Virulence 5:6, 645–647; August 15, 2014; © 2014 Landes Bioscience

Intracellular *Haemophilus influenzae* invades the brain

Is zyxin a critical blood brain barrier component regulated by TNF-α?

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Keywords: *Haemophilus influenzae*, CBO, CNS, intracellular pathogen, zyxin

In this issue of *Virulence*, Miyazaki et al.[1] provide a proof-of-principle study suggesting that zyxin, a cytoskeletal protein thought to provide protection in the tight junctions (TJs) of the blood–brain barrier (BBB), plays a vital role in the inhibition of *Haemophilus influenzae* (*Hi*) transmigration across the BBB by way of phagocytic cells as a cell-bound organism (CBO). A deficiency in zyxin in response to the production of tumor necrosis factor-α (TNF-α) during primary respiratory infection with *Hi* resulted in increased permeability of the BBB and therefore the transmigration of intracellular *Hi* organisms as a CBO across the BBB causing meningitis. This study provides fundamental evidence that zyxin is essential for the integrity of the BBB and protection against intracellular bacteria that use the "Trojan horse" (TH) mechanism to invade the central nervous system (CNS).

Hi is a gram-negative coccobacillus found in the microbiota of the human upper respiratory tract.[2] *Hi* is classified in two groups: polysaccharide encapsulated and acapsular strains. The group with capsule consists of serotypes a–f with variety b (*Hi*b) being a common etiology of invasive disease in children.[3,4] *Hi*b is responsible for causing respiratory infections and meningitis.[5] The initial infection with *Hi*b begins in the respiratory tract where the organism attaches to the epithelial cells and colonizes on the mucosal surface. As the infection progresses, bacteria reaches the bloodstream and get disseminated to the CNS causing meningitis. Before the approval of the first *Hi*b vaccine in the United States (US) in 1985, *Hi*b was the main cause of bacterial meningitis in children less than five years old.[4] Two-thirds of children with *Hi*b disease developed meningitis with 4% of the cases resulting in fatalities and leaving 15–30% of survivors with sequelae.[4] In addition, the annual mortality rate for *Hi*b meningitis infection in children under five years old in the western hemisphere was an estimated two deaths per 100 000 cases.[6] Today, *Hi*b disease in the US mainly affects unvaccinated infants and children.[4] *Hi*b-acquired disease is uncommon in children over five years old and adults, except in those cases with immunocompromised conditions such as AIDS patients that are susceptible to increased risk for systemic disease.

The non-encapsulated group of *Hi* is known as non-typeable *H. influenzae* (NT*Hi*) which is less invasive but can opportunistically colonize the upper respiratory tract of healthy people.[3,7] NT*Hi* is transmitted by way of respiratory droplets or secretions[4] resulting in mucosal infections such as otitis media, pneumonia, sinusitis, conjunctivitis, chronic bronchitis, and exacerbations in chronic obstructive pulmonary disease (COPD).[2-4,7] NT*Hi* adheres to epithelial cells in the respiratory tract and form biofilms, increasing the bacterium's ability to resist killing by the host's cellular and antimicrobial defenses. Similarly, biofilm formation allows high rate of genetic material exchange among neighboring cells due to the proximity of bacterial cells to each other resulting in a diverse and impervious microbial population to antimicrobials. The host immune response also plays an important role in NT*Hi* pathogenesis. In COPD patients, neutrophils and their antimicrobial molecules are highly present in sputum.[2] When NT*Hi* is phagocytized by neutrophils, a respiratory burst and high levels of neutrophil-chemoattractant IL-8 are produced. Nevertheless, instead of killing the microbes, the cells of innate immunity are killed mostly by necrosis whereas IL-8, produced during the host–pathogen interaction, stimulates the massive recruitment of neutrophils into the pulmonary system, thus intensifying the damaging inflammatory response.

Since *Hi* is an intracellular organism found in the adenoid tissue of non-diseased children,[5] Miyazaki et al.[5,8] hypothesized that epithelial cells from the oropharynx containing bacterial cells are able to escape the host immunological response and establish infection in the pulmonary system. To test this hypothesis, these investigators have previously developed a novel murine infection model that involves intranasal administration of CBO to induce bronchopneumonia. Interestingly, they showed that free *Hi* did not induce respiratory disease whereas

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Submitted: 08/08/2014; Accepted: 08/14/2014; Published Online: 08/14/2014
http://dx.doi.org/10.4161/viru.36086
Comment on: Miyazaki Y, Yusa T, Matsuo S, Terauchi Y, Miyazaki S. Zyxin modulates the transmigration of *Haemophilus influenzae* to the central nervous system. Virulence 2014; 5:665-72; PMID:25025691; http://dx.doi.org/10.4161/viru.29786

phagocytic CBO was able to cause bronchopneumonia further demonstrating the importance of this bacterium's association to host cells. Since *Hi* is an opportunistic pathogen, most likely free bacteria were eliminated by host cells. However, in the setting of immunosuppression engulfed bacteria can survive inside of phagocytic cells and persist in the pulmonary system.

