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DNase Treatment Prevents Cerebrospinal Fluid Block in Early Experimental Pneumococcal Meningitis

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Objective: Streptococcus pneumoniae is the most common cause of bacterial meningitis, a disease that, despite treatment with antibiotics, still is associated with high mortality and morbidity worldwide. Diffuse brain swelling is a leading cause of morbidity in S pneumoniae meningitis. We hypothesized that neutrophil extracellular traps (NETs) disrupt cerebrospinal fluid (CSF) transport by the glymphatic system and contribute to edema formation in S pneumoniae meningitis.

Methods: We used DNase I treatment to disrupt NETs and then assessed glymphatic function by cisterna magna injections of CSF tracers in a rat model of S pneumoniae meningitis.

Results: Our analysis showed that CSF influx into the brain parenchyma, as well as CSF drainage to the cervical lymph nodes, was significantly reduced in the rat model of S pneumoniae meningitis. Degrading NETs by DNase treatment restored glymphatic transport and eliminated the increase in brain weight in the rats. In contrast, first-line antibiotic treatment had no such effect on restoring fluid dynamics.

Interpretation: This study suggests that CSF accumulation is responsible for cerebral edema formation and identifies the glymphatic system and NETs as possible new treatment targets in S pneumoniae meningitis.

In many countries, Streptococcus pneumoniae is the most common pathogen in bacterial meningitis, which has a high mortality rate.3 Central nervous system (CNS) complications are the leading cause of mortality, and brain edema is a major cause of morbidity in bacterial meningitis. The mechanisms by which S pneumoniae meningitis induces edema are only partly elucidated, but are believed to involve the inflammatory response mounted against bacterial components sensed by CNS resident and circulating immune cells. These immunological reactions result in vascular leakage, inflammatory disruption of the blood–brain barrier (BBB), and extravasation of neutrophils and of protein-rich fluid to the infected site in the brain as early as 4 hours after meningeal infection.7,8

The pathological cellular hallmark of bacterial meningitis due to S pneumoniae is invasion of neutrophils into the brain parenchyma, periventricular spaces, and meninges.9 Neutrophils can eliminate pathogens through several biological mechanisms, including phagocytosis, release of reactive oxygen species, and secretion of antimicrobial proteins such...
as elastase, myeloperoxidase, heparin-binding protein, and histones. Also, neutrophils can release neutrophil extracellular traps (NETs), consisting of extracellular DNA fibers with cytoplasmic granule proteins. NETs are formed by neutrophils expelling their DNA and enzymes to form sticky extracellular traps that can directly immobilize and kill pathogens by means of antimicrobial activity of their constituent proteins and histones. The immobilization of pathogens by NETs also facilitates local phagocytosis. In the context of pneumococcal infection, NET formation was first identified in the lungs during pneumococcal pneumonia, in the middle ear during pneumococcal otitis media, and in the cerebrospinal fluid (CSF) during S pneumoniae meningitis. However, it is not known whether NETs form within the brain during bacterial meningitis.

The glymphatic system is the highly organized fluid transport of the brain. The basic principle of glymphatics is that CSF is pumped into the brain along periarterial spaces and disperses into the neuropil in a process supported by the polarized expression of aquaporin 4 (AQP4) water channels in the vascular endfeet of astrocytes. Next, extracellular fluid will leave the brain along the perivenous spaces, followed by drainage along cranial nerves. Meningeal and cellular fluid transport has recently been shown to be responsible for acute edema formation in the setting of stroke. In the present study, we pose the question of whether abnormalities in glymphatic transport are involved in the rapidly developing swelling of brain tissue in rats with S pneumoniae meningitis. We undertook experiments showing that formation of intracerebral NETs occurs in parallel with a significant decrease in CSF transport and suppression of efflux to the cervical lymph nodes in the setting of S pneumoniae meningitis. Elimination of NETs with DNase I treatment restored CSF dynamics, suggesting that NETs could be a potential pharmacological target to reduce the severity of acute brain edema in bacterial meningitis.

Materials and Methods

Animals

Adult male Sprague Dawley rats (350 ± 50g) purchased from Taconic Biosciences (Lillé Skensved, Denmark) were housed 4 per cage under a normal daylight cycle and fed ad libitum with free access to water. All surgical procedures performed in this study were conducted at the Department of Experimental Medical Science, Lund University, in accordance with the Swedish National Institutes of Health guidelines for the care and use of laboratory animals (license M143-16, approved by the local ethical committee for animal research).

Bacterial Culture

For animal infection, a strain of S pneumoniae (serotype 18b; referred to herein as SP001) had previously been obtained from CSF of a patient diagnosed with pneumococcal meningitis, as previously described. Bacteria were taken from glycerol stocks stored at −80°C, cultured overnight, and used for infecting the rats as described in Mohanty et al.

