoniae* and bacterial loads were determined 6, 24 and 48 h after infection in (A) BALF, (B) lung, (C) blood and (D) spleen. Data are expressed as means ± SEM; n = 8 mice/group, * $P < 0.05$, ** $P < 0.01$ as compared to WT mice. N.D. means not detectable.

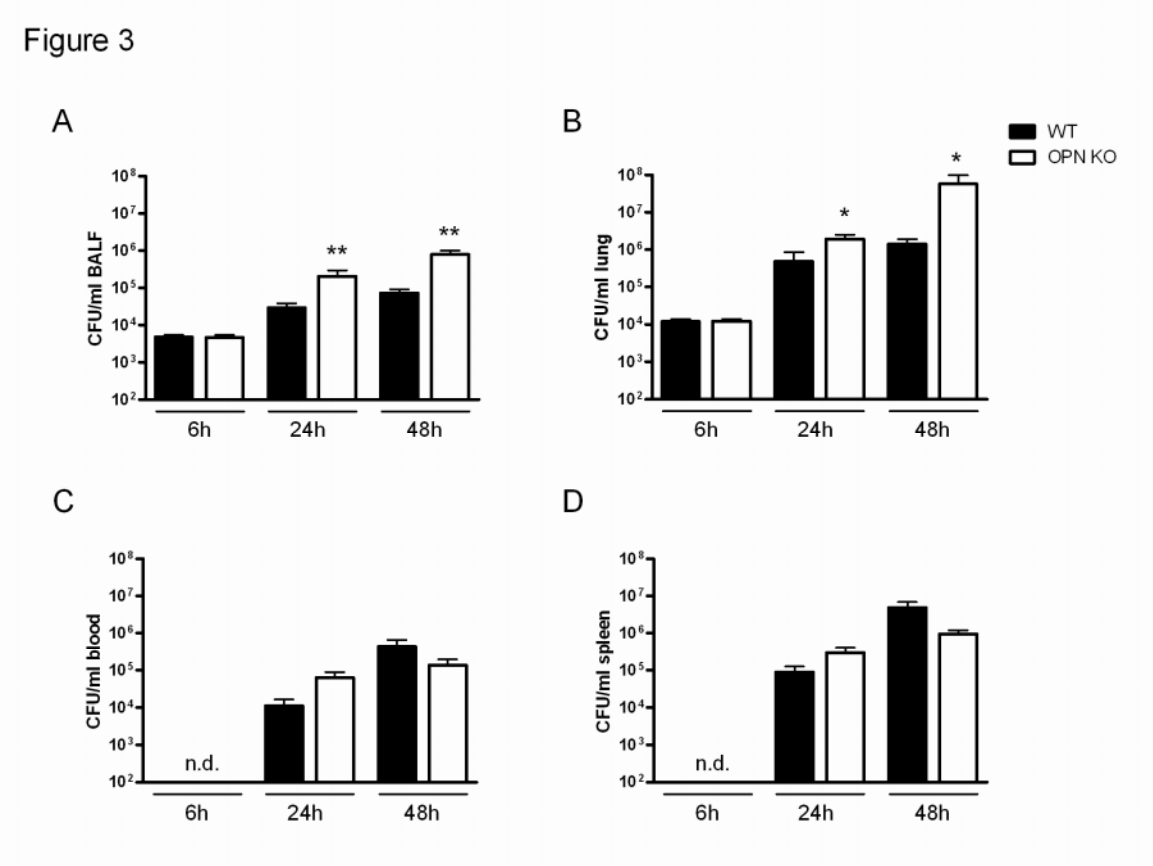


Figure 4: Similar lung histopathology. Representative lung histology of WT (A, D, G) and OPN KO (B, E, H) mice at 6 (A-C), 24 (D-F) and 48 (G-I) h after intranasal infection with 10^4 CFU of *K. pneumoniae*. The lung sections are representative for 8 mice per group per time point. H&E staining, original magnification 10x. Pathology scores are expressed as means ± SEM (WT mice: black bars; OPN KO mice: white bars; n = 8 mice/group).

