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Chapter 8

Osteopontin impairs host defense during pneumococcal pneumonia

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Abstract

Streptococcus (S.) *pneumoniae* is the most frequently isolated pathogen responsible for community-acquired pneumonia. Osteopontin (OPN) is a phosphorylated glycoprotein that is involved in inflammatory processes during both innate and adaptive immunity. The aim of this study was to determine the role of OPN in the host response during pneumococcal pneumonia. OPN was constitutively present in lungs and plasma of naive wild-type (WT) mice. Upon intranasal infection with a lethal dose of *S. pneumoniae* pulmonary OPN concentrations were significantly elevated at 6 h and further increased at 48 h after infection, whereas plasma OPN levels increased more slowly and were elevated only at 48 h. OPN knock-out (KO) mice showed a prolonged survival relative to WT mice, which was accompanied by a diminished pulmonary bacterial growth and reduced dissemination to distant body sites. In addition, at 48 h post infection pulmonary inflammation was reduced in OPN KO mice as reflected by lower inflammation scores and reduced chemokine concentrations. In contrast to pneumococcal pneumonia, OPN deficiency did not influence bacterial growth in primary pneumococcal sepsis induced by direct intravenous infection, suggesting that the detrimental effect of OPN on antibacterial defense during pneumonia primarily is exerted in the pulmonary compartment. Moreover, recombinant OPN stabilized *S. pneumoniae* viability *in vitro*. These results suggest that the pneumococcus misuses OPN in the airways for optimal growth and to cause invasive disease after entering the lower airways.

Introduction

Bacterial pneumonia is a common and serious illness that is a leading cause of morbidity and mortality. *Streptococcus* (S.) *pneumoniae* (the pneumococcus) is the most frequently isolated pathogen responsible for community-acquired pneumonia (1, 2) and in recent sepsis trials this gram-positive bacterium was an important causative organism especially in the context of pneumonia (3). This, together with the increasing incidence of antibiotic resistance in this pathogen, stresses the importance to expand our knowledge of the host defense mechanisms that influence the outcome in *S. pneumoniae* pneumonia (1, 4).

Osteopontin (OPN) is a phosphorylated glycoprotein, expressed by a broad range of tissues and cells, that has been implicated as an important regulator of inflammation (5-7). OPN has an important role in both innate and adaptive immunity by mediating inflammatory cell differentiation, maturation and migration, and cytokine production (5, 7-11). OPN especially seems to be involved in lung inflammation. Indeed, patients suffering from diverse pulmonary diseases, including interstitial pneumonia, tuberculosis, silicosis and sarcoidosis, displayed enhanced OPN expression in their lungs (12-16), whereas patients with idiopathic pulmonary fibrosis demonstrated increased OPN levels in bronchoalveolar lavage fluid (17). In addition, plasma OPN levels were dramatically elevated in patients with interstitial pneumonia (18) and in patients with sepsis, of whom almost half suffered from pneumonia as the primary site of infection (19). Moreover, a functional role for OPN has been described in several experimental models of lung disease, including allergy and asthma (20-22), acute respiratory distress syndrome (16) and fibrosis (23, 24).

Considering the association between lung disease and sepsis on the one hand and pulmonary and systemic expression of OPN on the other hand, we here sought to determine the potential role of OPN in the host response during pneumococcal pneumonia.

Materials & Methods

Mice

Nine to twelve 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) 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 *S. pneumoniae* serotype 3 (ATCC 6303; American Type Culture Collection), as described (25, 26). Sepsis was induced by intravenous injection in the tail vein of 5 x 10^5 CFU of the same *S. pneumoniae* strain. The inoculum was plated on blood agar plates to determine viable counts. After 6, 24 or 48 h mice were sacrificed, blood was drawn and organs were removed aseptically, and homogenized in 5 volumes of sterile 0.9% NaCl. To determine bacterial loads ten-fold dilutions were plated on blood agar plates and incubated at 37°C for 16 h. For survival studies mice (n = 14) were monitored for 4 days after infection.