Surgical Procedures and Treatments

Rats were weighed prior to the surgery and again after 24 hours postinfection. Anesthesia was induced by intraperitoneal injection of a mixture of ketamine (100mg/kg; Ketaminol Vet; Intervet International, Boxmeer, the Netherlands) and xylazine (20mg/kg; Rompun Vet; KVP Pharma + Veterinär Produkte, Kiel, Germany). The acute bacterial meningitis was induced by subarachnoid injection of S pneumoniae (3 × 106 bacteria) in a volume of 20μl at a rate of 2μl/min, or saline as control, as described in Mohanty et al. For treatment experiments, rats that received subarachnoid injection of S pneumoniae were divided into 3 groups: DNase (DNase group), ceftriaxone (antibiotic group), or saline (SP001 group). The treatments were administered at 6 hours after administration of S pneumoniae using a bolus dose of 225μl of bovine DNase (3500 units; Worthington Biochemical Corporation, Lakewood, NJ), antibiotic (ceftriaxone, 50mg/ml), or saline (control), respectively. Following the bolus dose, rats were infused intravenously (i.v.) in the jugular vein using a syringe pump, followed by a continuous infusion of the treatments, using 780 units/h of DNase I or equal volumes of 50mg/ml antibiotic or saline vehicle at a flow rate of 0.05ml/h for the next 18 hours, as previously described (n = 4–5 per group). Twenty-four hours after infection, when neutrophils can be detected in the CSF of infected animals, rats received a cisterna magna injection of 20μl of CSF tracers, performed as in Ramos et al. The CSF tracers were 1% ovalbumin Alexa Fluor 647 conjugated (Ova647; 45kDa; O34784; Thermo Fisher Scientific, Waltham, MA) or 1% cadaverine Alexa Fluor 647 conjugated (A30679; Thermo Fisher Scientific) diluted in artificial CSF (aCSF). The tracer was allowed to circulate for 30 minutes while the rats were resting on a heating pad, whereupon they were euthanized by decapitation while still under deep anesthesia.

Tissue Harvesting and Immunohistochemistry

Brains were extracted and weighed. Brains and lymph nodes were immersion fixed in 4% paraformaldehyde overnight at 4°C and imaged using a Leica (Wetzlar, Germany) M205 FA microscope with a Leica Plan APO ×1.0 objective. Brains were cut using a vibratome (100μm thick; Leica VT1200S). Immunohistochemistry was performed as previously described. The primary antibodies used were chicken IgY anti-glial fibrillary acidic protein (GFAP; 1:500, PA1-10004, Thermo Fisher Scientific); rabbit anti-AQP4 (1:500, AB3594; Millipore, Billerica, MA); rabbit anti–ionized calcium-binding adapter molecule 1 (Iba1; 1:500, 019–19741; Wako, Osaka, Japan), mouse IgG1 anti-myeloperoxidase (MPO; 1:100, NB1-51148; Novus Biologicals, Centennial, CO), and rabbit anti–S pneumoniae capsule antigen (1:100, PA1-7259, Thermo Fisher Scientific). The secondary antibodies used were Alexa Fluor (AF) conjugated (Thermo Fisher Scientific), goat antimouse IgG1 (AF568,
1:500), goat antirabbit AF488, goat antirabbit AF568, and goat antichicken AF568. The slices were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:1,000, 1 μg/ml, Thermo Fisher Scientific). Slices were mounted with ProLong Gold Antifade mounting medium (Thermo Fisher Scientific).

Image Acquisition and Analyses

CSF tracer influx into the brain was imaged ex vivo by whole-slice conventional epifluorescence microscopy using a Nikon (Tokyo, Japan) Ni-E microscope with a Plan Apo λ ×4/0.20 objective. Tracer influx into the lymph nodes was imaged.

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ex vivo using a macroscope (M205 FA, Leica) with a ×1.0 objective (Planapo M-series, Leica) and an sCMOS camera (ORCA-flashv4.0 V2; Hamamatsu Photonics, Hamamatsu, Japan). Images were acquired at constant exposure levels throughout the study. CSF tracer influx was quantified with Fiji/ImageJ software (v1.47, National Institutes of Health, imagej.nih.gov/ij).

Coronal brain sections and whole lymph nodes were manually outlined based on the DAPI counterstaining, and the percentage of the region of interest area covered by CSF tracer (with uniform thresholding at a pixel intensity of 50 out of 255, and subtraction of background fluorescence) was measured. For whole sections and hippocampal or cortical regions, the mean percentage area covered by the tracer was calculated for 16 slices spaced at intervals along the anteroposterior axis for a single animal (2 sections for each of the following coordinates: +2.0, +1.0, 0.0, −1.0, −2.0, −3.0, −4.0, −5.5 mm from bregma, n = 9 per group for Ova647, n = 4–5 for cadaverine Alexa Fluor 647 conjugated [Cad647]).

For the lymph nodes, the average percent area with tracer coverage was calculated in 2 lymph nodes per animal, resulting in a single biological replicate.

GFAP, AQP4, Iba1, and MPO immunofluorescence images were collected using a Nikon Eclipse Ti2 microscope with Plan Fluor, ×20/0.75 numerical aperture (NA), ×40/1.30 NA, or ×100/1.25 NA oil objectives. For image quantification, serial confocal images were acquired with the ×40/1.30 NA objective (0.3μm, 80 steps, excitation sources 405, 488, and 565 nm solid-state diode laser lines). The average percentage of 2 images per animal (maximum intensity projection, hippocampal CA2 region or neocortical layers I–III) covered by GFAP/Iba1/AQP4 signal was calculated with ImageJ, resulting in a single biological replicate.

For MPO+ cell quantification, z-stacks containing the neocortical layers I–III were reconstructed 3-dimensionally using Imaris software (v9.1.2). MPO+ cells were counted manually, and the average values were obtained from the counting of 2 images per rat (n = 11 rats per group).

For quantification of AQP4 polarization, representative 50μm segments centered around capillaries and small arterioles (Ø < 15μm) and large arterioles (Ø > 15μm), as identified by vascular-shaped AQP4 localization with a contiguous pixel value > 50, were analyzed using the line-plot tool in ImageJ. Four vessels for each size category were chosen for each animal. For index of polarization quantification, the baseline was calculated as the average intensity of the segment over a 10μm distance extending from the peak intensity of the segment. The index of polarization was defined as the ratio between the peak intensity of the vascular endfoot and the average of the baseline, as previously described.19 The average index of polarization of 2 Ø < 15μm vessels and 2 Ø > 15μm vessels was calculated for each animal (n = 4 per animal) and used as a single biological replicate.

