

Journal Pre-proof

A critical role for host-derived cystathionine- β -synthase, in *Staphylococcus aureus*-induced udder infection

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PII: S0891-5849(23)01094-8

DOI: <https://doi.org/10.1016/j.freeradbiomed.2023.11.001>

Reference: FRB 16234

To appear in: *Free Radical Biology and Medicine*

Received Date: 2 September 2023

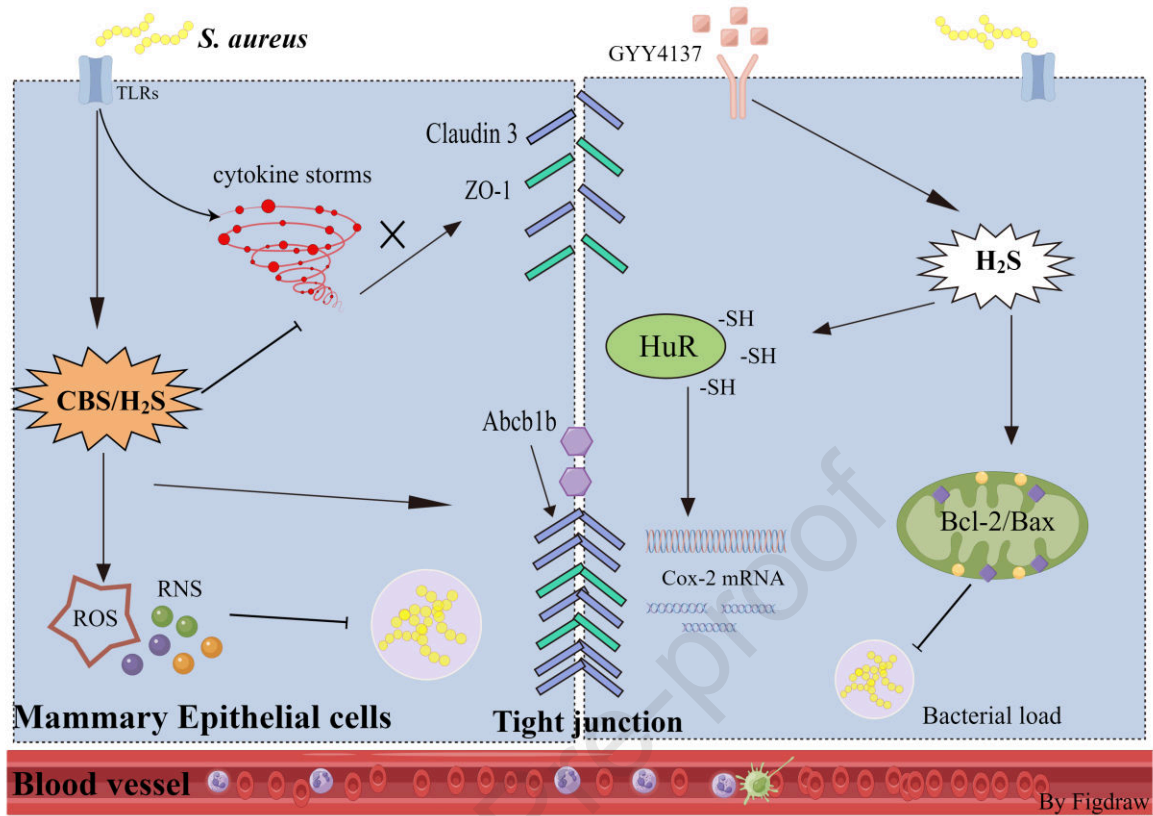
Revised Date: 31 October 2023

Accepted Date: 4 November 2023

Please cite this article as: S. Fu, B. Yang, Y. Gao, Y. Qiu, N. Sun, Z. Li, S. Feng, Y. Xu, J. Zhang, Z. Luo, X. Han, J. Miao, A critical role for host-derived cystathionine- β -synthase, in *Staphylococcus aureus*-induced udder infection, *Free Radical Biology and Medicine* (2023), doi: <https://doi.org/10.1016/j.freeradbiomed.2023.11.001>.

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ABSTRACT:

Cystathionine- β -synthase (CBS) catalyzes the first step of the transsulfuration pathway. The role of host-derived CBS in *Staphylococcus aureus* (*S. aureus*)-induced udder infection remains elusive. Herein, we report that *S. aureus* infection enhances the expression of CBS in mammary epithelial cells *in vitro* and *in vivo*. A negative correlation is present between the expression of CBS and inflammation after employing a pharmacological inhibitor/agonist of CBS. In addition, CBS achieves a fine balance between eliciting sufficient protective innate immunity and preventing excessive damage to cells and tissues preserving the integrity of the blood-milk barrier (BMB). CBS/H₂S reduces bacterial load by promoting the generation of antibacterial substances (ROS, RNS) and inhibiting apoptosis, as opposed to relying solely on intense inflammatory reactions. Conversely, H₂S donor alleviate inflammation via S-sulphydrating HuR. Finally, CBS/H₂S promotes the expression of *Abcb1b*, which in turn strengthens the integrity of the BMB. The study described herein demonstrates the importance of CBS in regulating the mammary immune response to *S. aureus*. Increased CBS in udder tissue modulates excessive inflammation, which suggests a novel target for drug development in the battle against *S. aureus* and other infections.