Assays

OPN, interleukin (IL)-1β, keratinocyte-derived cytokine (KC) and macrophage inflammatory protein (MIP)-2 were measured by ELISA using matched antibody pairs according to the manufacturer's instructions (R&D Systems, Abingdon, United Kingdom). Monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, IL-6, interferon (IFN)-γ and IL-10 were measured by cytometric bead array multiplex assay in accordance with the manufacturer's recommendations (BD Biosciences, San Jose, CA).

Immunohistochemistry

Lungs were harvested 6, 24 or 48 h after infection, fixed in 10% buffered formalin for 24 h, and embedded in paraffin. Hematoxilin and eosin stained slides were coded and scored from 0 (absent) to 4 (severe) for the following parameters: interstitial inflammation, endothelialitis, bronchitis, edema, thrombi, pleuritis and the 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.

Effect of OPN on *S. pneumoniae* viability, phagocytosis and phago-lysosomal fusion

S. pneumoniae (1 x 10^6/ml) was incubated in sterile normal saline in the presence of 800-0.8 ng/ml recombinant mouse OPN (rOPN; < 1.0 EU endotoxin per 1 µg as

determined by the Limulus amoebocyte lysate assay; R&D Systems, Minneapolis, MN), 800 ng/ml boiled rOPN (30 min 100°C), 800 ng/ml bovine serum albumin (BSA), or normal saline only at 37°C for 6 h. 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 *S. pneumoniae* was determined in essence as described (27). In brief, 10^6 CFU of growth-arrested (by mitomycin C, 50 µg/ml; Sigma) *S. pneumoniae* 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=8 per group) and incubated at 37°C or 4°C for 0, 20 or 60 minutes. Phagocytosis was stopped by placing cells on ice and erythrocytes were lysed with ice-cold isotonic NH_4Cl solution (155 nM NH_4Cl, 10 mM $KHCO_3$, 100 mM EDTA, pH 7.4). Neutrophils were labeled using anti-Gr-1-PE (BD Pharmingen, San Diego, CA). To determine phago-lysosomal fusion in neutrophils after phagocytosis of *S. pneumoniae*, the procedure was similar except for *S. pneumoniae* being labeled with pHrodo (Invitrogen) and neutrophils being labeled using anti-Gr-1-FITC (BD Pharmingen) (28). 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 data 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, survival was compared by Kaplan-Meijer analysis followed by log-rank test and comparisons of the numbers of positive and negative cultures were done using Chi-square tests, all using GraphPad Prism version 4.0, GraphPad Software (San Diego, CA). A value of $P < 0.05$ was considered statistically significant.

Results

Osteopontin levels increase during *S. pneumoniae* pneumonia

To obtain a first insight into a potential role for OPN during pneumococcal pneumonia, we measured OPN concentrations in lung and plasma from WT mice before, 6, 24 and 48 h after infection mice with 10^4 CFU S. *pneumoniae*. OPN concentrations in lung were significantly elevated at 6 h as compared to baseline and further increased

at 48 h after infection (Figure 1A). Plasma levels increased more slowly and were significantly elevated only at 48 h post infection as compared to baseline (Figure 1B).

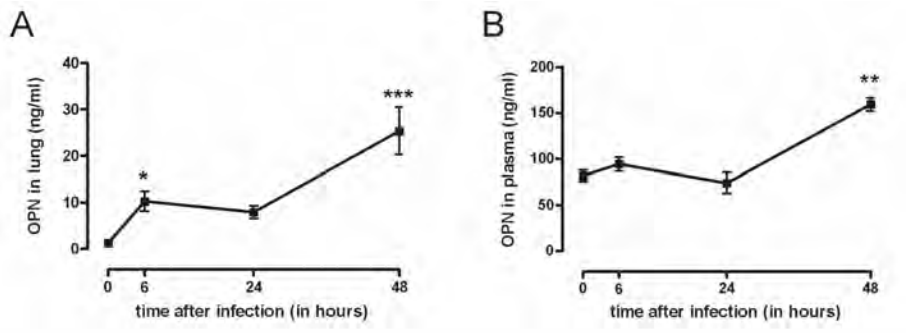

Figure 1: Pulmonary and plasma OPN concentrations are elevated during pneumococcal pneumonia. OPN concentrations in (A) lung and (B) plasma before, 6, 24 and 48 h after infection with 10^4 CFU of *S. pneumoniae*. Data are expressed as mean ± SEM; n = 8 mice/group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to t=0.

