Interaction of factor H-binding protein of *Streptococcus suis* with globotriaosylceramide promotes the development of meningitis

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**ABSTRACT**

*Streptococcus suis* is an important emerging zoonotic agent that causes acute bacterial meningitis in humans with high mortality and morbidity. Our previous work showed that factor H-binding protein (Fhb) contributed to virulence of *S. suis*, but the role of Fhb in the development of *S. suis* meningitis remained unclear. In this study, we demonstrated for the first time that Fhb contributed to the traversal of *S. suis* across the human blood–brain barrier by allelic-exchange mutagenesis, complementation and specific antibody blocking studies. We also showed that globotriaosylceramide (Gb3), the receptor of Fhb, was involved in this process and affected *S. suis* infection-induced activation of myosin light chain 2 through Rho/ROCK signaling in hCMEC/D3 cells. Using a murine model of *S. suis* meningitis, we further demonstrated that Gb3-deficiency prevented the mice from developing severe brain inflammation or injury. Our results demonstrate that the Fhb-Gb3 interaction plays an important role in the development of *S. suis* meningitis and might be a potential therapeutic target against *S. suis* infection.

**KEYWORDS**

blood–brain barrier; Fhb; Gb3; meningitis; *Streptococcus suis*

**Introduction**

Bacterial meningitis is a major cause of infection-related death and disability worldwide. Group B *Streptococcus*, *Escherichia coli* K1 strain and *Listeria monocytogenes* are the main causes of neonatal meningitis, and *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* type b cause most cases of adult meningitis. In recent decades, *Streptococcus suis* (*S. suis*) as an important emerging zoonotic pathogen, has gained increased attention because of its high prevalence in human meningitis cases in South East and East Asia. In China, the outbreak of *S. suis* serotype 2 in 2005 resulted in more than 200 human cases of infection with a fatality rate of nearly 20%. In Vietnam, *S. suis* has been the most frequent cause of bacterial meningitis in adults. To successfully induce meningitis, extracellular pathogens need to penetrate the physiologic barriers (the blood–brain barrier, BBB or the blood–cerebrospinal fluid barrier, BCSFB) between the bloodstream and the central nervous system (CNS). Most of previous works focused on studying how *S. suis* entered the brain via the BCSFB. In an inverted Transwell filter model of the BCSFB, *S. suis* invaded porcine choroid plexus epithelial cells specifically from the basolateral side in a capsule-dependent manner. Furthermore, *S. suis* infection significantly altered the tight junction morphology of these cells, and changes in protein expression and the actin cytoskeleton affected barrier function. However, the mechanism by which *S. suis* crosses the BBB is still unclear.

Many virulence factors, such as capsular polysaccharide (CPS), muramidase-released protein (MRP), suilysin (SLY), and factor H-binding protein (Fhb), have been demonstrated to be involved in the development of *S. suis* meningitis. *S. suis* Fhb has been identified as a novel immunogenic protein and a potential vaccine antigen. We previously reported that Fhb contributed to virulence and anti-phagocytosis by...
interacting with factor H to inhibit complement deposition on \textit{S. suis}.\textsuperscript{18} Crystal structure analysis showed that Fhb had abundant negative charges on the protein surface, which might facilitate the binding of Fhb to human factor H.\textsuperscript{22,23} In addition, Fhb was also shown to be a novel streptococcal adhesin P (SadP) that recognizes the galactosyl-\(\alpha1\)-4 galactose moiety of Gb3.\textsuperscript{24} Gb3, also known as CD77, is synthesized by the addition of galactose to lactosylceramide in a reaction catalyzed by Gb3 synthase (\(\alpha1\),4 galactosyltransferase) and is restricted in expression to certain cell types including some epithelial cells and endothelial cells.\textsuperscript{25-27}

Interactions between bacterial adhesion and host cell surface carbohydrates play an important role in infection and invasive diseases.\textsuperscript{28,29} The higher level of Gb3 expression on the cell surface of brain microvascular endothelial cells (BMECs) and \textit{S. suis} Fhb specific binding to Gal\(\alpha1\)-4Gal-oligosaccharides,\textsuperscript{24,30,31} prompted us to investigate the role of the Fhb-Gb3 interaction on the development of \textit{S. suis} meningitis. In this study, we used an immortalized human cerebral microvascular endothelial cell-line, hCMEC/D3, to build an immortalized human cerebral microvascular endothelial cell monolayer across the hCMEC/D3 monolayer (Fig. S1C). In addition, the transendothelial electrical resistance (TEER) across the hCMEC/D3 monolayer was evaluated to determine if it could be used as a monolayer cellular model of the human BBB model,\textsuperscript{32,33} and Gb3-deficient mice to develop a murine model of \textit{S. suis} meningitis. Our results showed that Gb3 was involved in Fhb-mediated translocation of \textit{S. suis} across the \textit{in vitro} human BBB and Gb3-deficiency protected the mice from developing severe brain inflammation or injury.

