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Hydrogen peroxide is responsible for the cytotoxic effects of *Streptococcus pneumoniae* on primary microglia in the absence of pneumolysin

Short title: *Cytotoxic effects of pneumococcal hydrogen peroxide on microglia*

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Abstract

Introduction: *Streptococcus pneumoniae* is the most common cause of bacterial meningitis and meningoencephalitis in humans. The bacterium produces numerous virulence determinants, among them hydrogen peroxide (H_2O_2) and pneumolysin (Ply), which contribute to bacterial cytotoxicity. Microglia, the resident phagocytes in the brain, are distinct from other macrophages, and we thus compared their susceptibility to pneumococcal toxicity and their ability to phagocytose pneumococci with those of bone marrow-derived macrophages (BMDM).

Methods: Microglia and BMDM were co-incubated with *S. pneumoniae* D39 to analyze survival of phagocytes by fluorescence microscopy, bacterial growth by quantitative plating, and phagocytosis by an antibiotic protection assay. Ply was detected by hemolysis assay and Western blot analysis.

Results: We found that microglia were killed during pneumococcal infection with a wild-type and an isogenic *ply*-deficient mutant, whereas viability of BMDM was not affected by pneumococci. Treatment with recombinant Ply showed a dose-dependent cytotoxic effect on microglia and BMDM. However, high concentrations of recombinant Ply were required and under the chosen experimental conditions, Ply was not detectable in the supernatant during infection of microglia. Inactivation of H_2O_2 by exogenously added catalase abolished its cytotoxic effect. Consequently, infection of microglia with pneumococci deficient for the pyruvate oxidase SpxB, primarily producing H_2O_2, resulted in reduced killing of microglia.

Conclusion: Taken together, in the absence of Ply, H_2O_2 caused cell death in primary phagocytes in concentrations produced by pneumococci.

Introduction

Streptococcus pneumoniae (pneumococcus) is a commensal bacterium on mucosal surfaces of humans, which, however, can become an invasive pathogen causing severe infections such as pneumonia, septicemia, and meningitis. Carriage of pneumococci is essential for subsequent infection [1]. Pneumococcal meningitis has case fatality rates of 20-37 % in high-income and up to 51 % in low-income countries [2]. A variety of factors is responsible for invasion, dissemination, and translocation into the central nervous system (CNS), yet the mechanisms of the pathogenesis of CNS infections are incompletely understood [3]. Two factors known to be involved in pneumococcal disease are hydrogen peroxide (H_2O_2) and pneumolysin (Ply) [4-10].

H_2O_2 is a metabolic by-product during aerobic growth, produced up to 90 % by the enzyme pyruvate oxidase (SpxB) [11] which was first described by Spellerberg *et al.* in 1996 [12]. Notably, the amount of released H_2O_2 is high enough to cause bacteriostatic and bactericidal effects on other bacteria in the upper respiratory tract [4]. Moreover, Ply release correlates with the amount and pyruvate oxidase enzymatic activity, *i.e.* H_2O_2 production [13]. In mice infection experiments, the production of H_2O_2 by SpxB led to prolonged colonization of the nasopharynx. During lung infection, the concerted action of Ply and SpxB contribute to bacterial replication in the lungs and translocation into the bloodstream [5]. Although powerful destructive effects were shown for H_2O_2 in mice *in vivo*, the knowledge about its role during brain infections remains limited. H_2O_2 caused ciliary stasis in a ciliated ependymal cell line [9] and played an auxiliary role in Ply toxicity on a microglial cell line [10]. With respect to primary brain cells, a cytotoxic effect of H_2O_2 has been shown for neurons and microglia [10].
Ply belongs to the group of cholesterol-dependent cytolysins (CDC). CDC-toxins are able to form pores in cholesterol-containing cytoplasmic membranes and thereby lead to lysis of host cells [14]. Ply production is necessary for colonization of the nasopharynx, the first step for pneumococcal infection [6]. Furthermore, Ply induces formation of nitric oxide, a key feature of septic shock [15], and in mouse infections it leads to lung damage [7]. Ply is a well-established major virulence factor on the path towards and during the progression of pneumonia. In a rat meningitis model, pneumococci deficient in Ply were reported to cause very mild diseases/symptoms in rats [8]. In a mouse model animals infected with Ply-deficient *S. pneumoniae* appeared healthier, had lower bacterial concentrations in blood and lived longer, whereas bacterial titers in the cerebellum, meningeal inflammation, and neuronal damage scores were comparable to mice infected with wild-type *S. pneumoniae* [16]. It is known that Ply and fragments of the pneumococcal capsule can inhibit motility of microglia [17] and that Ply is able to cause cell death in primary rat cortical neurons [18]. Furthermore, synaptic damage during meningitis is caused by Ply at disease-relevant non-lytic concentrations probably because it leads to a release of glutamate via pore-formation [19].
Microglia are the resident macrophages of the parenchyma of the CNS and make up 10-15 % of all glial cells, the most abundant cells in the CNS [20]. They are of different origin than other macrophages in the body. Microglia are derived from the yolk sac and are later replenished by resident progenitor cells, whereas almost all other macrophages are derived from bone marrow progenitors [21-24]. They have a key role during infections of the CNS as they display the first line of defense against invading pathogens. They express a broad range of receptors to recognize pathogens, they are able to phagocytose them and to produce cytokines which can recruit immune cells from the periphery and enables microglia to communicate with cells within the CNS [25].
In this study, we found that primary microglia, but not bone-marrow derived macrophages (BMDM) were killed during pneumococcal infection. This prompted us to find out, which factor is responsible for the rapid death of microglia. Our results indicate that cell death was mainly dependent on H_2O_2 under experimental conditions in which Ply was not released by pneumococci in sufficient concentrations to harm microglia. Both factors can be toxic for host cells, however, in relation to the concentrations produced by pneumococci during infection, very high concentrations of recombinant Ply and low concentrations of H_2O_2 were necessary. BMDM were more effective than microglia in killing pneumococci and, thereby, prevented accumulation of high concentrations of pneumococcal H_2O_2.