Keywords: *Staphylococcus aureus*; cystathionine- β -synthase; udder infection; blood–milk barrier

Introduction

Mastitis is the most costly infectious disease of dairy cows. *Staphylococcus aureus* (*S. aureus*) is one of the most important etiologic agents of mastitis [1, 2]. The intracellular survival nature of *S. aureus* is one of the key reasons why mastitis caused by it is so hard to cure. Unfortunately, there are currently no effective vaccines for *S. aureus* infections and antibiotics continue to be the mainstay of treatment. Antimicrobial resistance of *S. aureus* is believed to have a severe negative impact on human health [3, 4]. Therefore, it is essential to identify new molecular targets for drug development against *S. aureus*.

The transsulfuration pathway plays a central role in cellular sulfur metabolism and redox regulation. Cystathionine- β -synthase (CBS) catalyzes the first step of the transsulfuration pathway thereby limiting the rate of this fundamental biological process [5, 6]. CBS/hydrogen sulfide (H_2S) is an emerging gaseous signaling molecule that regulates many cellular processes. However, the effects of H_2S are often bell-shaped: at lower concentrations, it stimulates cellular bioenergetics, while at toxic concentrations, it suppresses mitochondrial function [7, 8]. Aberrant CBS expression and/or activity contributes to a wide range of disease development, including down syndrome, various cancers and infectious diseases [9-12]. Recently, mounting evidences focus on the importance of host-derived H_2S in microbial pathogenesis, however, research findings are somewhat controversial. For example, Li *et al.* found H_2S significantly impacts cellular responses and viral replication [13]. Similarly, Renieris *et al.* reported that H_2S affords protection against *Pseudomonas aeruginosa*

sepsis [14]. Conversely, Rahman *et al.* found that H₂S dysregulates the immune response by suppressing central carbon metabolism thus promoting *tuberculosis* [15]. Previous studies have shown that exogenous H₂S can prevent LPS-induced inflammation by blocking the TLR4/NF- κ B pathway and that endogenous H₂S can regulate LPS-induced inflammation through IL-8 signaling both in bovine mammary epithelial cells (MAC-T cells) [16, 17], little attention has been given to the interaction of host derived CBS/H₂S and mastitis pathogens, particularly *S. aureus*.

A previous study pointed out that exogenous H₂S protects the intestinal epithelial barrier function in Caco-2 monolayers from TNF- α and IFN- γ induced injury [18]. Like gut barrier, the blood-milk barrier (BMB) is a natural defense mechanism of mammary tissue that restricts the free exchange of cells and large molecules between blood and milk [19, 20]. The mammary epithelial barrier is formed by tight junctions between the monolayer of secretory mammary epithelial cells. Compromising the integrity of this barrier results in lower milk quality and volume. Additionally, pathogenic bacteria crossing this barrier may cause life-threatening systemic infections [21]. It is crucial to maintain the functional integrity of the BMB for optimal mammary gland health, but there is no data confirming a link between H₂S and the BMB.

And protein S-sulfhydration is a post-translational modification of a hydropersulfide moiety (-SSH) added to specific cysteine residues in targeted proteins [22]. This process is generally facilitated by H₂S and can regulate protein function in a reversible manner that is critical for precise regulation of homeostasis [23-25]. Recent

studies have proven that H₂S facilitates the S-sulfhydration of Human antigen R (HuR) , leading to a decrease in the stability of COX-2 mRNA in colitis, which confirmed the role of S-sulfhydration in epithelial cells [26]. Thus, we are curious to know if S-sulfhydration by H₂S plays a role in preventing mammary epithelial cell infection induced by *S. aureus*.

Herein, we focused on host-derived CBS/H₂S in the pathogenesis of *S. aureus* infections, and found that CBS is associated with inflammation and the BMB. We then investigated the underlying mechanism of action of CBS/H₂S in *S. aureus*-induced udder infection. These findings shed light on the molecular mechanisms involved in antimicrobial defense and regulation by host sulfur metabolism, providing a promising strategy to tackle the growing issue of mastitis.

Materials and methods

Bacterial strains, cell culture, and treatment

S. aureus ATCC 29213 and EpH4-Ev cells were purchased from American Type Culture Collection (Manassas, USA). Bacteria were inoculated into Luria-Bertani (LB) culture and incubated at 37°C on a shaker. Following overnight incubation, 30 µL of cultured material was transferred to 3 mL of fresh LB at 37°C with shaking until the optical density OD₆₀₀ reached 0.6-0.8. EpH4-Ev cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and plated at 80% confluence on 6-well plates. The monolayers were treated with or without 200 µM AOAA and 50 µM SAM for 4 h, 10 µM Vismodegib for 2 h,

or 50 μ M GYY4137 for 24 h at 37°C. The treated cells were infected with *S. aureus* at a multiplicity of infection (MOI) of 10 for 3 h at 37 °C. Supernatants and cells were collected separately and stored at –80 °C until use. Other reagents and materials are listed in **Table S1**.