Results

\textbf{Fhb mediates the traversal of \textit{S. suis} across the hCMEC/D3 monolayer}

The morphological and functional features of the hCMEC/D3 monolayer were evaluated to determine if it could be used as a monolayer cellular model of the human BBB. Fluorescence microscopy showed that the junction protein ZO-1 formed a characteristic “chickenwire” pattern at the margins of the cells (Fig. S1A). Transmission electron microscopy showed that confluent hCMEC/D3 cells formed a monolayer barrier with apical-basal polarization and tight junctions (Fig. S1B). In addition, the transendothelial electrical resistance (TEER) across the hCMEC/D3 monolayer (Fig. S1C) increased consistently and reached a steady value 7 d after seeding as previously reported.\textsuperscript{34} These features indicated that the hCMEC/D3 monolayer could form an effective barrier.

Next, we investigated whether \textit{S. suis} 05ZYH33 could pass through the hCMEC/D3 barrier. The apical surfaces of the hCMEC/D3 monolayers were challenged with \textit{S. suis} serotype 2 strain 05ZYH33 at a multiplicity of infection (MOI) of 100, samples were taken from the basal chambers of the Millicell\textsuperscript{®} inserts at 25 min post-infection (p.i.) to exclude the possible cytotoxicity effects caused by suilysin. Colony plate counts indicated that viable \textit{S. suis} 05ZYH33 cells that showed successful traversal constituted about 4% of the input (Fig. 1A). The infected monolayers were fixed and the integrity of the layers was examined by transmission electron microscopy. As shown in Fig. 2, the bacteria were often present at the margins of the cells and the tight junction structures were apparently disrupted/opened with gap formation. Laser scanning confocal microscopy (Fig. S2) also showed that \textit{S. suis} bacteria were present at the margins of the cells, indicating that \textit{S. suis} traverses the hCMEC/D3 cell monolayer via a paracellular route.

Next, we investigated which virulence factors might be involved in the traversal of \textit{S. suis} across the hCMEC/D3 monolayer. Using this barrier model, we screened the function of some surface proteins such as Fhb, SSU05\_1815, SSU05\_1664 and SSU05\_0186, which were identified as potential virulence factors in our previous study.\textsuperscript{19} We found that deletion of genes SSU05\_1815 or SSU05\_1664 or SSU05\_0186 did not affect the traversal ability of \textit{S. suis} (Fig. S1D). However, deletion of the gene \textit{fhb} significantly reduced the bacterial count in the basolateral chamber, and the complementation strain of the \textit{fhb} mutant showed restored traversal to the level of the wild-type strain 05ZYH33 (Fig. 1A). In addition, we found that blocking \textit{S. suis} with the Fhb-specific antibody could significantly reduce the traversal rate of \textit{S. suis} 05ZYH33 (Fig. 1B). These
results indicated that S. suis strain 05ZYH33 could traverse the hCMEC/D3 barrier early after infection and that Fhb contributes to this process.

Gb3 is involved in Fhb-mediated S. suis traversal across the hCMEC/D3 monolayer

Previous studies revealed that Fhb via its N terminus binds the Galα1–4Gal-oligosaccharides of glycolipid receptor Gb3.24 To investigate whether the interactions between Gb3 and Fhb could affect the adherence of S. suis to the hCMEC/D3 monolayer, we inhibited the Gb3 synthesis by incubation of cells for 3 d with 5 μM D-threo-1-phenyl-2-palmitoylarnino-3-morpholino-1-propanol (PPMP) as detected by flow cytometry (Fig. S3).35 As shown in Fig. 3A, the adherence of S. suis 05ZYH33 to the PPMP-treated hCMEC/D3 cells was significantly decreased compared with untreated hCMEC/D3 cells, whereas there was no significant change in the adherence of the fhb mutant to PPMP-treated cells compared with untreated hCMEC/D3 cells. These results indicated that the binding of Fhb to Gb3 may contribute to the adherence of S. suis 05ZYH33 to hCMEC/D3 cells.