Materials and Methods

Bacterial strains and culture conditions

All mutant strains used in this study were generated in *Streptococcus pneumoniae* D39 (NCTC 7466, serotype 2) in previous studies [26-29]. We used cryopreserved bacteria for all experiments. For preparation of cryostocks, *S. pneumoniae* was grown on Columbia blood agar plates (Oxoid™, Thermo Fisher Scientific) at 37 °C and 5 % CO_2 overnight and subsequently cultivated in Todd Hewitt Broth (Bacto™, Becton Dickinson) containing 0.5 % yeast extract (Carl Roth) (THY) until the optical density at 600 nm reached 0.6. Pneumococci were washed with phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific), diluted in medium with a final concentration of 15 % glycerol and snap-frozen in liquid nitrogen. Cryopreserved bacteria were stored at -80 °C, until they were thawed and diluted to the appropriate concentrations of colony forming units (CFU) needed in the experiments.

Eukaryotic cell culture

L929 cells (ATTC®: CCL-1) were cultured in 50 ml Dulbecco's Modified Eagle Medium (DMEM; low glucose (1 g/l), L-glutamine, pyruvate (Gibco, Thermo Fisher Scientific)) containing 5 % fetal calf serum (FCS; Biochrom) and 2 mM L-glutamine (Gibco, Thermo Fisher Scientific) in 175 cm^2 cell culture flasks at 37 °C and 8 % CO_2. Culture

supernatants were harvested weekly, centrifuged twice and frozen for later use to stimulate or differentiate microglia or BMDM.

The human laryngeal epithelial cell line HEp-2 (ATCC® CCL-23™) as well as the murine macrophage cell line J774A.1 (ATCC® TIB-67™) were cultured in DMEM (low glucose (1 g/l), L-glutamine, pyruvate (Gibco, Thermo Fisher Scientific)) containing 10 % FCS (Biochrom) and 2 mM L-glutamine (Gibco, Thermo Fisher Scientific) in 75 cm^2 cell culture flasks at 37 °C and 8 % CO_2.

Primary microglia were prepared from neonatal (0-2 days) mice as previously described [30, 31]. Briefly, after preparation, mixed glial cultures were cultured in poly-L-lysine (Merck) coated 75 cm^2 cell culture flasks with DMEM (high glucose (4.5 g/l), L-glutamine, without pyruvate (Gibco, Thermo Fisher Scientific)) containing 10 % FCS (Biochrom), 2 mM L-glutamine (Gibco, Thermo Fisher Scientific), and penicillin (50 units/ml)-streptomycin (50 µg/ml) (Gibco, Thermo Fisher Scientific) for 10 days or until confluency of the cell layer was reached at 37 °C and 8 % CO_2. To increase microglial cell division, 1/3 supernatants of the cell line L929 were added to the medium without antibiotics for 3-4 days at 37 °C and 8 % CO_2. For detachment of microglia, mixed glia cell culture flasks were shaken for 30 min at 37 °C at 130 rounds per minute (rpm) on an orbital shaker. Cells from supernatants were counted and microglia seeded as described for individual assays. Depending on the experiment, medium with or without phenol red was used for cultivation and seeding of microglia.

For obtaining primary bone-marrow-derived macrophages (BMDM), bone marrow cells (BMC) were prepared from mice and differentiated as previously described [32]. BMC were cultivated in DMEM (low glucose (1 g/l), L-glutamine, pyruvate (Gibco, Thermo Fisher Scientific)), supplemented with 10 % FCS (Biochrom), 2 mM L-glutamine (Gibco, Thermo Fisher Scientific), 1/5 M-CSF/L929 supernatant, and penicillin (50 units/ml)-streptomycin (50 µg/ml) (Gibco, Thermo Fisher Scientific). Cells were stimulated with 1/5 L929 culture supernatants for 3 days at 37 °C and 8 % CO_2. Then, the medium including stimulating supernatants was replaced and cells cultivated likewise for additional 6-7 days. Cells were detached via cold-shock for 3 min on ice and afterwards resuspended in warm medium without antibiotics and seeded according to the individual assays.

Phagocytosis and intracellular survival assay

Primary microglia or BMDM were seeded at 125000 cells/ml in DMEM without antibiotics in a 6-well plate (2 ml/well; Fig. 1 a, b) or 96-well plate (200 µl/well, in triplicates; Fig. 1 c, d) and incubated at 37 °C and 5 % CO_2. The following day, cells were infected *in vitro* at a multiplicity of infection (MOI) of 10 with *S. pneumoniae* D39, the capsule- (D39Δ*cps*), pneumolysin- (D39Δ*ply*), or double-knockout mutant (D39Δ*cps*Δ*ply*) for 1 h at 37 °C and 5 % CO_2 in the absence or presence of recombinant pneumolysin (rPLY; 1-1000 HU/ml). Subsequently, eukaryotic cells were washed with PBS containing $MgCl_2$ and $CaCl_2$ (Merck) and then incubated in medium containing final concentrations of 100 µg/ml gentamicin (Carl Roth) and 10 µg/ml penicillin G (Merck) for 1 h at 37 °C and 5 % CO_2. Afterwards, eukaryotic cells were washed with PBS containing $MgCl_2$ and $CaCl_2$, lysed with distilled water, and viable intracellular bacteria were plated at serial dilutions in triplicates. By using this plating method, a minimum of 100 CFU/well is detectable (indicated by the dotted line in the respective graph).

Assessment of cell viability by Hoechst/propidium iodide double staining

Primary microglia or BMDM were seeded at 100000 cells/ml in DMEM without antibiotics in a 24-well plate (1 ml/well) and incubated at 37 °C and 5 % CO_2. The following day, primary macrophages either were infected with *S. pneumoniae* D39, D39Δ*cps*, D39Δ*ply*, D39Δ*cps*Δ*ply*, or the pyruvate oxidase-knockout strain (D39Δ*spxB*), respectively, at a MOI of 3 or 10 or were treated with different concentrations of hydrogen peroxide (H_2O_2; Merck) for up to 6 h at 37° C and 5 % CO_2.

