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Polymorphonuclear leukocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis

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ABSTRACT

Background: Chronic lung infection with *Pseudomonas aeruginosa* is the most severe complication for patients with cystic fibrosis (CF). This infection is characterized by endobronchial mucoid biofilms surrounded by numerous polymorphonuclear leukocytes (PMNs). The mucoid phenotype offers protection against the PMNs, which are in general assumed to mount an active respiratory burst leading to lung tissue deterioration. An ongoing respiratory burst by the PMNs has, however, not been demonstrated previously in endobronchial secretions from chronically infected CF patients.

Objective: Based on the accumulating evidence for depletion of molecular oxygen (O_2) in the mucus in infected CF bronchi, we hypothesized that the O_2 depletion in the mucus in infected CF bronchi may be accelerated by the respiratory burst of the PMNs due to the reduction of O_2 to the superoxide anion (O_2^-) by the phagocyte NADPH-oxidase (Phox).

Methods: We established methods to isolate the O_2 consumption by the respiratory burst from aerobic respiration in freshly expectorated sputum from chronically infected CF patients.

Results: Inhibition of the Phox with diphenylene iodonium (DPI) delayed O_2 depletion, nearly abolished staining of O_2^- producing PMNs with hydroethidine and inhibited the rapid luminol-enhanced chemiluminescence in sputum. Furthermore, the total O_2 consumption was correlated to the concentration of PMNs in the sputum samples.

Conclusion: Our results demonstrate that CF sputum contains PMNs with an active consumption of O_2 for O_2^- production and suggest that the respiratory burst is ongoing and causes accelerated O_2 depletion due to formation of O_2^- in the lungs of chronically infected CF patients.

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INTRODUCTION

Chronic lung infection with *Pseudomonas aeruginosa* is the most severe complication in patients with Cystic fibrosis (CF).[1] The thick viscous endobronchial mucus hampers mucociliary clearance, which renders the CF lungs susceptible to chronic infections.[2] The chronic *P. aeruginosa* lung infection in CF patients is characterized by endobronchial mucoid biofilms surrounded by numerous polymorphonuclear leukocytes (PMNs).[3, 4] In spite of the bactericidal mechanisms of the PMNs and aggressive antibiotic treatments the biofilms persist. *P. aeruginosa* may obtain protection against the PMNs by formation of mucoid biofilm.[1] Thus, instead of clearing the bacteria the PMNs are suspected to promote the progressive lung tissue deterioration by releasing proteolytic enzymes and reactive oxygen species (ROS) responsible for the premature death in CF.[1, 5] Eventhough PMNs with evidence of priming and phagocytic activity have been isolated from CF airways, the activity of PMNs in the lungs of CF patients with chronic *P. aeruginosa* lung infection is far from clarified.[5] Interestingly, anaerobic conditions in the mucus have recently been demonstrated in non-obstructed bronchi of CF patients with chronic *P. aeruginosa* lung infection.[3] The cause of this O_2 depletion may be O_2 consumption by the epithelia and by *P. aeruginosa*.[3] We hypothesized that the respiratory burst of the PMNs contributes to the O_2 consumption in the mucus of the infected CF airways. The interaction between bacteria and the PMNs may increase O_2 consumption as a consequence of the extra respiration during phagocytosis.[6] This process is caused by the respiratory burst resulting from the one-electron reduction of O_2 by the Phox to O_2^-.[7] To evaluate the capability of the respiratory burst to deplete O_2, we constructed a simple reaction chamber that allowed real-time measurement of the O_2 concentration during phagocytosis of *P. aeruginosa* by PMNs. The respiratory burst was isolated from aerobic respiration by blocking the respiratory chain with KCN, which previously has been employed to demonstrate the resistance of the respiratory burst to inhibition of the aerobic respiration in PMNs.[8] The aerobic respiratory component was exposed by inhibiting the Phox with diphenylene iodonium (DPI). These *in vitro* findings were utilized to estimate the O_2 consumption and the ROS production by the respiratory burst of the PMNs in freshly expectorated sputum from CF patients with chronic *P. aeruginosa* lung infection.

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METHODS

Sputum samples

The study was performed on leftover sputum expectorated for routine bacteriology from 29 CF patients with chronic *P. aeruginosa* infections after obtaining permission by informed consent.

Table I Characteristics of the 29 CF patients

Characteristic	
Sex (M/F)	15/14
Age (y)*	32 (22-47)
Duration of chronic infection (y)*	19 (1-35)
FEV1 (%)*	48 (23-117)
FVC (%)*	89 (58-135)
During i.v. antibiotic treatment	21
In between i.v. antibiotic treatment	8

*Values are medians (range).

FEV1: Forced expiratory volume in 1 s. FVC: Forced vital capacity

Chronic *P. aeruginosa* infection was defined as the presence of *P. aeruginosa* in the lower respiratory tract at each monthly culture for ≥ 6 months or shorter in the presence of increased antibody response to *P. aeruginosa* (≥2 precipitating antibodies, normal: 0-1).[9] Before measurement of luminol-enhanced chemiluminescence (LEC) and dissolved O_2 the samples were brought into homogenous suspensions by gentle resuspension with a pipette. Normoxic condition was achieved by diluting 10 times with Krebs-Ringer buffer equilibrated in ambient air. The concentration of leukocytes in the diluted sputum was determined as previously described.[10, 11] The estimation of the frequency of PMNs is described in online supplement fig 5.

Preparation of PMNs

Human blood samples were obtained from normal healthy volunteers by venous puncture and the PMNs were isolated by gradient sedimentation and centrifugation as previously described before suspending the PMNs in Krebs-Ringer buffer with 10 mM glucose and 5 % (v/v) normal human AB positive sera at a density of 5 x 10^6 PMNs/ml.[10,11]

Bacteria

Inoculum for phagocytosis by PMNs was prepared from exponentially growing cultures of the widely studied *P. aeruginosa* wildtype strain (PAO1) (online supplement fig 1).

Estimation of the respiratory burst by LEC

The LEC from aliqouts of 100 µl isolated PMNs or sputum was determind as outlined in online supplement fig 3.

The concentration of dissolved O_2

Measurement of dissolved O_2 in sputum and during phagocytosis is described in supplement fig 2.

Visualization of O_2^- production

One µM hydroethidine (HE) (D7008, Sigma) was added to 100 µl sputum and the samples were incubated for 15 min at 37°C before examination with a fluorescence microscope (BX40,

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Olympus). To verify the origin of O_2^-, sputum was pretreated for 10 min with 50 µM DPI before addition of HE. The interaction between PMNs and bacteria in the sputum samples was visualized by adding HE (1 µM) together with the green permeable DNA dye SYTO 9® (Molecular Probe) (10 µM) and the samples were incubated for 15 min at 37°C before examination.

Statistical methods

Statistical significance was evaluated by two-way ANOVO test with Bonferroni post-tests for observations with parametric data. Nonparametric data were analyzed by Wilcoxon-signed rank test and Spearman rank correlation test. $P < 0.05$ was considered statistically significant. The tests were performed with Prism 4.0c (GraphPad Software, Inc.).

