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# Brain parenchymal TNF-α and IL-1β induction in experimental pneumococcal meningitis

Kaywan Izadpanah [a,b], Dorette Freyer [a], Joerg R. Weber [a,c], Johann S. Braun [a,d,*]

[a] Department of Neurology, Charité Universitätsmedizin Berlin, Germany
[b] Department of Orthopedics and Traumatology, University Freiburg, Germany
[c] Department of Neurology, Klinikum Klagenfurt, Austria
[d] Department of Internal Medicine, Division Neurology, College of Medicine and Health Sciences, United Arab Emirates University, United Arab Emirates

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ABSTRACT

Triggers of brain inflammation in pneumococcal meningitis are unknown. TNF-α and IL-1β were upregulated (real time PCR and in situ hybridization) in neurons and astrocytes time-dependently and maximally in the hippocampus during murine pneumococcal meningitis. Upregulation of TNF-α and IL-1β mRNA in the brain parenchyma was independent of cerebrospinal fluid leukocytosis, pneumococcal pneumolysin and $H_2O_2$, but it was potently induced by pneumococcal cell wall (PCW) fragments. Brain TNF-α mRNA was downregulated by a matrix metalloproteinases inhibitor. PCW fragments were located in the brain parenchyma. In conclusion, PCW fragments and matrix metalloproteinases trigger cytokine induction in the brain parenchyma during pneumococcal meningitis.



## 1. Introduction

High rates of neurological sequelae and mortality indicate that bacterial meningitis is still a medical challenge. Neurological morbidity and sequelae include pareses, seizures and cognitive deficits (Pfister et al., 1993). Pneumococcus is the most common and the most aggressive cause of acute bacterial meningitis. Neuronal cell death occurs in the hippocampus and in the cortex in experimental meningitis induced by *Streptococcus pneumoniae* (Braun et al., 1999a; Agyeman et al., 2014) or by pneumococcal cell wall (PCW) (Hoffmann et al., 2007a). Cerebral cortex and hippocampus exhibit selective vulnerability in pneumococcal meningitis. Neuronal apoptosis is also found there in meningitis patients (Nau et al., 1999).

The inflammatory cascades in the cerebrospinal fluid have been studied extensively. They are triggered by invading bacteria during meningitis. A plethora of pro- and anti-inflammatory host molecules are upregulated in the cerebrospinal fluid (Koedel et al., 2010; Agyeman et al., 2014). Limited knowledge exists about brain parenchymal inflammation and neuronal injury (Barichello et al., 2010). Soluble pneumococcal toxins or host inflammatory mediators may be responsible for neuronal damage and brain parenchymal inflammation.

Pneumococcal virulence factors (e.g., pneumolysin, hydrogen peroxide, and PCW) and host factors (e.g., TNF-α and IL-1β) in cerebrospinal fluid play key roles in triggering neuronal damage during pneumococcal meningitis (Braun et al., 2001, 2002; Agyeman et al., 2014). Pneumolysin, for example, is a multifunctional pneumococcal protein toxin of *S. pneumoniae* with proinflammatory and cytotoxic activities (Mitchell and Andrew, 1997; Braun et al., 1999b). Proinflammatory effects of pneumolysin are (in part) mediated by Toll-like-receptor 4 (TLR4) activation of microglia and release of TNF-α (Malley et al., 2003) whereas PCW fragments mediate inflammation by TLR2 (Bermpohl et al., 2005; Hoffmann et al., 2007b).

Cytokines and matrix metalloproteinases (MMPs) are essential host inflammatory mediators. They are released in large quantities by leukocytes which are invading the cerebrospinal fluid (CSF). TNF-α and IL-1β are markers of inflammatory activity in cerebrospinal fluid and related to clinical outcome parameters in bacterial meningitis. They also contribute to brain damage in other diseases, e.g., stroke or multiple sclerosis. MMPs are essential activators of cytokines in bacterial meningitis. They trigger intrathecal inflammation, leukocyte invasion, and brain edema (Koedel et al., 2010).

Our hypotheses were that inflammation during murine pneumococcal meningitis is not restricted to cerebrospinal fluid and that TNF-α and IL-1β are produced locally within the brain parenchyma. We also assumed that extent and localization of intraparenchymal TNF-α and IL-1β induction are determined by the stage of meningitis. Furthermore, we examined which host or bacterial components trigger brain in situ

* Corresponding author at: Charité Universitätsmedizin Berlin, Department of Neurology, Charitéplatz 1, D-10117 Berlin, Germany. Tel.: +971 37137 419; fax: +971 37672 995.
*E-mail addresses:* johann.braun@charite.de, johannb@uaeu.ac.ae (J.S. Braun).