Mice, genotype identification, and establishment of mastitis model

C57BL/6 (WT or CBS^{+/-}) mice were purchased from Cyagen Biosciences (Suzhou, China). Animals were maintained under standard conditions of humidity (50%) and temperature (25±2°C) in a 12 h light and 12 h dark cycle in the Laboratory Animal Center of Nanjing Agricultural University. Two females and 1 male were housed together in microisolator cages. Specific pathogen free-grade mice breeding fodder and water were supplied *ad libitum*. Offspring were removed from cages 24 h before bacterial administration. The parturient mice were anesthetized by ether and the teats as well as surrounding areas were disinfected with 75% ethanol. The fourth pair of mammary glands were held lightly with fine forceps and 50 μ L *S.aureus* (about 1×10^9 Colony Forming Units [CFU] mL⁻¹) was injected via the mammary ducts using a microsyringe. Twenty four h later, the mice were euthanized. Blood and mammary tissue were collected and stored at –80°C until use. A direct mouse genotyping kit (APExBIO, USA) was used to extract mouse tail DNA, and subsequently amplified using a specific primer pool via Accurate Taq Master mix (AG, Changsha, China). The products of PCR amplification were run on a 1.5% agarose gel to verify their size. All animal experiments were carried out in accordance with the guidelines of the Animal Welfare Council of China and approved by the

Ethical Committee for Animal Experiments of Nanjing Agricultural University (NJAU. No20220509099).

Lentivirus plasmid transfection and RNA sequencing

EpH4-Ev cells were transfected with CBS and mock vector and stably overexpressed cell lines were constructed after selection with puromycin. The expression effect was detected by qPCR. RNA sequencing was performed at Microeco Tech Co. Ltd. (Shenzhen, China). Briefly, RNA was extracted from cells using TRIzol reagent (AG, Changsha, China) and quality analysis was conducted using a Bioanalyzer 2100 (Agilent, Santa Clara, USA). Subsequently, PCR was conducted utilizing Phusion High-Fidelity DNA polymerase, universal PCR primers, and index (X) primer. The amplified PCR products were purified via the AMPure XP system and assessed for quality using a Bioanalyzer 2100. Following cluster generation, library preparation sequencing was done on an Illumina Novaseq platform, thereby generating 150 bp paired-end reads.

Assessment of biochemical parameters and relative enzyme activities

The protein samples from EpH4-Ev cells were extracted using cold lysis buffer and assayed using a BCA Protein Assay Kit. The levels of lactic dehydrogenase (LDH) in cell supernatant were measured using commercial kits according to the manufacturer's instructions. Pro-inflammatory factors (TNF- α and IL-1 β) in serum were measured by ELISA kit according to the manufacturer's instructions.

Viable bacterial count assay

The infected cells were washed thrice with PBS containing 100 µg/mL gentamicin for 15 min each at 37°C, followed by a subsequent wash with gentamicin-free PBS. The cells and the supernatant were collected, and an equal number of cells from each group were lysed with sterile water. Then, the samples were serially diluted 10 fold, and 100 µL aliquots of each dilution were inoculated on the LB plate media. CFUs were enumerated via the spread plate method after an 18 h incubation at 37°C.

Histopathologic assay

Tissues were preserved in 10% neutral buffered formalin, then washed with water before dehydration in alcohol solutions ranging from 75 to 100%, with a 5% increase every hour. Next, the tissue was soaked in xylene and embedded in wax for 3 h at 60°C. Three µm sections were stained with hematoxylin and eosin. The resulting histological changes were characterized by light microscopy.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent and subjected to reverse transcription using reverse transcriptase (AG, Changsha, China). An aliquot of the resulting cDNA was mixed with 5 µL TB Green PCR Master Mix (AG, Changsha, China) and 0.2 µL of each specific forward and reverse primer. The mixed systems were then analyzed using a Roche LightCycler 96. The reference gene was β -actin and fold changes were calculated as $2^{-\Delta\Delta C_t}$. All primer sequences (listed in **Table S2**) were synthesized by Tsingke Biological Company (Nanjing, China).