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RESULTS

O_2 consumption by PMNs and *P. aeruginosa* during phagocytosis

Estimations of the O_2 consumption during phagocytosis were initiated at atmospheric O_2 concentration. Accelerated initial reduction of the O_2 consumption with a reduced O_2 consumption rate at low O_2 concentrations during phagocytosis was seen when mixing PMNs with PAO1 (Fig. 1A). Nevertheless, the O_2 concentration decreased below the level of detection (0.01 mg O_2/ml). This was considered as an indication of anaerobic conditions, since similar recordings were obtained in buffer equilibrated in an anaerobic bench with growing strict anaerobes as control. The low O_2 consumption by unstimulated PMNs was also seen (Fig. 1A). Evidence of phagocytosis was provided by the reduced number of surviving *P. aeruginosa* in the presence of PMNs (online supplement fig 1) and by the GFP-tagged *P. aeruginosa* being ingested by or attached to the PMNs in the reaction chamber (online supplement fig 2).
The O_2 consumption of *P. aeruginosa* resulted in anaerobic conditions within 2 hours (Fig. 1B) and indicated that *P. aeruginosa* may consume a considerable part of the O_2 during phagocytosis.

Inhibition of the respiratory burst with DPI during phagocytosis of *P. aeruginosa* by PMNs

Treatment with 50 μM DPI reduced the chemiluminescence, which predominantly reflects O_2^- production during phagocytosis, to the level of unstimulated PMNs for up to 50 min (online supplement fig 3A). Thus, inhibition of the respiratory burst with 50 μM DPI during phagocytosis resulted in reduced O_2 consumption and prevention of anaerobic conditions (Fig. 1A). Since treatment with 50 μM DPI did not affect bacterial O_2 consumption (Fig. 1B), growth, and survival after phagocytosis (online supplement fig 1) significantly, 50 μM DPI was selected for estimating the O_2 consumption by the respiratory burst of the PMNs.

Inhibition of the respiratory chain with KCN during phagocytosis of *P. aeruginosa* by PMNs

Unexpectedly, the LEC was increased (online supplement fig 3B) without affecting the O_2 concentration (Fig. 1A) during phagocytosis inhibited with KCN. This could not be ascribed to *P. aeruginosa* since the bacteria ceased to consume O_2 shortly after addition of 2 mM KCN (Fig. 1B). Instead, the surprisingly high O_2 consumption and LEC during KCN treatment may result from a higher amount of O_2 being available for the respiratory burst due to the bacteria failing to consume O_2. In addition, KCN repression of aerobic respiration may decrease bacterial motility and facilitate capture of *P. aeruginosa* by the PMNs resulting in a stronger respiratory burst. Though KCN treatment may overestimate the O_2 consumption by the respiratory burst during, it was decided to use 2 mM KCN for the study of sputum. Lower concentrations may be insufficient to block the pseudomonal cyanide-independent terminal oxidase.[12]

O_2 consumption by the respiratory burst in sputum from CF patients with chronic *P. aeruginosa* lung infection

To ensure similar initial O_2 concentrations the expectorated sputum was diluted 10 times in the Krebs-Ringer buffer equilibrated in ambient air. O_2 consumption was observed in all sputum samples by the decreasing O_2 concentration and anaerobic conditions were established in 10 of the 23 sputum samples (Fig. 2A). The chemiluminescence was inhibited more than 95% after 10 minutes of treatment with DPI whereas KCN treatment had no effect (online supplement fig 4). Thus, the sputum was pretreated with DPI and KCN for 10 minutes to estimate the O_2 consumption rate during the following 30 min before the O_2 was depleted. The O_2 consumption rate was reduced by DPI treatment, indicating an active respiratory burst in the sputum samples, and resulted in a mean inhibition of the O_2 consumption rate by 56 % (SD: 20 %) (Fig. 2B), suggesting that the respiratory burst consumed the major part of the O_2. Active O_2 consumption by the respiratory

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chain was also observed by the reduced O_2 consumption rate caused by addition of KCN (Fig. 2C). The consequence of inhibiting the respiratory burst with DPI was a delayed generation of anaerobic conditions (Fig. 2D) whereas inhibition of the respiratory chain with KCN was insufficient to delay the depletion of O_2 significantly. DPI treatment allowed us to detect comparable levels of the O_2 consumption rate by the burst per PMN in CF sputum and in the phagocytosis experiment using normal PMNs and PAO1. In the sputum we found 1.7 nmol $O_2/10^6$ PMNs/min (SD= 1.6 nmol $O_2/10^6$ PMNs/min) and in the experimental phagocytosis assay we found 1.5 nmol $O_2/10^6$ PMNs/min (SD= 0.4 nmol $O_2/10^6$ PMNs/min). These data make it unlikely that the O_2 consumption rate of sputum PMNs is a CF-specific phenomenon.

PMNs with respiratory burst in sputum from CF patients with chronic *P. aeruginosa* lung infection

The mean fraction of PMNs constituted 90 % ± 7 % (SD) of the host cells in the sputum samples. Therefore, the concentration of PMNs was compared to the O_2 consumption rate in the diluted sputum samples. A positive correlation was observed supporting the involvement of PMNs in the O_2 consumption (Fig. 3A). Positive correlations were also observed between the concentration of CFUs and the O_2 consumption rate (Fig. 3B) and between the concentration of PMNs and the concentration of CFUs (Fig. 3C). No significant correlation was observed between the O_2 consumption rate and FEV1 or FVC.

To avoid unintended effects of the Krebs-Ringer buffer, the respiratory burst in undiluted sputum samples was estimated by LEC. As in diluted sputum, DPI treatment resulted in more than 95% inhibition after 10 minutes in undiluted sputum (Fig. 4A) indicating an active respiratory burst that persisted during the 2 hours of observation. Furthermore, the LEC was resistant to KCN suggesting an origin that may function independently of the respiratory chain such as the Phox of the PMNs.[8] To further verify the PMNs as the source of ROS in sputum, O_2^- production was visualized by staining with HE, which is oxidized by O_2^- to generate 2-hydroxyethidium that emits intensified red fluorescence when complexing with DNA.[13] In the untreated sputum, several fluorescent objects were seen at low magnification. Higher magnification revealed these objects to be PMNs (Fig 4B). In DPI treated sputum, only faint fluorescence was seen (Fig. 4C). By applying the validated HE to fresh, expectorated sputum together with the permeable DNA stain, SYTO 9®, we found bursting PMNs with ingested planktonic bacteria (Fig. 4D) and bursting PMNs failing to engulf bacteria from a biofilm (Fig. 4E).