For experiments including catalase pre-treatment, the enzyme (Merck) was used in a concentration of 2000 U/ml in cell culture medium and added 30 minutes prior infection with pneumococci or treatment with H_2O_2 [33]. After the respective incubation time, nuclei of these cells were stained with 15 nmol/ml propidium iodide (PI) (Merck) to label the nuclei of dead cells and 1.78 nmol/ml Hoechst 33342 (Merck) to label the nuclei of all cells for 10 min at 37° C. Three images from different areas per time point and condition (on average resulting in 1200 cells) were recorded at 200-fold magnification with a Nikon Eclipse Ti-S microscope (Nikon) using NIS-Elements BR 4.51.01 (Nikon) software. Cells were later analyzed using CellProfiler 3.0 (Broad Institute) [34] and ImageJ/Fiji (NIH) [35].

Hydrogen peroxide assay

Primary microglia were seeded at 100000 cells/ml in DMEM without antibiotics and without phenol red in 24-well plates (1 ml/well) and incubated at 37 °C and 5 % CO_2. The following day, microglia were infected with *S. pneumoniae* D39, D39Δ*cps*, D39Δ*ply*, D39Δ*spxB*, or the double knockout strains D39Δ*cps*Δ*ply* and D39Δ*cps*Δ*spxB* at a MOI of 3 for up to 6 h at 37° C and 5 % CO_2.

After centrifugation, supernatants were transferred to a 48-well plate (900 µl/well) and H_2O_2 levels were determined by the method described by Pick and Keisari [36] with the following modifications. Phenol red (Merck) was used at a final concentration of 0.072 mM, horseradish peroxidase (HRPO; Merck) at a final concentration of 9 U/ml, and the reaction was stopped by a final concentration of 10 mM NaOH (Carl Roth). Moreover, the experiment was performed in DMEM (high glucose (4.5 g/l) without L-glutamine, without phenol red, without pyruvate (Gibco, Thermo Fisher Scientific)) supplemented with 10 % FCS (Biochrom) and 2 mM L-glutamine (Gibco, Thermo Fisher Scientific). Changes in absorption due to oxidation of phenol red were detected at 605 nm in 96-well plates (200 µl/well) using a microplate reader (SpectraMax i3x, Molecular Devices). H_2O_2 concentrations in the supernatants were calculated by interpolation of absorption values to an H_2O_2 standard. If necessary, samples were diluted 1:4 or 1:6 with medium before performing H_2O_2 measurements.

In addition, the amount of H_2O_2 produced by pneumococci in pooled human cerebrospinal fluid (CSF) and in DMEM in the absence of microglia was compared. CSF was supplemented with the following amino acids: alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, histidine, hydroxyl proline, isoleucine, leucine, methionine, phenyl alanine, proline, serine, tryptophan, tyrosine, valine (final concentration 50 mg/l), glutamine and threonine (final concentration 100 mg/l) as well as adenine and uracil (final concentration 100 mg/l and 200 mg/l) (modified from [37]). Due to the limited amount of human CSF available, the sample volumes had to be adjusted for this assay. Briefly, *S. pneumoniae* D39 was incubated at a concentration of 10^6 CFU/ml (corresponding to a MOI of 10 in the presence of cells) in CSF or DMEM in a 48-well plate (250 µl/well) for up to 6 h at 37° C and 5 % CO_2. Supernatants were centrifuged and 100 µl of the supernatants were transferred to 96-well plates to perform the H_2O_2 assay as described above. If necessary, samples were diluted 1:2, 1:4, or 1:8 with medium before performing H_2O_2 measurement.

Cytotoxicity assay

A lactate dehydrogenase (LDH) release assay was performed to test the cytotoxicity of Ply, using rPly heterologously produced in *Escherichia coli* [38]. Primary cells were seeded at concentrations of 300000 cells/ml in 96-well plates (200 µl/well), whereas the cell lines were seeded at 150000 cells/ml (J774A.1) and 100000-cells/ml (HEp-2), respectively, resulting in similar amounts of cells on the next day. On the following day, cells were exposed for 2 h to 128-8200 HU/ml of rPly. The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used according to instructions of the manufacturer.

Hemolysis assay

The assay was performed to first determine the HU/mg of rPly [38] and later to determine the concentration of active Ply produced by pneumococci. Briefly, 100 µl sample with known concentration of rPly were serially diluted 1:2 in 0.9 % NaCl solution or DMEM (high glucose (4.5 g/l), L-glutamine, without pyruvate, without phenol red (Gibco, Thermo Fisher Scientific)) supplemented with 10 % FCS (Biochrom) and 2 mM L-glutamine (Gibco, Thermo Fisher Scientific), respectively, in conical shaped 96-well plates. Afterwards, 100 µl of a 2 % erythrocyte suspension, prepared from sheep blood (Fiebig Nährstoffe, Germany) in 0.9 % NaCl solution or DMEM was added. Completely lysed or untreated cells served as controls. The plates were incubated on an orbital shaker at 100 rpm for 2 h at 37 °C. Plates were centrifuged, supernatants transferred into flat bottom 96-well plates, and absorption was measured at 550 nm in a microplate reader (SpectraMax i3x, Molecular Devices). One HU/ml was defined as the concentration of toxin causing 50 % hemolysis of a 2 % sheep erythrocyte suspension. Depending on the lot and activity of rPly, one hemolytic unit corresponded to 2.2-5 ng rPly.