Total protein extraction and Western blotting

The cells and corresponding tissues were washed thrice with ice-cold PBS. They were lysed by incubating with RIPA lysis buffer, containing protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime, Nantong, China). The lysates were centrifuged at $12,000 \times g$ at 4°C for 15 min and supernatants collected. Protein concentration was determined using a BCA kit (Beyotime, Nantong, China). SDS loading buffer was added to the protein samples and then boiled at 100°C for 5 min. Separation of protein samples was done via 12.5% SDS-PAGE, and blotted onto PVDF membranes. To prevent nonspecific binding, the membranes were blocked with 10% non-fat powdered milk for 2 h. The blot was then probed overnight with relevant primary antibody at 4°C . After washing, the blots were incubated with HRP-conjugated secondary antibody at room temperature for 2 h. Signal detection was carried out using an ECL Western Blot Analysis System (Tanon, Shanghai, China). Bands were quantified using ImageJ 1.8 software (NIH, Bethesda, MD, USA).

Immunofluorescent staining

The cell-laden slides were fixed with 4% formaldehyde for 15 min, followed by 3 washes with PBS. Cell membranes were permeated with PBS containing 0.5% Triton-100 for 20 min. Subsequently, the cells were blocked with 5% BSA for 2 h at RT. The slides were incubated overnight at 4°C with diluted primary antibody. After washing, a secondary antibody was added and the slides were incubated for 2 h at RT. Following another 3 washes with PBS, the cells were exposed to $10 \mu\text{M}$ DAPI for 10

min. The slides went through another 3 washes with PBS, after which an anti-fluorescence quenching sealer was applied and the cells were photographed with an inverted fluorescence microscope (Invitrogen).

Immunohistochemistry

Paraffin-embedded specimens were deparaffinized in xylene. They were then hydrated using a series of graded ethanols and placed in 0.01 mol/L citrate buffer (pH 6.0). Subsequently, the samples were heated in a microwave oven over medium-high heat for 10 minutes, followed by incubation with a 3% H₂O₂-methanol solution at RT for 10 min. After primary and HRP-conjugated secondary antibody incubation, diaminobenzidine was used as a chromogen and hematoxylin as a counterstain. The resultant sections were observed with a light microscope and photographed.

Detection of reactive oxygen species (ROS)

The intracellular ROS of EpH4-Ev cells was stained by DCFH-DA according to the instructions (Beyotime, Nantong). Briefly, cells were loaded with 10 μ M DCFH-DA for 30 min at 37°C and washed 3 times with PBS. The entire process was protected from light. The cell samples were immediately analyzed by inverted fluorescence microscopy (Invitrogen).

Biotin switch assay of S-sulfhydration

A biotin switch assay was conducted with standard procedures [27]. The cells were lysed by incubating with RIPA lysis buffer containing PMSF. The lysates were centrifuged at 12,000 $\times$ g at 4°C for 15 min and supernatants collected. Protein concentrations were measured via BCA assay. Next, the lysates were added to HEN

buffer (250 mM HEPES-NaOH supplemented with 1 mM EDTA and 0.1 mM neocuproine) supplemented with 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS). The samples were shaken at 50°C for 30 min. Acetone was added to remove MMTS, and proteins precipitated by storing them at -20°C for 30 min. The pellets were resuspended in HENS buffer containing 4 mM biotin-HPDP. After incubating overnight at RT, the proteins were purified with acetone. Finally, the proteins were dissolved in HENS buffer, purified with streptavidin magpoly beads and subjected to western blotting with an anti-HuR antibody.

Statistical analyses

Data were expressed as the mean±standard error and analyzed by independent samples t-tests or one-way ANOVA followed by the Tukey's multiple comparison test as appropriate. All statistical analyses was performed using GraphPad Prism version 8.0.1 software. A value of $P<0.05$ indicated statistical significance.

Results

CBS expression is elevated in *S. aureus* mastitis

Firstly, we analyzed the relative expression of three crucial enzymes involved in the transsulfuration pathway in mammary gland and EpH4-Ev cells under normal state, including cystathionine c-lyase (CSE), 3-mercaptopyruvate sulphur transferase (3-MPST) and CBS. We found that CSE was primarily expressed in the cells, while CBS dominated in the tissue (**Figure 1A-B**). However, when EpH4-Ev cells were exposed to *S. aureus*, we observed that CBS gene expression was increased by hundreds times, but CSE and 3-MPST presented downward trend and displayed

small amplitude variations (**Figure 1C-E**). Since CBS plays a role in the conversion of homocysteine to cystathionine, which is a crucial precursor for the production of glutathione (a classic antioxidant), protein synthesis, and H₂S production, it suggests that CBS is a major contributor to the production of H₂S during this period (**Figure 1F**). Further analysis revealed a sharp increase in CBS protein expression in both EpH4-Ev cells and MAC-T cells. (**Figure 1G**). To validate *in vitro* findings, we established a *S. aureus*-induced mastitis model. Western blot analyses and qPCR assays demonstrated higher CBS levels after *S. aureus* stimulation (**Figure 1H-I**). To further corroborate the results described above, we performed immunohistochemistry on mammary tissue sections after *S. aureus* challenge (**Figure 1J**). Overall, these experiments demonstrate that up-regulation of CBS is induced by *S. aureus* both *in vitro* and *in vivo*.

