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Suppression of pulmonary innate host defense in smokers

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ABSTRACT

Background: Smoking increases the susceptibility to pulmonary infection and is a risk factor for the development of chronic obstructive pulmonary disease (COPD). We postulated that cigarette smoke suppresses the activation of the innate immune system in response to bacterial infection.

Methods: With sensitive ex-vivo analysis we measured the level of the endogenous antibiotic peptide human beta-defensin-2 in pharyngeal washing fluids and sputum of patients with community acquired pneumonia. The regulation of antibacterial host defense molecules was studied in vitro. The effect of cigarette smoke on the antibacterial activity of differentiated airway epithelium and the expression of host defense molecules was studied in an infection model in vitro.

Results: Current or former smoking was associated with significantly reduced hBD-2 levels in pharyngeal washing fluid and sputum from patients with acute pneumonia. Exposure of airway epithelium to smoke *in vitro* inhibited the induction of hBD-2 by bacteria. This correlated with decreased antimicrobial activity. This effect was mimicked by H₂O₂ and catalase blunted the smoke-induced inhibition of epithelial host defense.

Conclusions: Smoke exposure suppresses the induction of epithelial antibacterial host defenses. These findings link smoking with increased susceptibility to infection. This mechanism may be important in the pathogenesis of pneumonia and COPD.

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INTRODUCTION

Cigarette smoking and exposure to environmental tobacco smoke increase the risk of pulmonary infections in general and the risk to contract invasive pneumococcal disease by a factor of 4.1¹. In addition, other pulmonary infections are more frequent in smokers including influenza and tuberculosis². A population-based case control study revealed that the risk of community acquired pneumonia (CAP) attributable to the consumption of any type of tobacco was 32.4 % in patients with COPD³. Also animal experiments showed that smoke exposure results in decreased host defense of the lung. Cigarette smoke applied to mice for 6-8 weeks resulted in a delayed rate of bacterial clearance as compared to sham-exposed animals⁴. Smoke decreases the function of the mucociliary clearance⁵, promotes adhesion of bacteria to the airway epithelia⁶, and affects the function of pulmonary host defense cells⁷.

Cigarette smoke is the most important risk factor for the development of chronic obstructive pulmonary disease (COPD). COPD is a chronic progressive lung disease with large medical and economical burden that develops in approximately in 15-20 % of all smokers. The pathogenesis of COPD is not clear. Chronic smoke exposure results in an inflammatory process characterized by the presence of neutrophils, macrophages, and CD8⁺ lymphocytes. Detailed morphological analysis of tissue samples from COPD patients revealed the presence of lymphoid aggregates containing follicles in the peripheral airways⁸. Recurrent or chronic infections may be a driving force in the development of COPD. Smoking and COPD are related to bacterial colonization of the pulmonary tract. Rising airway bacterial load is associated with greater airway inflammation and accelerated decline in FEV1⁹. The diversity of the local microflora appears to change during the development of COPD

¹⁰. One hypothesis to describe the progression of COPD includes chronic or recurrent infections of the airways with subsequent activation of innate and adaptive immune mechanisms.

The innate host defense system of the lung is a first line protection system. Antimicrobial peptides (AMPs) are important effector molecules of the innate immune system ¹¹. The defensins and cathelicidins are the principal families of AMPs that are expressed in the respiratory tract. The β -defensins are mainly produced by epithelial cells and are secreted onto the airway surface where they have a broad spectrum of antimicrobial activity. In vitro studies and clinical studies show that the expression of several AMPs is induced by bacterial products and inflammatory mediators ^{11;12}.

The aim of the present study was to characterize the effect of smoking on functions of innate host defenses and to correlate findings from clinical studies with results from in vitro experiments.

METHODS

Patients from CAPNETZ

CAPNETZ is the German community acquired pneumonia-network that investigates the epidemiology, microbiology, and clinical course of CAP¹³ (URL: www.capnetz.de). Patients are identified by clinical signs and a positive X-ray of the lungs. The program was approved by local ethics committees and informed consent was obtained from the patients. Material used for this study was obtained from 27 consecutive patients with community acquired pneumonia where the necessary samples (pharyngeal washing fluid, sputum) were available. Patient characteristics are summarized in Table 1. Former smokers are defined as abstinent during 12 months; other groups are never smokers and current smokers. Pharyngeal washing fluid was collected from 10 non-smoking and 10 smoking individuals with no current symptoms of infections. Pharyngeal washings were obtained by rinsing the mouth and gargling for 10 sec. Pharyngeal washing fluid was used because the procedure can be performed noninvasively in sick patients and the material is suitable for the applied assays. Pharyngeal washings are commonly used to test for pathogens in lower airway infection¹⁴.