To determine the amount of active Ply produced by pneumococci, *S. pneumoniae* D39 was incubated at a concentration of 10^6 CFU/ml (corresponding to a MOI of 10 in the presence of cells) in THY or DMEM in 24-well plates (1 ml/well) for up to 6 h at 37° C and 5 % CO_2. The supernatants of five wells were pooled, centrifuged and 4 ml of the supernatant were concentrated using Amicon® Ultra-4 centrifugal filter unit (Merck). Hemolysis assay was conducted with 100 µl of the concentrated supernatant (concentration factor 10) as described above, and the

HU/ml supernatant was calculated based on the volume of concentrated supernatant that induced 50 % hemolysis of the 2 % sheep erythrocyte suspension. A minimum of 1 HU/ml supernatant could be detected by this method.

Western blot analysis

Concentrated supernatants obtained for the hemolysis assay were separated electrophoretically using a 5 % stacking and a 10 % running sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and were transferred to a polyvinylidene fluoride (PVDF) membrane (Merck). The membranes were blocked for 1 h at room temperature (RT) with 5 % skimmed milk powder in Tris-buffered saline (TBS) with 1 % Tween®20 (Carl Roth). Afterwards, they were incubated with a polyclonal rabbit antiserum raised against pneumolysin (Davids Biotechnologie GmbH, Germany; dilution 1:500 in 1 % skimmed milk powder in TBS with 1 % Tween®20) [38] overnight at 4°C, followed by incubation with HRP-linked goat anti-rabbit IgG (Cell Signaling; dilution 1:5,000 in 1 % skimmed milk powder in TBS with 1 % Tween®20) for 1 h at RT. The membranes were developed with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and the chemiluminescent signal was detected with ChemoCam Imager 3.2 (Intas, Germany).

Flow cytometric analysis

For the detection of rPly binding to microglia and J774A.1 macrophages and the detection of viable cells, 5×10^5 cells were co-incubated with rPly (1-1000 HU/ml) in the respective cell culture medium for 1 h at 37°C. Afterwards, cells were centrifuged (300 × *g*, 5 min, RT), the supernatant was removed and cells were washed in PBS, followed by a live/dead staining using the Zombie NIR Fixable Viability Kit (BioLegend) for 10 min at RT. Cells were washed and incubated with a primary polyclonal serum raised against rPly in rabbits (Davids Biotechnologie GmbH; dilution 1:250 in PBS + 2 % FCS) for 30 min at 37°C. After washing, cells were incubated with a secondary anti-rabbit Alexa-Fluor 488 conjugated antibody (Dianova) for 10 min on ice. Then, cells were washed and resuspended in 2 % paraformaldehyde (PFA). Samples were acquired on a BD LSRII cytometer (BD Biosciences). All data evaluation was performed using FlowJo software (FlowJo LLC).

The following controls were included in the analysis: cells were incubated with 0 HU/ml rPly + primary polyclonal serum raised against rPly in rabbits + secondary anti-rabbit Alexa-Fluor 488 conjugated antibody (refers to "-") and cells were pre-incubated with 1000 HU/ml rPly + a naïve rabbit serum + secondary anti-rabbit Alexa-Fluor 488 conjugated antibody (refers to "antibody control").

Statistical analysis

All experiments were performed at least three times unless otherwise indicated and results are shown as mean ± standard deviation (SD). All statistical analyses were carried out using GraphPad Prism (version 9.0.0; GraphPad Software, USA). Normal (Gaussian) distribution of the data was tested with Shapiro-Wilk test and statistical significant differences were identified by unpaired *t* test or one-way ANOVA followed by Tukey's, Sidak's, or Dunnett's multiple comparison test. Statistically significant differences are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Results

S. pneumoniae grows in the presence of microglia, but is killed by BMDM

Primary microglia and primary bone marrow derived macrophages (BMDM) were infected *in vitro* with wild-type or non-encapsulated *S. pneumoniae* D39 (D39Δ*cps*) at a MOI of 10. Non-encapsulated pneumococci were included, because they occasionally show different growth kinetics, depending on the medium used, and are usually phagocytosed more efficiently by macrophages than encapsulated pneumococci. Both effects could result in differences in bacterial numbers during growth in cell culture medium or in the presence of macrophages. Remarkably, equal concentrations (colony forming units per well (CFU/well)) of pneumococci were recovered when bacteria were grown in the presence of microglia or in medium (DMEM 4.5 g/l glucose) without eukaryotic cells (Fig. 1 a). Similar bacterial densities were reached, when pneumococci were grown in DMEM supplemented with 4.5 g/l glucose (medium used for cultivation of microglia) or 1 g/l glucose (medium used for cultivation of BMDM) (Fig. 1 a, b). In the presence of BMDM, the number of recovered pneumococci from the supernatant was significantly lower after 4 hours (h) and 8 h of incubation compared to growth in medium alone (Fig. 1 b). Taken together, this indicates that pneumococci were killed by BMDM, but not by microglia, and pneumococcal growth was independent of the glucose concentration.

To evaluate how pneumococcal survival in the presence of microglia or BMDM was influenced by phagocytic uptake and killing, a penicillin-gentamicin protection assay was performed to determine the number of intracellular pneumococci (Fig. 1 c). Phagocytic uptake was observed in BMDM and microglia after 1 h of infection followed by 1 h of antibiotic treatment and was similar for non-encapsulated pneumococci, but the number of intracellular encapsulated bacteria was lower in BMDM when compared to microglia suggesting a faster intracellular killing of the pneumococci by BMDM. Additionally, we observed killing of pneumococci in cell-free supernatant of BMDM (Fig. S1), indicating that also extracellularly secreted factors of BMDM are involved in killing of pneumococci. Thus, killing of *S. pneumoniae* was delayed in microglia compared to BMDM and pneumococci were killed extra- and intracellularly by BMDM.