To investigate the role of Gb3 in the traversal of S. suis across the hCMEC/D3 monolayer, we added Galα1–4Gal disaccharide (final concentration 0.5 mM), a competing ligand to Gb3, to the upper chamber of Millicell® inserts during the bacterial infection. As shown in Fig. 3B, Galα1–4Gal disaccharide significantly reduced the traversal of S. suis 05ZYH33 across the hCMEC/D3 monolayer, but had no significant influence on the traversal of the S. suis 05ZYH33 fhb-deletion mutant. These results indicated that binding of Fhb to Gb3 may contribute to the traversal of S. suis 05ZYH33 across the hCMEC/D3 monolayer.

Gb3 deficiency protects the mice from S. suis meningitis

To examine the role of Gb3 in S. suis meningitis, we constructed Gb3-deficient mice by disrupting the A4galt gene using the TALEN-mediated knockout method.36 After DNA sequencing, 10 of the offspring were found to carry the A4galt deletion mutants. Three of these 10 mice carried frameshift mutations and were selected for further breeding and characterization. Homozygous Gb3-deficient (A4galt−/−) mice
were healthy, fertile, and did not display any physical abnormalities.

We infected the wild-type C57BL/6 mice and the A4galt<sup>-/-</sup> mice intraperitoneally with 5 × 10<sup>6</sup> colony forming units (CFU) of S. suis 05ZYH33 and monitored their survival over time (Fig. 4A). At day 1 to day 2 p.i., 4 of the 14 wild-type mice (28.6%) died, but all of the Gb3-deficient mice and 71.4% of the wild-type mice survived until the end of the 10-day monitoring period. We repeated this experiment with S. suis strain S735, an ST1 European reference strain isolated from a diseased pig with meningitis, and also found that the wild-type C57BL/6 mice deteriorated rapidly and died during the first day after infection, but 66.7% of the Gb3-deficient mice survived the 5-day monitoring period (Fig. S4A). These results indicated that Gb3 deficiency might protect mice from septicemia.

Most of the surviving wild type-mice infected with S. suis 05ZYH33 developed apparent symptoms of meningitis, such as moderate or severe lethargy, rear limb weakness and an inability to walk. All Gb3-deficient mice infected with S. suis 05ZYH33 infection survived without severe behavioral abnormalities, or recovered quickly from lethargy within one to 2 d. We also recorded the weight loss of infected mice and found that surviving wild-type mice and Gb3-deficient mice showed similar weight loss curves (Fig. 4B). At day 2 p.i., both surviving wild-type mice and Gb3-deficient mice suffered high weight loss from their initial body weight. At day 8 p.i., the remainder of the surviving mice had recovered their initial body weight.

After intraperitoneal infection with S. suis 05ZYH33, both wild-type mice and Gb3-deficient mice developed bacteremia quickly, as demonstrated by the recovery of bacteria from infected mice at 1, 3 and 5 h p.i. (Fig. 4C). The bacterial loads in the blood of wild-type mice and Gb3-deficient mice decreased at 24 h p.i., then remained constant up to 72 h p.i. (~1 × 10<sup>4</sup> CFU/mL). There were no statistical differences in the bacterial loads in the blood of wild-type mice and Gb3-deficient mice at different p.i. time points. The bacterial loads in different organs were also detected and no statistically significant differences between perfused mice and unperfused mice were found at day 3 (Fig. S4B) and day 5 (Fig. S4C p.i.; subsequent studies were therefore performed in unperfused mice. Bacterial loads in the livers (Fig. 4D) and spleens (Fig. 4E) were essentially identical between wild-type mice and Gb3-deficient mice. However, the bacterial loads in the brains of wild-type mice were significantly higher than in Gb3-deficient mice from 48 to 120 h p.i. (Fig. 4F).

Histologically, the brains of wild-type mice and Gb3-deficient mice injected with PBS showed normal morphology with no signs of inflammation or injury (Fig. 5A and 5B). By contrast, the brains of wild-type mice infected with S. suis demonstrated the classical features of meningitis, such as meningeal thickening (Fig. 5D, G), hemorrhage (Fig. 5E, H) and inflammatory cell infiltration (Fig. 5F, I), that were absent or less severe in Gb3-deficient mice infected with S. suis (Fig. 5C). Semi-quantitative histological assessment (Fig. S4D and Table S1) also demonstrated that S. suis developed more severe brain inflammation or injury in wild-type mice than that in Gb3-deficient mice both at 72 and 120 h p.i.