Osteopontin negatively affects mortality and pulmonary bacterial growth during pneumococcal pneumonia

To investigate a potential role for OPN during the host response against pneumococci, we intranasally infected WT and OPN KO mice with a lethal dose of *S. pneumoniae* and followed them for 4 days. OPN deficiency significantly prolonged survival: the median survival time was 61.5 h for WT mice versus 70.5 h for OPN KO mice (Figure 2A, $P = 0.01$). Next, we wondered whether OPN deficiency impacts on bacterial growth in lungs. Therefore, we infected WT and OPN KO mice with 10^4 CFU *S. pneumoniae* and sacrificed them after 6, 24 or 48 h of infection. At 6 h, OPN KO mice displayed significantly decreased bacterial loads in their lungs as compared to WT mice (Figure 2B, $P < 0.01$). Although at 24 h pulmonary bacterial loads were similar in both groups, beyond this time point the number of pneumococci in lung homogenates only grew in WT mice and at 48 h post infection bacterial burdens were approximately 200-fold higher in WT mice than in OPN KO mice ($P < 0.001$). Remarkably, whereas none of the mice in either group displayed positive blood or spleen cultures 6 h post infection, OPN KO mice displayed a significantly diminished dissemination of the infection at 24 h as reflected by the fact that *S. pneumoniae* could be cultured from blood of only 3/8 OPN KO mice versus 7/8 WT mice and from spleen of only 1/7 OPN KO mice versus 7/8 WT mice ($P < 0.05$ and $P < 0.01$, respectively). In concurrence with

the data on pulmonary growth, bacterial loads in blood and spleen were further enhanced at 48 h after infection, but to a significantly lesser extent in OPN KO as compared to WT mice (Figure 2C-D, $P < 0.001$ and $P < 0.05$, respectively). Together these results suggest that OPN facilitates bacterial growth and early dissemination during severe pneumococcal pneumonia, which might explain the delayed lethality of OPN KO mice.

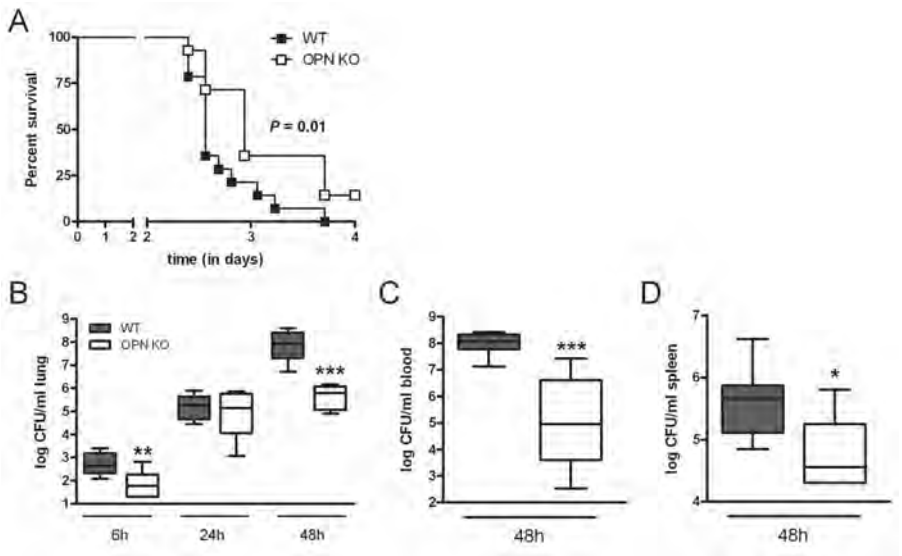

Figure 2: Prolonged survival and reduced bacterial growth in OPN KO mice. (A) Percentage survival of WT (closed symbols) and OPN KO (open symbols) mice after intranasal infection with 10^4 CFU of *S. pneumoniae*. n = 14 mice/group. *P* value indicates the difference between groups. WT (grey) and OPN KO (white) mice were infected with 10^4 CFU of *S. pneumoniae* and bacterial loads were determined 6, 24 and 48 h after infection in (B) lung, (C) blood and (D) spleen. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation; n = 8 mice/group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to WT mice.