As described in previous studies with *S. pneumoniae* and other streptococci, non-encapsulated pneumococci were phagocytosed in higher numbers by microglia as well as BMDM compared to encapsulated bacteria (Fig. 1 c). Conversely, no differences could be observed between the wild-type strain and the Ply-deficient isogenic mutant. To answer the question whether Ply affects phagocytosis under the chosen experimental conditions, recombinant pneumolysin (rPly) was added in increasing concentrations (up to 1000 hemolytic units per ml (HU/ml)) to microglia infected with *S. pneumoniae* D39Δ*cps*Δ*ply*. However, addition of rPly in the chosen concentrations did not alter bacterial uptake by microglia (Fig. S2).

Microglia are killed during in vitro pneumococcal infection, whereas BMDM survive

Next, we analyzed whether the observed differences in pneumococcal growth in the presence of microglia or BMDM (Fig. 1) were caused by differences in macrophage numbers due to cell death induced by pneumococci. Vital staining and microscopic analysis were performed to assess cell death of primary phagocytes during infection with pneumococci (Fig. 2). Remarkably, a significant decrease in the percentage of viable microglia but not of BMDM was detectable over time. At 6 h post infection, less than 20 % of microglia were still vital (Fig. 2 a), whereas BMDM retained almost full viability over time in the presence of pneumococci. Only non-encapsulated pneumococci were able to induce approximately 10 % cell death in BMDM (Fig. 2 b). The lethal effect of pneumococcal infection on microglia was independent of the capsule and Ply as the reduction of microglial cells during infection was similar for all strains (Fig. 2 a). The percentage of microglial cell death depended on the relation of bacteria and phagocytes for infection of microglia with D39Δ*cps.* After 4 h of infection, at MOI 3 approximately 40 % viable microglia could be detected, but only 20 % when infected at MOI 10 (Fig. 2 a and Fig. S3 a). Notably, the differences were not a consequence of different growth behavior, because comparable amounts of *S. pneumoniae* were recovered from the supernatants throughout the experiments (Fig. S3 b and Fig. 1 a). Taken together, *S. pneumoniae* infection did not impair BMDM viability but led to dose- and time-dependent cell death in microglial cells, which was independent from the presence of the pneumococcal capsule or Ply.

Cytotoxic effect of pneumolysin on phagocytes is only apparent at high concentrations

To analyze possible cytotoxic effects of Ply on phagocytes in more detail, we added different concentrations of rPly to primary microglia and BMDM as well as the murine macrophage cell line J774.A1 and the human laryngeal epithelial cell line HEp-2 for reasons of comparison. Dose-dependent cytotoxic effects on macrophages were only detected at very high concentrations (1025-8200 HU/ml) of rPly (Fig. 3 a). Cytotoxic effects that caused more than

20 % cell damage in macrophages were only observed at concentrations of 4100 and 8200 HU/ml rPly. Moreover, at these high rPly concentrations, BMDM and J774.A1 cells were significantly more susceptible towards the toxin than microglia. Notably, the epithelial cell line HEp-2 was more susceptible towards rPly than BMDM and J774A.1 cells, because 2050 HU/ml rPly caused 45 % cytotoxicity in HEp-2 cells and only 10-15 % cytotoxicity in all other cell types tested (Fig. 3 a).

To prove that rPly can bind to microglia under the chosen experimental conditions, a flow cytometric analysis with microglia, and in comparison with J774.A1 macrophages, was conducted (Fig. S4). Using concentrations that are sublytic for microglia, a dose-dependent binding of rPly was detected to J774.A1 and microglia. In contrast to the cytotoxicity assay (Fig. 3 a), the highest concentration (1000 HU/ml rPly) induced cell death in J774.A1 macrophages (Fig. S4 d) but not in microglia (Fig. S4 c).

As the activity of rPly might be influenced by the culture conditions, the hemolytic activity typically tested in a 0.9 % sodium chloride (NaCl) solution was determined in cell culture medium (DMEM) as well as in bacterial growth medium (THY). Indeed, the activity of rPly was significantly higher in 0.9 % NaCl compared to DMEM and THY, respectively (Fig. S5).

To correlate the concentrations of rPly used to induce cytotoxic effects in microglia and BMDM to the concentration of active Ply released by pneumococci *in vitro*, HU/ml of Ply released by *S. pneumoniae* D39 were determined after 6 h of incubation in DMEM only and in the presence of microglia, respectively (Fig. 3 b). No active toxin was detectable in the supernatant of infected microglia. In addition, we determined the HU/ml of Ply produced by *S. pneumoniae* D39 in THY medium in comparison to DMEM (Fig. S6 c) and found that the protein itself as well as its hemolytic activity was only detectable in THY (2-8 HU/ml) but not in DMEM. Plating of the supernatants revealed that bacteria did not grow beyond 10^8 CFU/ml during incubation in DMEM, whereas in THY medium we counted more than 10^8 CFU/ml after 6 h of incubation (Fig. S6 d and e). Even though the bacterial number in THY medium is high enough to induce autolysis of the pneumococci and release Ply, the measured hemolytic activity was far below the concentration of active toxin necessary to damage microglia. Taken together, Ply is not present under the given experimental conditions (DMEM) and therefore cannot be responsible for microglial cell death.

Hydrogen peroxide produced by S. pneumoniae kills microglia

Because Ply seems not to be responsible for pneumococcal cytotoxicity in microglial infection, we tested possible effects of hydrogen peroxide (H_2O_2) in a next step. The ability of pneumococci to produce high concentrations of H_2O_2 is well known and H_2O_2 is a frequently mentioned virulence determinant of pneumococci. To analyze the concentration of H_2O_2 produced during pneumococcal infection of microglia, the supernatants of infected cells were tested in a phenol red-horseradish peroxidase assay (Fig. 4 a). The wild-type and the non-encapsulated mutant strains produced up to 2.5 mM H_2O_2 in the presence of microglia after 6 h of incubation, whereas the Ply-deficient mutant strain produced only up to 1.5 mM. As expected, D39Δ*spxB* released only a limited amount of H_2O_2 (0.2 mM), with SpxB (pyruvate oxidase) being the enzyme that produces the vast amount of H_2O_2 during pneumococcal metabolism. In order to prove that measurable H_2O_2 was mainly produced by the pneumococci and not by the microglia themselves, H_2O_2 production was also analyzed in the absence of microglia, and comparable amounts of H_2O_2 were detected in medium plus pneumococci only (Fig. S7).

