Brain, Behavior, and Immunity xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Antibody-induced neutrophil depletion prior to the onset of pneumococcal meningitis influences long-term neurological complications in mice

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ARTICLE INFO

Article history:
Received 9 December 2015
Received in revised form 23 January 2016
Accepted 30 January 2016
Available online xxxx

Keywords:
Neutrophils
Streptococcus pneumoniae
Pneumococcal meningitis
Neurological sequelae
IntelliCage
Behaviour
Cognition
Infectious disease
Central nervous system

ABSTRACT

During pneumococcal meningitis, clearance of bacteria by recruited neutrophils is crucial for host protection. However, these innate immune mechanisms are often insufficient and treatment with antibiotics is necessary to prevent death. Despite this antibiotic treatment, approximately half of all survivors suffer lifelong neurological problems. There is growing evidence indicating the harmful effects of neutrophils on CNS integrity. Therefore, the present study investigated the roles of neutrophils in the acute inflammatory response and the resulting long-term neuropsychological effects in murine pneumococcal meningitis. Long-term behavioural and cognitive functions in mice were measured using an automated IntelliCage system. Neutrophil depletion with antibody 1A8 as adjunctive therapy was shown to remarkably impair survival in meningitic C57BL/6J mice despite antibiotic (ceftriaxone) treatment. This was accompanied by increased bacterial load in the cerebrospinal fluid (CSF) and an increase in IL-1β, but decrease in TNF, within the CSF at 20 h after bacterial inoculation. In the longer term, the surviving neutrophil-depleted post-meningitic (PM) mice displayed reduced diurnal hypolocomotion compared to PM mice treated with an isotype antibody. However, they showed nocturnal hyperactivity, and greater learning impairment in a patrolling task that is believed to depend upon an intact hippocampus. The data thus demonstrate two important mechanisms: 1. Neutrophil extravasation into the CNS during pneumococcal meningitis influences the pro-inflammatory response and is central to control of the bacterial load, an increase in which may lead to death. 2. Neutrophil-mediated changes in the acute inflammatory response modulate the neuropsychological sequelae in mice that survive pneumococcal meningitis.

1. Introduction

Fatalities caused by meningitis occur in all age groups. As one of the commonest bacterial meningitic pathogens, *Streptococcus pneumoniae* infection in the central nervous system (CNS) leads to life-threatening meningeal inflammation, known as pneumococcal meningitis. Even when accurately diagnosed and promptly treated, a large proportion of patients cured of pneumococcal meningitis suffer neurological damage (Jit, 2010; Weisfelt et al., 2006). Bilateral or unilateral hearing loss, psychological distress and cognitive impairments are the major neurological sequelae observed among survivors of pneumococcal meningitis (Christie et al., 2011).

It has long been argued that bacterial toxins are not the primary cause of poor prognosis in bacterial meningitis (Koedel et al., 2002). Cytotoxicity induced by the host immune response during bacterial infection is the key factor causing acute death or long-term neurological deficits (Gerber and Nau, 2010; Koedel et al., 2010). The recognition of pneumococci by host immunity initiates a cascade of inflammatory events that recruits leucocytes to the site of infection for bacterial clearance. Numerous studies have shed light on the pathologic effects of this pathogen-clearing process. In particular, immuno-active pneumococcal components are released during phagocytosis, which can result in dysregulated host immune activation (Mook-Kanamori et al., 2011). Highly immunogenic products also can be released via autolysis of bacteria, which results in further recruitment of leucocytes that may become pathologic (Barichello et al., 2015; Mook-Kanamori et al., 2011). Besides leading to the production of an excessive amount of toxic reactive oxygen species in the process of phagocytosis,

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http://dx.doi.org/10.1016/j.bbi.2016.01.021
0889-1591/

increased leucocytosis also may deregulate the host inflammatory response, leading to an imbalanced production of pro-inflammatory factors. In general, such dysregulated inflammation within the CNS leads to complications, including cerebral ischaemia, brain oedema, blood–brain barrier breakdown and hydrocephalus, causing secondary brain injury accompanying lifelong neuropsychological dysfunctions (Gerber and Nau, 2010).

Circulating leucocytes, including neutrophils, monocytes and macrophages, enter the CNS to arrive at infectious loci in response to chemotactic stimuli released by host immune cells that are capable of recognising an intruding bacterium or its components. It has increasingly become clear that leucocytosis during acute pneumococcal meningitis does not only eradicate bacteria but also damages brain cells, primarily by releasing free radicals and matrix metalloproteinases during the process of phagocytosis of the bacteria (Gerber and Nau, 2010; Koedel et al., 2010). During bacterial meningitis, the majority of recruited leucocytes are neutrophils as well as a smaller number of monocytes (Mildner et al., 2008; Polfliet et al., 2001). In a study investigating experimental pneumococcal meningitis induced through lumbar puncture, a remarkable dependence on neutrophils, rather than monocytes, for pneumococcal clearance was demonstrated (Mildner et al., 2008). Later, Koedel et al. (2009) showed beneficial effects on inflammatory resolution in an *in vivo* model of pneumococcal meningitis when neutrophil apoptosis was triggered by roscovitine. Conversely, inhibiting neutrophil apoptosis by over-expressing Bcl-2 protein in haematopoietic cells exacerbated inflammation. A recent study reported the elevation of a rapidly-formed chemokine, CCL20, also known as macrophage inflammatory protein 3α (MIP-3α), in the cerebrospinal fluid (CSF) of patients with pneumococcal meningitis, and that this chemokine could attract neutrophilic granulocytes *in vitro* (Klein et al., 2014). In the same study, mice deficient in the CCL20 receptor CCR6 were found with decreased CSF white blood cell counts, worsened clinical status and survival, as well as increased brain bacterial titre, BBB disruption and brain oedema, without affecting peripheral bacterial load and intracranial haemorrhage (Klein et al., 2014).