Measurements of hBD-2 concentrations and Western blotting

hBD-2 levels in pharyngeal washings obtained from patients were measured applying an ELISA that detects hBD-2 in a range of 0.15-12.5 ng/ml. 96 well microtiter plates (Nunc, Wiesbaden, Germany) were coated with a polyclonal antibody¹⁵ against hBD-2 in 50 mM carbonate / bicarbonate buffer, pH 9.6, and the plates were blocked with 1 % gelatin / PBS. 100 µl of samples were added into each well and incubated over night at 4°C. A series of seven dilutions of recombinant hBD-2¹⁵ was included as

standard. Wells were washed and incubated with 100 µl of a second polyclonal antibody against hBD-2 (0.5 µg / ml, AB 9871, Abcam, Cambridge, UK) and subsequently with 100 µl of a HRP labeled anti-goat antibody (1 : 40000, SigmaAldrich, Munich, Germany). Wells were washed again and 100 µl TMB + substrate solution (Dako, Carpinteria, CA, USA) was added and incubated for 10 min. 100 µl stop solution (H₂SO₄, 3 N) were added and the optical density of each well was determined using a microplate reader set to 450 nm. For test evaluation, we performed spiking experiments (recovery between 79 and 81 %).

Sputum from patients with pneumonia was analyzed for hBD-2 by Western analysis. Samples were normalized to the weight of the initial sputum probe, measurements of protein before loading revealed equivalent protein quantities with a small variation (SD < 15 % of the mean). 10 µl complete protease inhibitor cocktail (Roche, Mannheim, Germany) was added per 1 mg sample. To homogenize the sample, 10 µl 100 mM 1,4-Dithiothreitol (DTT, Roth, Karlsruhe, Germany) were added per 100 mg of sputum. 5 samples from smokers and 5 from non-smokers were pooled, extracted with 0.1 % trifluoroacetic acid (TFA) and 10 % acetonitrile. The material was applied to Sep-Pak Plus C18 cartridges (Waters, WAT020515, Milford, USA) and eluted with 0.1% TFA and 80% acetonitrile. The eluate was dried in a speed-vac concentrator and reconstituted in 20 µl distilled water. The extraction procedure of hBD-2 from sputum was validated by spiking experiments using commercially available hBD-2 peptide (Abcam plc., Cambridge, UK). The detection limit was 2.5 ng hBD-2. The samples were separated on a 10 - 20% tris-tricine gel (Anamed, Darmstadt, Germany) and blotted on nitrocellulose membranes using Dunn-Carbonate buffer in a tank-blotting system (Bio-Rad Laboratories, Hercules, CA, USA). For autoradiographic detection a polyclonal hBD-2 antibody¹⁵ and a horseradish

peroxidase (HRP)-labelled secondary rabbit-IgG antibody (GE-Healthcare, Little Chalfont UK)) were used.

Cells and tissue culture, bacterial strain

Human bronchial epithelial cells (HBEC) were isolated from large airways resected during surgery and cultivated as submerged or air liquid interface (ALI) cultures as described previously ¹⁶. Donors (42, 39, 53 years of age) underwent lung transplantation due to pulmonary fibrosis. Results did not differ between cells from the donors. The protocol was approved by the ethics committee of the University of Marburg and informed consent was obtained from the patients. *Pseudomonas (P.) aeruginosa* PAO1 bacteria were grown to an OD₆₀₀ = 1.00 in LB media. For experiments with heat inactivated bacteria, dilutions of 10⁷ CFU/ml were prepared in PBS and incubated for 30 minutes at 96°C. Experiments with viable bacteria were done with a dilution of 10⁵ CFU/ml in PBS. *P. aeruginosa* was used as a model organism because this bacterium is known to colonize the respiratory tract of COPD-patients and is sensitive to epithelial host defense factors. The amount of CFUs used to infect cells was determined by plating an aliquot of the inoculum and varied between experiments.