To test whether H_2O_2 itself is sufficient to kill primary macrophages, a concentration-effect curve of H_2O_2 with microglia was performed (Fig. 4 b). The tested concentrations were chosen according to the concentrations of H_2O_2 released by pneumococci during infection (Fig. 4 a). After 4 h, less than 40 % of the microglia were still alive and after 6 h, the cells were efficiently killed even by the lowest concentration of H_2O_2 (0.25 mM). Therefore, concentrations of H_2O_2 produced by pneumococci are sufficient to kill the microglia. Likewise, H_2O_2 was able to kill BMDM, yet the cells were not as susceptible for H_2O_2 as microglia (Fig. S8).

Finally, we treated microglia with catalase prior to pneumococcal infection and tested cell viability at 6 h post infection (Fig. 4 c). After infection with D39, less than 5 % viable cells were detected, conversely, when pre-treated with catalase, significantly more viable microglia (approximately 80 %) were observed after 6 h. As a control, microglia were incubated with 2.5 mM H_2O_2. Prior treatment with catalase retained cell viability of H_2O_2-treated microglia at the level of uninfected control cells (Fig. 4 c). Consequently, we also tested the pneumococcal *spxB* mutant, which produced smaller amounts of H_2O_2 during the infection of microglia (Fig. 4 a). After 6 h of infection with D39Δ*spxB,* approximately 70 % microglial cells were still viable. Moreover, when pre-treated with catalase, microglial viability could be restored to the level of the uninfected control (Fig. 4 c).

These results show that pneumococcal H_2O_2 is responsible for microglial cell death under the chosen experimental conditions and sufficient concentrations of H_2O_2 are released by pneumococci during microglial infection.

***S. pneumoniae* produces H_2O_2 in medium and in human CSF**

To assess if the observed effects of strong H_2O_2 production by *S. pneumoniae* also occur under more physiological conditions, pneumococci were incubated in pooled human CSF and for comparison in DMEM for up to 6 h (Fig. 5). Pneumococci grew to higher concentrations (CFU/ml) in CSF compared to DMEM (Fig. 5 a) and comparable amounts of H_2O_2 were produced by the bacteria under both conditions (Fig. 5 b). This indicates that H_2O_2 is released in cytotoxic amounts under physiological conditions.

Discussion

Here, we demonstrate that pneumococcal infection was cytotoxic for primary microglia, while it was not for BMDM. In contrast to microglia, BMDM efficiently killed *S. pneumoniae* intra- and extracellularly. The observed cell death was independent of Ply, as under our experimental conditions Ply was not detectable in the supernatants of infected cells, whereas catalase (an H_2O_2-degrading enzyme) treatment of cells diminished the cytotoxic effect of pneumococci on microglia. H_2O_2 was cytotoxic to microglia in concentrations released by pneumococci under the given experimental conditions. We detected up to 2 mM H_2O_2 produced by the wild-type pneumococcal strain D39 in our experimental set-up (Fig. 4 a). These concentrations correspond to an earlier finding, reporting that 1.8 mM H_2O_2 were formed by 5×10^7 CFU *S. pneumoniae* within 30 min [11]. Moreover, mutant strains lacking the major producer of pneumococcal H_2O_2, the pyruvate oxidase, were not cytotoxic to microglial cells in our experimental setting. After H_2O_2 titration on primary microglia, the resulting concentration-dependent cell death curve reflected the curve of microglial cell death caused by pneumococci (Fig. 2 a). H_2O_2 concentrations as low as 0.25 mM were able to cause microglial cell death in our experiments. A slightly lower concentration of H_2O_2 (approximately 0.2 mM after 6 h of incubation) was produced by D39Δ*spxB* during growth in the presence of microglia (Fig. 4 a) and a reduction of approximately 30 % in cell viability was observed after co-cultivation of microglia with D39Δ*spxB* for 6 h in comparison to microglia treated with catalase (Fig. 4 c). Under long term exposure of cells to H_2O_2, concentrations of 10 nM were sufficient to induce eukaryotic cell death [39, 40]. Microglia are, compared to other CNS cell types such as oligodendrocytes, astrocytes, and neurons, the slowest in degrading H_2O_2 [41]. It needs to be determined *in vivo*, whether microglia have a similar sensitivity to H_2O_2 as *in vitro* since microglial survival is regulated by CSFR1 and CSFR1 antagonists which make microglia more susceptible towards cell death induced by H_2O_2 [42].

In contrast to microglia, BMDM efficiently killed pneumococci extra- and intracellularly. Exposing BMDM to increasing concentrations of H_2O_2 revealed that they also can be harmed by it. Nevertheless, higher concentrations are needed, because BMDM are more resistant towards H_2O_2 toxicity than microglia and able to survive up to 0.25 mM H_2O_2 for 6 h. Even after treatment with 0.5 mM H_2O_2 for 6 h, survival is only reduced by approximately 25 % (Fig. S8). In addition to a higher H_2O_2 tolerance, BMDM are obviously able to prevent the accumulation of harmful pneumococcal H_2O_2 by efficiently killing the bacteria.