Reduced pulmonary inflammation in OPN KO mice

To further analyze lung pathology in response to *S. pneumoniae* pneumonia we determined pulmonary inflammation by the semi-quantitative scoring system described in the Methods section. Already at 6 h after infection in all mice mild pulmonary inflammation was present that slightly increased towards 24 h; at these early time points the extent of lung inflammation was similar in both groups (Figure 3A-F). At 48 h pulmonary inflammation had strongly increased in both groups; however, at this late time point the extent of lung pathology was much more severe

in WT mice than in OPN KO mice, which was especially due to more endothelialitis and bronchitis and an enhanced percentage of the lung that was affected (Figure 3G-I, $P < 0.01$ as compared to WT). In line, OPN KO mice showed significantly lower lung weights at 48 h after infection (0.20 ± 0.01 versus 0.26 ± 0.01 g in WT mice, $P < 0.01$), supporting our data on reduced pulmonary inflammation in the absence of OPN.

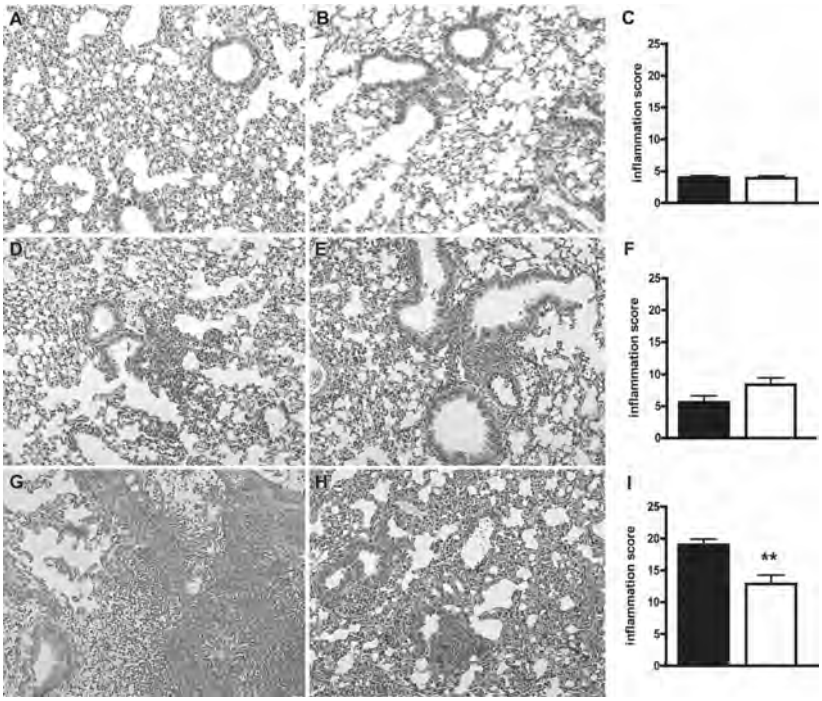

Figure 3: Reduced lung histopathology in OPN KO mice. Representative lung histology of WT (A, D and G) and OPN KO (B, E and H) mice at 6 (A-C), 24 (D-F) and 48 (G-I) h after intranasal infection with 10^4 CFU of *S. pneumoniae*. The lung sections are representative for 8 mice per group per time point. H&E staining, original magnification 10x. Inflammation scores are expressed as mean ± SEM (WT mice: black bars; OPN KO mice: white bars, n = 8 mice/group). ** $P < 0.01$ as compared to WT mice.