CBS expression is correlated with *S. aureus*-induced inflammatory injury

To gain mechanistic insights into CBS, we used CBS inhibitor aminooxyacetic acid (AOAA) and agonist s-adenosyl methionine (SAM) to treat EpH4-Ev cells. Since the NF- κ B pathway plays an important role in oxidative stress and inflammation, we tested marker proteins of this signaling pathway. Western blot was used to determine the protein expression of I κ B α , p-I κ B α and (p)65. The data indicated markedly increased phospho-p65/p65 and phospho-I κ B/I κ B ratios (**Figure 2A-C**), followed by a decrease in total I κ B α in the AOAA-treated group compared with the control group (**Figure 2D**). This phenomenon was reversed after pretreatment with SAM. We also documented changes in the expression of

inflammation-related molecular markers. The gene expression of pro-inflammatory factors, except IL-6, was up-regulated after AOAA administration compared with control groups. SAM treatment can significantly decrease gene expression of pro-inflammatory factors, particularly TNF- α . To that end, lactate dehydrogenase (LDH) release may serve as a potential marker of cell injury and death. We documented the release of LDH in culture medium in agreement with previous observations. AOAA can exacerbate EpH4-Ev cells damage and SAM largely reverses these changes (**Figure 2E**). Taken together, our study reveals that CBS may enhance the host defense response to *S. aureus*, thus attenuating inflammatory injury.

CBS agonist reduces bacterial burden by an inflammation-independent pathway

The inflammatory response is a double-edged sword since it has the power to cause damage to an organism while also possessing the capability to eliminate harmful intracellular bacteria [28]. Reactive oxygen (ROS) and nitrogen species (RNS) have intracellular bactericidal properties. Our data indicate that CBS inhibition exacerbates inflammatory responses. We therefore studied this effect on *S. aureus* growth and ROS/RNS production in EpH4-Ev cells. In addition, we examined the effect of CBS on inducible nitric oxide synthase (NOS2) expression on both gene and protein levels. Unexpectedly, we found almost complete abolition of NOS2 by AOAA. In contrast, the addition of SAM significantly promotes NOS2 expression (**Figure 3A-C**). Then we evaluated plate counts of intracellular bacteria after gentamicin incubation to kill extracellular bacteria, and

found that there was a significant decline in mean bacteria loads after treatment with AOAA comparing with control groups. These results are the opposite of what we had expected no significant difference between the AOAA and SAM groups (**Figure 3D-E**). This may be due, in part, to intense inflammation resulting in a decrease of mean bacteria loads post AOAA administration. However, alternative inflammation-independent pathways may exist to compensate for this physiological process in SAM treatment groups. Finally, we quantified intracellular ROS levels. We observed essentially the same data. Cell morphology using bright field microscopy indicated that the AOAA group underwent cell shrinkage compared to the other groups. This surprising outcome suggests that CBS may ameliorate harmful inflammatory responses while promoting otherwise protective innate responses.

CBS contributes to the maintenance of BMB integrity

In order to confirm whether AOAA pretreatment can cause a stronger inflammatory response that exacerbates pathological disorders we measured the expression of ZO-1 and Claudin 3 by western blot analysis. We found a significant downward trend in ZO-1 and Claudin 3 levels with AOAA, whereas SAM displayed the opposite effect (**Figure 4A-C**). Consistent with these results, as presented in **Figure 4D**, ZO-1 immunofluorescent staining also showed decreased expression of ZO-1 with AOAA pretreatment, but effectively recovered ZO-1 expression after SAM pretreatment of *S. aureus* infected EpH4-Ev cells. As a

result, we propose that CBS may contribute to the maintenance of BMB integrity of inflammation-induced permeability.

CBS induced *Abcb1b* expression is critical for improving BMB integrity

Since CBS can affect the BMB, what could be the underlying mechanism? We created CBS overexpression in EpH4-Ev cells and confirmed that it does (**Figure 5A-B**). Subsequently, we discovered that *S. aureus* infection causes a considerable decrease in the expression of ZO-1 and Claudin 3 tight junction proteins. CBS in EpH4-Ev cells exhibits greater resistance to *S. aureus* infection, particularly an almost complete restoration of Claudin 3 protein expression (**Figure 5C**). Transcriptomic data suggests that normal EpH4-Ev cells display 593 up-regulated and 992 down-regulated genes when exposed to *S. aureus*, while those in CBS EpH4-Ev cells were 558 up-regulated and 992 down-regulated genes (**Figure 5D**). A thorough analysis of differential gene expression identified a significant change in the *Abcb1b* gene as shown in both the volcano plot and the heatmap (**Figure 5E-F**). To determine whether the *Abcb1b* gene plays a direct role in maintaining BMB integrity induced by *S. aureus*, we employed the inhibitor Vismodegib, and found that Claudin 3 protein expression was significantly reduced, even in CBS overexpressed EpH4-Ev cells, indicating that *Abcb1b* inhibition reversed the protective effect of CBS overexpression on the BMB. Overall, these data provide additional evidence of the crucial role of CBS in the maintenance of the BMB, potentially by modulating the expression of *Abcb1b* gene.