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inflammation and if the responsible bacterial toxin is detectable within the brain parenchyma.

In the present work, we examined in situ production of TNF-α and IL-1β in the brain parenchyma during experimental pneumococcal meningitis in mice. We detected robust in situ induction of these cytokine-mRNAs and we characterized their spatiotemporal expression. Furthermore, we identified bacterial and host components which determined expression of brain parenchymal TNF-α and IL-1β.

## 2. Materials and methods

### 2.1. Bacterial cell culture

The following wild-type strains and isogenic mutant of *S. pneumoniae* were used: encapsulated (D39) and unencapsulated (R6) pneumococci (*S. pneumoniae*, *S. p.*) (Rockefeller University, New York, NY, USA) and the *plnA*$^{-}$/*spxB*$^{-}$ double mutant defective in pneumolysin (*plnA*$^{-}$) and hydrogen peroxide (*spxB*$^{-}$) production (Braun et al., 2002). Pneumococci were grown in C + Y medium (Lacks and Hotchkiss, 1960), pelleted at midlogarithmic growth phase, resuspended in PBS, and the bacterial inoculum was prepared. The viable number of cells/mL was adjusted to $1 \times 10^7$ CFU/mL. Pneumococcal cell wall (PCW) fragments were prepared as described previously and resuspended in PBS (Tuomanen et al., 1985; Weber et al., 2003). For heat-inactivation, bacteria were grown to mid-logarithmic phase, harvested by centrifugation, washed in PBS, and heat-killed in a water bath at 60 °C for 60 min. The heat-inactivated bacterial inoculum consisted only of heat-killed whole bacteria but not of PCW fragments. Heat-killed *S. pneumoniae* contain PCW but do not release cell wall components. Optical density (OD) of live and heat-killed pneumococci at 620 nm was adjusted to 0.01 which is equivalent to $10^7$ colony forming units (CFU)/mL.

### 2.2. Model of murine S. pneumoniae meningitis

Pneumococcal meningitis was induced in mice as described previously (Hoffmann et al., 2007a). C57BL/6 male mice with an average weight of 20 g were anesthetized with intraperitoneal ketamin (35 mg/kg) and xylazine (5 mg/kg). Meningitis was induced by lumbar intrathecal injection of $3 \pm 0.5 \times 10^5$ CFU viable wildtype or mutant *S. pneumoniae* or PCW in 30 μL PBS (for controls only 30 μL PBS) with a 32 G butterfly needle (n = 5 mice per group). Meningitis was induced in the presence or absence of the combined broad spectrum matrix metalloproteinase (MMP)-inhibitor GM 6001 (Calbiochem, i.p., 100 μL of 10 mg/mL) or anti-mouse CD18 antibodies (BD Pharmingen, i.v., 30 μL of 1 mg/mL) (n = 5 mice per group). Mice were anesthetized 24 h later, and CSF was taken from the cisterna magna and cultured on blood agar plates to confirm meningitis. Then, mice were perfused transcardially with 4% paraformaldehyde in PBS pH 7.4. Brains were removed from post-autopsied mice, postfixed and embedded in paraffin, and cut in 10 μm coronar sections. The meninges were carefully removed before processing the brains for RNA isolation. All experiments were approved by institutional and governmental committees and performed according to their guidelines.

### 2.3. In situ hybridization

Non-radioactive in situ hybridization was performed following published protocols (Roche Applied Science) (Breitschopf et al., 1992). Brain sections were deparaffinized and proteins denatured (with 200 mM HCl). Nonspecific background staining was decreased with acetylation. The sections were permeabilized with proteinase k, dehydrated and rehydrated. The digoxigenin-labeled TNF-α oligonucleotides were purchased from DNA-Technology (Aarhus, Denmark). For TNF-α hybridization, a mixture of two digoxigenin-labeled mRNA probes was used (probe 1: 5′-CT TCT CAT CCC TTT GGG GAC CGA TCA CC-3′, probe 2: 5′-CG TAG TCG GGG CAG CCT TGT CCC TTG AA-3′) (Jensen et al., 2000). For IL-1β hybridization, the following mRNA probe was used: 5′-GC TTG TGA GGT GCT GAT GTA CCA GTT GGG G-3′ (Clausen et al., 2005). The digoxigenin-labeled cRNA IL-1β probes were produced by in vitro transcription according to the manufacturer's protocol (Roche, USA). For negative controls, sections were incubated with hybridization buffer alone (without antisense nucleotides), an excess (×100) of unlabeled TNF-α or IL-1β probe or by pre-treatment with RNase A prior to hybridization (Gregersen et al., 2000). For positive control, sections were hybridized for glutaraldehyde-3-phosphatedehydrogenase (GAPDH) (5′-CC TGC TTC ACC ACC TTC TTG ATG TCA-3′) (not shown) (Clausen et al., 2005).