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DISCUSSION

PMNs in BAL-fluids from chronically infected CF patients and in sputum from healthy individuals have the potential to mount a respiratory burst upon stimulation.[14, 15] In contrast, the presence of PMNs with an active respiratory burst was demonstrated in fresh sputum from CF patients with chronic *P. aeruginosa* lung infection in this study. Both the inhibition of O_2 consumption and ROS generation with DPI as well as the correlation of the PMN concentration to the O_2 consumption rate are indications of an ongoing respiratory burst by the PMNs in sputum from chronically infected CF patients. PMNs with an active respiratory burst were not demonstrated directly in the lungs of the patients in this study. Therefore, our findings do not necessarily reflect the situation of all PMNs in the endobronchial mucus of infected CF lungs since the pO_2 may differ at various sites in the sputum plugs. However, the rapid, high chemiluminescence, observed in undiluted sputum ex vivo, may exclude the possibility, that adding Krebs-Ringer buffer, equilibrated to ambient air, causes the oxidative burst. In this context, the initial, high O_2 consumption and ROS generation seen in untreated sputum samples within 30 minutes after expectoration suggests that the respiratory burst of the PMNs was already active in the mucus in the lungs. Thus, our results indicate that the respiratory burst of the PMNs is the main contributor to the intensive O_2 depletion inside the mucus of the bronchi.[3] The respiratory burst of the PMNs in the sputum may be activated by direct contact with *P. aeruginosa*.[5, 16] and by the binary signalling from the engagement of the integrins and binding to inflammatory cytokines.[17] Interestingly, we have recently found levels of TNF-α, whith the potential to augment the respiratory burst, in sputum from several infected CF patients attending The Copenhagen CF Centre as did the patients in the present study.[18] In addition, comparable levels of TNF-α in sputum from infected CF patients are reported from other CF centers.[19] Besides TNF-α, platelet-activating factor, leukotriene B4, IL8, lipopolysaccharide, antibodies and alginate have been suggested to be major triggers for the induction of the respiratory burst of airway PMNs. [1, 5, 18, 19] On the contrary, the respiratory burst of the PMNs may be repressed by quorum sensing-regulated secretion of rhamnolipid, by exoprotease from *P. aeruginosa*, by antibiotic treatment, and by the high levels of apoptotic or necrotic cells in the sputum samples.[10, 11, 20-22] These opposing mechanisms may contribute to the variation observed in the correlation between the concentration of PMNs and the O_2 consumption in the sputum. Our results imply that at least in some parts of the CF mucus O_2 is present which allows PMNs to create a respiratory burst. This is in accordance with the steep pO_2-gradients in CF mucus. [3] Furthermore, based on the substantial fraction of PMNs able to create a significant respiratory burst found in CF sputum in this study, our previous demonstrations of the toxic effects of *P. aeruginosa* rhamnolipid on PMNs, may not apply to the entire population of PMNs in the sputum. [10, 11]

The demonstration of PMNs with an active respiratory burst in sputum from chronically infected CF patients may have been anticipated due to the ROS lesions in the infected CF lungs.[5, 23] But it could not have been taken for granted. In fact, the ROS in the CF lungs may be derived from other sources than the Phox of the PMNs, including epithelial cells, alveolar macrophages, and from mitochondrial respiration.[23-25] Due to the predominance of PMNs in the sputum samples the contribution of other cells to the O_2 metabolism was regarded to have low impact on the results in this study. In contrast to the Phox, mitochondrial activity is unlikely to influence our observations in the sputum samples significantly. PMNs contain only few and poorly developed mitochondria with a low activity of the respiratory chain.[26] In addition, sputum from chronically infected CF patients may contain *P. aeruginosa* producing pyocyanine, which may also cause O_2^- formation.[27] Thus, O_2^- formation by pyocyanine may be a reason for why DPI failed to block ROS generation completely in the sputum samples.

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Previously, faster O_2 depletion has been achieved when PMNs were added to *P. aeruginosa* growing in experimental biofilms due to increased O_2 consumption of both the bacteria and the PMNs.[16] However, in the present study the ability of the PMNs *per se* to create anaerobic conditions by an active respiratory burst was observed in the KCN-resistant O_2 depletion during the phagocytosis of PAO1 and in the sputum samples. This may, however, be expected, since PMNs are able to produce a substantial part of the energy needed for the respiratory burst by anaerobic glycolysis resulting in L-lactate formation.[28] Accordingly, our suggestion of an active respiratory burst by the PMNs in the CF lungs is supported by the increased glucose uptake in PMNs in CF lungs and the high concentration of L-lactate in sputum from CF patients with chronic *P. aeruginosa* lung infection.[29, 30]

The demonstration of PMNs with an active respiratory burst in sputum samples from CF patients with chronic *P. aeruginosa* lung infection verifies the frequent assumption that the PMNs induce ROS lesions in the lungs. Sufficient O_2 to produce O_2^- by the respiratory burst of the PMNs in sputum may be supplied by the daily inhalation of 8000 L of O_2-rich air.[23] The result of the fast dismutation of O_2^- is H_2O_2, which may lead to the formation of myeloperoxidase derived oxidising species found in CF airways.[5] Considering the short half-lifes of the ROS generated by PMNs, the tissue adjacant to the mucus is most likely to be exposed to PMN-derived ROS. Therefore, ROS generated in the mucus may be a reason for the affected bronchi in CF patients with chronic *P. aeruginosa* lung infection.[31] But it is less obvious how ROS generated in the mucus may reach more distant lung tissue. Since the included CF patients show a broad range of FEV1 values, one may suggest that O_2 consumption rate might be linked to pulmonary function in these patients. However, no significant correlation was observed between the O_2 consumption rate and FEV_1 or FVC. This may be due to the condition of the infected patients, which has been stabilized for years by elective antibiotic treatment resulting in a slower decline of lung function and less excerbations. Thus, the lung function of the included patients may primarily result from slowly accumulated deterioations of the lung tissue rather than the ongoing inflammation in the endobronchial mucus. Our results provide arguments to control the extent of ROS by antibiotic treatment, since O_2 consumption was mainly caused by ROS formation of the PMNs and was correlated to the concentration of *P. aeruginosa* in the sputum samples. The latter correlation probably results from a balanced accumulation of PMNs in response to the concentration of *P. aeruginosa* in the sputum samples.

As indicated in fresh sputum samples, the ROS from the respiratory burst of the PMNs in the mucus may reach the bacteria, due to the ingestion by PMNs with a respiratory burst and the intimate surrounding of the endobronchial biofilms by the PMNs.[4] Though the killing of *P. aeruginosa* by PMNs was apparently independent of ROS formation in this study, the surviving bacteria may experience ROS lesions, which may affect virulence. In particular, PMN-derived ROS may cause mutations in the *mucA* gene, leading to formation of the crucial mucoid phenotypes. [32] In fact, mutations in the *mucA* gene were found in the majority of the mucoid isolatets from 91 Scandinavian patients with chronic *P. aeruginosa* infection.[33] In addition, the PMN-derived ROS may induce highly mutagenic lesions in the bacterial DNA that are associated with resistance to antibiotics and accumulation of hypermutable isolates.[34]

The accelerated O_2 depletion by the PMNs probably affects the O_2 distribution in the endobronchial mucus. Though the precise O_2 profile of the endobronchial mucus is not determined, the activity of the PMNs is sensitive to the local O_2 tension. In particular, the accumulation of PMNs in the lung secretions may be increased due to the postponing of apoptosis bye hypoxia.[35] Sputum from chronically infected CF patients may also contain biomarkers for the bacterial response to O_2 depletion such as slow growth and cyanide secretion.[12, 36]

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This study demonstrates an ongoing respiratory burst of the PMNs in sputum from CF patients with chronic *P. aeruginosa* lung infection that may induce environmental changes in the lungs with the potential to generate virulent and resistant bacterial phenotypes, to facilitate colonization by anaerobic bacteria, and to cause oxidative lesions in the lung tissue of the patients.