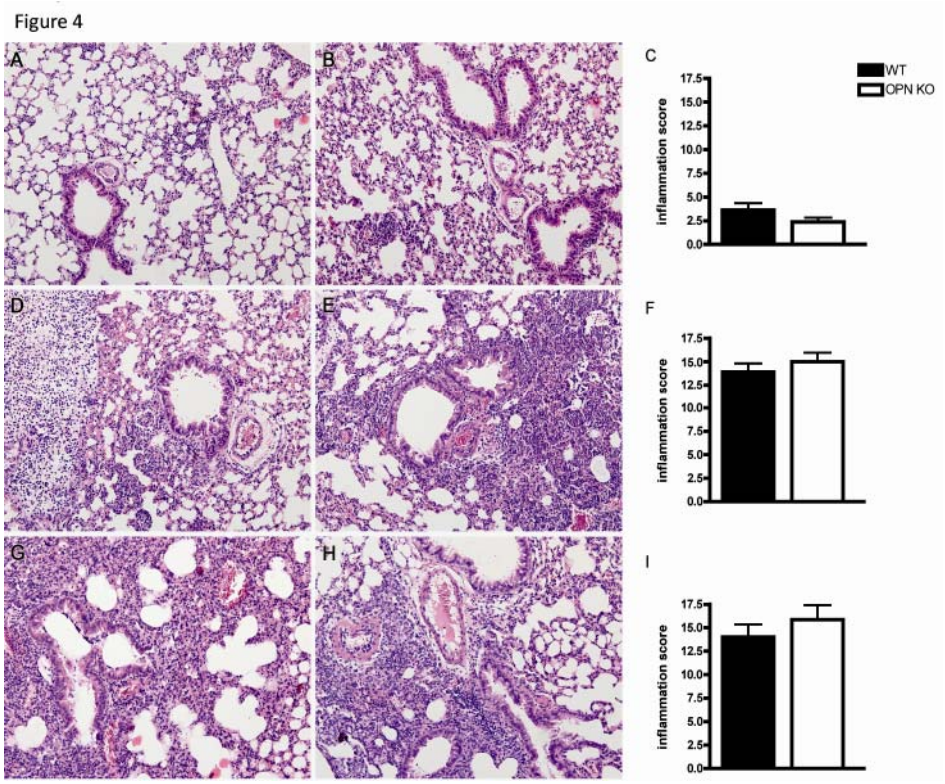


Figure 5: Impaired early neutrophil recruitment in bronchoalveolar lavage fluid of OPN KO mice. Neutrophil influx into BALF (A), MPO concentrations in lung homogenates (B), Ly6+ cell quantification in lung tissue slides (C), and BALF levels of KC (D), MIP-2 (E) and LIX (F) at 6 h after infection with 10^4 CFU of *K. pneumoniae*. Data are expressed as means ± SEM; n = 8 mice/group, * $P < 0.05$, *** $P < 0.001$ as compared to WT mice.