Smoke exposure and infection models

Tissue cultures were exposed to volatile cigarette smoke (CS) as described previously ¹⁷. Briefly, tissue cultures were exposed to CS for 15 min (= 3 cigarettes). After the exposure, the medium of the cultures was replaced immediately. Control cultures were incubated in the exposure chamber for the same time period without burning a cigarette. To determine effects of cigarette smoke on expression and

release of hBD-2 and IL-8, heat inactivated *Pseudomonas aeruginosa* PAO1 (10^7 CFU / ml in 30 μ l) was applied to the apical side of the cultures for 8 hours.

To investigate the effects of reactive oxygen species on the β -defensin expression in airway epithelium, cells were incubated with or without H_2O_2 for 1 hour. After the exposure, the medium was replaced and the cells were stimulated with heat inactivated *P. aeruginosa* PAO1 (10^7 CFU / ml) for 8 hours. Catalase (Sigma-Aldrich, Steinheim, Germany) was added at a concentration of 6400 U/ml immediately before and after smoke exposure to the culture medium.

Bacterial survival assays

The apical surface of differentiated epithelium was exposed to the smoke as described. After smoke exposure, the cells were infected with $1 \times 10^{4-5}$ CFU of viable *P. aeruginosa* in 15 μ l PBS. To determine the numbers of viable bacteria 6 hours after infection, the apical surfaces of the cultures were washed with 100 μ l of PBS and serial dilutions were plated onto LB agar plates. 5 μ g of recombinant hBD-2 in 200 μ l¹⁸ was applied to the apical surfaces of the cultures in some experiments based on concentrations calculated for airway secretions¹⁹

Cytokine measurements

Levels of human IL-8 in tissue culture medium were determined by commercially available ELISAs, according to the manufacturer's instructions (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Quantitative PCR

RNA isolation, reverse transcription, and quantitative PCR were performed as described elsewhere ¹⁷. GAPDH primer (sense, 5'-GAAGGTGAAGGTCGGAGTC-3'; antisense, 5'-GAAGATGGTGATGGGATTTC-3'), hBD-1 primer (sense, 5'-GCCTCAGGTGGTAACTTTCTCA -3'; antisense 5'- GCGTCATTTCTTCTGGTCACT -3'), and hBD-2 primer (sense 5'- TCAGCTCCTGGTGAAGCTC -3'; antisense 5'-GGGCAAAGACTGGATGACA -3') were purchased from TIB Molbiol (Berlin, Germany).

Statistical methods

Values are displayed as mean \pm SD. Comparisons between groups were analyzed by t test, or ANOVA for experiments with more than two subgroups. Post hoc range tests were performed with the t test with Bonferroni adjustment. Results were considered statistically significant for $P < 0.05$.

RESULTS

Epithelial defensin is decreased in smokers with CAP

To determine whether smokers have decreased levels of mucosal AMPs in their airway secretions, we measured the amount of hBD-2 in pharyngeal flushing fluid and sputum from 27 patients with CAP by ELISA. We found significantly decreased concentrations of hBD-2 in pharyngeal flushing fluids of current smokers and former smokers as compared to never smokers (Fig. 1A). Healthy never smoking and healthy current smoking controls showed low levels of defensin. In a next step we analyzed whether hBD-2 is present in sputum of these patients. Due to its physical properties, sputum could not be used for ELISA and therefore hBD-2 was analyzed by Western blotting in extracts of pooled sputum samples. No sputum was available from healthy controls. hBD-2 was present in the sputum of never-smoking patients. In contrast, no peptide was found in the sputum of pneumonia patients that were current smokers (Fig. 1B). It is likely that Western blotting is not sensitive enough to reveal small differences as determined by ELISA. These data show that epithelial antimicrobial defensins are induced during pneumonia and that current and former smoking suppresses this induction.

Smoke exposure suppresses the induction of epithelial defensin by bacteria

We next asked whether the volatile cigarette smoke modulates the response of differentiated primary bronchial airway to *P. aeruginosa*. Smoke exposed cells showed decreased antimicrobial activity resulting in significantly increased numbers

of viable bacteria after the infection with *P. aeruginosa* (Fig. 2A). The treatment of the cells with volatile cigarette smoke did not cause an increase in LDH release (data not shown).