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Legends to figures.

Figure 1. O_2 consumption by the respiratory burst and aerobic respiration in the reaction chamber. (A) Increased O_2 consumption during phagocytosis ($p<0.001$), inhibition of the respiratory burst during phagocytosis by treatment with 50 μM DPI ($p<0.001$), and unchanged O_2 consumption during phagocytosis by treatment with 2 mM KCN during phagocytosis of PAO1 (15×10^6/ml) by normal PMNs (5×10^6/ml). (B) Decreased O_2 consumption by PAO1 (15×10^6/ml) during treatment with 2 mM KCN ($p<0.001$) and unaffected bacterial O_2 consumption during treatment with 50 μM DPI. Statistical analysis was performed by two-way ANOVA test.

Figure 2. O_2 consumption and ROS generation in diluted sputum from CF patients with chronic *P. aeruginosa* lung infection. (A) The decreasing O_2 concentration as a function of incubation time measured in the reaction chamber (n=23). (B) Reduced O_2 consumption rate in sputum treated with 50 μM DPI (n=16). (C) Reduced O_2 consumption rate in sputum treated with 2 mM KCN (n=15). (D) Postponing of O_2 depletion in sputum treated with 50 μM DPI. B-D data were analysed by Wilcoxon signed rank test.

Figure 3. Correlations between the O_2 consumption rate, the concentration of PMNs, and CFUs in diluted sputum from CF patients with chronic *P. aeruginosa* lung infection. The O_2 consumption rate was determined in the reaction chamber, the concentration of PMNs was estimated by flow cytometry, and the CFUs were counted by plating of serial dilutions. (A) O_2 consumption rate vs the concentration of PMNs. (B) O_2 consumption rate vs the concentration of CFUs. (C) The concentration of PMNs vs the concentration of CFUs. Statistical analysis was performed by Spearman Rank Correlation test.

Figure 4. ROS generation in sputum from CF patients with chronic *P. aeruginosa* lung infection. (A) The effect of treatment with KCN and inhibition with DPI ($p<0.001$) on ROS generation as determined by LEC (n=10). Statistical analysis was performed by two-way ANOVA test (n=10). (B and C) Visualization of O_2^- production by fluorescence microscopy of sputum stained with HE. (B) Untreated sputum. (C) Sputum treated with 50 μM DPI to inhibit the Phox. (D) PMNs with ingested planktonic bacteria (arrow) with green fluorescence from SYTO 9®. (E) Bursting PMN (arrow) engaged with a biofilm PMN with bacteria emitting green fluorescence from SYTO 9®.

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Figure 1.

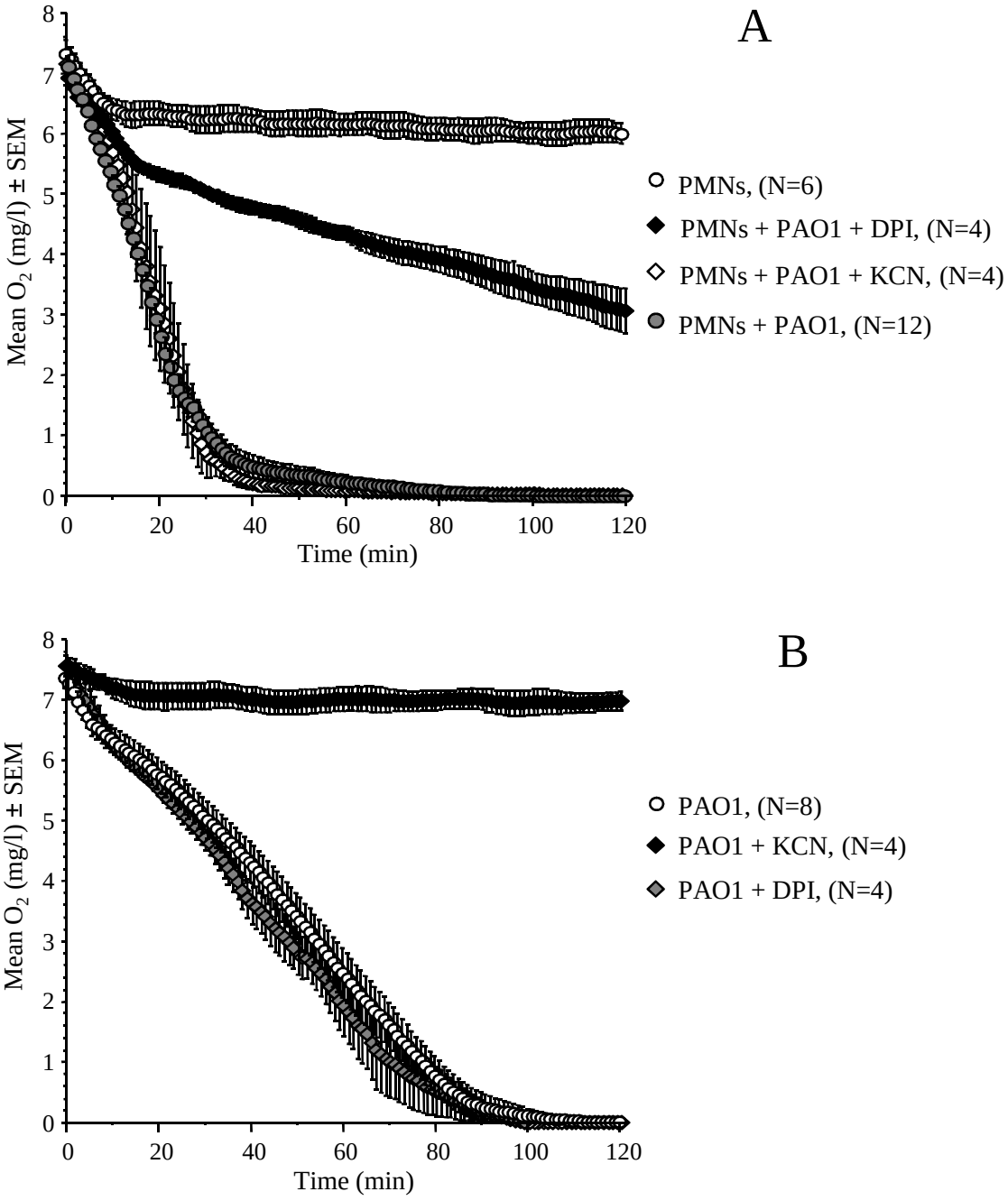

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Figure 2.

