Studies on coagulation-induced inflammation in mice
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Chapter 8

A hypoxic episode boosts pulmonary host defense during 
Pseudomonas Aeruginosa pneumonia

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Abstract

Cytokines play an important role in the pathogenesis of Pseudomonas aeruginosa pneumonia. Indeed, tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine, and interleukin (IL)-10, an anti-inflammatory cytokine, improve host defense during pneumonia with P. aeruginosa. Previously, we reported that exposure of mice to hypoxia leads to an altered cytokine expression pattern in different body tissues. Levels of TNF-α were elevated in lung tissue, and remained elevated for at least 10 days after re-exposure to normal oxygen levels. During and after hypoxia, no cytokines could be detected in the circulation. Therefore, we hypothesized that a period of hypoxia preceding pneumonia would enhance host defense against P. aeruginosa. To test this hypothesis, mice were exposed to 8% O₂ for 24 hours; five days later, these mice were inoculated with P. aeruginosa. Normoxic mice inoculated with P. aeruginosa and hypoxic mice inoculated with saline served as controls. Inoculation with P. aeruginosa resulted in pneumonia, with elevated wet lung weights, histological signs of inflammation, and local bacterial growth. Elevated levels of TNF-α and IL-10 were present in lungs. Although wet lung weights and histological signs of inflammation did not differ between the two pneumonia groups, bacterial outgrowth from lung tissues was lower in hypoxic mice than mice that were held in normal oxygen tensions before instillation of bacteria.

In summary, a preceding hypoxic episode boosts host defense during P. aeruginosa pneumonia, most likely via local induction of cytokine levels in the lung.

Introduction

In pneumonia, the initiation, maintenance, and resolution of inflammation are dependent upon a complex network of pro-inflammatory and anti-inflammatory cytokines. Much of our knowledge about the role of cytokines in the pathogenesis of pneumonia is derived from animal studies of experimental pneumonia. In contrast to systemic infection where excessive production of pro-inflammatory cytokines is detrimental by leading to organ failure and death,¹,² local production of pro-inflammatory cytokines is required for adequate pulmonary host defense against respiratory pathogens, such as Streptococcus pneumoniae³,⁴ and Klebsiella pneumoniae.⁵ During P. aeruginosa pneumonia, both administration of low doses of TNF-α, and augmentation of local expression of TNF-α in the lungs through gene therapy, significantly diminished mortality and enhanced bacterial clearance from the pulmonary compartment.⁶-⁸ In contradiction with a beneficial role of TNF, administration of P. aeruginosa to mice lacking the type 1 TNF receptor show enhanced bacterial clearance from their lungs, indicating a negative contribution of TNF receptor type 1 in host defense against P. aeruginosa.⁹ Surprisingly, administration of the anti-inflammatory cytokine IL-10 also improves host defense in a murine model of pneumonia with P. aeruginosa, as shown by decreased lung injury and mortality.¹⁰
Hypoxia and *P. aeruginosa* pneumonia

We recently demonstrated that exposure of mice to low levels of oxygen (hypoxia) results in rapid and prolonged elevation of TNF-α and IL-10 levels in different body tissues, including lungs.\(^{11}\) Levels of TNF-α remained elevated for more than 10 days after re-exposure to ambient oxygen levels. This change in expression of cytokines was restricted to organ tissues, since hypoxia did not induce systemic cytokine production. *Ex vivo* cytotoxicity studies showed that TNF-α produced during hypoxia induces cell death of WEHI cells, implying that hypoxia-induced cytokines are biologically active.

Based on the persistently high cytokine levels, we hypothesized that a preceding hypoxic period would influence pulmonary host defense during *P. aeruginosa* pneumonia. Therefore, in the present study, we exposed mice to 8% O\(_2\) for 24 hours and five days later inoculated them with *P. aeruginosa*. Pulmonary cytokine levels, histological signs of inflammation, and local bacterial growth were determined to visualize the influence of a preceding hypoxic period on pulmonary host defense.

**Methods**

**Animals**

Female C57BL/6 wild-type mice were purchased from Charles River (Zeist, The Netherlands). All mice were housed in the same temperature-controlled room with alternating 12h light/dark cycles, and were allowed to equilibrate for at least 5 days before the study. Animals were provided regular mouse chow (SRM-A; Hope Farms, Woerden, The Netherlands) and water *ad libitum*. Mice were used at 8-10 weeks of age. The experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands.