To investigate whether this defect of epithelial host defense after smoke exposure is correlated with decreased production of defensin, hBD-2 expression levels were measured after smoke exposure and in control cells. Smoke exposed airway epithelium showed a significantly suppressed hBD-2 mRNA induction in response to bacterial stimulation with heat-inactivated *P. aeruginosa* (Fig. 2B). Expression of hBD-1 was not regulated under the applied conditions (Fig. 2B). The suppressed induction of hBD-2 expression correlated with a decreased hBD-2 peptide secretion (Fig. 2C). Exogenously applied purified hBD-2 peptide significantly reduced the number of recovered bacteria from smoke exposed and infected cells (Fig. 2D). Additional experiments showed that most viable bacteria were washed off by the procedures and that the bacteria attached to the cells did not influence the results (data not show). Despite variations in the amount of CFUs in the inoculum applied in individual experiments, the outcomes were qualitatively equivalent.

To test whether reactive oxygen species are involved in the mechanisms of defensin suppression, we applied H₂O₂ before exposing the airway epithelium to bacteria and found significant suppression of hBD-2 mRNA induction by heat inactivated *P. aeruginosa* (Fig. 3A) without elevating the release of LDH from these cells (data not shown). The use of catalase as antioxidant partly abrogated the effects of cigarette smoke on the induction of hBD-2 expression (Fig.3B).

To test whether smoke exposure resulted in a general decrease of cellular responses, we measured the release of IL-8 after exposure to smoke and heat inactivated bacteria. Combination of smoke exposure and administration of *P. aeruginosa* resulted in increased release of IL-8 (Fig. 3C).

DISCUSSION

The main finding of the present investigation is that cigarette smoke suppresses the activation of the epithelial innate host defense system of the lung. Oxidative stress appears to play a role because H₂O₂ exposure mimics the suppressive effect of cigarette smoke and antioxidant treatment weakens this effect. Defensin appears to have a specific role in this mechanism because its expression correlates with host defense in tissue culture and clinical samples. External application of the peptide hBD-2 restored antimicrobial activity.

An increasing body of data supports the role of defensins in pulmonary host defense. In vitro experiments showed a wide antimicrobial activity²⁰. Also animal studies with mice deficient in mouse β -defensin 1 (mBD-1) showed increased susceptibility to infection^{21;22}. The number of defensin gene copies varies between individuals (copy number variants). Individuals with low numbers of gene copies of the defensin locus are predisposed for Crohn's disease²³. It is known that smoking alters the function of host defense components of the lung. Smoke exposed animals also reveal increased inflammation but delayed clearance of *Pseudomonas aeruginosa* after infection⁴. In addition, host defenses against respiratory syncytial virus appear suppressed after smoke exposure²⁴. The mechanism how cigarette smoke causes a breach of the host defense is unclear. Cigarette smoke is known to inhibit the function of professional immune and host defense cells such as dendritic cells²⁵, T-cells²⁶, and alveolar macrophages²⁷.

Here we show that also the host defense function of airway epithelium is compromised by cigarette smoke. Smokers with pneumonia had significantly

decreased levels of hBD-2 in their mucosal secretion. Epithelial cells are the main cellular source of hBD-2 in the lung ¹⁵. Th2 biased inflammation during atopic dermatitis is correlated with decreased levels of antimicrobial peptides and skin infections ²⁸. Here we show that smoke exposure decreases epithelial host defenses in the pulmonary system. In the study population, current smoking was associated with lower age and less severe pneumonia. The mechanistic interaction between these factors and their impact on AMP expression is largely unclear.