CBS upregulated genes associated with the innate immune response and pathways related to cell receptor interactions.

In order to further define the underlying mechanisms of Abcb1b maintenance of BMB integrity, the Interacting Genes/Proteins database (STRING v11.5) was used to construct the PPI network (**Figure 6A**). After obtaining the differentially expressed genes (DEGs), we conducted GO and KEGG enrichment analyses to construct a bubble diagram that helped identify the functional relevance of these genes (**Figure 6B-C**). The GO enrichment analysis data indicates that the DEGs were mostly associated with the collagen-containing extracellular matrix (ECM), immune system processes and innate immune responses. The KEGG pathway analysis data showed that DEGs were mainly concentrated in cytokine-cytokine receptor interactions, NOD-like receptor signaling pathways, and the coronavirus disease-COVID-19 pathway. Taken together, the CBS genes had increased activity related to innate immune responses, as well as in pathways related to cell receptor interactions.

H₂S donor can significantly reduce inflammation and cell damage induced by *S. aureus*

We found that 100 μ M GYY4137 does not have a cytotoxic effect on EpH4-Ev cells (**Figure 7A**). GYY4137 treatment increased the expression of ZO-1 and Claudin 3. There is not a statistically significant difference in p65 when challenged by *S. aureus* (**Figure 7B-E**). While GYY4137 significantly reduces the expression of these proinflammatory factors, it amplifies TNF- α (**Figure 7F**). As expected, western blotting indicates a significant increase in NOS2 protein expression following

GY4137 treatment compared with the *S. aureus* infection group (**Figure 7G-H**).

Due to its direct bactericidal activity, we examined the intracellular bacterial load, and there was a decrease after GY4137 treatment (**Figure 7I**). Besides the cytotoxic effect mediated by *S. aureus* infection on epithelial cells via inducing apoptosis, the process involves the balanced transcription of anti-apoptotic and pro-apoptotic proteins. We detected the expression of apoptosis-related proteins, Bax and Bcl-2. The ratio of Bcl2/Bax was significantly increased with GY4137 treatment (**Figure 7J-M**). The studies described above suggest that an H₂S donor significantly reduces inflammation and cell damage induced by *S. aureus* via promoting NO production and inhibiting apoptosis.

CBS-H₂S axis decreases COX-2 expression and the stability of its mRNA by S-sulphydrating HuR

Given that S-sulphydration of HuR mediated by endogenous H₂S leads to the amelioration of COX-2 expression in colitis [26]. We found that the total protein expression of HuR increases significantly during *S. aureus* infection, whereas GY4137 fails to lower it. Conversely, the levels of COX-2 protein (**Figure 8A-D**) and mRNA (**Figure 8E**) decreases markedly, accompanying a significant increase in S-sulphydrated HuR. Conclusively, HuR S-sulphydrylation is observed in EpH4-Ev cells, and may potentially play a critical role in the body's response to inflammation.

CBS^{+/-} mice develop severe tissue damage as a result of *S. aureus* infection

Data from experiments in CBS^{+/-} mice examined the effects of CBS on *S. aureus* infection *in vivo*. The mammary glands were congested and swollen following *S.*

aureus infection. Hemorrhage was present in the CBS^{+/-} group compared with the WT group (**Figure 9A**). Histologic examination indicated that *S. aureus* infection caused mammary epithelial cells to be disordered and detached accompanied by marked inflammatory cell infiltration. These changes were more pronounced in CBS^{+/-} group (**Figure 9B**). Macrophage infiltration was detected via immunohistochemical staining for CD68 (**Fig 9C**). Serum pro-inflammatory factors TNF- α (**Fig 9D**) and IL-1 β (**Fig 9E**) were detected via ELISA. Additionally, serum levels of oxidative stress factor MDA were quantified (**Fig 9F**). The levels of pro-inflammatory factors TNF- α and IL-1 β and the oxidative stress marker MDA were remarkably increased in the *S. aureus* infection group. The increase in CBS^{+/-} mice was more pronounced compared to wild-type mice. Conversely, the bacterial loads in mammary tissue were reduced in the CBS^{+/-} group. To determine BMB integrity, we assayed the expressions of Zo-1 and Claudin 3 tight junction proteins. Consistent with the *in vitro* results, a highly significant reduction in the expression of these tight junction proteins was present in *S. aureus* infected CBS^{+/-} mice. In sum, these results indicate that CBS negatively regulates the magnitude of mammary gland inflammation thereby decreasing tissue damage.

Discussion

S. aureus is a significant globally-distributed zoonotic pathogen that can infect a variety of epithelial cell types [29,30]. The mammary gland is a vital organ in mammals, responsible for milk production and delivery, and udder infections are a

major health and economic concern in dairy animals. Identifying molecules involved in host defense against pathogen entry is a promising approach to treatment and prophylaxis.