Cytokine and chemokine levels during pneumococcal pneumonia

Cytokines and chemokines play an important role in host defense during bacterial pneumonia (1, 29). Therefore we measured the concentrations of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IFN-γ), the anti-inflammatory cytokine IL-10 and chemokines (KC, MIP-2 and MCP-1) in lungs obtained 6, 24 and 48 h after infection. At 6 h after infection all mediators were similar in WT and OPN KO mice or below detection limits (data not shown). Similarly, at 24 h no differences were found between

the two groups in the pulmonary levels of any of these mediators (Table I). Whereas lung cytokine levels remained similar in both mouse strains at 48 h, chemokine levels were decreased in lungs from OPN KO as compared to WT mice (Table I; KC: $P = 0.06$, MIP-2: $P < 0.01$ and MCP-1: $P < 0.05$). In addition, we measured concentrations of TNF-α, IL-6, IFN-γ and MCP-1 in plasma and spleen. These mediators were similar in WT and OPN KO mice at 24 h; however, at 48 h post infection OPN KO mice displayed strongly reduced concentrations of all four mediators in plasma (Table I) and spleen (data not shown).

Table I: Pulmonary and plasma cytokine concentrations during pneumococcal pneumonia.

	24h		48h	
pg/mL	**WT**	**OPN KO**	**WT**	**OPN KO**
Lung				
TNF-α	46 ± 35	32 ± 13	354 ± 63	508 ± 232
IL-1β	815 ± 493	839 ± 335	3405 ± 404	4251 ± 1235
IL-6	324 ± 211	356 ± 162	2278 ± 411	1483 ± 457
IFN-γ	8 ± 5	6 ± 2	56 ± 16	36 ± 10
IL-10	51 ± 33	170 ± 55	177 ± 15	226 ± 18
KC	2753 ± 1053	1195 ± 297	12258 ± 1717	7170 ± 1419
MIP-2	1389 ± 984	1365 ± 356	62208 ± 10105	18125 ± 4834 **
MCP-1	1623 ± 490	1432 ± 412	4782 ± 833	2465 ± 341 *
Plasma				
TNF-α	7 ± 2	7 ± 2	268 ± 71	38 ± 17 **
IL-6	146 ± 53	156 ± 61	6124 ± 1488	437 ± 104 ***
IFN-γ	15 ± 6	17 ± 7	670 ± 285	70 ± 20 *
MCP-1	213 ± 73	202 ± 70	2293 ± 477	295 ± 68 ***

Proinflammatory cytokine (TNF-α, IL-1β, IL-6, IFN-γ), antiinflammatory IL-10 and chemokine (KC, MIP-2 and MCP-1) levels in lung and plasma at 24 and 48 h after intranasal *S. pneumoniae* infection in WT and OPN KO mice. Data are expressed as mean ± SEM; n = 8 mice/group/time point. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. WT at the same time point.

Osteopontin stabilizes *S. pneumoniae* viability *in vitro*

The reduced bacterial outgrowth in the absence of OPN might be explained by an effect of OPN on phagocytosis capacity of or phago-lysosomal fusion in OPN KO neutrophils or by a direct effect of OPN on *S. pneumoniae*. Phagocytosis capacity and phago-lysosomal fusion were not altered in OPN KO as compared to WT neutrophils (Figure 4). To establish a possible direct effect of OPN on pneumococcal growth, we incubated *S. pneumoniae* in sterile normal saline at a concentration similar to the inoculum we used for infection experiments for 1-6 h in the presence or absence of

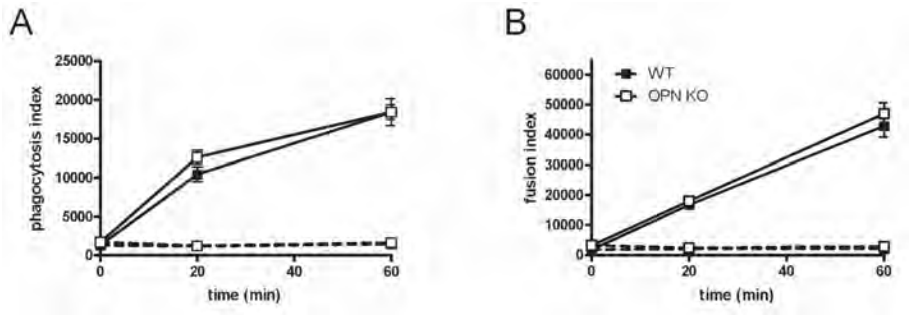

Figure 4: No effect of OPN on neutrophil phagocytosis and phago-lysosomal fusion. Phagocytosis (A) of growth-arrested *S. pneumoniae* and phago-lysosomal fusion upon phagocytosis (B) was determined in neutrophils from WT (closed symbols) and OPN KO (open symbols) blood at 37°C (solid lines) and 4°C (dashed lines). Data are expressed as mean ± SEM; n = 4-8 mice/group.

