Basic Medical Research

NF-κB, neutrophil extracellular traps, and microglial in mice with *Streptococcus suis* serotype 2 meningitis infection

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ABSTRACT

BACKGROUND *Streptococcus suis* is the most frequent etiology of zoonotic bacterial meningitis, potentially initiating an outbreak. Acute bacterial meningitis caused by *S. suis* has various manifestations, often accompanied by sepsis with multiple organ involvement. This study aimed to evaluate the pattern of *S. suis* outgrowth in the brain, which is associated with nuclear factor-κappaB (NF-κB) activation, neutrophil extracellular traps (NETs) release (NETosis), and microglial activation as three crucial pathological mechanisms of bacterial meningitis.

METHODS This study used 64 female BALB/c mice aged 6 weeks and weighed 18–20 g, grouped into infected and non-infected as the control group. Both groups were administered 1 ml of *S. suis* serotype 2 suspension (1×10^7 colony forming-unit/ml) and normal saline intraperitoneally. The bacterial colony count of *S. suis* was evaluated, along with NF-κB and NET levels in blood and brain, as well as meningeal inflammation and microglial activation in the brain at Days 1, 3, 5, and 7 post-infection.

RESULTS The invasion of *S. suis* into the brain slightly induced NF-κB activation, leading to a burst of inflammatory responses, neutrophil infiltration with NET releases, and microglia activation that co-occurred, showing their peaks on Days 3 and 5 after onset.

CONCLUSIONS The *S. suis* invasion into the mice's brain increased NF-κB activation, NETosis, and microglia activation during *S. suis* meningitis infection.

KEYWORDS brain, central nervous system, infection, inflammation, *Streptococcus suis*

Several reports have revealed an increased occurrence of *Streptococcus suis* infections in areas with dense pig populations, especially in Asian countries including Thailand, Vietnam, China, and Indonesia (Bali Province). *S. suis* serotype 2 is the primary pathogenic cause of bacterial meningitis in these areas[1–4] with various clinical manifestations, including systemic infections (i.e., sepsis), pneumonia, endocarditis, arthritis, and cellulitis.[1,5,6]

A primary mechanism of bacterial meningitis is the pathogens' ability to invade the brain, which induces an inflammatory response via nuclear factor-κappaB (NF-κB) activation or the migration of neutrophils through the blood-brain barrier (BBB).[7] In contrast, microglia, the resident macrophages within the brain parenchyma, are critical cells of the innate immune system. Consequently, bacterial invasion changes microglial morphology to its active form, facilitating movement towards the area of infection following a chemotaxis gradient and promoting proliferation to elevate defense against pathogens, protect neurons, and restore brain tissues.[8] Active microglia tend to induce various proinflammatory mediators that increase BBB permeability, enhance migration of

peripheral immune cells to the central nervous system, and initiate inflammatory processes.[9,10]

Previous work has shown that mice injected intraperitoneally (IP) showed a pattern of *S. suis* infection that spreads through a hematogenous pathway, leading to bacteremia and meningoencephalitis. Additionally, NF-κb and microglia activation have been associated with meningitis. However, no studies have ever associated NF-κB, neutrophil extracellular traps (NETs), and microglial activation in a meningitis model. Thus, a detailed study of developing systemic infection leading to meningitis is required to understand the pathogenesis of *S. suis* meningitis comprehensively and should encompass NF-κB-induced inflammatory response, neutrophil activation to release NETs (NETosis), and microglial activation.

METHODS

This experimental study has been ethically declared by the Research Ethics Committee of the Faculty of Medicine, Universitas Udayana/Sanglah Hospital, Denpasar (No: 497/UN14.2.2.VII.14/LP/2020) and was conducted under guidelines and policies.

