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Chapter

Osteopontin promotes host defense during Klebsiella pneumoniae induced pneumonia

Gerritje J.W. van der Windt^{1,2} Jacobien J. Hoogerwerf^{1,2} Alex F. de Vos^{1,2} Sandrine Florquin³ Tom van der Poll^{1,2}

¹Center of Infection and Immunity Amsterdam (CINIMA), ²Center for Experimental and Molecular Medicine, and ³Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Submitted

Abstract

Klebsiella (K.) pneumoniae is a frequently isolated causative pathogen in respiratory tract infection. Osteopontin (OPN) is a phosphorylated glycoprotein that is involved in inflammatory processes during both innate and adaptive immunity. Circulating OPN levels are elevated in patients with sepsis and pneumonia. The aim of this study was to determine the role of OPN during K. pneumoniae induced pneumonia. Therefore, we intranasally infected wild-type (WT) and OPN knockout (KO) mice with 10⁴ colony forming units of K. pneumoniae, or administered them with Klebsiella LPS. In other experiments WT mice and mice deficient for the OPN receptor CD44 received PBS or rOPN. WT mice displayed elevated pulmonary and plasma OPN levels during Klebsiella pneumonia; OPN levels increased faster in the bronchoalveolar space than in lung tissue. 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. Early neutrophil recruitment into the bronchoalveolar space was impaired in the absence of OPN after intrapulmonary delivery of either intact Klebsiella or Klebsiella LPS. Moreover, rOPN induced neutrophil migration into the bronchoalveolar space, which was independent from the presence of CD44. OPN did not affect K. pneumoniae growth in vitro or phagocytosis and phagolysosomal fusion by neutrophils. 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.

Introduction

Pneumonia is a common and serious illness that is a major cause of morbidity and mortality in humans. *Klebsiella* (*K*.) *pneumoniae* is a frequently isolated causative pathogen in respiratory tract infection (1, 2). The high incidence of pneumonia and the increasing resistance to antimicrobial agents stress the importance of gaining more insight into the pathogenesis of this infectious disease (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 (3-5). 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 (3, 5-9). 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 (3, 10-12). Animal studies have especially pointed to a role for OPN in lung inflammation, such as seen in allergy and asthma (13-15), acute respiratory distress syndrome (16), fibrosis (17) and parasitic infection (18-20). Furthermore, patients suffering from diverse pulmonary diseases, including tuberculosis, silicosis and sarcoidosis, displayed enhanced OPN expression in their lungs (16, 21-23), and patients with tuberculosis or interstitial pneumonia had dramatically elevated plasma OPN levels as compared to healthy controls (24-26). 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 (27). 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 & Methods

Mice

Nine to eleven week old 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 (28)), 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 (29). After indicated time points mice were sacrificed, blood was drawn in heparin containing tubes and organs were removed aseptically, and homogenized in 5 volumes of sterile 0.9% NaCl using a tissue homogenizer (Biospec Products, Bartlesville, OK). In separate experiments for bronchoalveolar lavage (BAL), the trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland). BAL was performed by instilling two 0.5 ml aliquots of sterile saline. To determine bacterial loads ten-fold dilutions of blood, BAL fluid (BALF) and lung and spleen homogenates were plated on blood agar plates and incubated at 37°C for 16 h. Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA) and differential cell counts were performed on cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). In some experiments K. pneumoniae LPS (100 µg; L1519, Sigma, St. Louis, MO) or recombinant mouse OPN (rOPN, 1 or 10 μ g; < 1.0 EU endotoxin per 1 μ g 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 (30); 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).

Immunohistochemistry

Lungs were harvested 6, 24 or 48 h after infection, fixed in 10% buffered formalin for 24 h, and embedded in paraffin. H&E stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, thrombi, pleuritis and percentage of the lung surface demonstrating confluent (diffuse) inflammatory infiltrate by a pathologist blinded for groups. The total "lung inflammation score" was expressed as the sum of the scores for each parameter, the maximum being 28. For Ly-6 staining, Ly-6G monoclonal antibody was used (BD Pharmingen, San Diego, CA) as described (31). Ly-6G stained slides were photographed with a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Wetzlar, Germany). Ten random pictures were taken per slide. Colored areas were analyzed with Image Pro Plus (Media Cybernetics, Bethesda, MD) and expressed as percentage of the total surface area. The average of ten pictures was used for analysis.