A major conclusion of the present study is that CBS plays a vital role in host defense against *S. aureus*. Firstly, we observed CBS protein and gene levels increased significantly during *S. aureus*-infection *in vitro* and *in vivo* suggesting a direct link between *S. aureus* infection and CBS/H₂S. We subsequently found the level of expression of CBS was inversely proportional to the degree of inflammatory injury caused by *S. aureus*. The role of CBS in inflammation is controversial so far. Phillips *et al.* noted that upregulation of CBS in colonic epithelium reprogramed metabolism and promoted carcinogenesis [12], Zhu *et al.* found that CBS is essential for AKT-induced senescence and suppresses the development of gastric cancers associated with PI3K/AKT activation [31]. Vila *et al.* reported that CBS gene knockdown promotes inflammation and oxidative stress in immortalized human adipose-derived mesenchymal stem cells [32]. This finding highlighted the strong cell type specificity of the role of CBS in the occurrence and development of inflammation. Besides, in intracellular bacterial infections, inflammatory responses aim to clear pathogens yet they often lead to tissue damage. There needs to be a delicate balance between eliciting sufficient protective immunity and causing irreparable tissue damage. Host cells that produce ROS and RNS have bactericidal activity [33]. In this study, we found that CBS inhibitor reduced bacterial burden by exacerbating inflammation leading to a decrease of ROS and RNS.

CBS agonists not only reduce inflammation, but also decrease bacterial load in part because of their moderate production of ROS and RNS. The above results suggest that up-regulated CBS is able to balance host defense and tissue damage. In general, once bacteria enter a cell, they can trigger host cell apoptosis, restrict the expression of self-toxic factors, halt early cytotoxicity, and adapt to the intracellular environment in order to evade host defenses [34,35]. We suggest that an H₂S donor may prevent apoptosis, thereby limiting bacterial growth. Overall, our data indicate that *S. aureus* infection triggers upregulation of CBS, reducing inflammation and bacterial burden.

Based on the observed changes in cell morphology, we focused on the integrity of the BMB. This barrier is a crucial structure for mammary gland function. Tight junctions prevent invasion of microorganisms and regulate the exchange of various substances within the mammary gland [36]. Little is known regarding the correlation between the transsulfuration pathway and tight junction proteins in mammary gland tissue. Most studies have centered around the gastrointestinal system. It has been confirmed that exogenous H₂S can help alleviate damage to the intestinal barrier [37]. The data generated in the current study indicates that after exposure to *S. aureus*, upregulation of CBS expression enhances the expression of tight junction proteins, particularly Claudin 3, these experiments employed CBS agonists and inhibitors, H₂S donor and the creation of cell lines that overexpress CBS. RNA-seq transcriptomics show that Abcb1b was the most abundant and was therefore selected for further study. Abcb1b codes P-gp protein. P-gp presence in the blood-brain barrier

(BBB) limits drug accumulation in the brain [38]. It also plays a vital role in preserving intestinal homeostasis by transporting xenobiotics and bacterial toxins [39]. And P-gp/Abcb1b is essential for junction reassembly at the blood-testis barrier [40]. Vismodegib, a potent inhibitor of P-gp, has undergoing clinical trials for a the treatment of a variety of solid tumors [41]. In the study described herein, the overexpression of CBS on Claudin 3 protein was partially abolished after employing Vismodegib, which indicates that Abcb1b may be the downstream molecule of CBS in the progression of maintaining the BMB. Moreover, the protein-protein interaction networks of Abcb1b were constructed by STRING and detected CD44 protein. Previous studies confirmed that CD44 could activate macrophages upon *S.aureus* infection triggering release of ROS that mediates activation of acid sphingomyelinase releasing ceramide that causes alterations of the cytoskeleton and internalization of pathogens [42]. As known that maintaining the structure of the cytoskeleton is vital for the proper functioning of the blood-milk barrier. It seems that the regulatory influence of CBS on Abcb1b might be connected to CD44, and is worth further research. Similarly, GO term analysis revealed that many of the DEGs are enriched in the immune process and collagen-containing extracellular matrix suggesting that CBS is important to host immune defense and the extracellular components that play crucial roles in resistance to infection. Enriched KEGG pathways of the DEGs are supported by the three KEGG signaling pathways in which CBS participate, including cytokine-cytokine receptor interactions, coronavirus disease COVID-19 and NOD-like receptor signaling, which in line with previous results in current study that

CBS primarily impacts the structure and functionality of cell membranes, all of these tight junction proteins were predominantly localized to the cell membrane at cell-cell contacts.

Since S-sulfhydration of proteins plays a vital role in the functioning of both endogenous and exogenous H₂S, we investigated whether S-sulfhydration in *S. aureus* infected EpH4-Ev cells occurs, and if so, what is the target protein? Previous studies proved that inhibition of COX-2 mRNA stability by increasing the S-sulfhydration of human antigen R (HuR) may be a mechanisms underlying the anti-inflammatory effect of the CBS-H₂S axis in colitis and other disorders [26], Our data indicates that S-sulfhydration of HuR protein is not cell-type specific in its functionality.