Experimental approach: bacteria and growth conditions

All experimental groups used *S. suis* serotype 2 obtained from clinical meningitis isolates in the Laboratory of Clinical Microbiology, Faculty of Medicine, Universitas Udayana, and registered in GenBank (accession number MN395406-34). Bacteria quantity was based on a previous study by Domínguez-Punaro et al.[11] The bacteria were cultivated overnight at 37°C on sheep blood agar plates, and isolated colonies were injected into 5 ml of tryptic soy broth (TSB) and kept at 37°C for 18–24 hours with agitation. The bacterial pellet was resuspended in TSB until it had an optical density of 600 nm of 0.4. Phosphate-buffered saline (PBS; pH 7.3) was used to wash stationary phase bacteria twice. The bacterial pellet was resuspended and adjusted to a concentration of 1×10^7 colony-forming unit (CFU)/ml.

Mice and experimental models of *S. suis* meningitis

Six-week-old female BALB/c mice, obtained from the Division of Drug Development and Experimental Animal, Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Udayana, were housed in standard laboratory conditions of 12-hours light or 12-hours dark cycles with free access to rodent chow and water for 1 week. Mice without clinical symptoms were randomly selected for sampling. For the intervention group, 32 mice were infected with 1 ml of 1×10^7 CFU/ml of *S. suis*, an established model of systemic IP meningitis. Eight mice were assigned to each treatment group. In total, 64 mice were assigned to treatment groups. The control group received an IP injection of 1 ml of 0.9% NaCl. All animals were observed daily before being anesthetized intramuscularly with a cocktail of ketamine (35 mg/kg) and xylazine (5 mg/kg) and euthanized on Days 1, 3, 5, or 7 after inoculation.

Determination of viable bacteria in the blood and brain

Blood (100 µl) was collected by retro-orbital puncture at each designated time point, and the brain (0.05 g) was obtained aseptically. The brains were trimmed, placed in 500 µl of PBS (pH 7.3), and homogenized with a vortex. Then, serial dilutions of the homogenate in PBS (50 µl) were plated onto blood agar plates. Both brain and blood samples were incubated overnight at 37°C. Colonies were counted and expressed as CFU/0.05 g for the brain samples and CFU/ml for the blood samples.

Histology

The brains were removed, post-fixed in 10% buffered formaldehyde, and processed in paraffin. The paraffin-embedded mouse brains were then deparaffinized, rehydrated, stained with hematoxylin and eosin according to the standard protocol, and examined under a light microscope (Olympus Cx21; Olympus, China). Meningitis was characterized by thickening of the meninges and inflammatory cell infiltration in either the brain parenchyma or perivascular areas, with hyperemic brain vessels and necrotic areas.

Immunohistochemistry

Brain tissue (5 mm thick) was fixed in paraffin in a coronal plane from the frontal lobe. Sections were selected across the brain and placed on slides, with 12 sections per mouse. Tissue slices were immunostained with the primary antibody rabbit anti-transmembrane protein 119 (TMEM119) (ab209064; Abcam, UK) and secondary antibody Dako EnVision®+ Dual Link System-HRP (Agilent Technologies Inc., USA) proportional to the absorbance, and the findings were represented

in arbitrary units. A light microscope (Olympus Cx21; Olympus, China) was used to examine the tissue slices.

Assessment of microglial activation

The activation of microglial cells was characterized as follows: (1) immunohistochemical staining indicating active microglia morphology; (2) a substantial increase in the number and size of microglia compared to the control group.[12] Kreutzberg's activation stages were used to analyze microglial morphology, as follows: stage 0, fully resting microglia with multiple branches; stage 1, a mix of resting and mildly activated microglia, demonstrating shortened processes with increased thickness; stage 2, mildly activated microglia with shortened processes with increased thickness; stage 3, a mix of mildly and strongly activated ameboid microglia without processes; stage 4, ameboid microglia with processes.[13]

Enzyme-linked immunosorbent assay (ELISA) assessment of NETs

The NF-κB (E-EL-M0838; ElabScience®, USA) and NET Mouse ELISA Kit® (H00004353-M01; Abnova, Taiwan) were used for the ELISA following the manufacturer's protocol. Sandwich ELISA was used to detect NETs using anti-myeloperoxidase and anti-neutrophil elastase monoclonal antibodies and a peroxidase-conjugated anti-DNA monoclonal antibody. In contrast, NF-κB was quantified using a commercial kit. Briefly, the capture antibody was coated onto a 96-well ELISA plate. The values of NF-κB and NETs were calculated as ng/ml.