Due to the fact that *Hi* is an intracellular pathogen, CBO can migrate from the lungs to the bloodstream and disseminate to the CNS. Hence, the authors investigated the factors that enhance *Hi*-associated meningitis and the molecules associated with phagocytic-infected cell transmigration across the BBB.[1] Proteomic analyses of membranous extracts from TNF-α-treated human brain endothelial cells identified zyxin as a novel regulatory molecule of the BBB permeability. Then, a zyxin-deficient and wild-type murine pneumonia model intranasally inoculated with dendritic cells containing phagocytized *Hi* were compared resulting in significantly greater mortality for the knockout animals than wild type mice, suggesting that zyxin is an essential component of the cells that make up the BBB.

The BBB is a highly selective network of capillaries that separates the CNS from circulation. This physical barrier regulates the passage of polar materials from the blood into the CNS and eliminates toxic compounds from the brain to maintain homeostasis.[3] The BBB consists of endothelial cells and it is regulated by pericytes, microglia, astrocytes, and neurons. BBB's specialized endothelial cells contain in their membranes, TJs (occludin, claudins, and junctional adhesion molecules) and water channels that have essential biological functions as a gate to transport polar substances[4] and infectious organisms and a wall inhibiting the horizontal movement of lipids and transmembrane proteins that helps to keep an intact cellular structure and signaling network.[7] TJs in endothelial cells are closely connected and cooperating with framework proteins in the cytoplasm such as zonula occludens (ZO) proteins, and actin cytoskeleton and related proteins (e.g., zyxin) involved in cell communication pathways.[5,6] Several studies have shown that alteration of TJs results in weakened BBB integrity facilitating the entrance of infectious microbes to the CNS.[8,9]

BBB dysfunction most likely involves the action of multiple pro-inflammatory cytokines on the endothelial cell lining cerebral capillaries. TNF-α has a direct pathogenic role in inflammatory, infectious, and neurodegenerative CNS diseases. Neurological defects described in a transgenic mouse overexpressing TNF-α demonstrate that high levels of this cytokine are detrimental for the host.[10] High TNF-α expression stimulates lymphocyte penetration of the brain causing detrimental effects in the CNS. TNF-α-dependent activation of NADPH oxidase induces reactive oxygen species (ROS) generation which reduces the expression of adhesion and TJ proteins disrupting the integrity of the BBB.[11] In the manuscript discussed here, it was demonstrated how the breakdown of the BBB integrity is a result of TNF-α released from the primary infection site in the respiratory tract. High TNF-α production in the blood directly alters the permeability of the BBB suggesting that this cytokine plays an important role in the induction of meningitis by inhibiting the expression of TJs. Although previous studies have shown that elevated TNF-α levels increase the BBB permeability[12-14] and the essential role of zyxin on bacterial dissemination,[15,16] this is the first study that shows that TNF-α reduces zyxin expression on the surface of brain endothelial cells exacerbating *Hi* CNS transmigration using the TH mechanism leading to increased mortality.

The article by Miyazaki et al.[1] is a major contribution to scientists investigating CNS microbial invasion because it identifies zyxin as a critical factor in maintaining the BBB integrity. This cytoskeletal protein is known to primarily control cellular motility by modulating the organization of actin fibers. In addition, zyxin regulates cellular gene expression due to its close relationship with protein regulators of signal transduction. This study is timely and provides abundance of questions for future investigations to decipher the mechanisms by which an opportunistic intracellular pathogen takes advantage of the host's immune response and overcomes a physical barrier such as the BBB to get access to the CNS and cause disease. In this regard, this study can be extended to determine how different pro-inflammatory cytokines (e.g., IL-6) may potentially alter zyxin expression causing BBB dysfunction (e.g., cytokine-mediated signaling events or ROS generation). Also, it is of great interest to acquire a better understanding of the microbes' ability (e.g., secreted virulence factors) to modify the BBB components and enhance CNS invasion and disease with the future goal of developing prophylactic measures for combating these infections that annually affect 1.2 million individuals and kill 120000 people worldwide.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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