**S. suis infection induces phosphorylation of myosin light chain 2 in a Gb3-dependent manner**

Using the murine model of S. suis meningitis, we found that S. suis infection caused substantial hemorrhage in the parenchyma of wild-type mice.
but not in the Gb3-deficient mice (Fig. 5C). We observed cerebral parenchymal hemorrhage in 66.7% of mice at 72 h p.i. and 71.4% of mice at 120 h p.i. in the wild-type group, but only 16.7% and 12.5% of mice respectively in the Gb3-deficient group (Table S1). These results indicated that the Fhb-Gb3 interaction might affect S. suis infection-induced permeability changes in the endothelial barrier. To test this hypothesis, we infected the hCMEC/D3 monolayer with S. suis 05ZYH33 or the fhb-deletion mutant, and investigated changes in the TEER and the permeability of the hCMEC/D3 monolayer to Lucifer Yellow (LY), a marker of passive paracellular diffusion. Compared with wild-type infection, infection with the fhb-deletion mutant significantly reduced the increase in LY permeability coefficients (Fig. 6A) and restored the decrease in the TEER (Fig. 6B) induced by S. suis 05ZYH33.

Figure 4. Gb3 deficiency contributes to the survival of S. suis meningitis. Wild-type C57BL/6 mice and A4galα knockout mice were injected intraperitoneally with 5 × 10⁶ CFU S. suis 05ZYH33. (A) Kaplan–Meier survival curve. The survival of mice (n = 14 per group) was monitored and analyzed using the Log-rank test, *P < 0.05. (B) The weight loss curve of mice after S. suis 05ZYH33 infection (n = 14 per group). (C–F) From a total of 38 wild-type and 40 A4galα knockout mice infected by S. suis, samples were taken aseptically of the blood (C), liver (D), spleen (E) and brain (F), and homogenated to perform colony plate counts. Each point represents one animal. The differences between wild-type and Gb3-deficient mice were analyzed using the Mann–Whitney test, *P < 0.05.

It has been established that the paracellular permeability of endothelial cells can be modulated by different molecular mechanisms ranging from the regulation of actomyosin contraction and actin polymerization to the direct regulation of junctional membrane proteins. Among them, the phosphorylation of myosin light chain 2 (MLC2) is associated with increased endothelial permeability. In this study, we examined the MLC2 phosphorylation of Ser-19 by western blotting. As shown in Fig. 6C, S. suis 05ZYH33 infection and Fhb protein treatment induced a significant increase in MLC2 Ser-19 phosphorylation in hCMEC/D3 cells, but not in Gb3-deficient hCMEC/D3 cells (PPMP-treated), whereas infection with the fhb-deletion did not cause significant MLC2 phosphorylation in hCMEC/D3 cells with or without Gb3. These results suggested that interactions between Fhb and Gb3 might activate...
some signaling effectors to phosphorylate and activate MLC2.

It has been shown that both myosin light chain kinase (MLCK) and Rho-associated protein kinase (ROCK) can induce the phosphorylation of MLC2. We therefore used the ROCK inhibitor Y-27632 and myosin light chain kinase inhibitor ML-7 to determine which kinase can mediate the activation of MLC2 phosphorylation induced by S. suis infection.

The inhibitors, at the concentrations used, exhibited no cytotoxicity to hCMEC/D3 cells (Fig. S5). Confluent hCMEC/D3 monolayers were pretreated with Y-27632 or ML-7 for 1 h, then infected with S. suis 05ZYH33 or vehicle alone for 30 min. Similar to Gb3-deficient cells (PPMP-treated), the S. suis infection-induced increase in MLC2 phosphorylation was significantly inhibited in Y-27632-treated hCMEC/D3 cells, but not in ML-7-treated cells (Fig. 6D). These results indicated that S. suis infection may induce Gb3-dependent activation of MLC2 through Rho/ROCK signaling.