Statistical analysis

Data are presented as mean (standard error of measurement). Student's *t*-test was used for NET, NF-κB, and microglial activation, while the Mann–Whitney *U* test was used for bacterial load. Statistical analyses were performed to compare variables between the interventional and control groups. Statistical significance was set at $p<0.05$. All analyses were performed using the SPSS software version 22 for Mac (IBM Corp., USA).

RESULTS

Bacteremia and acute bacterial meningitis after IP induction of *S. suis* serotype 2 in BALB/c mice

S. suis bacterial colonies was grown in the brain tissue and blood of the intervention groups on Days 1, 3, 5, and 7. However, no evidence of *S. suis* growth was observed under sterile conditions in the control group. The *S. suis* colony count in the blood peaked on Day 3, then gradually decreased by Day 5 (mean 6.22×10^4 CFU/ml) and 7 (mean 1.96×10^4 CFU/ml). The growth of *S. suis* colonies within the brains of mice in the intervention groups was significantly different from that of the control group on Days 3 and 5 post-infection, whereas no significant differences were found on Days 1 and 7. The growth pattern of brain *S. suis* colonies was the same as that found in the blood post-infection, with the highest colony count on Day 3 (mean 1.57×10^5 CFU/ml) and the lowest on Day 7 (mean 1.25×10^3 CFU/ml) (Figure 1a).

Microscopic changes within the brains of mice in the infection and control groups were categorized as meningitis or meningoencephalitis and normal, respectively. No pathological characteristics were identified within the brains of mice from the control group (Figure 2a). Meningitis was characterized by thickening of the meninges and inflammatory cell infiltration in the brain parenchyma and perivascular areas (Figure 2b), hyperemic brain vessels (Figure 2c), and necrotic areas (Figure 2d).

The pattern of NF-κB activation in blood and brain after IP induction of *S. suis* serotype 2 in BALB/C mice

Mean NF-κB levels in the blood and brain on Days 1, 3, 5, and 7 were higher in the infection group than those in the control group. This indicated a host inflammatory reaction in response to IP exposure to *S. suis*. The mean value of brain NF-κB levels was higher in the infected group than in the control group, with a significant difference on Day 3 post-infection and no difference on Days 1, 5, and 7 (Figure 1b). There was a significant correlation in the amount of NF-κB in blood and brain ($r = 0.409$, $p = 0.002$).

The pattern of NETosis in blood and brain after IP induction of *S. suis* serotype 2 in BALB/C mice

Figure 1c shows that the mean NET levels in the blood and brain of the treatment groups were significantly higher than those of the control group on Days 3, 5, and 7. These results indicated increased neutrophil activity in the blood and brain after IP injection of *S. suis*. The correlation between NET levels in the blood and brain was not statistically significant ($r = 0.129$, $p = 0.123$).