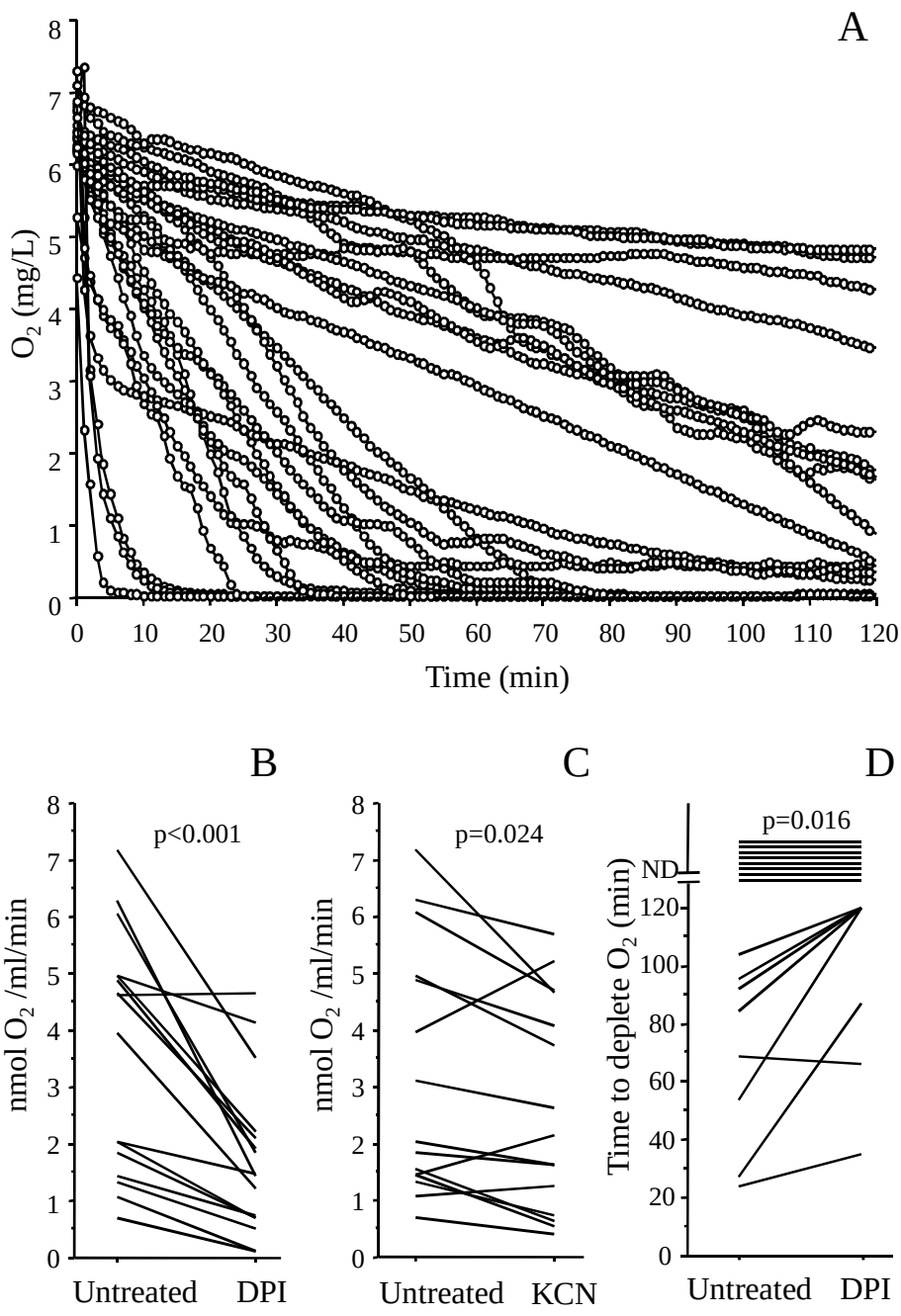

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Figure 3.