increasing concentrations of rOPN (0.8-800 ng/ml) and determined the viability of the bacteria. The number of viable pneumococci decreased over time when incubated in normal saline only. rOPN 800 ng/ml stabilized the viability of *S. pneumoniae*, whereas lower OPN concentrations had no effect (Figure 5A). In a separate experiment we confirmed the effect of rOPN on the viability of *S. pneumoniae* and further showed that boiled OPN or BSA did not exert such an effect (Figure 5B). These data suggest that OPN serves as a growth factor for *S. pneumoniae in vitro*.

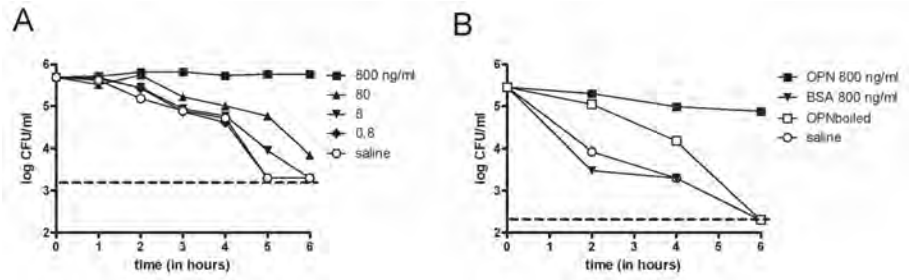

Figure 5: OPN stabilizes *S. pneumoniae* viability *in vitro*. (A) *S. pneumoniae* in saline (10^6 CFU/ml) was incubated with increasing doses (0.8-800 ng/ml) of recombinant OPN (black symbols) or saline (white symbols) and the viability of *S. pneumoniae* was determined over 6 h at 37°C. (B) *S. pneumoniae* in saline (10^6 CFU/ml) was incubated with 800 ng/ml recombinant OPN (■), 800 ng/ml boiled recombinant OPN (□), 800 ng/ml BSA (▼), or saline (○) and the viability of *S. pneumoniae* was determined over 6 h at 37°C. Dashed lines depict detection limits.

OPN does not impair the immune response upon intravenously administered *S. pneumoniae*

We wondered whether the detrimental effect of OPN on host defense against *S. pneumoniae* was primarily present in the lungs (with as a consequence thereof enhanced bacterial dissemination to distant organs) or also was demonstrable in the systemic compartment. To study this, we injected *S. pneumoniae* directly intravenously into OPN KO and WT mice, thereby bypassing the role of OPN in the lungs upon primary pulmonary infection. OPN was detectable at relatively high concentrations in the circulation of uninfected WT mice, confirming the data presented in figure 1B; intravenous *S. pneumoniae* injection resulted in a modest rise in the plasma concentrations of OPN which did not reach statistical significance (0 h: 69 ± 6 ng/ml; 48 h: 95 ± 11 ng/ml; non significant). Unlike the clear phenotype of OPN KO mice in pneumococcal pneumonia, OPN KO and WT mice displayed similar bacterial loads in blood, lung, spleen and liver at 24 and 48 h after intravenous administration of *S. pneumoniae* (Figure 6). In addition, TNF-α, IL-6, IFN-γ and MCP-1