Statistics
All statistical analyses were performed using GraphPad (San Diego, CA) Prism software. Data were tested for normality by visual examination of quintile–quintile plots and Shapiro–Wilks test. When comparing 2 groups, unpaired t test or Mann–Whitney U test were used. For the t tests, variances were compared using an F test, and Welch correction was applied in cases of unequal variance. Where multiple comparisons were made for similar analyses (eg, comparison of the same cell marker in multiple brain regions), p values were adjusted for multiple comparisons using the Holm–Šidák method. For CSF tracer measurements, data were log-transformed to ensure normality and tested by 2-way analysis of variance with Geisser–Greenhouse correction for nonsphericity and Tukey post hoc multiple comparisons test. The statistical treatment of each dataset is described in the corresponding figure legends. A p value of <0.05 was considered significant for rejection of the null hypothesis. The number of animals that were used per group is stated in each figure legend.

Results
Induction of Bacterial Meningitis in Rats Using Patient-Derived S Pneumoniae
To induce bacterial meningitis, a clinical isolate of S pneumoniae was injected into the subarachnoid space of adult rats (Fig 1). After 24 hours, infected animals had lost 3.6% more body weight than controls (mean baseline SP001: 341 ± 6g; control: 347 ± 5g; mean after

FIGURE 1: Validation of a rat model of acute bacterial meningitis infection by Streptococcus pneumoniae. (A) Schematic representation of the experimental procedure. (B) Change in body weight of infected and control rats 24 hours after subarachnoid injection of S pneumoniae strain SP001 as a percentage of baseline body weight (unpaired t-test, ***p = 0.0017, n = 11 in each group, mean ± standard deviation [SD]). (C) Brain weight of infected and control rats (unpaired t-test, **p < 0.01, n = 5 in each group, mean ± SD). (D, E) Quantitation of myeloperoxidase (MPO)+ neutrophils in the cortex (D) and in the meninges (E) of infected and control rats (Welch t-test with Holm–Šidák multiple comparison adjustment, ****p < 0.0001, n = 11 rats in each group, 2 z-stacks per region for each animal, mean ± SD). (F) Twenty-four hours after infection, S pneumoniae was present in the meninges and in the parenchyma close to the bacterial injection site (left, upper panel), and also in the ventricular system (left, lower panel). S pneumoniae was completely absent in control rats, and few neutrophils (MPO+ cells, green) were found only near the injection site (right, upper panel) but not in the ventricular wall (right, lower panel). (G) In infected animals, an orthogonal view of cortical images depicted 3 neutrophil killing strategies: (1) neutrophil extracellular trap (NET) formation characterized by DNA (blue) colocalized with MPO (green; left panel), (2) neutrophil degranulation, and (3) bacterial phagocytosis (right panel) revealed by MPO and SP001 colocalization in neutrophils, within the cytoplasm of neutrophils. (H) NETosis was visualized for the first time in the meninges of infected rats. Scale bars: F, 100μm; G, H, 10μm. CFU = colony forming unit; Ctrl = control; DAPI = 4',6-diamidino-2-phenylindole; Kx = ketamine xylazine; MPO = myeloperoxidase; PFA = paraformaldehyde; SP001 = S pneumoniae strain 001.
FIGURE 2: *Streptococcus pneumoniae* meningeal infection promotes gliosis in the brain parenchyma. (A–D) Confocal microscopy of coronal brain sections obtained from *S. pneumoniae*-infected (A, C, upper panel) and control rat brains immunolabeled for glial fibrillary acidic protein (GFAP; A, C; red), microglial/macrophage ionized calcium-binding adapter molecule 1 (Iba1; B, D; yellow), and neutrophil myeloperoxidase (MPO; A; green) and counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). (A, C) In infected animals, astrogliosis was observed in brain areas with bacterial proliferation and recruitment of neutrophils such as the meninges and the lateral ventricle (LV), and also in areas where bacteria and neutrophils were not detected, namely the hippocampus (Hpc; C, arrow). (A) Direct cell–cell contacts between astrocytes and neutrophils were observed in the meninges and in the lateral ventricle. (C, lower panel) Astrocytes were not activated in the meninges, lateral ventricle, and hippocampus of control animals. Activated and hypertrophic microglia were observed in the meninges of infected rats (B, D, yellow inset, arrows), in direct contact with infiltrating neutrophils. There was also a massive presence of meningeal macrophages (B, D, blue inset, arrows), directly interacting with neutrophils (B, arrows). In sharp contrast, there was no sign of microglial activation in control animals (D, lower panel, arrows), which had only sporadic macrophages present in the meninges. (E–G) Quantification of GFAP immunoreactivity in the cortex (CTX; E) and hippocampus (F) and Iba1⁺ immunoreactivity in the cortex (G; unpaired t-test with Holm–Šidák multiple comparison adjustment, *p < 0.05, **p < 0.01, ***p < 0.001). Error bars represent ± standard deviation, n = 8 in each group. Scale bars: 10 μm. Lm = leptomeninges; SP001 = *S. pneumoniae* strain 001.
FIGURE 3: *Streptococcus pneumoniae* meningeal infection reduces aquaporin 4 (AQP4) polarity. (A) Immunostaining for AQP4 (green) and (B) quantitation thereof in cortex of infected and control rats (unpaired t-test with Holm–Šidák multiple comparison adjustment, n = 8 per group). (C) Scheme of a representative 50μm segment centered around capillaries and small arterioles (Ø < 15μm) and large arterioles (Ø > 15μm) used for measuring AQP4 intensity and thereby to quantify AQP4 polarization. (D) Line plots of AQP4 immunointensity across large arteries in the neocortex and (E) calculation of the AQP4 polarization index (unpaired t-test with Holm–Šidák multiple comparison adjustment, n = 4/group, 4 vessels/animal). (F) Line plots of AQP4 immunointensity across small arteries in the cortex and (G) calculation of the peak-to-baseline level (AQP4 polarization index; Mann–Whitney U-test with Holm–Šidák multiple comparison adjustment, n = 4/group, 4 vessels/animal). Data are mean ± standard deviation, *p < 0.05. Scale bars: 50μm. Ctrl = control; CTX = cortex; ns = nonsignificant; SP001 = *S. pneumoniae* strain 001.
euthanasia SP001: 324 ± 6g control: 336 ± 5g; \( p = 0.001, n = 11 \) per group) and had 29.2 ± 2.0% higher brain weight compared with saline-injected animals (SP001: 2.02 ± 0.03g, control: 1.6 ± 0.04g; \( p < 0.0001; n = 10 \) SP001, \( n = 10 \) control). The loss in body weight and increase in brain weight are comparable to earlier reports.\(^{13,20}\) Quantification of MPO\(^{+}\) cells indicated an increased number of neutrophils on the pial surface (SP001: 460.5 ± 114.6 cells/mm\(^2\), control: 159.2 ± 27.0 cells/mm\(^2\), \( p < 0.0001; \) mean ± standard error of the mean; \( n = 11 \) per group) and dura mater (SP001: 3,289.0 ± 310.9 cells/mm\(^2\), control: 880.4 ± 86.6 cells/mm\(^2\), \( p < 0.0001; n = 11 \) per group). Also, immunolabeling vibratome sections for \( S \) pneumoniae.