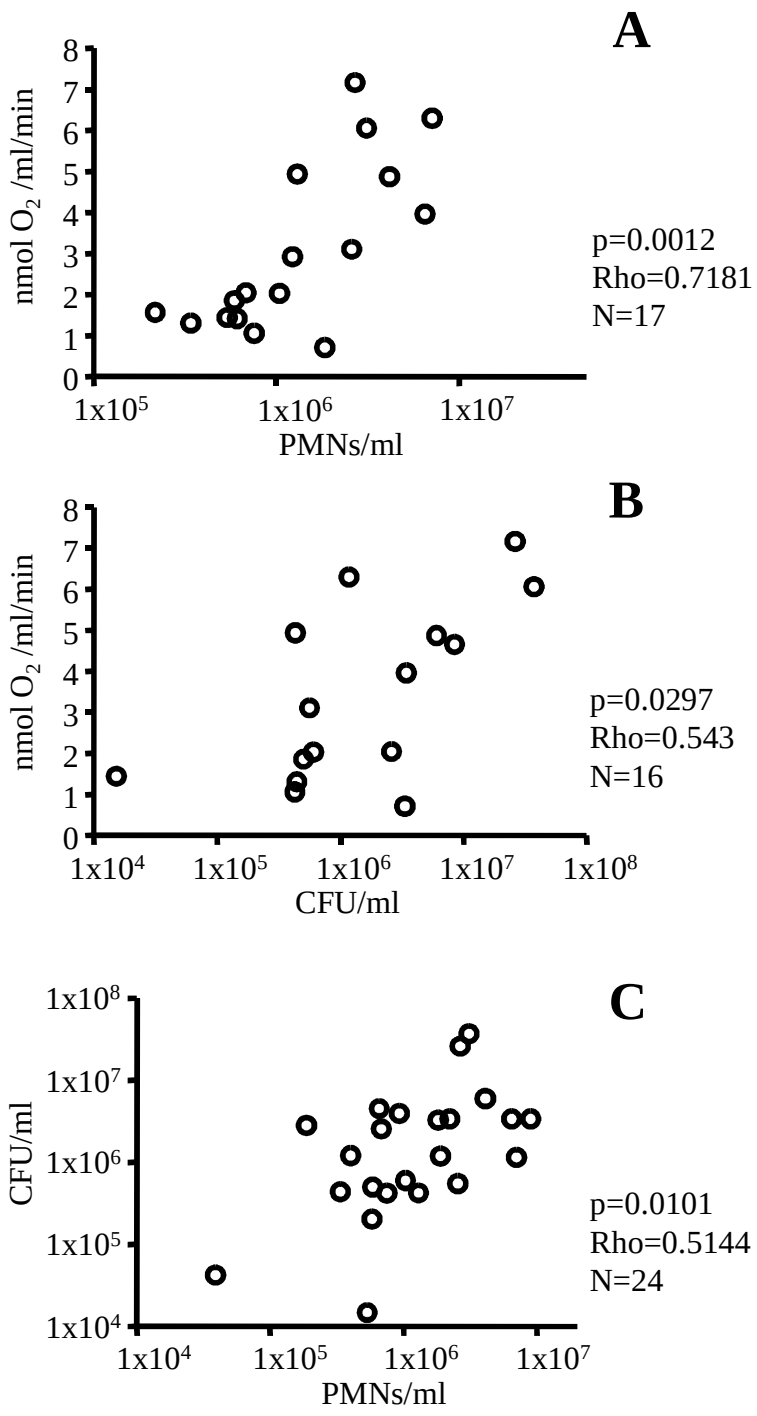

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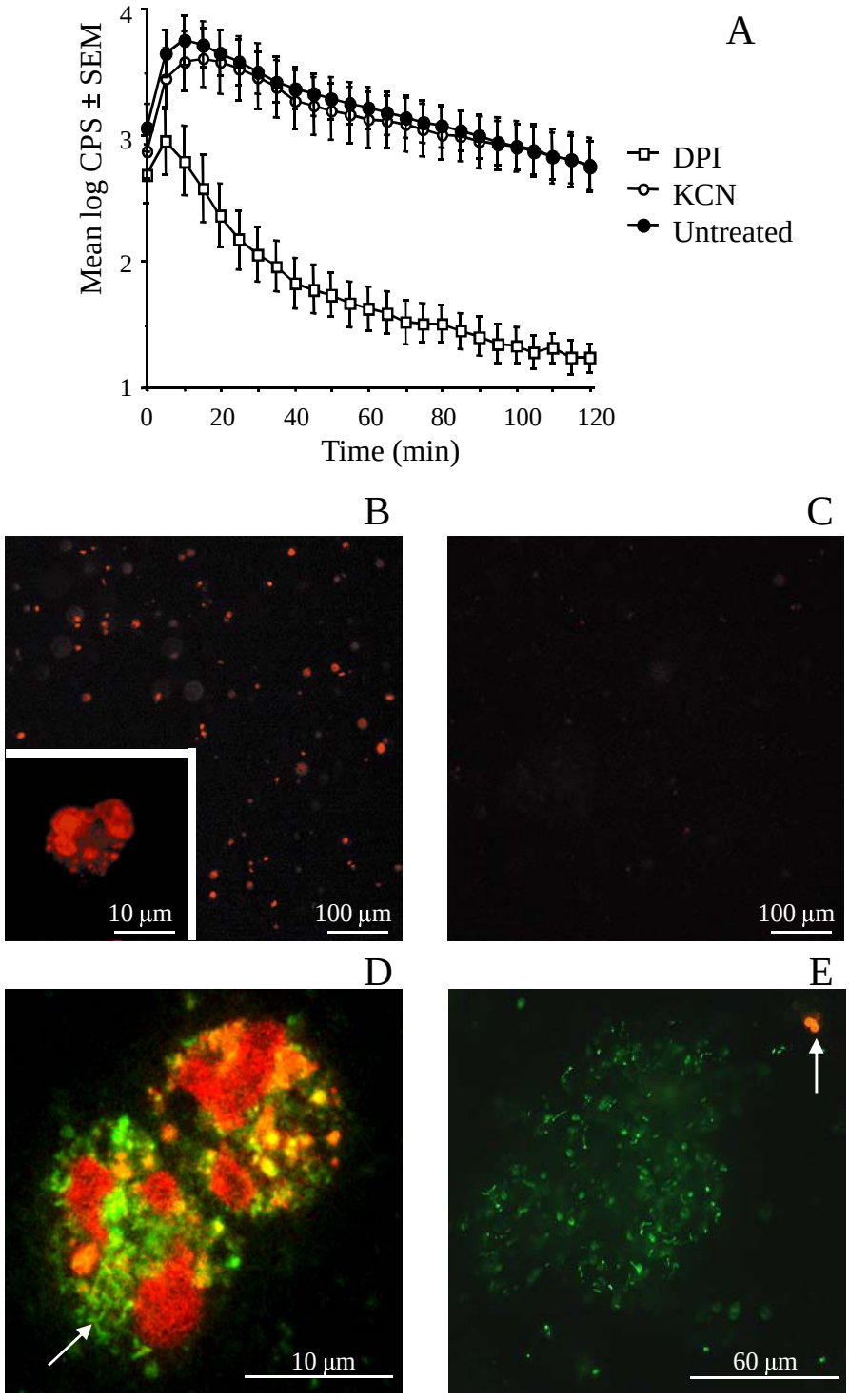
Figure 4.



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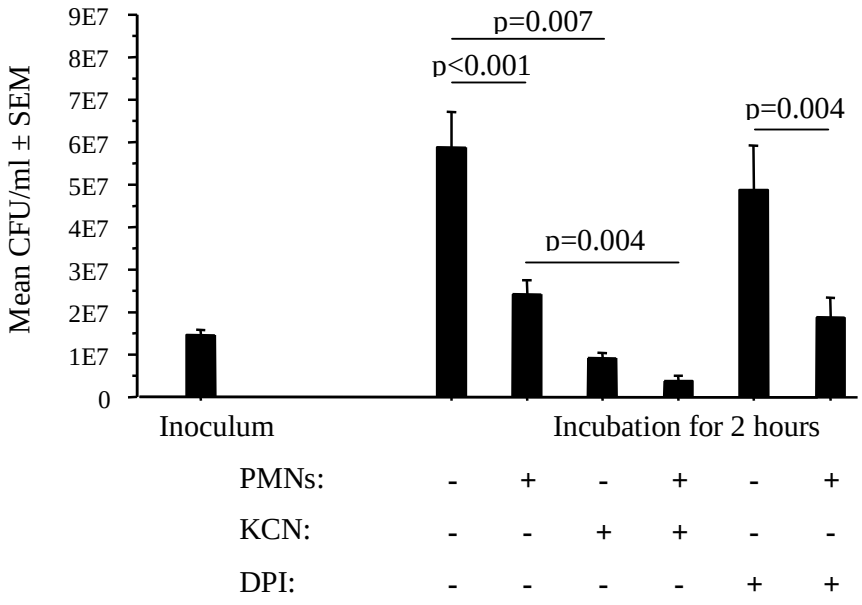
Online supplement Figure 1 The outcome of incubating PAO1 alone or with PMNs during treatment with 50 μM DPI and 2 mM KCN. The reaction chamber was loaded with 5 x 10^6 PMNs/ml and 15 x 10^6 PAO1/ml and was incubated for 2 hours. The colony-forming units (CFUs) of samples with PAO1 before and after incubation were estimated by serial dilution in 0.9 % NaCl and plated on blue agar plates (SSI) that are selective for Gram-negative rods. To prevent agglutination the initial dilution step was performed in 1 % Triton X-100 (234729, Sigma). The plates were incubated overnight at 37°C before counting of the CFUs. Statistical analysis was performed by two-tailed, unpaired student t-test on 4 to 12 independent experiments.



Preparation of the inoculum for phagocytosis by PMNs. *P. aeruginosa* wildtype strain (PAO1) was obtained from Professor Barbara Iglewski (University of Rochester Medical Center, NY, USA) and was grown over night in shake flasks (180 r.p.m.) with ox broth (State Serum Institute (SSI)) at 37 °C. The culture was diluted in ox broth to OD_{600} = 0.05 and grown in shake flasks until OD_{600} = 0.5 was reached. Again the culture was diluted to OD_{600} = 0.05 and grown until OD_{600} = 0.5 before centrifugation (10 min, 5000 x *g*) and resuspension in 0.9 % NaCl to a density of 9 x 10^8 PAO1/ml.

