Replication in perivascular meningeal macrophages precedes meningitis in mice

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Background

Streptococcus pneumoniae is the leading cause of bacterial meningitis in children younger than 5 years of age in the US, and is the leading cause of death due to communicable disease (CDC, 2020; Wang *et al.,* 2016). Following the onset of bacteraemia, haematogenous spread of pneumococci across the blood brain barrier (BBB) results in the invasion of the CSF and meninges, which precedes the secretion of pro-inflammatory cytokines and neutrophil influx resulting in the onset of meningitis and brain injury (McNeela *et al.,* 2010; Hassane *et al.,* 2017). Pneumococci are known to adhere to endothelial cells prior to translocation across the endothelial barrier, but knowledge on the interaction of pneumococci with macrophages and microglial cells following this translocation event is scarce (Iovino *et al.,* 2017; Uchiyama *et al.,* 2009). To study these interactions, we utilised an *in vivo* murine model in which cefazolin was administered 12 hours after the induction of bacteraemia to prevent septicaemia without penetrating the BBB.

Pneumococci replicate within perivascular macrophages following translocation of the endothelium →

Cefazolin was administered at 12h post-infection (PI) following intravenous infection of mice with type 2 pneumococcal strain D39. Pneumococci were recovered from the brain by 24h PI, and counts increased significantly up to 72h PI alongside consistently negative pneumococcal blood counts (Fig 1A). Confocal image analysis of brain samples revealed preferential localisation of single pneumococci with endothelial cell membranes within the choroid plexus (CP) and meninges at earlier time points (Fig 1BC), before being associated primarily with perivascular (PV) macrophages at later time points, presenting as intracellular clusters (Fig 1BD). During this intracellular period, neutrophils were absent from the CP and meninges and mice displayed no signs of meningeal inflammation. At 72h PI, pneumococci were predominately extracellular, and an influx of neutrophils was visualised in close proximity to extracellular pneumococci (Fig 1EF). These findings imply that following endothelial translocation by pneumococci, PV macrophages permit intracellular replication which allows bacterial numbers to increase before lysis and release into the CSF, therefore inducing neutrophil influx.