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pneumoniae was found on the pial surface and in cortex near the bacterial injection site, in close association with infiltrating neutrophils as previously reported. Sporadic S pneumoniae was present also at the ventricular walls, where infiltrating neutrophils were also observed. Bacteria colonies were absent in brain coronal sections obtained from control rats. A minor neutrophilic infiltration was noted near the injection site in the control rats, as expected after the invasive procedure.

**Neutrophils Recruited in the Brain from NETs**

We previously identified NETs, which are webs of extracellular DNA in association with antimicrobial proteins and proteases, in the CSF in pneumococcal meningitis. Here, we asked whether NETs also develop in the meninges and brain of infected rats and investigated the interaction between S pneumoniae and neutrophils. In the brain parenchyma and within the ventricular walls, we saw clear signs of neutrophil release of antimicrobial proteins and phagocytosis. This may reflect the mixing of CSF between the subarachnoid space and ventricular system, delivering proliferating pneumococci to the ventricular system. S pneumoniae and MPO+ granules colocalized both in the extracellular space and within neutrophils cytoplasm (see Fig 1G). Formation of bona fide NETs was demonstrated by colocalization of MPO+ neutrophils with decondensed chromatin structures and fibrous protrusions of DNA. Massive NETosis and bacterial phagocytosis was most prominent in the dura and arachnoid matter (see Fig 1H) and brain.

**S Pneumoniae Infection Promotes Gliosis**

Next, we asked whether intrinsic CNS immune effectors, namely microglia and astrocytes, were activated by S pneumoniae infection (Fig 2). Astrogliosis, characterized by increased expression of GFAP, astrocytic hypertrophy, and overlapping of the astrocytic domains was observed in the brain of infected rats, as previously described. Astrogliosis was not restricted to bacteria proliferation sites, which included glia limitans, cortical layers I–III, and the ventricular walls, but was also detected in the hippocampus, where no bacteria were present. At the site of bacteria injection and in the lateral ventricle, MPO+ infiltrating neutrophils and GFAP+ activated astrocytes were in direct cell–cell contact. Such an interaction regulates neutrophil function in vitro by attenuating neutrophil degranulation, while boosting neutrophil phagocytic capability and proinflammatory cytokine release, and has recently been indicated to promote astroglia activation in vivo. In contrast, the noninflamed brain of control rats contained quiescent nonreactive astrocytes with low GFAP immunoreactivity, multiple nonoverlapping radial processes within their individual domains, located in the glia limitans, the ventricular walls, and hippocampus. Similarly, in the meninges of infected animals, activation of microglia, identified by Iba1+ immunoreactivity, was identified by cell body hypertrophy and the presence of short, thick processes. Numerous Iba1+ macrophages were also observed in the meninges, identified by their spherical morphology. Notably, Iba1+–ramified microglia cells in the parenchyma and Iba1+ meningeal macrophages directly interacted with MPO+ infiltrating neutrophils, which can influence microglia and neutrophil immune responses.
responses. In contrast, the CNS parenchyma of control animals contained resting nonactivated microglia cells, with highly ramified processes and smaller cell bodies. Meningeal macrophages were sporadically observed in control rats.