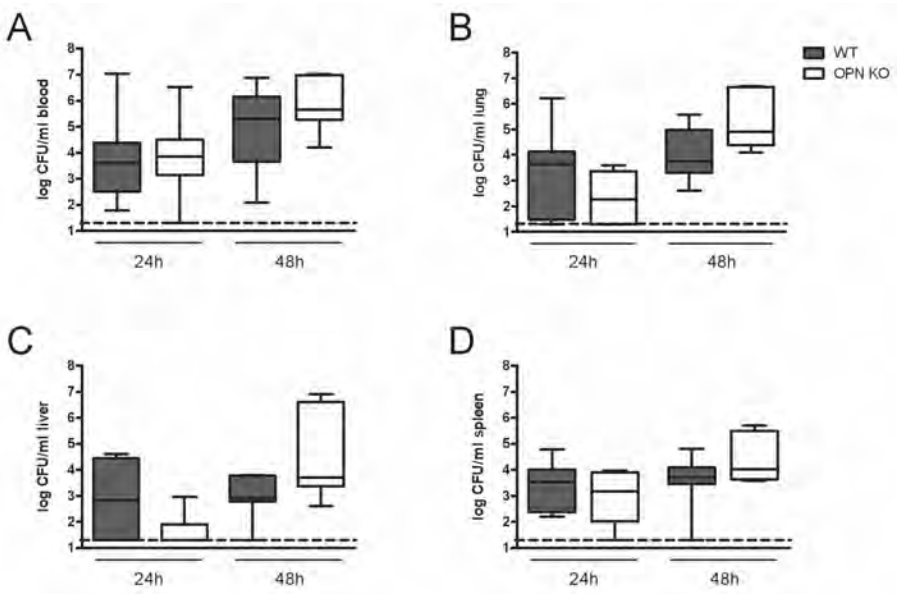

Figure 6: Similar bacterial growth during pneumococcal sepsis. Bacterial loads in (A) blood, (B) lung, (C) liver and (D) spleen from WT (grey) and OPN KO (white) mice at 24 and 48 h after intravenous infection with 10^5 CFU of *S. pneumoniae*. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation; n = 8 mice/group. Dashed line depicts detection limit.

concentrations in plasma (Table II) and spleen (data not shown) were indistinguishable between both mouse strains at both time points. Taken together, these data suggest that the negative effect of OPN on the antibacterial response during pneumococcal pneumonia is due to a pulmonary rather than a systemic effect of OPN.

Table II: Plasma cytokine concentrations after intravenous infection with *S. pneumoniae.*

	24h		48h	
pg/mL	**WT**	**OPN KO**	**WT**	**OPN KO**
Plasma				
TNF-α	18 ± 10	21 ± 6	31 ± 10	66 ± 23
IL-6	236 ± 171	202 ± 60	325 ± 76	1001 ± 323
IFN-γ	157 ± 121	178 ± 94	222 ± 119	389 ± 116
MCP-1	607 ± 261	650 ± 176	552 ± 278	844 ± 290

Proinflammatory cytokine (TNF-α, IL-6 and IFN-γ), and chemokine (MCP-1) levels in plasma at 24 and 48 h after intravenous *S. pneumoniae* infection in WT and OPN KO mice. Data are expressed as mean ± SEM; n = 8 mice/group/time point.

Discussion

The present study is the first to investigate the functional role of OPN during gram-positive bacterial pneumonia and sepsis. In pneumococcal pneumonia OPN KO mice displayed a survival benefit and an improved antibacterial defense, as reflected by lower bacterial loads in their lungs, reduced dissemination to distant body sites and less pulmonary inflammation. In contrast, during primary pneumococcal sepsis OPN deficiency did not affect bacterial growth. We further showed that OPN stabilizes *S. pneumoniae* viability *in vitro*. Our data suggest that OPN facilitates the growth of pneumococci in the lungs after primary infection of the respiratory tract, subsequently resulting in enhanced dissemination of the infection.

Knowledge of the production of OPN during acute inflammatory diseases is limited. Recently, patients with bacterial sepsis, who predominantly suffered from pneumonia, were reported to have elevated circulating levels of OPN (19). Although several investigations demonstrated enhanced OPN expression during subacute and chronic pulmonary inflammation (12, 14-17, 21, 30), so far only one study examined the impact of bacterial respiratory tract infection on OPN production, showing induction of OPN mRNA in the lungs of mice exposed to the gram-negative pathogen *Francisella novicida* via the airways (31). In accordance, in preliminary experiments our group observed elevated pulmonary levels of OPN in mice with gram-negative

pneumonia caused by either *Klebsiella pneumoniae* or *Burkholderia pseudomallei* (data not shown, see Chapter 7 and 9). To date studies on OPN levels during gram-positive bacterial infection have not been reported. We show here that airway infection with viable *S. pneumoniae* results in a rapid increase of OPN in lung tissue with a more gradual rise in plasma OPN levels.