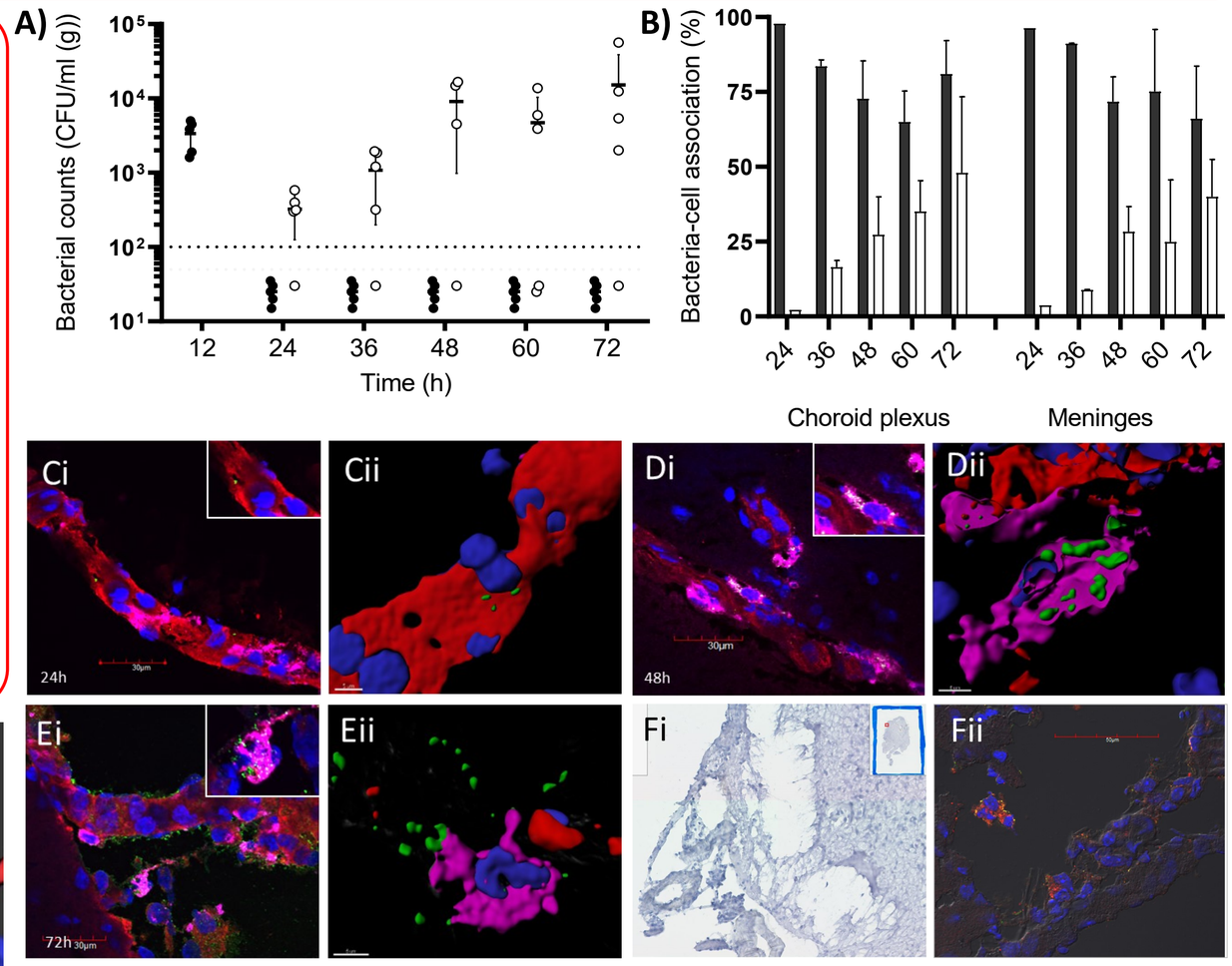

Figure 1: Pneumococcal blood/brain counts, localisation within the brain, and representative confocal microscopy images. A) Blood (CFU/ml) and brain (CFU/g) bacterial counts throughout the time course are represented by filled and open circles respectively. Dotted line shows the limit of detection. B) The percentage of bacteria associated with endothelial cells (filled bars) or PV macrophages (open bars) within the CP and meninges throughout the time course. C, D, E) Confocal microscopy of brain samples at 24h (C), 48h (D) and 72h (E) PI. Nuclei=blue, pneumococci=green, endothelial cells=red, PV macrophages=magenta. F) H&E (Fi) and confocal microscopy (Fii) showing neutrophil influx in proximity to extracellular bacteria at 72h PI. Fii: nuclei=blue, pneumococci=green, neutrophils=red.