**S Pneumoniae Infection Promotes Perivascular AQP4 Depolarization in the Brain Parenchyma**

To further investigate changes in astrocytes, we analyzed the expression of the astrocytic water channel AQP4, which plays a pivotal role in glymphatic system function. In particular, predominant AQP4 localization to astrocytic parenchymal processes rather than vascular endfeet correlates with poor glymphatic function, that is, low polarization index. Acute *S pneumoniae* meningitis did not increase the overall AQP4 immunoreactivity in the cortex (Fig 3; \( p = 0.1143; n = 4 \) per group) or hippocampus (data not shown). However, AQP4 polarization to vascular endfeet processes around arterioles with
Ø > 15µm was decreased by 39.6 ± 6.5% in pneumococcal meningitis rats (p = 0.0286; n = 4 per group), but was unchanged around capillaries and small arterioles with Ø < 15µm (p = 0.886; n = 4 per group). The observations of activated astrocytes with depolarized AQP4 expression suggests that bacterial inflammation may suppress glymphatic transport.

A previous study indicated that lack of AQP4 function by genetic deletion prevents BBB breakdown and formation of edema in acute bacterial meningitis; however, this could be because AQP4 also plays a role in immune activation. Our study suggests that the suppression in CSF efflux contributes to edema, as lack of efflux of CSF to the periphery correlated with increased brain weight.

S Pneumoniae Infection Decreases Glymphatic Influx into Brain and CSF Drainage to Cervical Lymph Nodes

Brain edema is one of the most threatening clinical complications of bacterial meningitis. Nonetheless, the impact of meningitis on the glymphatic system has not been examined so far. Perivascular fluid transport by the glymphatic system is dependent on proper astrocytic localization of AQP4. As S pneumoniae meningitis causes gliosis and disturbance of AQP4, we investigated the distribution of a fluorescent tracer (Ova647, 1% in aCSF; molecular weight [MW] = 45kDa) 30 minutes after cisterna magna tracer injection in rats 24 hours after injection of S pneumoniae or saline into the subarachnoid space (Fig 4). Strikingly, the CSF tracer distribution in the brain was largely excluded from the parenchyma in infected animals. Instead, the CSF tracer aggregated along the middle cerebral artery and its branches. In saline-injected controls, the CSF tracer also entered the perivascular spaces, and penetrated diffusely distributed in the brain parenchyma. Quantitation across the anterior–posterior axis revealed that overall CSF tracer influx was reduced in the infected rats compared to healthy controls (SP001: 2.133 ± 0.575, control: 7.632 ± 1.545; p = 0.0019; n = 9 per group). All the coronal sections showed significantly decreased CSF tracer influx in infected rats compared to controls (n = 9 per group). The infected rats also displayed a striking decrease in CSF tracer influx in the hippocampus and along the cortex compared to healthy controls (hippocampus, SP001: 2.12 ± 0.91, control: 19.4 ± 4.31; cortex, SP001: 1.06 ± 0.328, control: 4.46 ± 0.58; n = 9 rats per group). No difference in CSF tracer distribution was found between ipsilateral and contralateral hemispheres, suggesting a brain-wide effect of bacterial meningitis (p > 0.99; n = 9 per group).

We next evaluated tracer accumulation in cervical lymph nodes in both treatment groups (see Fig 4H–J). S pneumoniae infection suppressed efflux of CSF tracer to the lymph nodes by 42 ± 33% compared with control rats (see Fig 4J; SP001: 1.21 ± 0.32, control: 2.85 ± 0.49; p = 0.00018; n = 9 per group). The impaired CSF drainage to the cervical lymphatics may contribute to both the brain edema and the elevation of intracranial pressure that characterize bacterial meningitis. In the dura and arachnoid matter of S pneumoniae–infected rats, phagocytic neutrophils and microglia contained the Ova647 CSF tracer. Higher magnification imaging with an orthogonal view showed tracer associated with neutrophils (Fig 5). Conversely, in control rat brains,

FIGURE 5: Cadaverine distribution is impaired in Streptococcus pneumoniae–infected animals, and cerebrospinal fluid (CSF) tracer is taken up by infiltrating immune cells. (A) Schematic representation of the experimental procedure. Twenty-four hours after S pneumoniae or saline subarachnoid injection, a CSF tracer (amino acid derivative cadaverine–Alexa Fluor 647) was injected into the cisterna magna of anesthetized rats. (B, C) Representative images reveal that infected animals had impaired CSF penetration into the brain parenchyma along perivascular spaces. Colocalization of CSF tracer (magenta) with myeloperoxidase (MPO; B; green) and ionized calcium-binding adapter molecule 1 (Iba1; C; yellow) immunostaining reveals that CSF tracer was taken up by both neutrophils and microglia/macrophages. In control rat brain sections, CSF tracer is distributed into the brain parenchyma along the perivascular space of brain penetrating vessels (B; lower panel, arrow), neutrophils are absent (C; lower panel), and only sporadic meningeal macrophages are observed. (D) CSF tracer is trapped in neutrophil extracellular traps (NETs) in the meninges of infected animals, highlighted by the orthogonal view of confocal images (E) showing colocalization of decondensed DNA (blue), MPO (green) and CSF tracer (Alexa Fluor 647–conjugated ovalbumin, magenta; white arrows). (F) Representative images of brain coronal section from SP001-infected and control rats, upon injection of cadaverine. In infected animals, cadaverine distribution was impaired, recapitulating the results obtained with the CSF tracer ovalbumin–Alexa Fluor 647. (G) Cadaverine distribution in 8 coronal sections across the anteroposterior axis of the brain. Cadaverine was quantified as the amount of signal above a fixed threshold and background subtracted. The percentage area (area fraction %) covered by cadaverine was calculated for each section. Y-axis indicates the distance in millimeters in relation to bregma (2-way analysis of variance with Geisser–Greenhouse correction and Tukey post hoc test, ***p < 0.01, ****p < 0.0001, mean ± standard deviation [SD], n = 4 Ctrl and n = 6 SP001). (H) Cadaverine distribution in S pneumoniae–infected and control groups (Mann–Whitney t-test, **p = 0.0049, mean ± SD, n = 4 Ctrl and n = 6 SP001). CSF tracer was quantified as the amount of signal above a fixed threshold and background subtracted. The average percentage area (area fraction %) covered by tracer was calculated. Scale bars: B, C, 100µm; B, C (insets), 20µm; D, E, 10µm; F, 1mm. aCSF = artificial CSF; CFU = colony-forming unit; Ctrl = control; Ctx = cortex; DAPI = 4’,6-diamidino-2-phenylindole; Kx = ketamine xylazine; Lm = leptomeninges; ns = nonsignificant; PFA = paraformaldehyde; SP001 = S pneumoniae strain 001.
neutrophils were absent, and no Ova647 CSF tracer uptake was observed.