**Exposure of mice to hypoxia**

Mice were exposed to normobaric hypoxia for 24 hours using the method described previously.\(^ {11}\) In brief, mice were placed in a custom-made hypoxia-chamber containing an oxygen sensor (Marin Assist, Hazerswoude, The Netherlands) and the oxygen level was lowered to 8% within one hour. After 24 hours at 8% O\(_2\) the mice were re-exposed to ambient oxygen levels for 5 days. Mice exposed to ambient oxygen levels alone were used as controls.

**Induction of pneumonia**

*P. aeruginosa* (strain PA103) pneumonia was induced as described.\(^ {12,13}\) Briefly, bacteria were grown to mid-logarithmic phase in Luria Broth for 6 hours at 37°C, harvested by centrifugation at 1,500 g for 15 min, washed twice in pyrogen-free 0.9% NaCl and resuspended in 10 ml of 0.9% NaCl. The number of bacteria was determined by serial dilution in sterile 0.9% NaCl and subsequent culturing on blood agar plates for 16 hours. Before the intranasal administration of bacteria (50
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μl inoculum containing $5 \times 10^5$ CFU/ml, mice were anesthetized by inhalation of isoflurane (Forene, Abott, Queensborough, Kent, UK). Mice exposed to hypoxia but inoculated with 50 μl of sterile 0.9% NaCl alone were used as hypoxic controls. All mice were sacrificed after 24 hours, because former experiments using this model of acute pneumonia did not show any significant changes at earlier time points, and because mice would die rapidly at later time points.\textsuperscript{14,15}

*Preparation of blood samples and lung homogenates*

Twenty-four hours after inoculation, mice were bled from the vena cava inferior after being anesthetized by i.p. injection of FFM (1:1:2 hypnorm (Janssen Pharmaceutical, Beerse, Belgium), dormicum (Roche, Mijdrecht, The Netherlands), H$_2$0 (sterile water for injection, Braun Melsungen AG, Melsungen, Germany); 0.1 mL per 10 grams body weight). Whole lungs were harvested and weighed. One lung was used for histology and the other one was homogenized in 5 volumes sterile 0.9% NaCl. Serial 10-fold dilutions were made in sterile 0.9% NaCl, plated onto sheep blood agar plates and incubated at 37°C. Colony-forming units were counted after a 16-hour-incubation period.

For cytokine measurements, lung homogenates were diluted in equivolunteers of lysis buffer (pH 7.4, yielding final concentrations of 150 mmol/L NaCl, 15 mmol/L Tris-HCl, 1 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 1% Triton (v/v), 10 pmol/L pepstatin A, 10 pmol/L leupeptin and 10 pmol/L aprotinin), and centrifuged twice (1,780 g and 20,800 g, respectively). Supernatants were stored at -20°C until cytokine measurement.

*Cytokine measurements*

IL-10 and TNF-α levels were measured using commercially available ELISA kits (R&D Diagnostics, Minneapolis, USA). Detection limits were 31 pg/mL.

*Histology*

Shortly after sacrificing the mice, lungs were removed, fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. 5-μm-thick sections were stained with hematoxylin and eosin according to standard protocols. All slides were coded and scored by a pathologist for the presence of pneumonia, interstitial inflammation, endothelialitis, bronchitis, edema, thrombosis and pleuritis. All characteristics were rated on a 0-4 scale.

*Statistics*

Data are expressed as means ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann-Whitney U test in case of histology data and using the Student’s t-test in case of the other data. A value of p < 0.05 was considered to represent a statistically significant difference.
Hypoxia and *P. aeruginosa* pneumonia

**Results**

*Exposure of mice to hypoxia*

To study the effect of preceding hypoxia on pulmonary host defense during *P. aeruginosa* pneumonia, mice were exposed to 8% O₂ for 24 hours. In accordance with previous results, hypoxic mice showed signs of dyspnea and did not eat, drink or move about normally resulting in loss of body weight and hypothermia. Within minutes after re-exposure to ambient oxygen levels, body temperature and behavior normalized and the previous hypoxic mice were indistinguishable from normoxic controls.

*Induction of pneumonia*

Five days after a 24-hour-exposure to hypoxia, mice were inoculated with *P. aeruginosa* (for clarity, these mice will be indicated as pneu-hypoxic) or saline (hypoxic controls). Normoxic mice inoculated with *P. aeruginosa* served as additional controls (pneu-normoxic). Inoculation with *P. aeruginosa* induced signs of pneumonia in all mice. Twenty-four hours after inoculation with *P. aeruginosa*, lungs appeared swollen and reddish, with multiple hemorrhages on the surface. As is shown in figure 1, wet lung weights of pneu-hypoxic mice were two times higher than that of hypoxic controls without pneumonia (342 ± 29 mg vs 186 ± 5 mg). Wet lung weights of pneu-hypoxic and pneu-normoxic mice inoculated with *P. aeruginosa* did not differ (342 ± 29 mg vs. 368 ± 21 mg, respectively).