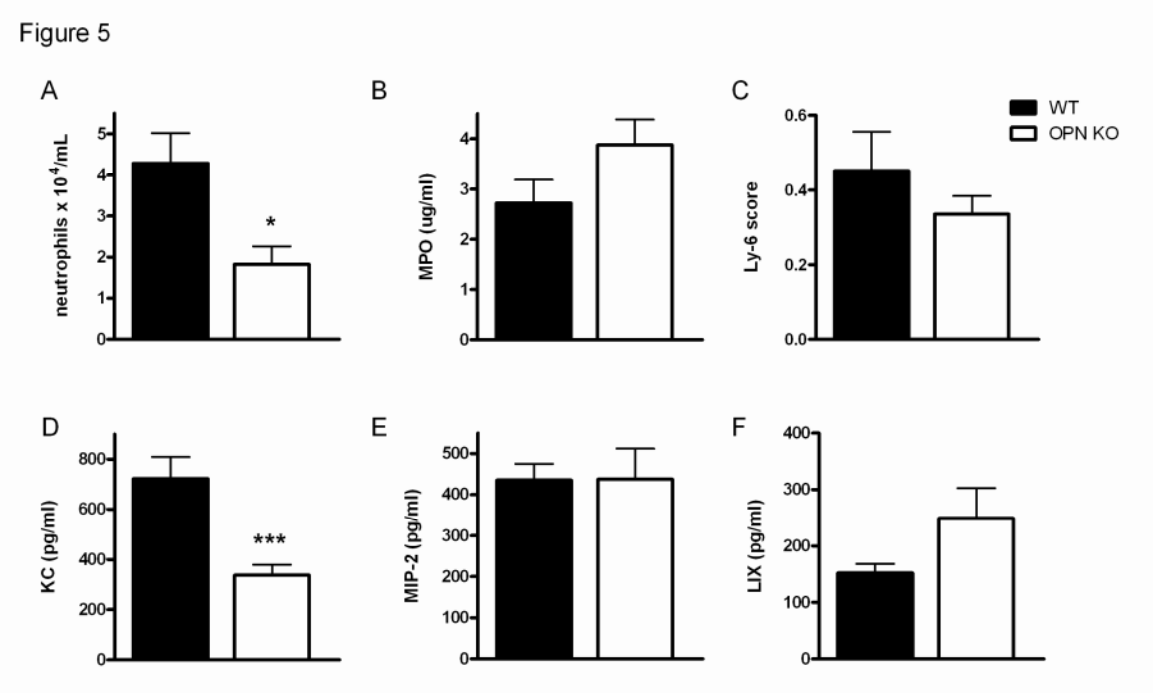


Figure 6: OPN induces neutrophil recruitment into the bronchoalveolar space. OPN levels in BALF of WT mice (A) and neutrophil influx into BALF of WT (black bars) and OPN KO (white bars) mice (B) 6 and 24 h after intranasal administration of 100 µg *Klebsiella* LPS. Data are expressed as means ± SEM (n = 6-8/group). ** $P < 0.01$ as compared to t=0 for (A) and ** $P < 0.01$ as compared to WT for (B). (C-E) Neutrophil influx into BALF at 6 h after intranasal PBS, 1 µg or 10 µg recombinant OPN administration in WT mice (C). Neutrophil influx into BALF at 6 h after intranasal 10 µg rOPN administration in WT mice that received 50 µg KC neutralizing antibody or isotype control intraperitoneally 2 h before rOPN (D) and in WT (black bars) and CD44 KO (white bars) mice (E). Data are expressed as means ± SEM (n = 5-8/group). *** $P < 0.001$ as compared to PBS.

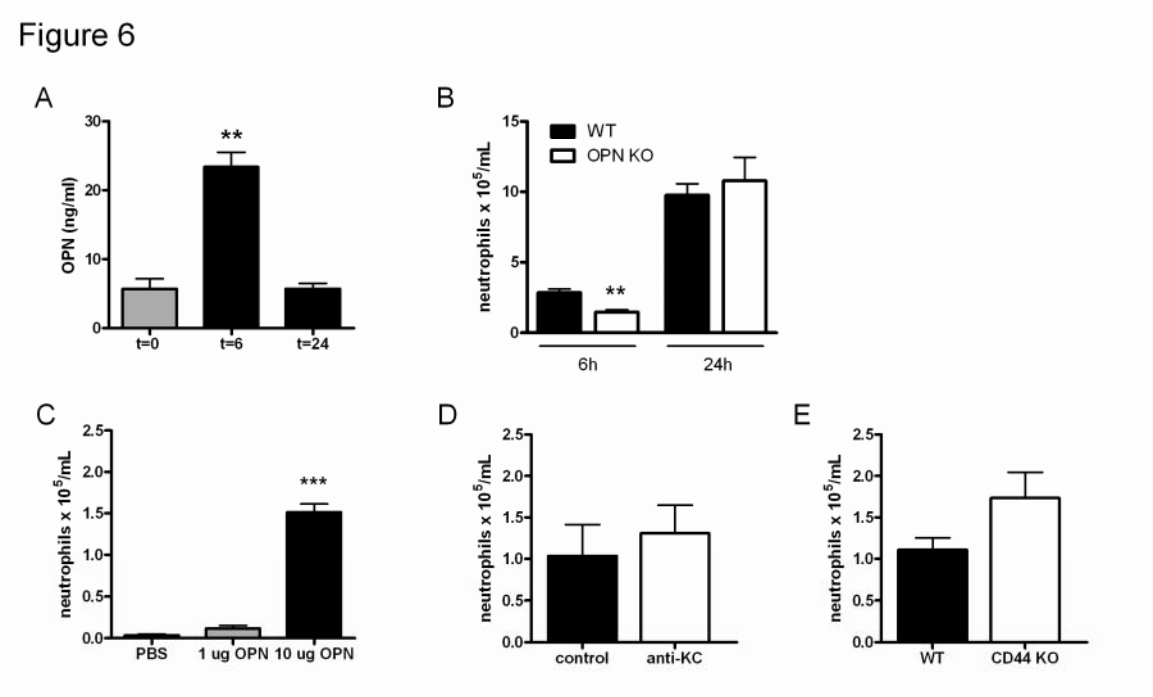


Figure 7: No bactericidal effect of OPN on *K. pneumoniae in vitro* and unaltered neutrophil phagocytosis and phago-lysosomal fusion. Increasing doses of recombinant OPN do not influence the growth of *K. pneumoniae* (A). Phagocytosis (B) of growth-arrested *K. pneumoniae* and phago-lysosomal fusion upon phagocytosis (C) was determined in neutrophils from WT (black symbols) and OPN KO (white symbols) blood at 37°C (solid lines) and 4°C (dashed lines). Data are expressed as means ± SEM; n = 5 mice/group.

