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STREPTOCOCCUS PNEUMONIAE INFECTION REGULATES EXPRESSION OF NEUROTROPHIC FACTORS IN THE OLFACTORY BULB AND CULTURED OLFACTORY ENSHEATHING CELLS

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Abstract—*Streptococcus pneumoniae* is the causative agent of numerous diseases including severe invasive infections such as bacteremia and meningitis. It has been previously shown that strains of *S. pneumoniae* that are unable to survive in the bloodstream may colonize the CNS. However, information on cellular components and pathways involved in the neurotropism of these strains is still scarce. The olfactory system is a specialized tissue in which olfactory receptor neurons (ORNs) are interfacing with the external environment through several microvilli. Olfactory ensheathing cells (OECs) which also form the glial limiting membrane at the surface of the olfactory bulb (OB) are the only cells that ensheathe the ORNs axons. Since previous data from our group showed that OECs may harbor *S. pneumoniae*, we decided to test whether infection of the OB or OEC cultures modulates the expression levels of neurotrophic factor's mRNA and its putative effects on the activation and viability of microglia. We observed that neurotrophin-3 (NT-3) and glial cell-line-derived neurotrophic factor (GDNF) expression was significantly higher in the OB from uninfected mice than in infected mice. A similar result was observed when we infected OEC cultures. Brain-derived neurotrophic factor (BNDF) expression was significantly lower in the OB from infected mice than in uninfected mice. In contrast, *in vitro* infection of OECs resulted in a significant increase of BDNF mRNA expression. An upregulation of high-mobility group box 1 (HMGB1) expression was observed in both OB and OEC cultures infected with *S. pneumoniae*. Moreover, we found that conditioned medium from infected OEC cultures induced the expression of the pro-apoptotic protein cleaved-caspase-3 and an apparently continuous nuclear factor-kappa B (NF-κB) p65 activation in the N13 microglia. Altogether, our data suggest the possible existence of an OEC–pathogen molecular interface, through which the OECs could interfere on the activation and viability of microglia, favoring the access of non-hematogenous *S. pneumoniae* strains to the CNS in the absence of bacteremia.

Key words: pneumococcal meningitis, nasopharyngeal colonization, HMGB1, NF-κB, BDNF, cleaved-caspase-3.

INTRODUCTION

Streptococcus pneumoniae is the causative agent of numerous diseases including severe invasive infections, such as bacteremia and meningitis. The relationship between bacteremia and meningitis is not yet completely understood and remains as a highly controversial issue (Brandt et al., 2008; Weiser, 2010). It has been previously shown that strains of *S. pneumoniae* that are unable to survive in the bloodstream may have access to the CNS even in the absence of immunosuppression, causing meningitis (van Ginkel et al., 2003). However, there is still scarce information on cellular components and pathways involved in the neurotropism of *S. pneumoniae* strains that are incapable to perform hematogenous dissemination. Depending on the immune status of the host, *S. pneumoniae* can only colonize the respiratory tract without causing any damage. However, under still unknown circumstances, these bacteria can subvert local immune system defenses, leading to the establishment of meningitis (Neill et al., 2014). Thus, given the increasing number of *in vivo* and *in vitro* evidences, the olfactory system and the trigeminal nerve have been shown as two important pathways and the most likely routes of bacterial invasion (van Ginkel et al., 2003; Macedo-Ramos et al., 2011, 2014; Panni et al., 2013).

The olfactory system is a specialized tissue in which olfactory receptor neurons (ORNs) are interfacing with

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Abbreviations: BNDF, Brain-derived neurotrophic factor; CM-I-OEC, conditioned medium from infected; CM-OEC, conditioned medium from uninfected; CNTF, Ciliary neurotrophic factor; DF, dermal fibroblast; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GDNF, Glial cell-line-derived neurotrophic factor; HMGB1, High-mobility group box 1; NFκB, nuclear factor-kappa B; NGF, Nerve growth factor; NT-3, Neurotrophin-3; OECs, Olfactory ensheathing cells; ORNs, olfactory receptor neurons.