![Figure 1. Wet lung weights 24 hours after inoculation with *P. aeruginosa* are not influenced by a previous hypoxic period. Pneu-hypoxic mice are shown as black bars, pneu-normoxic mice as dashed bars, and hypoxic mice inoculated with 0.9% NaCl as white bars (n=10 per group). Indicated is mean +/- SEM. * P < 0.05 between indicated groups](image)

Twenty-four hours after inoculation, lungs of all mice displayed interstitial inflammation, endothelialitis and pleuritis (figure 2). Bronchitis, edema and thrombi were found to a lesser extent. No difference between pneu-hypoxic and pneu-normoxic mice was observed. Hypoxic controls showed only minor signs of interstitial inflammation, endothelialitis, pleuritis and thrombus formation and no signs of bronchitis or edema.
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Bacterial clearance

To study the consequences of a preceding hypoxic episode on antibacterial defense to *P. aeruginosa*, we compared bacterial outgrowth in lung and blood 24 hours after infection (figure 3). Bacterial loads in lung of pneu-hypoxic mice were five times lower than in lung of pneu-normoxic mice. Bacterial loads in blood did not differ between the two groups but bacterial counts in blood were very low and not all mice were bacteremic (50% of the hypoxic mice and 60% of the normoxic mice showed bacterial outgrowth from blood).

Cytokine levels

Local production of TNF-α and IL-10 within the pulmonary compartment influences antibacterial host defense during pneumonia, whereas exposure to hypoxia elevates local levels of these cytokines for a prolonged period.
Hypoxia and *P. aeruginosa* pneumonia

Therefore, we measured the concentrations of IL-10 and TNF-α in lung homogenates after inoculation with *P. aeruginosa*. As is shown in figure 4, IL-10 and TNF-α levels were increased in pneu-hypoxic mice compared to pneu-normoxic mice (208 ± 15 pg/ml vs. 130 ± 16 pg/ml and 1.3 ± 0.13 ng/ml vs. 0.80 ± 0.14 ng/ml, respectively). IL-10 and TNF-α levels in lung homogenates of hypoxic control mice were, respectively, 1.78 and 1.38 times higher than in pneu-hypoxic mice.

![Figure 4. Cytokine levels 24 hours after *P. aeruginosa* administration are influenced by a previous hypoxic period. IL-10 (A) and TNF-α (B) levels in lung homogenates 24 hours after inoculation with *P. aeruginosa* or saline. Pneu-hypoxic mice are shown as black bars, pneu-normoxic mice as dashed bars, and hypoxic mice inoculated with 0.9% NaCl as white bars (n=10 per group). Indicated is mean +/- SEM. * P < 0.05 between indicated groups.](image)

**Discussion**

In the present study we investigated whether a preceding hypoxic episode influences host defense during *P. aeruginosa* pneumonia. To address this question, we exposed mice 24 hours to 8% O₂. Five days later, the mice were inoculated with *P. aeruginosa* or saline. Normoxic mice inoculated with *P. aeruginosa* served as additional controls. Inoculation with *P. aeruginosa* resulted in elevated wet lung weights, interstitial inflammation, endothelialitis, pleuritis, local bacterial growth and elevated levels of TNF-α and IL-10 in the lungs. Wet lung weights, and histological signs of inflammation did not differ between the two pneumonia groups; however, bacterial outgrowth was lower in lungs of pneu-hypoxic mice than of pneu-normoxic mice, while TNF-α and IL-10 levels were higher in lungs of pneu-hypoxic mice. Overall these data indicate that hypoxia-induced cytokine levels are biologically active and improve host defense during *P. aeruginosa* pneumonia.