To rule out the possibility that reduced tracer movement in *S. pneumoniae* meningitis was due to engulfment of tracer, we carried out experiments as above, but with a smaller nonprotein tracer, cadaverine (Cad647, 1% in aCSF; MW = 1kDa). Cadaverine was not observed in perivascular cells, but similar to Ova647 did not enter...
the brain parenchyma (see Fig 5F–H; SP001: 7.23 ± 1.35, control: 13.86 ± 2.04; p = 0.038; n = 4 SP001, n = 6 control), demonstrating that glymphatic transport is significantly suppressed by S pneumoniae meningitis.

Dissolving NETs Relieve CSF Circulation Blockage in Bacterial Meningitis

In peripheral tissues, NET components are proinflammatory and promote an immune response to resolve the infection.31,32 However, our recent work showed that NETosis is harmful in the CNS, as DNase I treatment effectively reduced the bacterial load in both the brain and peripheral organs in S pneumoniae meningitis.13 To understand the mechanism of the DNase treatment, we assessed the effect of i.v. DNase I treatment on glymphatic fluid transport and compared DNase treatment to i.v. antibiotic treatment (ceftriaxone) in infected rats (Fig 6). DNase treatment prevented the increase in brain weight caused by S pneumoniae meningitis, indicating that edema was ameliorated by dissolving NETs (average brain weight, SP001: 1.94 ± 0.05g, control: 1.55 ± 0.04g, antibiotic: 1.83 ± 0.06g, DNase: 1.56 ± 0.06g). In contrast, the mean brain weight in the antibiotic treatment group was significantly higher compared to noninfected controls, showing that conventional antibiotic treatment is less efficient than DNase treatment in alleviating brain swelling (n = 15 SP001, n = 14 saline, n = 4 antibiotic, n = 5 DNase). To understand whether a restoration of glymphatic transport played a role in edema prevention, we tracked glymphatic fluid transport using CSF-injected tracers. CSF tracer influx was significantly reduced in vehicle-treated rats with S pneumoniae meningitis, but also in rats receiving antibiotic treatment. CSF tracer influx was significantly reduced in rats with S pneumoniae meningitis treated with vehicle or with antibiotics, compared to controls. CSF tracer influx in rats treated with DNase was comparable to that in control rats without bacterial meningitis (SP001: 0.63 ± 0.34, control: 5.46 ± 1.86, antibiotic: 0.86 ± 0.34; DNase: 4.31 ± 2.01; n = 4 SP001, n = 4 control, n = 4 antibiotic, n = 5 SP001 DNase). CSF tracers were found in superficial cervical lymph nodes of healthy rats. The blockage of CSF tracer efflux to the cervical lymph nodes in bacterial meningitis rats was not relieved by antibiotic treatment, whereas DNase treatment normalized efflux of CSF tracer to cervical lymph nodes (SP001: 0.23 ± 0.18, control: 11.85 ± 3.77; antibiotic: 0.41 ± 0.23, DNase: 17.50 ± 2.08; n = 4–5 rats per group), thus demonstrating that dissolving NETs restored both CSF influx and efflux, and confirming that targeting NETosis, a specific neutrophil function, improves intracerebral CSF transport in bacterial meningitis. In contrast, ceftriaxone treatment had no effect on perivascular CSF transport, although its antibacterial activity effectively reduced the brain bacteria burden.13 Interestingly, ceftriaxone treatment did not decrease neutrophil invasion, microglia, or astrocyte response compared to untreated rats infected by S pneumoniae. Dissolving NETs by DNase treatment did not reduce neutrophil invasion, but did reduce reactive microglial activation (MPO+ cells/mm2, SP001: 991.8 ± 86.4 cells/mm2, antibiotic: 478.4 ± 120.6 cells/mm2, DNase: 575.9 ± 148.0 cells/mm2; Iba1 immunoreactivity, SP001: 11.8 ± 2.5, antibiotic: 5.4 ± 2.0, DNase: 4.4 ± 1; GFAP immunoreactivity, SP001:

FIGURE 6: Targeting neutrophil extracellular traps (NETs) with DNase I restores glymphatic influx of cerebrospinal fluid (CSF) tracer and normalized brain weight. (A) Schema of the experimental procedure to disrupt NETs; 6 hours after subarachnoid injection of Streptococcus pneumoniae or saline, rats received treatment with DNase I or antibiotic ceftriaxone or vehicle (saline). Thereafter, rats were injected in the cisterna magna with CSF tracer and euthanized 30 minutes after the injection. (B) Brain weight of control, S pneumoniae SP001-infected saline treated, antibiotic-treated, and DNase-treated rats (p-values for 1-way analysis of variance [ANOVA] with Tukey post hoc test comparison, ***p < 0.01, ****p < 0.0001, mean ± standard deviation [SD], n = 14 saline-treated, n = 15 SP001 saline-treated, n = 4 SP001 antibiotic-treated, n = 5 SP001 DNase I-treated). (C) Representative images of a whole brain. (D) Coronal sections and cervical lymph nodes (left and right middle). (E) Representative graph of brain-wide CSF tracer distribution (average over all sections) in control, S pneumoniae-infected, antibiotic-treated, and DNase-I-treated groups (p-values from a 2-way ANOVA with Tukey post hoc test and Greenhouse–Geisser correction, analyzing the main treatment effect across all sections. ***p < 0.01, ****p < 0.0001, mean ± SD, n = 4 saline-treated, n = 4 SP001 saline-treated, n = 4 SP001 antibiotic-treated, n = 5 SP001 DNase I-treated). (F) Quantification of total CSF tracer drainage of 2 lymph nodes per rat (left and right middle; p-values from a 2-way ANOVA with Tukey post hoc test correction, *p < 0.05, ****p < 0.0001, mean ± SD, n = 4 saline-treated, n = 4 SP001 saline-treated, n = 4 SP001 antibiotic-treated, n = 5 SP001 DNase I-treated). (G, I, K) Confocal microscopy of coronal brain sections obtained from S pneumoniae-infected and control rat brains immunolabeled for (G) myeloperoxidase (MPO; green), (I) microglial/macrophage ionized calcium-binding adapter molecule 1 (Iba1; yellow), and (K) glial fibrillary acidic protein (GFAP; red), counterstained with 4’,6-diamidino-2-phenylindole (DAPI; blue). (H, J, L) Quantification of (H) number of MPO+ neutrophils, (J) Iba1 microglia/macrophages immunoreactivity, and (L) GFAP immunoreactivity in the cortex (p-values from a 1-way ANOVA with Tukey post hoc test correction, *p < 0.05). (M) Representative images of AQP4-labeled coronal brain section images. (N) Quantification of AQP4 immunoreactivity (1-way ANOVA with Tukey post hoc test correction). Scale bars: C, 5mm; D, 1mm; G, I, K, M, 50µm. AQP4 = aquaporin 4; Cb = cerebellum; CFX = ceftriaxone; Ctx = cortex; I.V. = intravenous; MCA = middle cerebral artery; ns = nonsignificant; OB = olfactory bulb; SP001 = S pneumoniae strain 001.
4.8 ± 0.6, antibiotic: 2.3 ± 1.2, DNase: 1.29 ± 0.5; n = 4–5 rats per group). Neither AQP4 expression (see Fig 6M, N) nor polarization (Fig 7) changed upon antibiotic or DNase treatment (n = 4–5 rats per group). Overall, these findings suggest that CSF stasis is a major contributing cause in edema formation and that the glymphatic system and NETs could be promising new treatment targets in *S. pneumoniae* meningitis.

**Discussion**

Here, we showed that *S. pneumoniae* meningitis significantly suppressed CSF drainage toward the cervical lymphatic system (Fig 8), possibly explaining why glymphatic transport of CSF into the rat brain was almost absent in this rat model of bacterial meningitis. Dissolving NETs by DNase I treatment prevented the disease mechanisms...
leading to CSF outflow resistance and eliminated brain swelling postinfection. This observation opens a new perspective on cerebral edema, as it suggests that blockage of CSF transport contributes to acute brain swelling in *S. pneumoniae* meningitis. Interestingly, CSF tracer influx was also severely disrupted in the hippocampus, where bacteria and neutrophils were absent. This is in line with the hippocampus being a prominently affected site in bacterial meningitis and the association of neurological dysfunction with the extent of hippocampal neuron apoptosis. Thus, the hippocampal gliosis and glymphatic dysfunction observed in *S. pneumoniae* meningitis, bacteria proliferate mainly in the subarachnoid space (SAS) and the perivascular compartment, and circulating neutrophils are recruited in large numbers into the central nervous system, where they form neutrophil extracellular traps (NETs). Glymphatic function is impaired, possibly due to NETosis, followed by accumulation of immune cells and bacteria, along with astrocytosis and aquaporin 4 (AQP4) depolarized from the vascular endfeet processes. Reduced glymphatic clearance leads to cerebral edema and accumulation of waste products and metabolites in the perivascular space and the brain parenchyma. ISF = interstitial fluid; SP001 = *S. pneumoniae* strain 001.

**FIGURE 8:** Glymphatic system function is impaired in pneumococcal meningitis. Our proposed model of glymphatic activity in *Streptococcus pneumoniae*-infected animals and controls is shown. In healthy animals, cerebrospinal fluid (CSF) enters the brain parenchyma via periarterial pathways, washes out metabolites and waste products from the interstitial space, and exits the brain parenchyma along the perivenous pathway. Neutrophils, the predominant immune cell population in the blood, are absent from the healthy brain parenchyma. In *S. pneumoniae* meningitis, bacteria proliferate mainly in the subarachnoid space (SAS) and the perivascular compartment, and circulating neutrophils are recruited in large numbers into the central nervous system, where they form neutrophil extracellular traps (NETs). Glymphatic function is impaired, possibly due to NETosis, followed by accumulation of immune cells and bacteria, along with astrocytosis and aquaporin 4 (AQP4) depolarized from the vascular endfeet processes. Reduced glymphatic clearance leads to cerebral edema and accumulation of waste products and metabolites in the perivascular space and the brain parenchyma. ISF = interstitial fluid; SP001 = *S. pneumoniae* strain 001.