Microglia play a key role during infections of the CNS as they represent the first line of defense against invading pathogens. They express a broad range of receptors for the recognition of pathogens and they are able to phagocytose them and to produce cytokines which can recruit immune cells from the periphery [25]. On the other hand, activation of microglia, the release of pro-inflammatory cytokines and chemokines, and the recruitment of peripheral leukocytes can result in an excessive inflammatory response and the breakdown of the blood brain barrier [43]. In addition, microglial activation by bacterial products contributes directly to neuronal death during bacterial meningitis [44], leading to neurological sequelae in up to 50 % of survivors of pneumococcal meningitis [45-47]. Thus, activation of microglia by bacterial pathogens might facilitate invasion of the CNS and manifestation of bacterial meningitis. However, an increased inflammatory response poses a risk for the pathogen and therefore induction of microglial cell death might represent a strategy to ensure bacterial survival. Studies by Braun *et al.* 2002 demonstrated that pneumolysin and H_2O_2 both can induce neuronal and microglial apoptosis *in vitro* and mediate brain cell apoptosis during bacterial meningitis *in vivo* [10]. Mutants lacking pneumolysin and H_2O_2 significantly reduced neuronal damage during pneumococcal meningitis in rabbits and in the absence of pneumolysin, catalase treatment reduced neuronal damage *in vivo* [10]. Moreover, H_2O_2 acts as an important vasodilator in early cerebral hyperfusion during pneumococcal meningitis [47]. Catalase treatment of rats reduced the regional cerebral blood flow and brain water content in the early phase of pneumococcal meningitis [48], but did not reduce the intracranial hypertension which appears to depend on the inflammatory host response such as

the production of nitric oxide [49]. For that reason, catalase treatment might be, in combination with modulation of the host response, an effective treatment to reduce neuronal damage and the regional cerebral blood flow. Previous studies revealed that the production of H_2O_2 by pneumococci is directly correlated to the release of Ply, as strains lacking pyruvate oxidase (and lactate oxidase) showed decreased cytotoxicity and hemolytic activity [13, 50]. Notably, in our study, we have measured up to 2 mM H_2O_2 in the supernatant of infected microglia but we were not able to detect any Ply in the cell culture medium with a hemolysis assay (limit of detection 1 HU/ml; Figure 3 b). Additionally, we analyzed whether Ply is released in the absence of microglial cells in the eukaryotic cell culture medium (DMEM) compared to a classical bacterial growth medium (THY). Indeed, after 4 and 6 h of growth, Ply was detectable in THY inoculated with *S. pneumoniae* D39 and D39Δ*cps* by a hemolysis assay and Western blot analysis (Fig. S6), but not when bacteria were cultured in the medium used for microglial cultivation and infection (DMEM). One reason for the lack of Ply in the eukaryotic cell culture medium might be the density of bacteria reached under these experimental conditions. In THY, approximately 5×10^8 CFU/ml were detected after 6 h of incubation in contrast to 5×10^7 CFU/ml in DMEM. It is known that pneumococci undergo autolysis when the stationary growth phase (> 10^8 CFU/ml) is reached, and that Ply is mainly released during autolysis [51]. We speculate that in DMEM the stationary growth phase is reached less rapidly and therefore Ply is not released. Furthermore, we observed that the primary phagocytes used in our study are more resistant to the actions of rPly compared to the cell lines J774A.1 and HEp-2 (Figure 3 a). It is well known that cell lines often react differently compared to primary cells. It has been shown for Anthrolysin O (ALO), another CDC, that in contrast to concentrations required for toxicity seen on the monocyte cell line THP-1, 10-fold higher doses of toxin were required in primary human monocyte-derived macrophages [52].

A concentration of at least 1025 HU/ml rPly (reflecting 2.2-5 µg/ml rPly) was required to measure cytotoxic effects of approximately 10 % in primary microglia (Figure 3 a). Our results are in line with other studies in which Ply was cytotoxic to primary microglia in concentrations of 60 and 200 nM (3 and 10 µg/ml) [53]. In humans, 0.85-180 ng/ml Ply were found in CSF during meningitis [54]. Another study in humans suffering from *S. pneumoniae* meningitis revealed, that Ply concentrations in CSF were higher (median 1 mg/ml) than concentrations inducing microglial cell injury *in vitro*. Moreover, in survivors, Ply levels significantly declined between the first and second lumbar puncture (interval approximately 48 h), while in non-survivors Ply levels remained high [55]. This suggests that Ply is produced *in vivo* and it is very likely that it exerts important functions during CNS infections. However, under our *in vitro* conditions, it was not possible to reproduce the release of these high concentrations of Ply. Ply has been shown to be toxic to brain cells, in particular neurons [10, 18, 19, 56, 57] and is able to inhibit microglial taxis [17]. An effect of Ply on microglial cell death has been attributed to different cell death mechanisms in microglial cell lines, namely, apoptosis, autophagy, and pyroptosis [10, 58]. At sublytic concentrations, Ply is able to induce changes of the cytoskeleton via RhoA and Rac1 [59] as well as a direct transmembrane actin interaction [60] and to enhance dynamin endocytosis in primary microglia [61]. However, effects of Ply on microglia at sublytic concentrations were not addressed in the present study.

In order to show that Ply can bind under the chosen experimental conditions to microglia and is active in the used cell culture medium, we performed a flow cytometric analysis (Fig. S4) and determined the hemolytic units of rPly in DMEM (Fig. S5), respectively. Indeed, the activity of rPly was decreased in cell culture medium compared to a sodium chloride solution, but binding of Ply to microglial cells and, in comparison to the macrophage cell line J774.A1, was detectable.

In conclusion, rPly is able to cause dose dependent cytotoxic effects on microglia, however, the required concentrations were very high and in the absence of Ply, H_2O_2 affected strongly eukaryotic cell viability.

As the observed effect of high H_2O_2 production by pneumococci in medium, was also seen in human CSF *ex vivo*, this effect is likely of physiological relevance in the brain. We show that primary microglia, brain phagocytes that should clear a pneumococcal infection, are rapidly and effectively killed by pneumococcal H_2O_2, presumably affecting the ability of the brain to prevent the entrance of pneumococci into nervous tissue.