Interestingly, the release of the proinflammatory cytokine IL-8 from epithelial cells is synergistically increased by the combination of smoke exposure and bacterial infection. In parallel, the host defense function and the expression of hBD-2 are decreased. Smokers have decreased levels of host defense peptide and the amount of inflammation in COPD is correlated with the magnitude of the bacterial load ⁹. Smoke is generally thought to affect proinflammatory pathways including MAP kinases p38, ERK1/2, and JNK or NF- κ B ¹⁷. Acrolein is a major product of organic combustions and inhibits NF- κ B-activation by interaction with I κ B kinase (IKK) ²⁹. The smoke component acrolein inhibits the baseline expression of IL-8 and hBD-2 in sinonasal epithelial cells ³⁰. Smoke also inhibits LPS-induced production of inflammatory cytokines by suppression of the activation of activator protein-1 in bronchial epithelial cells ³¹. Also the downregulation in TLR4 mRNA and protein expression in an epithelial cell line by cigarette smoke extract could contribute to the effect of smoke on hBD-2 expression ³². Our data provide evidence that reactive oxygen species are involved in this process. H₂O₂ could mimic the effect of smoke in epithelial host defense and catalase significantly increased the expression of hBD-2. The lack of LDH release in our studies excluded frank cell necrosis. Apoptosis is a further mechanisms that could result from smoke exposure ³³. The increased

production of IL-8 after smoke exposure showed that the effect of smoke on defensin expression is not due to an overall decrease in cell viability. The effect of smoke components on various signaling processes results in the differential expression of individual genes as shown here for IL-8 and hBD-2.

The clinical implication of smoke-induced inhibition of pulmonary defenses and the subsequent susceptibility to infection is obvious. In addition, a breach in innate host defense likely contributes to the pathogenesis of COPD. This chronic inflammatory lung disease is correlated with colonization and increased bacterial load. A further feature of COPD is a self-perpetuating inflammatory process. Consistent with these findings, depression of hBD-2 induction was also found in former smokers, who stopped smoking more than 12 months before. Smoke-induced decreased endogenous host defenses might lead to an altered microflora of the lungs with increased numbers of microorganisms. Smoke is also known to break down the epithelial barrier that is essential to provide a physical barrier for microorganisms and to limit a host defense reaction³⁴. The combination of a broken epithelial barrier and the presence of microorganisms could result in a vicious cycle of inflammation, structural damage, and infection. The contribution of antibacterial host defense molecules in this process has been suggested by an association study that showed that a genetic variation of human beta-defensin 1 (hBD-1) is found more frequent in COPD patients as compared to controls³⁵.

In conclusion, smoking decreases the epithelial host defense. Smoke exposure reduces epithelial host defense potentially resulting in increased susceptibility to

colonization and infection. This mechanism is likely involved in increased susceptibility to pulmonary infection.

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COMPETING INTEREST

The authors declare no competing interest.

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Tables

Table 1: Patient characteristics. Figures for age and CRB-65 indicate mean (std. deviation). p values were calculated using 1 way ANOVA. n.s. = not significant.

Item	Current smokers	Former smokers	Never smokers	p-value
n	12	6	9	-
Age	49.9 (12.8)	70.9 (14.6)	67 (14.6)	0.0021
Sex (m/w)	7/5	4/2	5/4	n.s.
CRB-65	0.33 (0.5)	1.2 (0.4)	1,1 (0.6)	0.0043
Mortality	0	0	0	-

Figure legends

Figure 1 **Smoking inhibits the mucosal host defense system in patients**

with community acquired pneumonia. hBD-2 peptide concentration was measured in pharyngeal washing fluids of patients with CAP by ELISA. (A) Current or former smokers have decreased levels of the antimicrobial peptide hBD-2 in their pharyngeal washings as compared to never smokers; numbers of patients = 10, 6, 12, 6, and 9 for the healthy non-smokers, healthy smokers, current smokers, former smokers, and never smokers (last three groups with CAP), respectively. $P = 0.0024$ (ANOVA) with $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ for Bonferroni post hoc test where indicated. (B) Proteins were extracted from sputum as described in the methods section, pooled, and separated by SDS-gel electrophoresis and blotted onto nitrocellulose. In never smokers, hBD-2 is present in the size of the active form (arrow). In current smokers, hBD-2 is not detectable. The blot is representative of several experiments and shows pooled material from 5 persons.

Figure 2 **Smoke exposure of differentiated airway epithelium results in a**

breach of epithelial host defense. Cell cultures were smoke exposed (CS) and subsequently infected with 1×10^5 viable *P. aeruginosa*. The data in panels A-C were obtained from the same experiment. (A) Smoke exposure reduces antimicrobial activity of epithelial cells. Washes from the apical surface were prepared 6 h after infection and the number of viable bacteria determined. (B) Smoke exposure

suppressed the induction of hBD-2 by bacteria. hBD-1 and hBD-2 mRNA levels were determined by quantitative RT-PCR. (C) hBD-2 peptide concentration in apical washings was measured by ELISA and found to be significantly decreased after smoke exposure. (D) Exogenously applied hBD-2 (5 µg per well) was added to infected, smoke exposed cells and restored the epithelial antimicrobial activity. Cells = untreated control, PAO = infected cells, CS = smoke exposed cells; $n = 8$ per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (t-test).

