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The pro-inflammatory cytokine interferon-gamma is an important driver of neuropathology and behavioural sequelae in experimental pneumococcal meningitis

L.K. Too [a], Helen J. Ball [a], Iain S. McGregor [b], Nicholas H. Hunt [a,*]

[a] Molecular Immunopathology Unit, Bosch Institute and School of Medical Sciences, University of Sydney, Sydney, New South Wales 2006, Australia
[b] School of Psychology, University of Sydney, Sydney, New South Wales 2006, Australia

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ABSTRACT

Interferon-gamma is known to play a complex modulatory role in immune defence during microbial infections. Its actions in pneumococcal meningitis, however, remain ill-defined. Here, a pathological role for IFN-γ was demonstrated using a murine model of pneumococcal meningitis, in that C57BL/6J mice deficient in this pro-inflammatory cytokine (IFN-$\gamma^{-/-}$) showed less severe acute and long-term neuropathology following intracerebral challenge with *Streptococcus pneumoniae*. The absence of IFN-γ significantly lengthened the survival of mice that otherwise would have developed fatal clinical signs within two days of CNS infection. Compared to their wild-type counterparts, IFN-$\gamma^{-/-}$ mice showed a diminished inflammatory response (attenuated levels of pro-inflammatory cytokines in the cerebrospinal fluid) and milder brain pathologies (less BBB permeability to protein and brain haemorrhage) during the acute phase of disease. Following a full regime of antibiotic treatment, we found substantial brain injuries in the wild-type mice 10 days after infection. IFN-$\gamma^{-/-}$ mice, however, showed decreased neuronal damage in both hippocampus and cortex. In the longer term (≈10 weeks p.i.), the wild-type mice that had survived meningitis due to antibiotic treatment had neurobehavioural abnormalities including diurnal hypoactivity, nocturnal hyperactivity and impaired performance in a discrimination reversal task. IFN-$\gamma^{-/-}$ mice, concomitantly tested in the automated IntelliCage platform, had reduced behavioural and cognitive disorders compared to wild-type mice. Both IFN-$\gamma^{-/-}$ and wild-type survivors of pneumococcal meningitis showed impaired working memory in the IntelliCage-based complex patrolling task. These observations indicate an association between IFN-γ-driven acute brain pathology and the long-term neurological sequelae resulting from pneumococcal meningitis.

1. Introduction

Pneumococcal meningitis is an acute purulent infection of the central nervous system (CNS) caused by the Gram-positive coccus, *Streptococcus pneumoniae*. This bacterium induces inflammation in the arachnoid, subarachnoid space and pia mater, which in turn elicits a severe life-threatening medical condition (van de Beek et al., 2004). The disease represents a major form of adult bacterial meningitis (BM), with reported case-fatality rates of up to 37% in developed countries and as high as 51% in resource-poor settings (Brouwer et al., 2010). Even after recovery following optimal treatment, a quarter of the patients suffer from lifelong neurological deficits (van de Beek et al., 2002; Weisfelt et al., 2006). Deafness, visual impairment and loss of cognitive performance occur in approximately half of the patients who survive pneumococcal meningitis, despite appropriate antibiotic treatment (Weisfelt et al., 2006).

During pneumococcal meningitis, acute innate immunity is triggered when cells involved in immune and inflammatory responses, such as astrocytes, dendritic cells, cerebral endothelial cells, microglia, monocytes, neutrophils, and perivascular and meningeal macrophages, sense the live pneumococci or their immuno-stimulatory pneumococcal components (Mook-Kanamori et al., 2011). Pneumococcal recognition occurs through interactions of microbes or their products with pattern recognition receptors, upon which numerous cytokines and chemokines are released from innate immune cells to perform a diverse spectrum of

* Corresponding author. Address: Molecular Immunopathology Unit, Medical Foundation Building (K25), The University of Sydney, New South Wales 2006, Australia. Tel.: +61 2 90363242; fax: +61 2 90363286.
E-mail address: Nicholas.hunt@sydney.edu.au (N.H. Hunt).

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immunological actions aimed at host protection. For instance, two early response pro-inflammatory immune mediators, interleukin (IL)-1β and tumour necrosis factor (TNF), propagate and amplify host inflammatory actions to combat bacterial challenge and are responsible for recruiting phagocytic effector cells to the site of infection so as to facilitate bacterial clearance (Koedel et al., 2002b; Saukkonen et al., 1990). On the other hand, unabated inflammatory responses lead to pathological conditions — either death or neurological sequelae.

Pneumococcus-induced immunological processes and the ensuing pathological events that underlie the neurological sequelae of pneumococcal meningitis arise from a complex interplay between the pathogen and host defence mechanisms (Barichello et al., 2012; Mook-Kanamori et al., 2011). The migration of leukocytes to the infectious and inflammatory focus exerts double-edged effects. While controlling the rapid proliferation of invading microbes, localised leukocytes also play a central role in causing neurological complications and brain damage by generating damaging toxic agents, such as reactive oxygen species (de Menezes et al., 2009; Kastenbauer et al., 2002; Leib et al., 1996). Many experimental studies have identified neuronal damage in the hippocampus as the major contributing factor to the long-term cognitive impairments typical of pneumococcal meningitis (Leib et al., 2003; Loeffler et al., 2001; Wellmer et al., 2000). Therefore, intervention in the interplay of bacterial and host-driven toxicity has the potential to alleviate brain injury, hence avoiding impairment of important neurological functions. One of the avenues to achieve this involves re-programming the production of inflammatory cytokines.