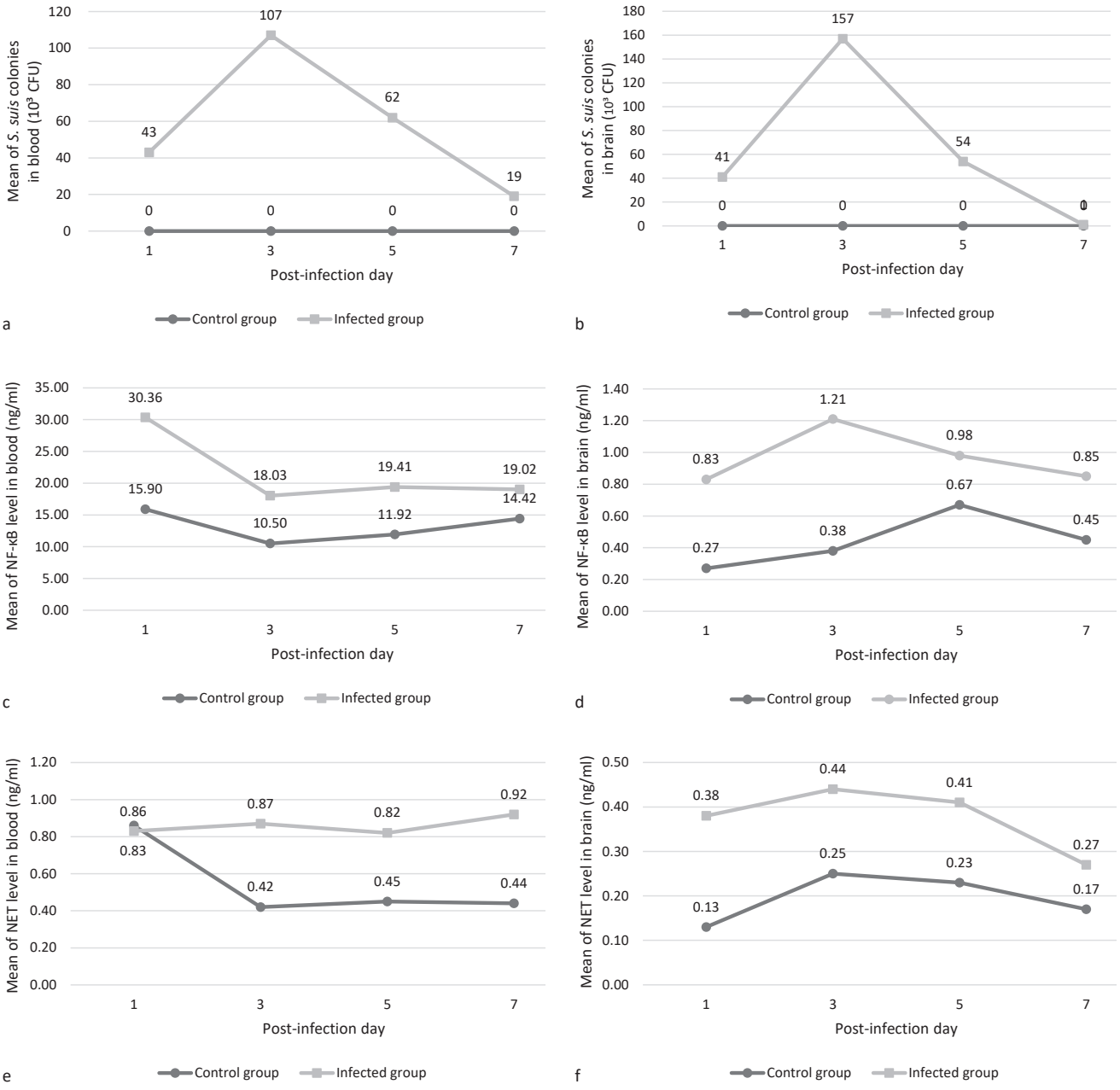

Figure 1. Inflammation induces BBB breakdown, facilitates the bacteria, neutrophils, and proinflammatory markers to enter the brain and exacerbates the infection. Mean of *S. suis* colonies in blood (a) and brain (b), NF-κB levels (b), and NET levels in blood (e) and brain (f). BBB=blood-brain barrier; CFU=colony-forming unit; NET=neutrophil extracellular trap; NF-κB=nuclear factor-κappaB

The pattern of microglia activation and proliferation after IP induction of *S. suis* serotype 2 in BALB/c mice

Table 1 shows the distribution of microglial activation in the infected and control groups, calculated based on the number of microglia per high-power field at 400x magnification in the frontal lobe of the cortex. TMEM 119 was used as a microglia surface marker. The mean number of microglia in the treatment group on Days 1, 3, 5, and 7 was higher than that in the control group. Most of the microglia in the control group were inactive or resting (Figure 3a), except on the third day when inactive microglia appeared to be mixed with mildly activated microglia. In contrast, activated microglia were primarily observed in the infected group. Strongly activated microglia (Figure 3b) were found in the infected group on Days 3 (29%) and 5 (38%), along with mildly activated microglia (Day 3: 43%; Day 5: 50%). In contrast, mildly activated microglia were mainly observed on post-infection Days 1 (40%) and 7 (50%).