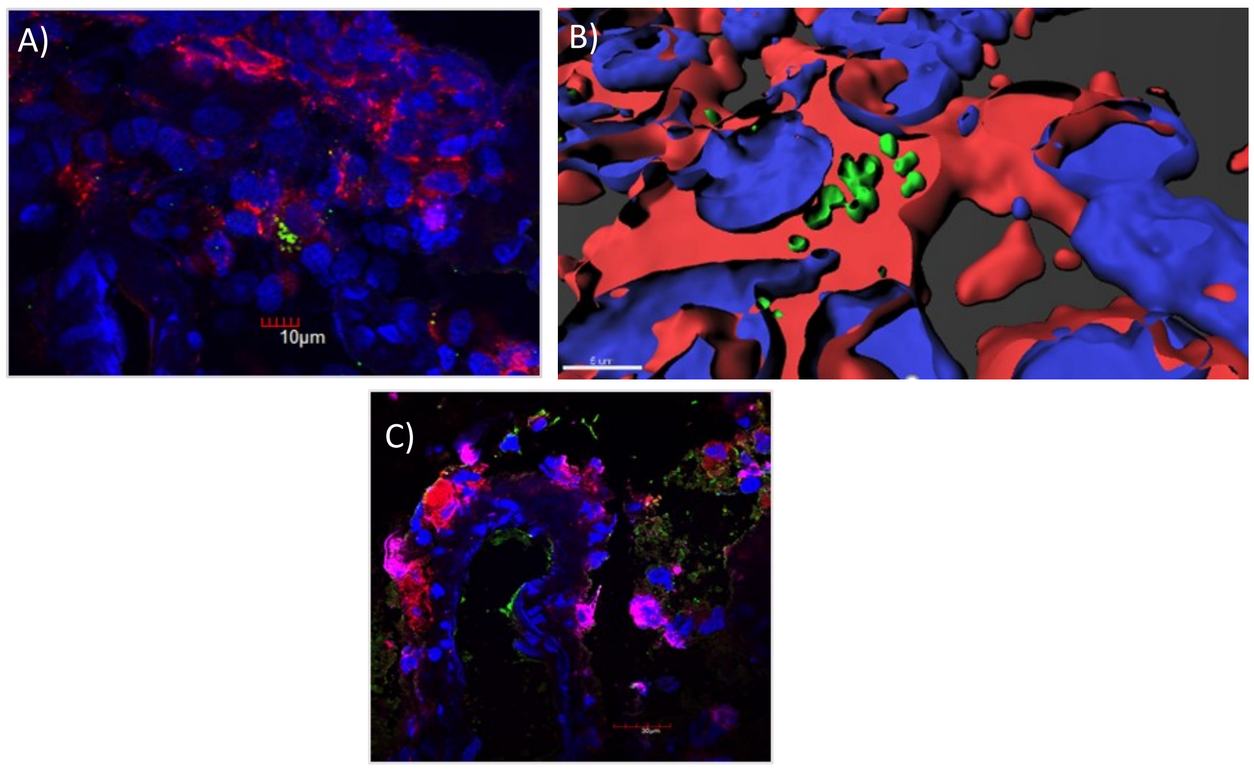

Figure 2: Confocal microscopy of human brain samples from patients with late-stage pneumococcal meningitis. IHC staining of human brain samples from patients who succumbed to pneumococcal meningitis. Nuclei=blue, pneumococci=green, PV macrophages=red, neutrophils=magenta. A) Pneumococcal cluster within a PV macrophage in the PV tissue of the hippocampus. B) 3D reconstruction of a pneumococcal cluster within a PV macrophage in the PV tissue of the hippocampus. C) Neutrophils surrounding extracellular pneumococci in the PV tissue of the temporal cortex.

PECAM blockage, Caspase-1 inhibition and prevention of pneumococcal uptake into PV macrophages decreases brain bacterial counts →

To investigate potential approaches that could lower pneumococcal brain counts, we administered an anti-PECAM antibody prior to infection and an anti-CD169 antibody after 8h infection (to negate effects on CD169+ macrophages in the spleen prior to brain invasion) to prevent pneumococcal endothelial adhesion and uptake into PV macrophages respectively. Pneumococci also induce NLRP3 inflammasome formation in brain phagocytes which, through the action of Caspase-1 and subsequent pyroptotic cell death, results in the release of pro-inflammatory cytokines (Witzenrath *et al.,* 2011; Hoegen *et al.,* 2011). Thus, we also administered VX765 Caspase-1 inhibitor prior to infection to prevent pyroptosis in PV macrophages. Administration of anti-CD169 antibody significantly lowered the association of pneumococci with PV macrophages and significantly reduced brain pneumococcal counts at 48h PI (Fig 3AB). This implies that uptake into PV macrophages is essential for an increase of pneumococcal numbers in the brain. Anti-PECAM administration also resulted in ~62% of mice yielding no pneumococci in the brain, therefore demonstrating that a blockage of endothelial adhesion can successfully reduce the numbers of pneumococci crossing the BBB. Further, inhibition of Caspase-1 through administration of VX765 resulted in negative brain pneumococcal counts in ~87% of mice. The mechanisms responsible for this reduction in counts is unknown, however we hypothesise that prevention of the pyroptotic pathway could result in the cells resorting to the apoptotic pathway which successfully kills intracellular bacteria.