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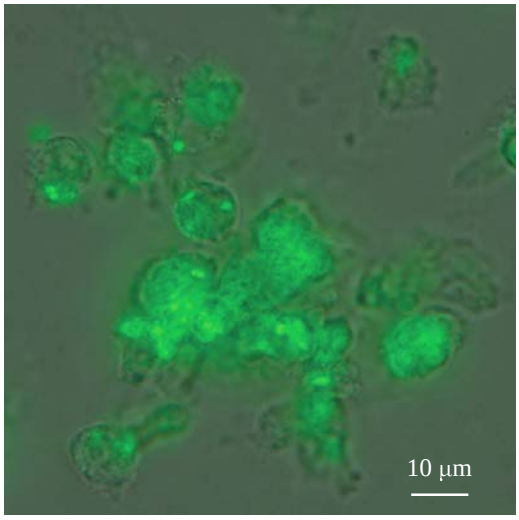
Online supplement Figure 2 Demonstration of PMNs with attached and ingested GFP-tagged PAO1 in the reaction chamber. PAO1 was tagged with a plasmid-based mini-Tn*7* transposon system (pBK-miniTn*7*-*gfp*3) constitutively expressing a stable green fluorescent protein (GFP) according to Koch *et al.*[1]. Inoculum for phagocytosis by PMNs was prepared as for untagged cultures and 5 x 10^6 PMNs/ml were incubated with 15 x 10^6 GFP-tagged PAO1/ml and incubated in the reaction chamber for 2 hours before examination by flourescence microscopy merged with light microscopy.



The concentration of dissolved O_2

In order to measurement of dissolved O_2 in sputum and during phagocytosis is described in supplement fig 3., reaction chambers with a volume of 4 ml were constructed from polypropylene tubes (91016, Techno PlasticProducts AG). Samples with PMNs (5 $\cdot 10^6$ PMNs/ml), PAO1 ($2 \cdot 10^7$ CFUs/ml) or diluted sputum were added to the reaction chamber followed by immediate immersing of a luminescent dissolved O_2 (LDO) sensor (HACH Company). Recording of the concentration of

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O_2 was initiated and the chamber was fixed with Tesapack (64014, Tesa AG) and sealed with sealing tape (3750, Scotch). To estimate the contribution of the respiratory burst and aerobic respiration, samples were pretreated for 10 min with 50 µM DPI and 2 mM KCN, respectively. To establish phagocytosis the LDO sensor with the mounted reaction chamber was shakened at 200 r.p.m at 37^{o}C for 2 hours.

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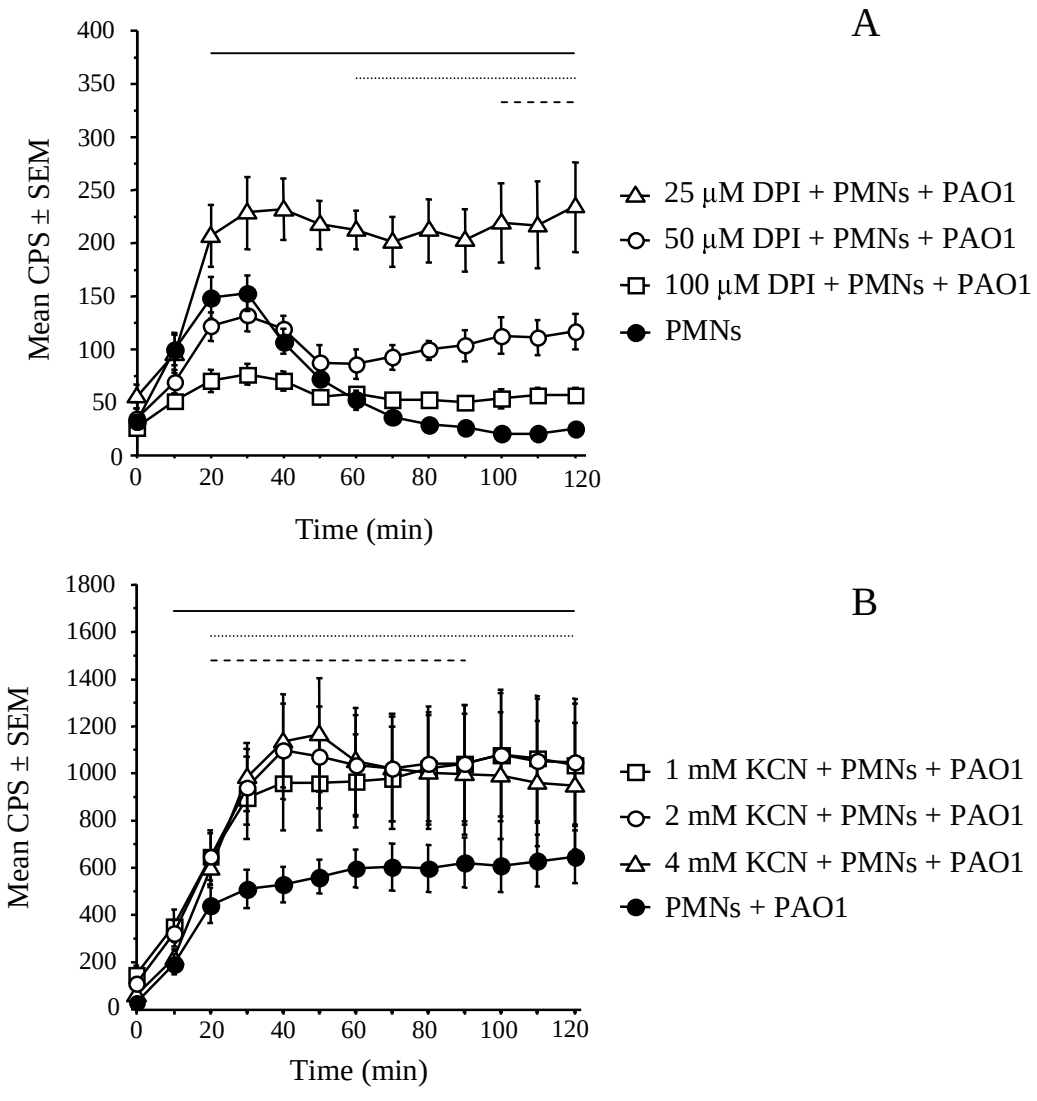
Online supplement Figure 3 ROS generation during phagocytosis of PAO1 (15 x 10^6/ml) by normal PMNs (5 x 10^6/ml) in the presence of DPI or KCN estimated by LEC. A. inhibition of ROS generation by treatment with DPI. Lines indicate intervals of significantly higher ROS generation between untreated PMNs and PMNs incubated with PAO1 in the presence of DPI. Solid line: PMNs vs PMNs + PAO1 + 25 μM DPI, dotted line: PMNs vs PMNs + PAO1 + 50 μM DPI, dashed line: PMNs vs PMNs + PAO1 + 100 μM DPI. B. Increased ROS generation during phagocytosis of PAO1 by normal PMNs in the presence of KCN. Lines indicate intervals of significantly higher LEC between phagocytic PMNs without KCN and with KCN. Solid line: 1 mM KCN, dotted line: 2 mM KCN, dashed line: 4 mM KCN. Statistical analysis was performed by two-way ANOVA test with Bonferroni post-tests (n=6).



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Estimation of the respiratory burst by Luminol-enhanced chemiluminescence (LEC).

Aliqouts of 100 µl isolated PMNs ($5 \cdot 10^6$ PMNs/ml) or sputum were added per well in a microtiter plate (Nunc). Phagocytosis was established by adding PAO1 to the PMNs at a final density of $2 \cdot 10^7$ CFU/ml. To assess the significance of inhibition of the respiratory burst, samples were added DPI (D2926, Sigma) that inhibits the Phox without affecting internalization of bacteria[1]. Aerobic respiration was assessed by treatment with KCN (60178, Fluka) that inhibits the reduction of O_2 to H_2O by cytochrome c[2]. Immediately after, the wells were added 175 µl luminol (30 nM) (A4685, Sigma) and the chemiluminescence was recorded for 2 hours at 37°C in a plate reader (Wallac 1420 Victor, PerkinElmer).