Discussion

The interaction between S. suis and the BBB is a crucial process in the development of S. suis meningitis. Studies have shown that various S. suis virulence factors, such as SrtA,41 DltA,42 Sseno43 and MRP,13 are involved in the interactions between S. suis and BMECs, either directly or indirectly,44,45 and several genes were preferentially expressed by S. suis upon interaction with BMECs.46 In addition, S. suis could acquire plasmin activity when in contact with cultured hBMECs and induce the release of arachidonic acid47,48 or the shedding of adhesion molecules from the cell surface of the hBMECs,49 although the molecular mechanisms for this remained to be elucidated. Our previous study also showed that suilysin could remodel the cytoskeleton of hBMECs by activating RhoA and Rac1 GTPase.16 These bacterial and/or cellular factors might be necessary for S. suis to overcome the BBB.

Meningeal pathogens can cross the BBB transcellularly, paracellularly and/or by the Trojan-horse mechanism.50 In this study, we demonstrated a S. suis serotype...
strain could paracellularly translocate the hCMEC/D3 cell monolayer and that the Fhb-Gb3 interaction contributed to regulation of the permeability of the BBB. Our in vitro results were in accordance with previous studies that showed that S. suis serotype 2 strains could adhere to human and porcine BMECs, but not invade human BMECs. A recent study also showed that S. suis translocated across human intestinal epithelial cells predominantly via a paracellular route.

In this study, we also demonstrated that the Fhb-Gb3 interaction contributed to the development of S. suis meningitis. Through recognition of E. coli PapG adhesion, Pseudomonas aeruginosa lectin I (LecA) and verotoxins, Gb3 plays an important receptor role in pathogen-induced infections. For example, the LecA-Gb3 interaction could sufficiently trigger plasma bending of host cells to allow for uptake of P. aeruginosa. Another study reported that the PapG-Gb3 interaction could activate the ceramide signaling pathway to induce cytokine responses in infected cells. These studies and our present results support the hypothesis that Gb3 provides sensitivity to bacterial infection and is involved in the pathogenesis.

Besides brain endothelial cells, Gb3 is also highly expressed in renal endothelial cells. We speculate Fhb-Gb3 interaction might contribute to the stable colonization in kidney because the high volume of blood flow and filtration rate could increase the chance of Fhb interaction with cells of the renal microvasculature and the filtration barrier. Previous studies and our results (Fig. S4B, S4C) also showed that S. suis could accumulate in mice kidney during infection. Indeed, kidney was one of prominently involved organs according to the autopsy report of cases died from Streptococcus suis septicemia.

We report, for the first time, that the Fhb-Gb3 interaction affects the activation of MLC2 through Rho/ROCK signaling. Phosphorylation of MLC2 plays a critical role in controlling actomyosin contractility in both smooth muscle and nonmuscle cells. ROCK can induce acto-myosin contractility by direct phosphorylation of MLC2 or the myosin phosphatase target subunit resulting in the inhibition of myosin phosphatase and hence hyperphosphorylation of MLC2. We supposed that MLC2 phosphorylation induced by Fhb-Gb3 interaction might play an
important role in the permeability change in BMECs. Related to this hypothesis, we found that S. suis infection caused Gb3-dependent severe hemorrhage in the brain parenchyma of mice. Future studies will aim to further elucidate the pathogenic process.

Since Fhb-Gb3 interaction is important to establish S. suis infection, we speculate that Fhb is a promising target for anti-adhesion therapy. Some carbohydrate-based anti-adhesive compounds could be developed to inhibit the infection of S. suis. Indeed, derivatives of galabiose, as well as polyvalent galabiose dendrimers could successfully inhibit the adhesion of S. suis in the nanomolar range.63 Besides, as an immunogenic protein, Fhb (HP0272, SSU0253) also showed good protection in mice and/or pigs against S. suis challenge.20,21

In summary, our results demonstrated, for the first time, that S. suis could traverse across the human in vitro BMECs monolayer through interactions with Fhb and Gb3 on the cell surface. This interaction was also important for the development of S. suis meningitis and subsequent disease progression.

Materials and methods

Ethics statement

All animals used in this study were housed at the animal center of the Academy of Military Medical Sciences (AMMS). Animals were cared for in accordance with the principles of laboratory animal care approved in China. All experimental procedures were approved by the Institutional Animal Care and Use Committee of AMMS.