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 in essence as described (32). In brief, growth-arrested (by mitomycin C, 50 µg/ml; Sigma) K. pneumoniae (10⁸ CFU/ml) labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE dye, Invitrogen, Breda, The Netherlands) were added to 50 µl heparinized whole blood from WT or OPN KO mice (n = 5 per group) and incubated at 37°C or 4°C for 0, 15, 30 or 60 minutes. Phagocytosis was stopped by placing cells on ice and erythrocytes were lysed with icecold isotonic NH₄Cl solution (155 nM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4). Neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen). To determine phagolysosomal fusion in neutrophils after phagocytosis of *K. pneumoniae*, the procedure was similar except for K. pneumoniae being labeled with pHrodo (Invitrogen) and neutrophils being labeled using anti-Gr-1-FITC (BD Pharmingen) (33). Phagocytosis and phago-lysosomal fusion were determined using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). 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 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 10⁴ 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).



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.

Osteopontin limits pulmonary bacterial growth

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 48h after infection. At 6 h after infection bacterial loads were similar in BALF and lungs from both mouse strains (Figure 2A-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 2A-B). To obtain insight into the dissemination of the infection we determined bacterial loads in blood and spleen.

At 6 h after infection 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 2C-D). Together these data suggest that OPN contributes to local host defense, within the pulmonary compartment, during *Klebsiella* pneumonia.



Figure 2: 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.

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 3).



Figure 3: 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).

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 4A, 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 4B-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 4D-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 ± 1.7 x 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 2), 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*.



Figure 4: 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.

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 (but not in OPN KO mice), LPS administration caused a significant rise in the BALF levels of OPN at 6 h after LPS (P < 0.01), whereas at 24 h OPN levels were back to baseline again (Figure 5A). 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 5B). 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.



Figure 5: 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 μ g *Klebsiella* LPS. Data are expressed as means \pm 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 μ g or 10 μ g recombinant OPN administration in WT mice (C). Neutrophil influx into BALF at 6 h after intranasal 10 μ g rOPN administration in WT mice that received 50 μ g 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 \pm SEM (n = 5-8/group). *** *P* < 0.001 as compared to PBS.

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 μ g of rOPN in WT mice. At 6 h after instillation we determined the numbers of infiltrated cells in BALF. Although 1 μ g of rOPN was not associated with influx of cells, instillation of 10 μ g rOPN resulted in the

recruitment of neutrophils to the bronchoalveolar space (Figure 5C, P < 0.001 as compared to PBS). Since the reduced neutrophil numbers in OPN KO mice early after *K. pneumoniae* infection was 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 (30, 34)) 2 h prior to intrapulmonary delivery of rOPN (10 µg). Anti-KC treatment did not affect neutrophil recruitment into BALF induced by rOPN (Figure 5D). CD44 is a transmembrane molecule mediating cellular migration and chemokine responses (11, 12), and an important receptor for OPN (10). 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 5E). 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 6A). In addition, phagocytosis capacity and phago-lysosomal fusion were not altered in OPN KO as compared to WT neutrophils (Figure 6B-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.



Figure 6: 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.

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 (27), 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. 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 (14, 16, 22, 23, 35-37). 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 (38). 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 (3, 16, 39). 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. As rapid neutrophil recruitment to the lungs is essential for effective clearance of K. pneumoniae (40, 41), 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 concanavalin A induced hepatitis (42-44). 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 (9). In accordance, OPN KO neutrophils were reported to exhibit reduced chemokinesis and chemotaxis towards N-formyl methionyl leucyl phenylalanine, whereas rOPN exerted direct chemotactic effects on neutrophils in vitro (9, 45). A recent study has indicated that polymerization of OPN results in an interaction with the α 9 β 1 integrin on neutrophils, which is essential for the chemotactic function of OPN (46). 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 (47), LIX (48) and MIP-2 (41) have been shown to mediate neutrophil recruitment during gram-negative lung inflammation. 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.

CD44 is an important receptor for OPN (3, 10) and involved in polarization and chemotaxis of neutrophils (49). In contrast to macrophages, the expression and localization of CD44 in neutrophils is independent of OPN expression (9). 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 (50).

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: phagocytosis of (IgG or IgM coated) sheep red blood cells, production of superoxide, cytokine release and matrix metalloproteinase-9 release upon phorbol 12-myristate 13-acetate (PMA) were all similar in WT and OPN KO neutrophils (9). Furthermore, we also did not find a direct bactericidal effect of any of the tested concentrations of OPN on *K. pneumoniae* growth *in vitro*. Recently, it has been shown that OPN is able to bind to *Staphylococcus aureus* and *Streptococcus agalactiae* and thereby mediates phagocytosis through $\alpha_x \beta_2$ integrins on monocytes (51). Whether OPN is also able to bind *K. pneumoniae* is unknown; however, our results demonstrate that for phagocytosis of this pathogen by neutrophils OPN is not crucial as the assay was performed in whole blood in which constitutive OPN was present in WT samples. Importantly, as bactericidal effects and phagocytosis are processes to occur rapidly upon infection, our *in vitro* results are in concurrence with similar bacterial loads at the early time point of 6 h after infection.

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.

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