← Human meningitis brain samples illustrate similar pneumococcal pathogenesis to mice

Brain samples from two patients who succumbed to pneumococcal meningitis were stained for IHC as above. Microscopy analysis revealed the presence of pneumococci associated with endothelial cells and in the CSF in the temporal cortex and hippocampus PV tissue. Large clusters of pneumococci were also found within PV macrophages in these regions (Fig 2AB), and neutrophils were found in sites of extracellular pneumococci accumulation, oftentimes in close proximity to macrophages that display morphological features in keeping with cell lysis (Fig 2C).

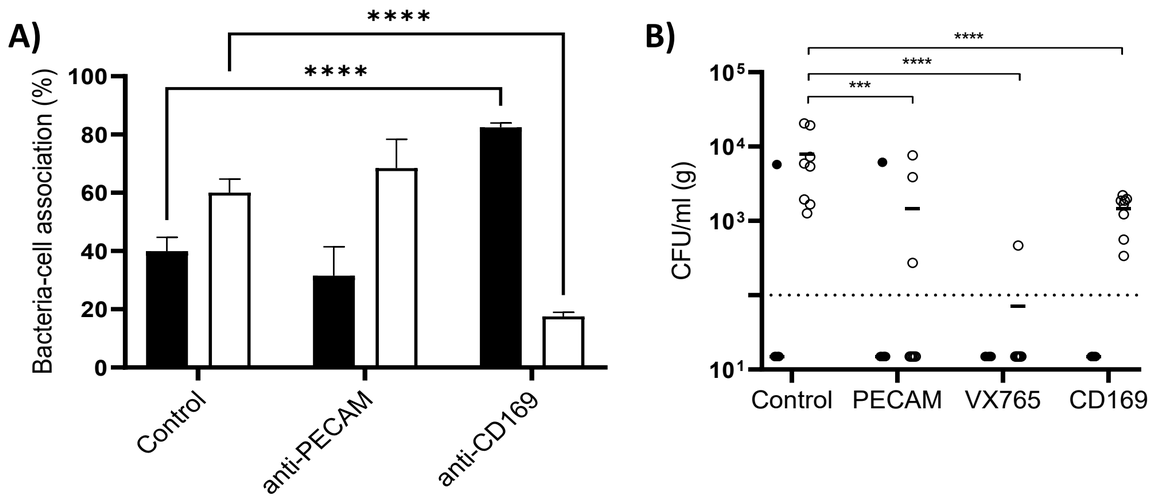

Figure 3: Pneumococcal CFU counts and cell association following administration of blocking antibodies and inhibitors. Anti-PECAM and VX765 NLRP3 inflammasome inhibitor were administered 30 minutes pre-infection, and anti-CD169 was administered 8h PI and compared against control mice treated with PBS. A) Brain bacterial association to endothelial cells (filled bars) and PV macrophages (open bars) at 48h PI. B) CFU counts in the blood (filled circles) and brain (open circles) were determined at 48h PI (***; P<0.001, ****; P<0.0001).

Summary

Following endothelial adhesion, pneumococci translocate the BBB, are phagocytosed by PV macrophages where they replicate, and are released following cell lysis resulting in neutrophil influx.

Blockage of endothelial adhesion, uptake into PV macrophages and inhibition of pyroptosis can significantly lower brain pneumococcal counts.

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