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Statement of Ethics
C57BL/6 WT mice were cared for in accordance with the principles outlined in the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" at the Institute for Biochemistry, University of Veterinary Medicine Hannover. The killing of neonatal mice for the extraction of primary cells, was approved by the Lower Saxony State Office for Consumer and Food Safety, Germany.
The anonymized leftovers of CSF samples from patients receiving diagnostic lumbar punctures were pooled. The patients did not suffer from any infectious disease and had no CSF pleocytosis. Written informed consent was obtained from the patients. This study protocol was reviewed and approved by the Ethics Committee of the University Medical Center Göttingen, Georg-August-University Göttingen, Germany (approval number: 34/1/05).

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Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Authors Contribution
FJ conducted experiments, took microscopic pictures, analyzed data, and drafted the manuscript. DS conducted experiments, analyzed data, and drafted the manuscript. RN supervised the study. TPK and SH produced rPly, constructed mutant and wild-type *S. pneumoniae* strains, and supported study design. DH contributed to design of the flow cytometric experiments and analyzed data. PVW conceived the study and drafted the manuscript. JS contributed to study design, performed experiments, analyzed data, and drafted the manuscript. All authors read and approved the final version of the manuscript.

Data availability statement
All relevant data are contained within the manuscript and supporting information files.

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Figures

Figure 1. Bacterial growth is impaired in presence of BMDM due to phagocytosis. (**a**) Microglia or (**b**) BMDM were infected with *S. pneumoniae* D39 or the capsule deficient mutant D39Δ*cps* at a MOI of 10 in a 6-well plate (2 ml/well). Growth of pneumococci was determined in medium only or in the presence of primary macrophages ("+ primary cells"). Colony forming units (CFU) are shown as mean ± SD of three independent experiments (one technical replicate each). Significance is indicated compared to bacteria grown in medium by ** $P < 0.01$ and **** $P < 0.0001$ (one-way ANOVA followed by Sidak's multiple comparison test). (**c**) In a penicillin-gentamicin protection assay, microglia and BMDM were infected with D39 or isogenic mutants, respectively, and the number of intracellular bacteria was determined via plating. Host cells were infected for 1 h at a MOI of 10 in a 96-well plate (200 µl/well) and afterwards treated with antibiotics for 1 h. The number of recovered bacteria (CFU/well, 100 µl/well) is shown as mean ± SD of at least three independent experiments (technical triplicates each). Significant differences between microglia and BMDM are indicated by ** $P < 0.01$ (unpaired *t* test). The dotted line represents the limit of detection for the applied plating method.

Figure 2. BMDM survive *in vitro* pneumococcal infection, while microglia die in a time-dependent manner. After infection of (**a**) microglia or (**b**) BMDM at a MOI of 10 with *S. pneumoniae* D39 or different mutant strains, cytotoxicity was visualized by propidium iodide-Hoechst-staining of the nuclei. On average 1200 cells were analyzed per condition by fluorescence microscopy to determine the number of dead cells. Results are shown as mean ±SD of at least three independent experiments (one technical replicate each). Significant differences between uninfected cells (control) and cells infected with *S. pneumoniae* are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison test).

Figure 3. Cytotoxic effect of pneumolysin on macrophages is only apparent at high toxin concentrations, which are not produced by pneumococci. (**a**) Primary microglia and BMDM as well as the J774A.1 macrophage cell line and the epithelial cell line HEp-2 were stimulated with recombinant Ply (HU: hemolytic units) for 2 h. Cytotoxicity was determined by LDH release assay. Results are shown as mean ±SD of at least three independent experiments (technical duplicates each). Significant differences between the cells are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (one-way ANOVA followed by Tukey's multiple comparison test). (**b**) HU/ml of Ply produced by *S. pneumoniae* D39 after 6 h of incubation in DMEM or in the presence of microglia were determined by hemolysis assay with 2 % sheep erythrocytes. Results are shown as mean ±SD of three independent experiments (one technical replicate each). The dotted line represents the limit of detection.

Figure 4. Hydrogen peroxide kills microglia in concentrations produced by *S. pneumoniae*. (**a**) Microglia were infected with *S. pneumoniae* D39 or different mutant strains, at a MOI of 3 for up to 6 h. Hydrogen peroxide (H_2O_2) concentration in the supernatant was determined via phenol red-horseradish peroxidase assay. Results are shown as mean ± SD of at least three independent experiments (one technical replicate each). Significant differences compared to D39 are indicated by **** $P < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison test). (**b**) Microglia were exposed to different concentrations of H_2O_2 in DMEM for up to 6 h. Cytotoxicity was determined by propidium iodide-Hoechst-staining of the nuclei. Results are shown as mean ±SD of three independent experiments (one technical replicate each). Significant differences between untreated cells (control) and cells treated with H_2O_2 are indicated by ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ (one-way ANOVA followed by Dunnett's multiple comparison test). (**c**) Microglia were infected with *S. pneumoniae* D39 or D39Δ*spxB*, at a MOI of 3 for 6 h after pre-treatment with catalase (2000 units/ml). Cytotoxicity was determined by propidium iodide-Hoechst-staining of the nuclei. Results are shown as mean ±SD of three independent experiments (with the exception of H_2O_2 + catalase, which was only performed twice; one technical replicate each). Significant differences between cells pre-treated with catalase and untreated cell are indicated by *** $P < 0.001$ and **** $P < 0.0001$ (unpaired *t* test).

Figure 5. *S. pneumoniae* produces comparable amounts of H_2O_2 in DMEM and CSF. *S. pneumoniae* D39 was grown in DMEM or human CSF in 48-well plates (250 µl/well) for 6 h, with a starting CFU of 1×10^6 CFU/ml. (**a**) Growth of pneumococci (CFU/ml) in DMEM and CSF was determined by plating. (**b**) Concentration of H_2O_2 (mM/well) produced by pneumococci during growth in DMEM and CSF was measured via phenol red-horseradish peroxidase assay. Results are shown as mean ± SD of three independent experiments (a: four technical replicates were pooled for plating, b: technical duplicates each). Significant differences between DMEM and CSF are indicated by * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ (unpaired *t* test).