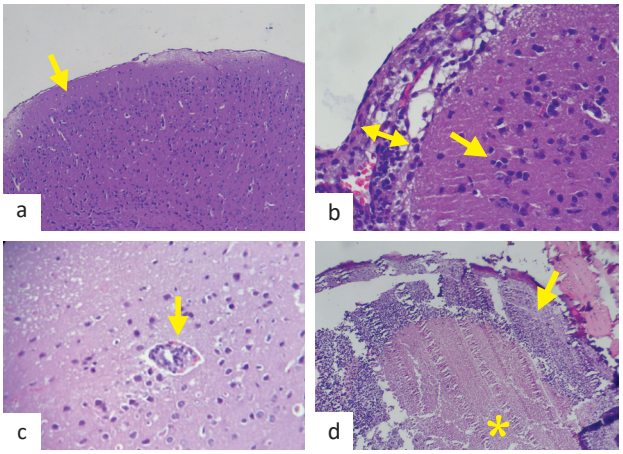

Figure 2. Microscopic alterations in the brains of infected and control mice. (a) Control mice brains with normal meninges (arrow) on Day 3 (100× magnification); (b) mice brains with meningitis (arrows) on Day 5 post-infection; (c) perivascular lymphocytic infiltration (arrow) on Day 3 post-infection (400× magnification); (d) mice's brain with dense neutrophil infiltrates with infiltration to the temporal bone (arrow) accompanied by areas of necrosis (asterisk) on Day 5 post-infection (100× magnification)

Table 1. The distribution of microglia activation in infected and control groups

Day	Stage of microglia activation	Infected, n (%)	Control, n (%)	N (%)
1	0	0 (0)	5 (100)	5 (50)
	1	2 (40)	0 (0)	2 (20)
	2	2 (40)	0 (0)	2 (20)
	3	1 (20)	0 (0)	1 (10)
	4	0 (0)	0 (0)	0 (0)
3	0	0 (0)	4 (57)	4 (29)
	1	2 (29)	3 (43)	5 (36)
	2	3 (43)	0 (0)	3 (21)
	3	2 (29)	0 (0)	2 (14)
	4	0 (0)	0 (0)	0 (0)
5	0	0 (0)	0 (0)	0 (0)
	1	1 (13)	8 (100)	9 (56)
	2	4 (50)	0 (0)	4 (25)
	3	3 (38)	0 (0)	3 (19)
	4	0 (0)	0 (0)	0 (0)
7	0	0 (0)	4 (50)	4 (25)
	1	4 (50)	4 (50)	8 (25)
	2	4 (50)	0 (0)	4 (25)
	3	0 (0)	0 (0)	0 (0)
	4	0 (0)	0 (0)	0 (0)

Transmembrane protein 119 (TMEM119) was used as a microglia surface marker

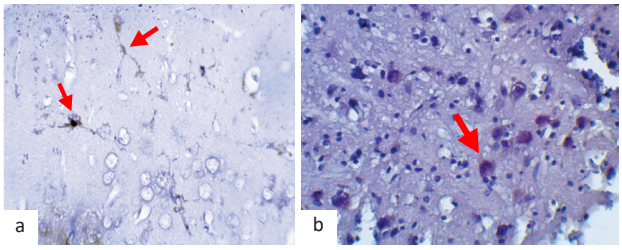

Figure 3. The microglia in the control and infected group (400× magnification). (a) Inactive/resting microglia with many branches in the control groups (arrows); (b) activated microglia (brown cells) with amoeboid-shapes and no processes in the brain parenchyma of meningitis mice (arrows)