Sections were incubated with the hybridization mixture (20 ng DIG-labeled TNF-α or IL-1β probe, 50% formamide, 0.2% SDS, 0.01% sheared salmon sperm DNA, 5 x SCC, 5% dextran sulfate), heated for 4 min at 95 °C and for 10 h at 60 °C. Control sections were incubated with a sense RNA probe. Sections were then incubated for 20 min in 20 × SSC, in 10 × SSC, in 5 × SSC and in 1 × SSC. Next, slides were washed in 50% formamide in 1 × SSC and incubated for 15 min with blocking reagent containing 10% fetal calf serum. Thereafter, slides were incubated for 60 min with alkaline-phosphate-conjugated anti-DIG antibody (1:500). The sections were then washed in TBS and developed with the alkaline phosphate substrate nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP).

### 2.4. Immunohistochemistry

Following perfusion fixation of control and meningitis mice, brains (n = 3) were frozen and cut in 10 μm sections. For localization of PCW, brain sections were incubated with a primary mouse IgA monoclonal antibody specific for pneumococcal cell wall phosphorylcholine, anti-TEPC-15 (1:200, Sigma Aldrich, St Louis, MO, USA) overnight at 4 °C. Primary antibody binding sites were visualized by a fluorescent secondary antibody Alexa Fluor 488 goat anti-mouse IgA (Molecular Probes, Göttingen, Germany). Some sections were stained with rabbit polyclonal anti-GFAP antibodies (1:300, Abcam, Cambridge, UK) following in situ hybridization. A Cy3 conjugated goat anti-rabbit IgG antibody (Molecular Probes) was used for visualization.

### 2.5. Quantitative real time PCR

Total cellular RNA of neocortex was extracted and cDNA synthesis was performed as described (Ruscher et al., 2002). Expression of the samples was normalized in relation to its ß-actin mRNA content. For isolation of RNA, frozen brains were homogenized in 1 mL ice cold Trizol (Gibco). Chloroform (200 μL) was added, mixed and centrifuged. RNA was transferred to isopropanol and precipitated by centrifugation. The pellet was washed with ethanol (75%), and then centrifuged again. The pellet was air-dried and redissolved in DEPC-$H_2O$. To exclude DNA contamination, DNA was digested with DNAse. Proteins were removed with phenol–chloroform–isoamyl alcohol precipitation.

Total RNA was reverse transcribed into cDNA which was quantified with semi-quantitative real time PCR; control was done without reverse transcriptase. PCR was performed with LightCycler FastStart DNA Master SYBR Green Hybridization Probes Kit (Roche Diagnostics, Mannheim, Germany). The PCR mix consisted of (in duplicate): forward and reverse primers (each 1 μL = 10 pmol), 2.4 μL $MgCl_2$ (25 mM), 2 μL Fast Start Reaction Mix with polymerase, 11.6 μL $H_2O$, and 2 μL cDNA. For amplification and detection, we used LightCycler Relative Quantification Software (Roche Molecular Biochemicals). Relative expression of the target gene was calculated in relation to ß-actin control gene which is not regulated during meningitis. The following sequence-specific primers (Biotez, Berlin, Germany) were used: β-actin forward: 5′-ctc aac tgt gaa atg cca cc-3′, β-actin reverse: 5′-gcc aca gga ttc cat acc ca-3′, TNF-α forward: 5′-agg cgg tgc ttg ttc ctc a-3′, TNF-α reverse: 5′-agg cga gaa gat gat ctg act gcc-3′, and IL-1β forward: 5′-ctc aac tgt gaa atg cca cc-3′, IL-1β reverse: 5′-tgt cct cat cct gga agg t-3′.