In light of the above, we hypothesised that neutrophils are major contributors to neurological damage during pneumococcal meningitis due to their pro-inflammatory roles, and that systemic depletion of neutrophils prior to pneumococcal meningitis induction accompanied with prompt antibiotic treatment may improve long-term neurological sequelae in surviving mice. In the present study, we aimed to determine the role of $Ly6G^+$ neutrophils in regulating some aspects of the acute inflammatory response, namely cytokine production, and in modulating long-term behavioural and cognitive functions in an established murine model, specifically in mice cured of pneumococcal meningitis by ceftriaxone (Too et al., 2014a). The behavioural and cognitive findings were evaluated in the context of the pathology of acute meningitis in this model. We found that depletion of neutrophils led to increased disease severity, altered pro-inflammatory cytokine profile, and an attenuated clearance of bacteria from the CNS. Decreased survival was seen in neutrophil-depleted infected mice, despite ceftriaxone treatment, when compared to controls. Crucially, neutrophil-depleted animals that did survive pneumococcal meningitis manifested long-term alterations in neuropsychological profile when compared to their neutrophil-replete equivalents.

2. Material and methods

2.1. Ethics statement

All experimental procedures involving animals were approved by the University of Sydney Animal Ethics Committee and adhered to the NSW Animal Research Act (1985 – Animal Research Regulation 2010) and the 2004 NHMRC 'Australian code of practice for the care and use of animals for scientific purposes'.

2.2. Mice and husbandry

C57BL/6J female mice, aged eight weeks old and weighed 17–21 g at the beginning of experiments, were purchased from the Animal Resources Centre (Perth, Western Australia). Since there is a high tendency for male mice to engage in fighting in the grouped house environment of the IntelliCage (Section 2.7), which might impair their well-being and the test outcomes (Voikar et al., 2005), only female mice were used in the study. Mice were allowed to acclimatise to the animal house environment at the University of Sydney, where the study was carried out, for at least a week prior to beginning experimentation. During non-testing periods they were housed in groups of 3–6 mice in individually-vented cages (Tecniplast, Buguggiate, Italy), and given *ad libitum* access to water and food in a temperature-controlled environment with a 12:12 h light/dark cycle (light on 6:00 am–6:00 pm).

2.3. Experimental model of pneumococcal meningitis

Similar to our previous studies (Too et al., 2014a,b,d), a clinical isolate of *S. pneumoniae* serotype 3 WU2 strain (courtesy of Prof. J. Paton, University of Adelaide, Australia) was used as the primary aetiological agent of experimental pneumococcal meningitis. Briefly, 10^5 colony-forming units (cfu) of the laboratory cultured *S. pneumoniae* in 10 μL Dulbecco's Phosphate-Buffered Saline (DPBS) were injected into the brain third ventricle of isofluorane-anaesthetised mice to induce central inflammation that mimicked clinical cases of pneumococcal meningitis. Sham-infected control animals received DPBS injection concurrently. While sham animals remained well, symptoms of early-stage sickness, including mild lethargy and immobility with hunched posture, were observed in the *S. pneumoniae*-infected mice beginning 20 h post-inoculation (p.i.), at which time ceftriaxone (CEFT) administration was initiated in both infected and uninfected animals according to a standardised treatment regime previously described (Too et al., 2014a,b,d). The disease course was monitored daily up to 10 days, unless otherwise specified, and mice that developed signs indicative of irreversible pneumococcal meningitis (marked lethargy and immobility, prominent gait disturbance, delayed righting reflex, or fitting) were euthanased. Throughout the text, mice cured of *S. pneumoniae* meningitis due to CEFT treatment are denoted as "post-meningitic" (PM), and sham-infected normal mice as "control".

2.4. Peripheral neutrophil depletion

Depletion of systemic circulating neutrophils in mice was performed 24 h prior to the induction of pneumococcal meningitis. A Ly6G-specific (1A8) monoclonal neutralising antibody (0.05 mg/-mouse) was administered once via the intraperitoneal (i.p.) route, while corresponding control mice received an IgG2a isotype control antibody (GL117) and therefore were not depleted of neutrophils. Both antibodies were purchased from the Walter and Eliza Hall Institute Biotechnology Centre (Bundoora, VIC, AUS). The depletion of $Ly6G^+$ neutrophils was verified by flow cytometric analysis of blood samples collected from the tail vein at the time of sample collections for Sections 2.5 and 2.6 or about 1–3 h prior to bacterial inoculation. An anti-mouse Gr-1 antibody (clone RB6-8C5) was used for flow cytometric verification of neutrophil depletion (Suppl. Fig. 1). The 1A8 antibody (Daley et al., 2008) depleted >98% of circulating neutrophils (Suppl. Fig. 1) but did not affect the numbers of monocytes (not shown).

Please cite this article in press as: Too, L.K., et al. Antibody-induced neutrophil depletion prior to the onset of pneumococcal meningitis influences long-term neurological complications in mice. Brain Behav. Immun. (2016), http://dx.doi.org/10.1016/j.bbi.2016.01.021