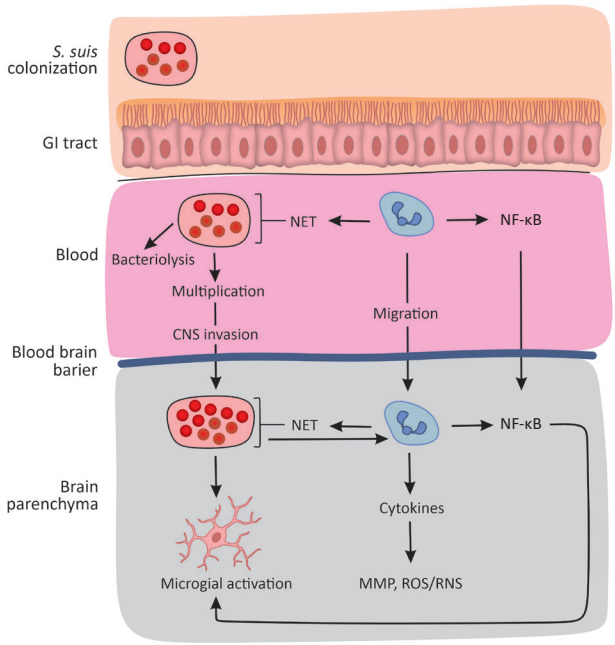

Figure 4. Proposed pathogenesis of *S. suis* meningitis based on data gained from our study. Bacteremia led to brain infection, in which NF-κB and NET play important roles. CNS=central nervous system; GI=gastrointestinal; MMP=matrix metalloproteinase; NET=neutrophil extracellular trap; NF-κB=nuclear factor-κappaB; ROS=reactive oxygen species; RNS=reactive nitrogen species

DISCUSSION

This study demonstrated that the spread of *S. suis* infection in the brain is hematogenous. Bacteremia occurred on Day 1, peaked on Day 3, and then decreased until the end of the observation period. Another study even reported that bacteremia occurred at 3 hours post-infection, with a peak on Day 3, decreased by Day 9, and disappeared on Day 12.[11] Due to the survivability of *S. suis* in the blood, this bacterium can spread to various organs, including the brain. The bacterial load is crucial to the incidence of meningitis, but other

factors, such as virulence and adhesion factors, are also important to consider.[14,15] The high release of various components of *S. suis* bacteria in the blood induces the production of inflammatory mediators, potentially leading to streptococcal toxic shock-like syndrome.[16]

The prevalence of *S. suis* colonies in the brain revealed the same pattern as that in bacteremia. The highest growth of *S. suis* colonies occurred on Day 3 which had a monophasic pattern of acute infection. However, other studies have shown that the growth pattern of *S. suis* colonies in the brain varies more than that in the blood and other organs. Moreover, brain pathology due to *S. suis* serotype 2 infection corresponds to acute inflammation with the infiltration of inflammatory cells (neutrophils and lymphocytes) in the meninges, brain parenchyma, and perivascular area, followed by thickening of the meninges, vascular hyperemia, and necrotic areas closely associated with brain inflammation and persistent bacteremia up to Day 5 post-infection.[11]

In this study, meningitis was accompanied by bacteremia and other organ infections in 15 mice (47%), whereas meningitis as a single manifestation occurred in three mice (9%). Moreover, the incidence of meningitis was 56%, which occurred on the first day post-infection, peaking on Days 3 and 5 compared to other studies by approximately an 11–40% increase.[11,17] The quantification of *S. suis* colonies within the brain revealed high colony growth in two areas: the frontal lobe and the temporal bone containing the meninges and cerebellar brain tissue. A high bacterial load of *S. suis* in the brain can also cause secondary bacteremia, aggravating the disease.[17]

This study discovered that the mortality rate before 72 hours post-infection was 12.5%. Other studies reached 20% before 48 hours and were associated with systemic disease and septic shock.[11,17] Mortality due to sepsis was associated with high levels of bacteria in circulation[17] and did not show significant histopathological changes in the brain, heart, liver, or spleen.[11]

High levels of NF-κB indicate a severe inflammatory process.[7] Indeed, blood NF-κB levels post-infection, especially at the beginning of the observation, indicated an inflammatory host response against *S. suis* invasion. NF-κB expression was chosen over cytokines due to its stability and earlier expression within the nucleus. NF-κB is a DNA-binding protein found in almost all immune cells and has pleiotropic properties with various roles in cell activity.[18,19] The accumulative effect of tissue damage due to excessive NF-κB activation reflects increased serum levels of NF-κB.[20]