Reagents

Galα1–4Gal disaccharide (G154755) was synthesized by Toronto Research Chemicals. PPMP, ML-7 and Y27632 were purchased from Sigma-Aldrich. Anti-CD77 antibody [38–13] (ab 19795), rat IgM kappa monoclonal [RTK2118]-isotype control (ab35768) and goat anti-rat IgM mu chain (Dylight® 488) preadsorbed antibody (ab98368) were purchased from Abcam. Phospho-myosin light chain 2 (ser19) mouse monoclonal antibody (#3675) and myosin light chain 2 rabbit monoclonal antibody (#3672) were purchased from Cell Signaling Technology. Anti-ZO-1 rabbit polyclonal antibody and Alexa Fluor 594 labeled goat anti-rabbit IgG antibody were purchased from Life Technologies.

Bacterial strains and cell line

S. suis strain 05ZYH33, Δfhb and CΔfhb have been described previously.18 The hCMEC/D3 cell line was obtained under license from INSERM, France, and cultured as described previously.54 To construct the in vitro BBB model, the cells (passage 25–35) were seeded at a density of 50,000 cells per cm² on collagen-coated Millicell®-PET inserts with 3 μm or 0.4 μm porosity and cultured for 7–8 d at 37°C in 5% CO₂.

Evaluation of bacterial traversal of the in vitro blood–brain barrier

The ability of S. suis to traverse the monolayers of hCMEC/D3 cells was tested using cells grown on Millicell® inserts of 3 μm pore size. The hCMEC/D3 medium was replaced with EBM-2 medium supplemented with 0.25% fetal calf serum without antibiotics 24 h before the infection. On the day of infection, mid-log phase S. suis cells were washed with PBS once, resuspended in EBM-2 medium, then added to the apical chamber of the inserts at a MOI of about 100, and the cultures were incubated for 25 min. After infection, the number of S. suis that traversed the insert membrane was counted by collecting the cell culture media from the basolateral chamber and plating the media onto THB agar.

Adhesion assay

The adhesion assay was performed as described previously.13,51,53 Briefly, mid-log phase S. suis were pelleted, washed once with PBS, and resuspended in EBM-2 medium without antibiotics. Confluent monolayers of hCMEC/D3 cells grown in 24-well plates were infected with the bacterial suspension at a MOI of 100:1. The plates were centrifuged at 800 × g for 10 min to bring the bacteria to the surface of the monolayer and incubated for 2 h at 37°C with 5% CO₂. After this incubation period, the cell monolayers were washed 4 times with PBS and then lysed with 0.1% saponin on ice for 20 min. The number of cell-adherent bacteria was determined by plating appropriate dilutions of the lysate on THB agar plates. Bacterial adherence was calculated as: (recovered CFU/initial inoculum CFU) × 100%.

Flow cytometry

HCMEC/D3 cells were stained with anti-CD77 antibody for 30 min at room temperature, followed by 3 times washes in staining buffer. The cells were then incubated with anti-rat IgM mu chain (Dylight® 488) for 30 min at room temperature. A control using isotype-matched antibody (rat IgM kappa monoclonal [RTK2118]) was performed in parallel. Flow cytometric acquisition was performed on FACSCalibur flow cytometer (BD Biosciences). The data was analyzed using FlowJo software.
**Cytotoxicity assay**

Lactate dehydrogenase (LDH)-release from hCMEC/D3 cells in the presence of Y27632 (10 μM) and ML-7 (1 μM) for 1 h, and PPMP (5 μM) for 3 days, was detected using a CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. The percentage of cytotoxicity was calculated as: LDH released in test sample (A490)/ maximum LDH release (A490) × 100. The maximum release was determined as the amount released by total lysis of untreated cells with 0.1% Triton X-100.

**Staining and microscopy assay**

Immunofluorescence staining was performed as described previously.16 An Olympus FV1000 confocal microscope was used for collecting fluorescence images. For transmission electron microscopy, sample preparation was performed as described previously.65 The ultrathin sections were examined with a Hitachi H1650 transmission electron microscope.