In this study we demonstrate that endogenous OPN impairs host defense during pneumococcal pneumonia. The experiments in which *S. pneumoniae* was injected directly into the circulation, revealing similar bacterial growth in OPN KO and WT mice in all body sites examined, suggest that the effect of OPN on pneumoccal infection primarily is exerted in the lungs. In accordance with this notion, bacterial loads in lungs of OPN KO mice with pneumococcal pneumonia were already reduced 6 h after infection, which at 24 h was associated with a clearly diminished spreading of pneumocci to blood and spleen in these animals. Of note, at 24 h pulmonary bacterial burdens were similar in OPN KO and WT mice, whereas at 48 h bacterial loads were approximately 200-fold higher in the lungs of the former mouse strain. These data suggest that the innate immune system is unable to compensate for the apparently detrimental effect of OPN on the growth of *S. pneumoniae* in the lungs in the early phase of pneumococcal pneumonia (when defense mechanism predominantly rely on resident cells such as alveolar macrophages and the respiratory epithelium) as well in the late phase of the infection (when the growing bacterial load overwhelms innate defense mechanisms provided by infiltrating neutrophils).

OPN did not impact on pulmonary inflammation during the early phase of pneumococcal pneumonia; however, at 48 h after infection less inflammation was present in lungs of OPN KO mice. The attenuated inflammatory response in OPN KO mice at this late time point was also reflected in reduced chemokine levels in lung homogenates and lower plasma concentrations of TNF-α, IL-6, IFN-γ and MCP-1. Likely, the diminished local and systemic inflammatory response associated with OPN deficiency was the result of the much lower bacterial loads in lungs and blood, providing a less potent proinflammatory stimulus. Indeed, earlier studies have demonstrated a clear positive correlation between the bacterial load and the extent of inflammation during pneumococcal pneumonia (32, 33). OPN may influence the production of cytokines and chemokines in various ways: OPN deficiency has been associated with an impaired T-helper 1 response upon several stimuli (34) (providing an additional explanation for the reduced IFN-γ levels in OPN KO mice in the current

study), whereas OPN stimulation induced a proinflammatory response in human monocytes (35).

OPN has been shown to be chemotactic for neutrophils *in vitro* and *in vivo* (see also Chapter 7) (11, 36-39) and neutrophil recruitment to the lungs is essential for a protective host response against *S. pneumoniae* (40-42). We found no difference in neutrophil content of lung tissue from WT and OPN KO mice, as reflected by similar MPO levels in lung homogenates (data not shown). The reduced bacterial growth in the absence of OPN might alternatively be explained by an altered phagocytosis capacity or phago-lysosomal fusion in OPN KO neutrophils. However, 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 (11). Recently, it has been shown that OPN is able to bind to *Staphylococcus aureus* and *Streptococcus agalactiae*, thereby mediating phagocytosis through $\alpha_x\beta_2$ integrins on monocytes (43). Whether OPN is also able to bind *S. pneumoniae* remains to be established; 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 in WT samples constitutive OPN was present. Interestingly, we found that OPN stabilizes the viability of *S. pneumoniae in vitro*. This effect was dose-dependent and specific as boiled OPN and BSA were not effective. OPN did not affect *S. pneumoniae* growth in Todd-Hewitt broth supplemented with yeast extract (a regular growth medium for pneumococci; data not shown), suggesting that OPN prolongs pneumococcal survival in non-optimal conditions. Together these data suggest that OPN, which is constitutively present in lungs of uninfected mice and is further induced by *S. pneumoniae*, is misused by the pneumococcus during infection of the lower airways.

In conclusion, we show here for the first time that OPN impairs the antibacterial response against *S. pneumoniae* in the lungs, at least in part by stabilizing the viability of this gram-positive pathogen.

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