Brain NF-κB levels increased from Day 1 post-infection and peaked on Day 3, which was associated with increased prevalence of *S. suis* bacteria in the brain. Another study also reported increased levels of proinflammatory mediator mRNA within the brains of mice infected with *S. suis*, where the expression of I-kappa beta (an indirect marker of NF-κB) was prevalent on Days 3 and 5 post-infection.[14] Activation of NF-κB plays various roles, including pleocytosis, BBB damage in bacterial meningitis, and the initial inflammatory process, by increasing the expression of genes encoding many proinflammatory mediators.[7,19] The excessive inflammatory response to meningitis has a severe prognosis because it can lead to intracranial complications, such as cerebral edema, high intracranial pressure, and cerebrovascular insult.[11]

The highest levels of NF-κB varied; it peaked on Day 1 in the blood and Day 3 in the brain. This was associated with a high level of bacteremia on Day 1, followed by a high incidence of meningitis on Days 3–5. However, NF-κB in blood and brain was significantly correlated, suggesting that we can also predict NF-κB levels in the brain through patient's blood samples. Studies comparing blood and cerebrospinal fluid cytokine levels in patients with sepsis and meningitis have concluded that blood and brain cytokine levels vary widely.[17]

The decrease in NF-κB levels on Day 5 post-infection is likely due to the initiation of the infection resolution process in which the number of pathogens was sufficiently reduced such that NF-κB-induced inflammation was attenuated. High NF-κB levels indicate an ongoing inflammatory response contributing to tissue damage and further complications of bacterial meningitis.[7]

The high levels of NETs in the blood and brain, especially on Days 3 and 5 post-infection, indicated a rapid neutrophil response to combat *S. suis* invasion and were associated with a high incidence of meningitis on the same day. The high number of *S. suis* bacteria in the blood and brain causes high mobilization of neutrophils in circulation and the brain, which increases in response to *S. suis* invasion. Neutrophils release of many NETs during infection can induce NETs and become trapped on them to limit their spread, thereby stimulating an inflammatory response.[21,22]

In contrast, blood NET levels on Day 1 post-infection were comparable in the infected and control groups. However, this does not correspond to the mechanism of NET formation that occurs at the onset of infection.

Microglia act as the guardians of the brain and local immune cells, which are activated in the event of infection or trauma.[8,23] In this study, *S. suis* induction led to microglial activation, characterized by morphological changes of microglia into an amoeboid form (phagocytosis), to facilitate movement toward the lesion following a chemotaxis gradient. *S. suis* induction is also accompanied by an increase in microglial density and has the highest incidence of meningitis. Another study showed that high bacterial antigen levels could increase microglia activation.[24] Studies on the pigs' brains infected with *S. suis* have also demonstrated increased microglia density and activation.[13] Microglial activation protects the brain parenchyma from bacterial invasion, produces proinflammatory mediators, increases the BBB's permeability, and induces the migration of neutrophil cells.[8,10] Furthermore, infectious conditions cause microglia proliferation, increasing microglia density for the defense, protection, and restoration of brain tissue.[8]

Systemic infection can lead to microglial activation due to the spread of proinflammatory mediators from circulation to the brain, which are carried out through various mechanisms: first, stimulation of afferent autonomic nerves, especially the vagus nerve; active transport through BBB and passive transport through circumventricular organs; finally, activation of inflammatory receptors on microvascular endothelial cells of the brain.[12] This study provides evidence for NF-κB, NETs, and microglial activation in a meningitis model. A brief explanation of how these factors interact is depicted in Figure 4.

One of the limitations of this study was the method used to induce meningitis. Owing to resource constraints (no stereotactic tools were available), IP was used in this investigation rather than intrathecal administration. In conclusion, this study comprehensively demonstrated the pattern of progressive systemic infection in *S. suis* invasion causing bacteremia and progressing to meningitis, which triggers NF-κB activation to induce inflammatory responses in the blood and brain. This facilitates the mobilization of neutrophils to the blood and migration of neutrophils to the brain with the release of NETs and the activation and proliferation of microglia that co-occur, with a peak on Days 3 and 5 post-infection.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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