Acute edema formation is a significant predictor of meningitis outcome, and its cause has previously been attributed simply to inflammation. Animal studies have shown promising results suggesting that brain edema and increase CSF pressure can be prevented by dexamethasone treatment. In the clinic, the recommended treatment is dexamethasone in combination with antibiotics, as some studies show reduction in hearing loss and mortality; however, it is not established whether dexamethasone reduces brain edema in patients. The search for therapeutics that can alleviate edema is still ongoing, demonstrating that edema remains an issue in bacterial meningitis. A novel aspect of our studies lies in the finding that NET formation is a key culprit of CSF blockage and edema formation in the context of meningitis. Although leukocytes in general are needed to resolve bacterial meningitis infection in the most effective way, our results suggest that NETs produced by neutrophils may be counterproductive. Studies on NETs in the CNS are still in their infancy, but the presence of NETs in *S. pneumoniae* meningitis has been demonstrated in the CSF of human patients and in our rat model of acute bacterial meningitis. Similarly, NET formation in the human CSF was
reported in viral CNS infections and nonpneumococcal bacterial infections, namely, Lyme neuroborreliosis, showing that cerebral NET formation occurs in several conditions. Here, we detected abundant NETs in the dura, pial surface, cortex, and ventricular system during acute S pneumoniae meningitis, suggesting that NET formation is a widespread phenomenon. Despite their antimicrobial properties through bacterial sequestration, NET components such as extracellular DNA and antimicrobial proteins including histones and neutrophil elastase have been shown previously to exacerbate inflammation.

We recently documented that degradation of NET-associated DNA using DNase I resulted in improved outcome of S pneumoniae meningitis in terms of bacterial burden in the brain, lungs, spleen, and blood, but this earlier study did not address its effects on brain edema formation and glymphatic function. Based on the observations reported here, we hypothesized that brain accumulation of NETs could sterically hinder CSF transport and thus the clearance of interstitial waste products, metabolites, bacterial debris, and excess fluid in meningitis. In the blood and sputum, NETs are known to activate the coagulation system, which changes the rheological properties of these fluids. In this regard, DNase I effectively promotes blood clot lysis. Similarly, NET accumulation in the subarachnoid space and ventricular system may augment CSF viscosity, thus obstructing its circulation within the CNS. We used intravenous delivery of DNase, as our previous study showed that this is just as effective as invasive intrathecal delivery when administered 6 hours after subarachnoid injection of bacteria. This is likely due to increased BBB permeability in meningitis resulting in overactivity of extracellular matrix metalloproteases, with onset as soon as 6 hours after infection. We found that NET disruption by intravenous administration of DNase I resolved glymphatic influx impairment and normalized brain weight, thus indicating an effective prevention of edema formation. In contrast, treatment with a β-lactam antibiotic (ceftriaxone), a first-line treatment used for meningitis, neither improved glymphatic function nor prevented edema formation. Our findings thus suggest that DNase I merits further exploration as a therapeutic target to reduce edema and thereby intracranial pressure in meningitis.

Cerebral edema is classically defined as cytotoxic, vasogenic, and interstitial edema. This study focused on the earliest stages of edema that occur at 24 hours after injection of S pneumoniae into the subarachnoid space. Our study suggests CSF transport failure as a source of edema formation. The finding that restoration of CSF efflux in this study prevented edema is in line with the occurrence of ventriculomegaly in one third of bacterial meningitis patients, which suggests that CSF efflux pathways are blocked. Our study may extend this observation by suggesting a blockage of intraparenchymal glymphatic CSF circulation and CSF efflux to extracranial lymphatics, rather than a blockage within the ventricular system. The glymphatic system is known to be impaired in other inflammatory conditions such as traumatic brain injury and by lipopolysaccharide injection alone. However, this study adds new understanding of NET-induced blockage of CSF circulation in meningitis, which is characterized by a massive neutrophil invasion. CSF efflux through dural lymphatic vessels is emerging as a key site for solute and peptide clearance, and interestingly, NETs were also observed in the dorsal dura in the present study. In humans, the site of convergence for transporting CSF from the CNS to the periphery appears to be located exactly at the parasagittal dorsal dura. Considering that we observed NETs both at the pial surface and in the dorsal dura, and that DNase I treatment restored CSF tracer efflux to cervical lymph nodes, we propose that NETs might block the narrow exit sites from where efflux of CSF in the subarachnoid space occurs in the meningeal and dural lymphatics.

Targeting NETs might also hold promise for improved therapy in bacterial meningitis, as targeting the neutrophil-mediated inflammation rather than the bacteria directly is less apt to be impacted by antimicrobial resistance and some patients are hypersensitive to β-lactams. Moreover, translation of the present findings to the clinic is a realistic prospect, because DNase I is already an approved drug for cystic fibrosis. However, further preclinical studies are certainly needed to prove its efficacy and confirm the absence of side effects in the context of meningitis. A particular topic for future research is to define in greater detail the localization of NETs within the CNS and ascertain the disruptive effects of NETs on CSF drainage to the spinal cord, lymphatic system, and peripheral organs. Our observations shed light on a new mechanism of brain edema formation during bacterial meningitis, suggesting that an intact glymphatic system is necessary for preventing edema in bacterial meningitis in rats. Finally, we propose that targeting NET formation might be a new strategy for preventing edema in bacterial meningitis.

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Author Contributions
C.P., A.L.R.X., A.L., P.B., M.N., and I.L. contributed to conception and design of the study; C.P., M.R., J.F., and M.K. contributed to acquisition and analysis of data; C.P., A.L.R.X., M.N., and I.L. drafted the manuscript and figures. All authors approved the manuscript.

Potential Conflicts of Interest
Nothing to report.

References