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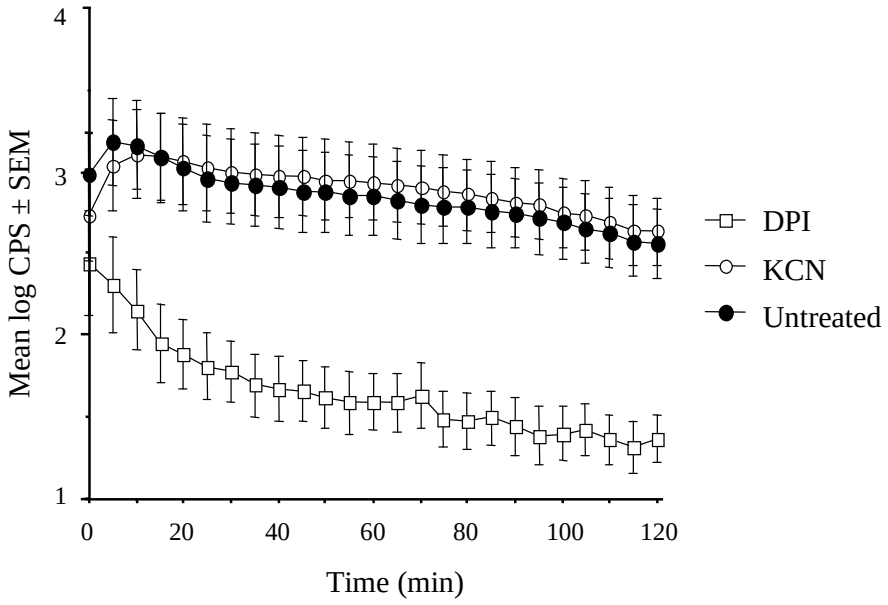
online supplement fig 4 the effect of treatment with DPI and KCN on ROS generation in diluted sputum from CF patients with chronic *P. aeruginosa* lung infection estimated by LEC. Lines indicate intervals of significantly different ROS generation ($p<0.001$). Statistical analysis was performed by two-way ANOVA test (N=10).



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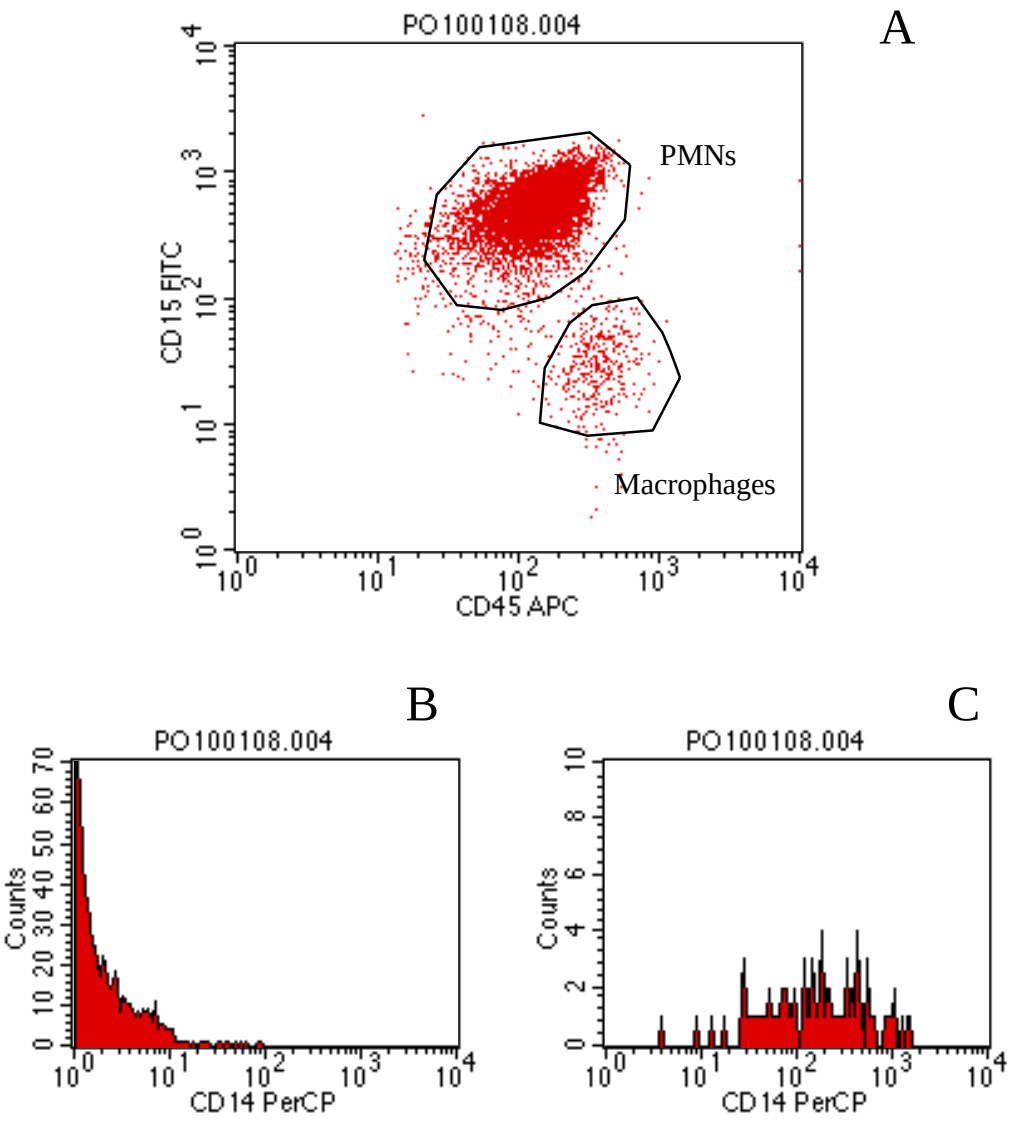
online supplement fig 5 Flow cytometric discrimination of PMNs and macrophages in CF sputum using fluorochrome-labeled specific monoclonal antibodies. A. Dot plot displaying CD15 and CD45 staining intensity of sputum cells gated in forward light scatter and side light scatter. Regions of PMNs and macrophages are indicated and used as gates for generation of histograms demonstrating low CD14 expression on cells in the PMNs region (B) and high CD14 expression on cells in the macrophages region (C).



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Estimation of the frequency of PMNs. To estimate the frequency of PMNs, 100 µl diluted sputum was added 5 µl of each of the following labeled mouse anti-human monoclonal antibodies from BD Bioscience: IgG1κ CD11b-PE, IgG2a CD14-peridinin chlorophyll A protein, IgMκ CD15-FITC 555401, IgG1κ CD45-allophycocyanin. The samples were incubated on ice for 30 minutes, washed with cold PBS, and fixated in PBS with 2 % paraformaldehyde (Sigma, 158127) before they were analyzed by flow cytometry using a FACSort (BD Bioscience).

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