Figure 7

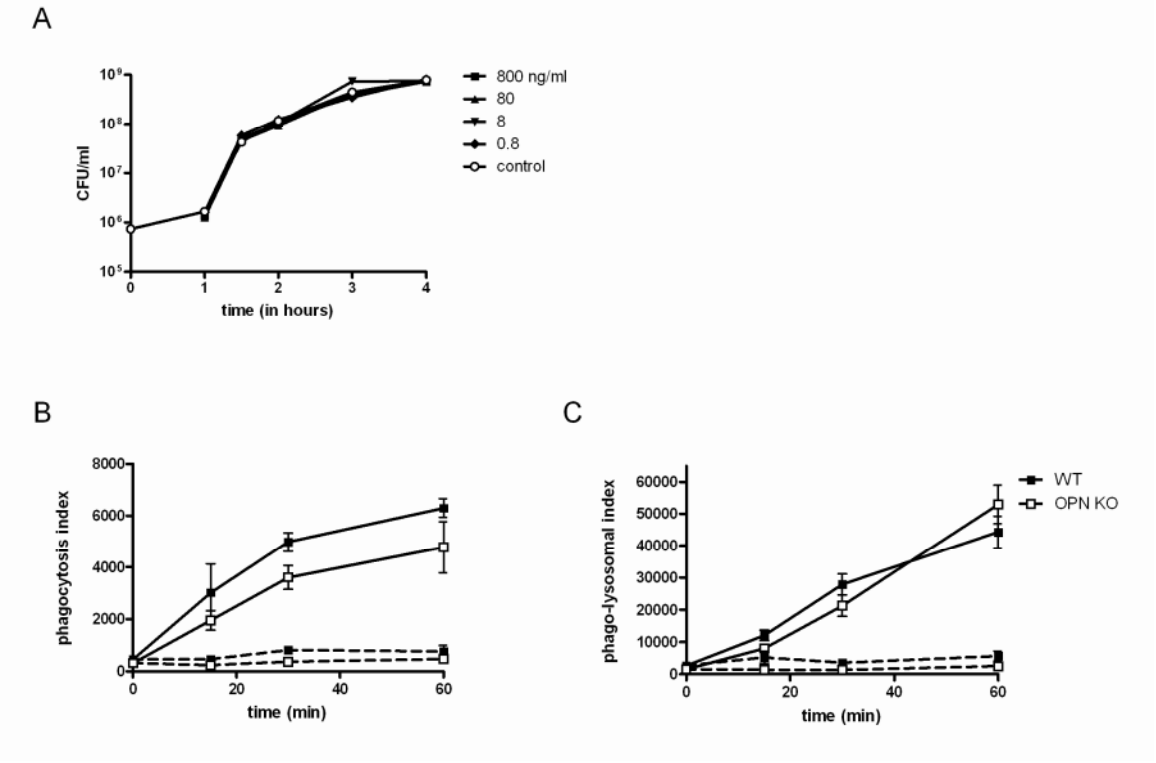

A
CFU/ml
time (in hours)
800 ng/ml
80
8
0.8
control
B
phagocytosis index
time (min)
C
phago-lysosomal index
time (min)
WT
OPN KO

Table I: No differences in plasma cytokine concentrations and organ failure in WT and OPN KO mice

	24 h		48 h	
Plasma mediators	**WT**	**OPN KO**	**WT**	**OPN KO**
TNF-α (pg/ml)	66.6 ± 18.4	93.3 ± 22.0	44.2 ± 8.0	35.2 ± 7.7
IL-6 (pg/ml)	264 ± 72	403 ± 112	353 ± 120	193 ± 34
ALAT (U/L)	17.6 ± 2.2	17.2 ± 3.3	666 ± 212	325 ±143
ASAT (U/L)	60.0 ± 7.4	50.6 ± 1.7	1125 ± 422	659 ± 332
Urea (mmol/L)	4.6 ± 0.4	5.7 ± 0.3	6.3 ± 0.2	6.6 ± 0.4
Creatinin (umol/L)	7.6 ± 0.5	6.7 ± 0.2	7.8 ± 0.6	8.0 ± 0.7

Plasma concentrations of TNF-α, IL-6, ALAT, ASAT, urea and creatinin in WT and OPN KO mice at 24 and 48 h after intranasal infection with 10^4 CFU *K. pneumoniae*. Data are expressed as mean ± SEM. n=6-8 mice/group.