The concentrations of a number of cytokines rapidly increase in the cerebrospinal fluid (CSF) of patients with BM. These include the pro-inflammatory cytokines TNF, IL-1β, IL-6, interferon-gamma (IFN-γ) and IL-12, anti-inflammatory IL-10 and transforming growth factor (TGF)-β, and the neutrophil chemoattractant IL-8, among others (Mook-Kanamori et al., 2011). In particular, the CSF levels of TNF, IL-1β, IL-1 receptor antagonist, IL-6 and TGF-α correlate positively with fatal prognosis in BM patients (Grandgirard et al., 2013). It also has been reported recently that IFN-γ is significantly higher in the CSF of patients with pneumococcal meningitis than in those with meningococcal or haemophilus meningitis (Coutinho et al., 2013; Grandgirard et al., 2013).

IFN-γ is a powerful mediator of multiple immune pathways during inflammation and bacterial infection (Schroder et al., 2004). It regulates the transcription of genes necessary for leukocyte trafficking, such as IFN-inducible protein 10 (IP-10/CXCL10) (Gil et al., 2001; Rollins et al., 1990), monocyte chemoattractant protein-1 (MCP-1/CCL2) (Gil et al., 2001; Rollins et al., 1990), monokine induced by IFN-γ (MIG/CXCL9) (Liao et al., 1995), intercellular adhesion molecule 1 (ICAM-1/CD45) (Hou et al., 1994) and macrophage-inflammatory protein-1α and 1β (Mip-1α/CCL3 and -1β/CCL4) (Gil et al., 2001). Moreover, IFN-γ is capable of modulating class I and II antigen presentation, cellular apoptosis, production of ROS and antimicrobial effects (Schroder et al., 2004). Since it is a primary inducer of the expression of indoleamine 2,3-dioxygenase-1 (IDO-1), IFN-γ also has been implicated as a key factor in causing multiple neurological disorders as a consequence of inflammation-induced dysregulation of the tryptophan-kynurenine metabolic pathway (Connor et al., 2008). We recently reported that IFN-γ plays a key role in causing acute death in experimental pneumococcal meningitis (Mitchell et al., 2012).

In this study, we aimed at testing the hypothesis that IFN-γ drives the immunopathological events associated with the long-term neurological sequelae resulting from pneumococcal meningitis. We found, by genetically disrupting IFN-γ function, that C57BL/6J mice showed diminished mortality, blood–brain barrier (BBB) permeability to plasma proteins, intracerebral haemorrhage and neuronal degeneration in the hippocampus and cortex. In line with this, IFN-γ-deficient mice were protected against developing behavioural abnormalities and certain cognitive changes that were detected in their WT counterparts.

2. Materials and methods

2.1. Mice

IFN-$\gamma^{-/-}$ mice on a C57BL/6 background (Too et al., 2014) were bred at the University of Sydney. Their aged-matched, wild-type (WT) counterparts, C57BL/6J mice, were purchased from the Animal Resources Centre (Perth, Western Australia). Female mice aged between 6 and 9 weeks were used and all were housed in a temperature- and humidity-controlled environment with a 12-h diurnal cycle commencing at 6 a.m. They were given *ad libitum* food and water prior to and during experimentation, except when they were tested in an automated home-cage system that required controlled water access (see below for details of the IntelliCage behavioural studies). Experiments were conducted in compliance with 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' with approval from the University of Sydney Animal Ethics Committee.

2.2. Experimental pneumococcal meningitis and antibiotic treatment

Pneumococcal meningitis was induced in mice as previously described (Too et al., 2014). The pathogenic *S. pneumoniae* serotype 3 WU2 strain (courtesy of Prof. J. Paton, University of Adelaide, Australia) was used throughout the study. In brief, an inoculum containing 5×10^7 colony forming units (cfu)/mL of pneumococci was prepared in sterile Dulbecco's phosphate buffer saline (DPBS) from an exponential-phase pneumococcal subculture. All bacterial culture was carried out in brain heart infusion broth (Oxoid, Australia) at 37 °C and 5% CO_2. To induce pneumococcal meningitis, mice were inoculated with a 10 μL bacterial suspension containing $\sim 5 \times 10^5$ cfu into the third ventricle under light anaesthesia with inhalant isofluorane. Retrospective quantification of the cfu in an inoculum was performed via serial dilution on horse blood agar plates (Oxoid, Australia) incubated overnight in a 5% CO_2 environment at 37 °C. Sham-inoculated mice received 10 μL DPBS concurrently.

Following pneumococcal inoculation, the health status of mice was monitored for up to 10 days post-inoculation (p.i.). All mice that developed signs indicative of irreversible pneumococcal meningitis were promptly euthanased. These clinical signs included marked lethargy with hunched posture, prominent gait disturbance, delayed righting reflex, and fitting. In this model, approximately 90% of mice were euthanased between 40 and 48 h p.i. To cure mice and thereby prevent them developing lethal illness, a third generation cephalosporin antibiotic, ceftriaxone (CEFT; C5793, Sigma–Aldrich), was administered. Antibiotic interventions, which were initiated at 20 h p.i. at 100 mg/kg (~2 mg per mouse) intraperitoneally and repeated daily three times, saved the majority of mice from developing the symptoms of severe disease. Therefore, experiments planned beyond 20 h p.i. necessitated 1–4 doses of antibiotic administration. Mild clinical signs were first evident in mice from around 16–20 h p.i. Throughout the text, mice that received the full CEFT treatment regime and thus survived *S. pneumoniae* are referred to as "post-meningitis" (PM), while those euthanased for investigation of acute pathology prior to completing CEFT treatment are termed "meningitis". Sham-inoculated mice received CEFT concurrently with the infected mice to control for any direct actions of the antibiotic on the animals.