Figure 3 Oxidative stress contributes to the smoke-dependent inhibition epithelial antimicrobial activity. (A) hBD-2 induction by application of 1×10^7 CFU/ml of heat inactivated *P. aeruginosa* (PAO) is inhibited by H_2O_2 (1 hour preincubation with the indicated concentrations). (B) Application of catalase (added immediately before smoke exposure) partly restored the suppressive effect of cigarette smoke (CS) on hBD-2 expression. (C) Measurement of IL-8 in culture medium. Cigarette smoke and bacterial exposure increase the release of the cytokine. $n = 8$ per group, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (t-test for A-B, ANOVA for C < 0.0001 ; Bonferroni post hoc test where indicated).

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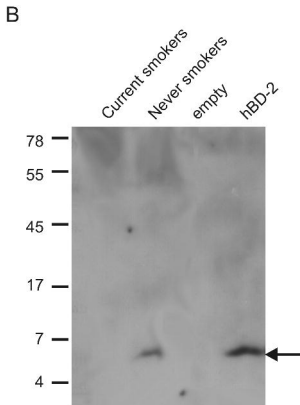
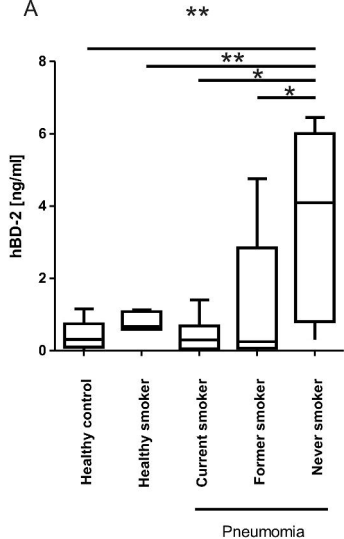
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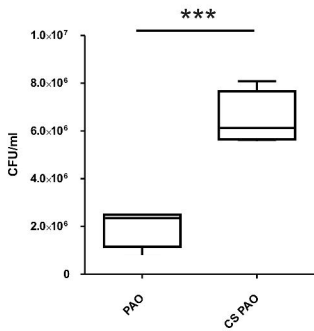
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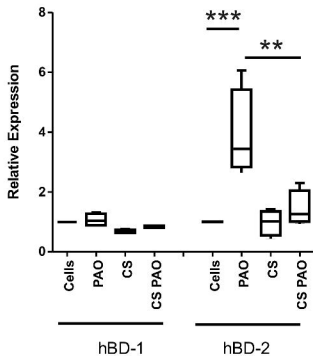
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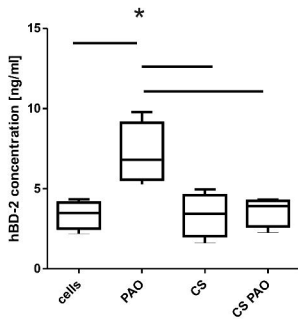
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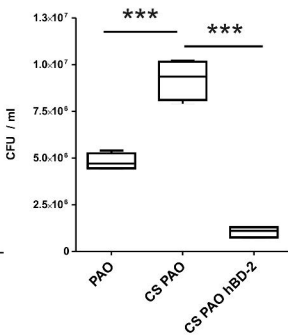
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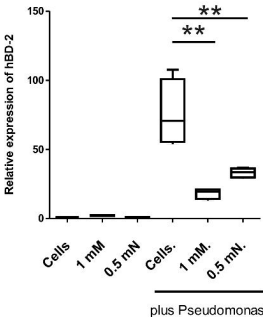
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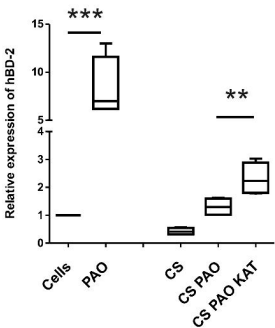
D



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B



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