Exogenous IL-10 and TNF-α have previously been shown to improve lung injury and survival during *P. aeruginosa* pneumonia.⁷,¹⁰,¹⁶ Our results show that not only exogenous TNF-α and IL-10 influence host defense against *P. aeruginosa*, but when present before onset of the infection, endogenous TNF-α and IL-10 also enhance host defense. The potential beneficial effect of TNF-α during *P. aeruginosa* pneumonia is rather puzzling. Previous work of our group showed that other pro-inflammatory cytokines have negative effects on host-defense during *P. aeruginosa* pneumonia. IL-1 receptor (IL-1R) knockout mice show
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increased resistance against \textit{P. aeruginosa} pneumonia, as reflected by an enhanced clearance of bacteria from the lungs, reduced cytokine production in the lungs and diminished neutrophil recruitment.\textsuperscript{12} Wildtype mice treated with IL-1 receptor antagonist (IL-1ra) show comparable results. Since IL-1β levels produced during hypoxia are diminished as compared to normoxic mice,\textsuperscript{11} it is possible that differences between hypoxic and normoxic mice in host defense against \textit{P. aeruginosa} are (partly) caused by hypoxia-induced alterations in IL-1. As for IL-1, IL-18 knockout mice and wildtype mice treated with an IL-18 binding protein that neutralizes IL-18, show increased resistance against \textit{P. aeruginosa}, as reflected by less bacteria in lungs, diminished cell influx in the pulmonary compartment and suppressed local cytokine production.\textsuperscript{13} Experiments using interferon-γ receptor α-subunit (IFN-γR) deficient mice demonstrate enhanced clearance from the lungs as well,\textsuperscript{15} again indicating that other pro-inflammatory cytokines than TNF-α impair host defense against \textit{P. aeruginosa}. However, a prominent role for IFN-γ in hypoxia-induced protection against \textit{P. aeruginosa} is not likely, since we previously demonstrated that hypoxia does not induce changes in IFN-γ levels in lungs.\textsuperscript{11} Future experiments using IL-10, TNF-α and IL-1β inhibitors or knockout mice should prove whether IL-10, TNF-α, and/or IL-1β are responsible for hypoxia-induced protection against \textit{P. aeruginosa}.

It has previously been shown that both hypoxia\textsuperscript{11} and \textit{P. aeruginosa} pneumonia\textsuperscript{14,15} result in elevated levels of TNF-α and IL-10 in pulmonary tissue. Surprisingly, our experiments show that the combination of hypoxia and pneumonia results in lower cytokine levels than hypoxia alone. The observed reduction of cytokine levels during pneumonia indicates that the pre-existing, hypoxia-induced cytokines disappear faster from the pulmonary compartment than pneumonia-induced production takes place. Since a previous hypoxic period enhances host defense against \textit{P. aeruginosa}, it seems that TNF-α (and/or IL-10) levels produced during pneumonia are not sufficient or too late for accurate host defense against \textit{P. aeruginosa}, thereby explaining the beneficial effect of TNF-α induction either by administration of exogenous TNF-α\textsuperscript{16} or by endogenous hypoxia-induced production before administration of \textit{P. aeruginosa}.

An intriguing observation is the lack of differences in histological signs of inflammation between hypoxic and normoxic mice after administration of \textit{P. aeruginosa}. Buret et al.\textsuperscript{16} showed that the working mechanism of exogenous TNF-α during \textit{P. aeruginosa} pneumonia does not involve the influx of neutrophils, but only the increased activity of infiltrated neutrophils. This suggests that in our mice the number of leukocytes infiltrated into lung tissue (and therefore the degree of interstitial inflammation, endothelialitis and pleuritis) is the same in both groups of mice, while the lower bacterial loads in the pneumonia flies can be explained by an increase in anti-bacterial capacity of the infiltrated leukocytes.

In the present work, we studied the influence of hypoxia on host defense. The model we chose is \textit{P. aeruginosa} pneumonia. \textit{P. aeruginosa} pneumonia is a nosocomial pneumonia, often associated with intubation and mechanical ventilation.\textsuperscript{17} Since the role of TNF-α and IL-10 in community acquired pneumonia and nosocomial pneumonia might be different,\textsuperscript{18} our results about the
Hypoxia and *P. aeruginosa* pneumonia

Influence of a hypoxic episode on host defense against *P. aeruginosa* should not be extrapolated to community acquired pneumonia. For instance, administration of exogenous IL-10 or neutralization of endogenous IL-10 during pulmonary infection with *Klebsiella pneumoniae* or *Streptococcus pneumoniae* enhances bacterial clearance and improves survival.\(^{19,20}\) The opposite holds true in *P. aeruginosa* pneumonia, since administration of exogenous IL-10 boosts host defense and promotes survival.\(^9\)

In summary, a preceding hypoxic episode boosts host defense during *P. aeruginosa* pneumonia, without affecting wet lung weights, interstitial inflammation, endothelialitis and pleuritis, most likely via upregulation of cytokines in the lung.

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