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the external environment through several microvilli, called olfactory cilia; that extend into a mucus layer in the olfactory epithelium of the nasal mucosa (Purves et al., 2001). A second cell population spatially and structurally related to the ORNs in the epithelium olfactory is the olfactory ensheathing glia (OECs) which also forms the glial limiting membrane at the surface of the olfactory bulb (OB) and are the only cells that ensheathe the olfactory axons (Doucette, 1984). Recent evidence suggests that OECs can function as a primary innate immunocyte in the olfactory pathway by which cell debris are removed during olfactory nerve homeostasis and/or immune function (Su et al., 2013). The homeostatic properties of the OECs involve secretion of neurotrophic molecules during both developmental and adult neurogenesis (Ramón-Cueto and Valverde, 1995; Woodhall et al., 2001; Lipson et al., 2003; Lu et al., 2014). Although some studies have shown evidence for a central role of the OECs in orchestrating the innate immune response and participating in host defense following microbial infections, the cellular and molecular mechanisms of this actions remain unknown (Vincent et al., 2007; Leung et al., 2008). It can be predicted that *S. pneumoniae* infection into the brain promotes inflammation, invasion of leukocytes and stimulation of microglia. Once activated, microglia release soluble effectors such as free radicals, proteases, cytokines, excitatory amino acids, and alarmin high-mobility group box 1 (HMGB1) that can directly or indirectly cause damage to neural cells (Bianchi, 2007; Gerber and Nau, 2010). Thus, it may well be that neurotrophic factors from the OB play a prominent role in this process considering its essential functions in the homeostasis of the central nervous system.

Since previous data from our group showed that OECs may harbor *S. pneumoniae*, we decided to test whether infection of the OB or OEC cultured from adult OB modulates the expression levels of mRNA for neurotrophins and/or the indirect microglial activation in cultures.

EXPERIMENTAL PROCEDURES

Pneumococcal strain

The encapsulated strain of *S. pneumoniae* (EF3030, serotype 19F) used in the present study was kindly donated by Dr. David E. Briles. The EF3030 strain was selected for performing interaction assays with OECs based on the fact that it colonizes the upper respiratory tract in the absence of spreading through the blood circulation (Briles et al., 1992) and it belongs to a serotype frequently associated with resistance to penicillin and other antimicrobial agents (Di Fabio et al., 2001).

S. pneumoniae infection of OB

The experiments were carried out on 4-week-old male BALB/c mice purchased from Animal Laboratory Breeding Center at Fundação Oswaldo Cruz (CECAL). Control and infected mice were housed in the National Center for Bioimaging (CENABIO) at Universidade Federal do Rio de Janeiro under specific pathogen-free conditions with environmental monitoring and in an animal care facility. The present study was approved by the Ethics Committee for Laboratory Animal Use in Research of the Federal University of Rio de Janeiro (UFRJ), Permit Number: 01200.001568/2013-87, in accordance with the International Guide for the Care and Use of Laboratory Animals (Committee, 2011). Body temperature and weight were measured before and after the bacterial challenge and the infection was followed for up to six days. To inoculum, 30 μL of a suspension of viable *S. pneumoniae* strain EF3030, serotype 19F in a concentration of 3×10^7 cfu/μL diluted in saline solution was applied intranasally with 15 μl per nares. Control mice were inoculated in the same way with sterile saline (Wu et al., 1997; van Ginkel et al., 2000). The OB were carefully excised from the brain with a dissection microscope, washed and stored at −80 °C until used for gene expression analysis.

Primary cultures of OECS

Primary rat OEC cultures were obtained according to a modification by our group (Macedo-Ramos et al., 2011) of the procedure described by (Nash et al., 2001). Eight-week-old male Wistar rats were obtained from the Centre for Experimental Surgery Laboratory at the Federal University of Rio de Janeiro. Animal euthanasia procedures followed the norms established by the Brazilian Society for Neuroscience (SBNeC) as well as by the ethics committees of the Federal University of Rio de Janeiro (UFRJ). OEC cultures were prepared by the tissue-harvesting procedure without trypsin using only an ethylenediaminetetraacetic acid (EDTA – Sigma Chemical Co., St. Louis, MO, USA) solution (Macedo-Ramos, Master's Thesis, 2012). In our experience, EDTA allows less rough dissociation and a better yield of OECs than trypsin. For fibroblast and astrocytes removal by adhesion, the cell suspension was seeded into an uncoated flask (Nash et al., 2001). Finally, the cells were plated in the desired density into the appropriate laminin-coated flasks (Sigma), plates or wells in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS – Cultilab, Campinas, SP, Brazil), 1 mM glutamine, 1000 U/mL penicillin, and 50 μg/mL streptomycin. All these chemicals were from Sigma. The cells were kept at 37 °C and 5% CO_2, and the medium was regularly exchanged.

Interaction assay of *S. pneumoniae* and OEC cultures

When the cell cultures had reached total confluence (four weeks), interaction assays of *S. pneumoniae* and OEC cultures were performed as previously described by us in detail (Macedo-Ramos et al., 2011). After repeated washing to remove any residual FCS, OEC cultures were infected for 3 h at 37 °C with suspensions of living *S. pneumoniae* cells in serum- and antibiotic-free DMEM F-12. Three hours later, the supernatants from OEC cultures maintained either in medium alone or in medium containing *S. pneumoniae* were collected and the coverslips containing adhered OEC cultures fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. This interaction time was chosen based on our previous time-course studies (Macedo-Ramos et al., 2011,