**Generation of Gb3-deficient mice by TALEN**

Exon 3 of the A4galt gene (ensemble ID: ENSMUSG0000047878) was selected as a Transcription Activator-Like Effector Nucleases (TALEN) target site. Three pairs of TALEN plasmids were constructed to bind exon 3 of the A4galt locus (Fig. S6A). TALENs were constructed using the Solid-phase Synthesis method by a commercial service (Viewsolid Biotech Company, China) and confirmed by sequencing. The 3 synthesized TALENs were evaluated for sequence-specific binding activity by the luciferase single-strand annealing (SSA) recombination assay and showed higher cleavage activity to the target site (Fig. S6B). Then, in vitro-transcribed mRNA from the TALENs was microinjected into fertilized eggs to generate A4galt knockout mouse. The founders were genotyped by PCR followed by DNA sequencing analysis. The C-base deletion at the 47th base downstream of the ATG site in the A4galt gene locus was identified (Fig. S6C) and thus mutated the truncated Gb3 synthase after Pro16 (Fig. S6D). The positive founders were bred to the next generation.

**Luciferase single-strand annealing (SSA) recombination assay**

The luciferase SSA recombination assay was performed according to described previously methods.66 The relative luciferase activity was measured using a dual-luciferase assay system (Promega) and detected on a GloMax® 96 Microplate Luminometer (Promega). Each experiment was repeated 3 times.

**Immunoblot analysis**

Cell lysis and immunoblot analysis were performed as described previously.16 Antibodies used for immunoblotting included rabbit anti-MLC2 antibody (1:1,100) and mouse anti-p-MLC2 (Ser19) (1:1,000).

**Experimental design of the mouse model of S. suis meningitis**

S. suis infection in adult C57BL/6 mice was performed as described previously with some modifications.67 Six- to 8-week-old (17 ± 2 g) A4galt-deficient mice or wild-type littermate controls were used and divided into 4 groups: (1) wild-type mice injected intraperitoneally with 1 mL of PBS (C57BL/6, n = 8); (2) wild-type mice injected intraperitoneally with S. suis (C57BL/6, n = 38); (3) Gb3-deficient mice (A4galt−/−, genetic background C57BL/6, n = 6) injected intraperitoneally with 1 mL of PBS; and (4) Gb3-deficient mice (A4galt−/−, genetic background C57BL/6, n = 6) injected intraperitoneally with 1 mL of PBS of S. suis (C57BL/6, n = 40). Prior to infection, mid-log phase S. suis strain 05ZYH33 was washed once and diluted in PBS. Mice were injected intraperitoneally with 5 × 10⁶ CFU bacteria in a 1-mL volume. At 1, 3, 5, 24, 48 and 72 h p.i., vein blood (10 μL) from infected mice was collected. At 24, 48, 72 and 120 h p.i., the organs (brain, liver and spleen) were harvested aseptically and homogenized with a Dounce homogenizer. In some experiments, mice were subjected to cardiac perfusion with PBS before tissue collection as described previously.68 The number of viable bacteria in the inoculum, blood and organ homogenates was determined by colony plate counts. To evaluate survival, wild-type and Gb3-deficient mice (14 mice per group for strain 05ZYH33, 6 mice per group for strain S735) were infected intraperitoneally with 5 × 10⁶ CFU of S. suis in 1 mL THY medium (THY plus 0.2% yeast extract), and mortality was recorded daily for 10 or 5 days, respectively.

**Histopathological assessment**

At day 3 or 5 p.i., 6 to 8 mice were randomly chosen from each experimental group (A4galt−/− and wild type controls) and killed. The brains were quickly removed and fixed with 4% paraformaldehyde. Sections were stained with hematoxylin and eosin and analyzed for brain injury using an Olympus BX53 microscope. To score brain inflammation and injury, a semi-quantitative
Scoring system was used as described previously. Each section was evaluated blindly for inflammation by 2 examiners (H.H. and D.K.). The degree of meningeal inflammation was scored according to the following parameters: meninges thickening, neutrophil infiltration and hemorrhage. Brain parenchymal inflammation and injury were scored according to the following parameters: neutrophil infiltration and hemorrhage. Each parameter was graded using a 0, 1, 2, 3 point system with 0 indicating no, 1 mild, 2 moderate, 3 marked histopathologic changes. The total “meningitis score” was expressed as the sum of the scores for each parameter, the maximum being 15.

Statistical analysis

All data were analyzed using GraphPad Prism software. The in vitro experimental data were analyzed using one-way or 2-way ANOVA analysis followed by Bonferroni’s multiple comparisons test or Holm–Sidak’s multiple comparisons test. In the mouse experiments, Kaplan–Meier method was used to draw survival curves and a log-rank test was used to compare survival rates. Mann–Whitney U tests were used to compare bacterial loads in blood and organ samples. For all tests, a P value < 0.05 was considered significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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