Finally, we further validated our in vitro results in EpH4-Ev cells utilizing an in vivo CBS^{+/-} mouse model. We found CBS^{+/-} mice suffered more significant injuries compared to control mice. This was confirmed via HE stained tissue examination, immunohistochemistry and Elisa. Due to the role of IL-1 β in infections caused by *S. aureus* is important only in the early stages[43]. so there is no statistical difference in serum. There is a decrease in the bacterial load of mammary tissue in the CBS^{+/-} group. The reduction of bacteria burden is achieved at the expense of increased inflammatory damage. To this end, the significantly reduced expression of tight junction protein ZO-1 and Claudin 3 after CBS knockdown is consistent with previous in vivo results.

The data from the current study details the importance of host CBS in anti *S. aureus* infection documenting a potential relationship between CBS and BMB for the first time. Mechanistically, CBS maintains a fine balance between eliciting effective

protective immunity and preventing excessive damage to tissues by the generation of direct antibacterial substances, regulating apoptosis related proteins and S-sulphydrating target proteins (**Fig 10**). With antibiotic resistance and evasion of vaccination on the rise, it is crucial to gain a deeper understanding of the interactions between hosts and pathogens. This will hopefully aid in the identification of new therapeutic targets. Our study helps to elucidate host defense mechanisms against pathogens and suggests that manipulating sulfur metabolism may be a promising tool for drug discovery and vaccine optimization.

Conflicts of interests

The authors declare no competing interests.

Acknowledgments

This project was supported by grants from the National Natural Science Foundation of China (No. 32072867, 32273013 and 32272963), the Jiangsu Province Key Research and Development Program (No. BE2022384) and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. The authors declare no competing interests. Dr. Howard Gelberg is thanked for his help in editing this manuscript.

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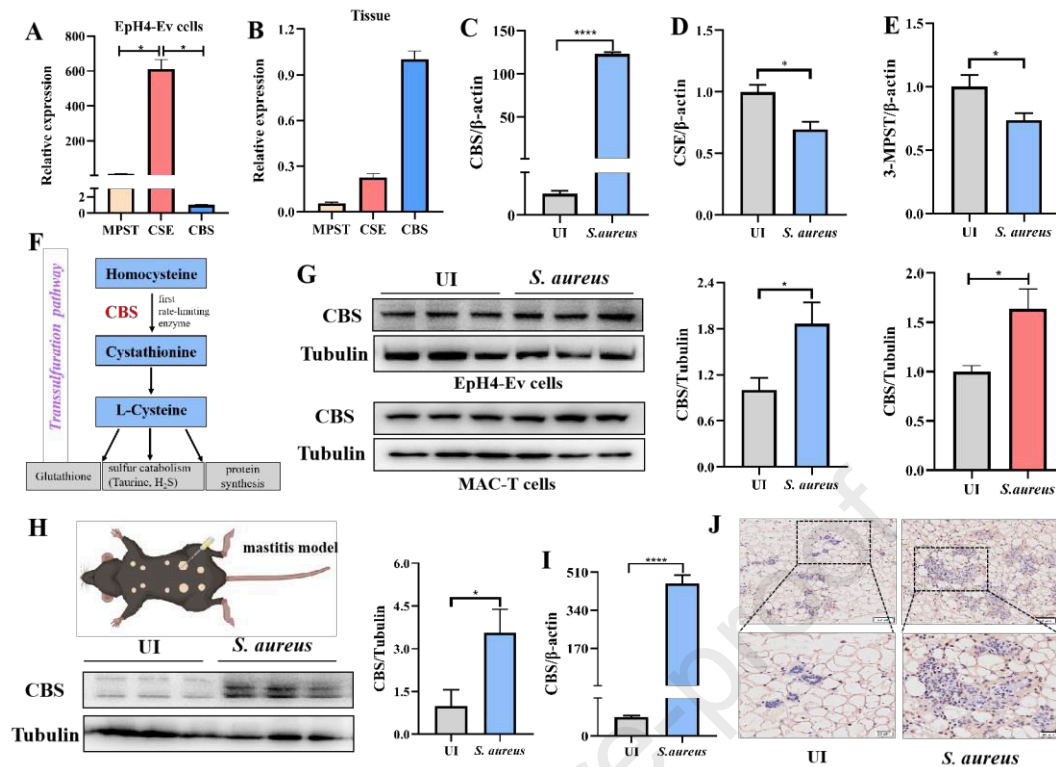


Figure 1. The expression of CBS is increased in mastitis caused by *S. aureus*.

Relative expression levels of three crucial enzymes involved in transsulfuration pathway in mammary gland tissue (A) and EpH4-Ev cells (B). Gene expression of CBS (C), CSE (D) and 3-MPST (E) in uninfected and *S. aureus*-infected EpH4-Ev cells. (F) Endogenous transsulfuration pathway that generates glutathione, taurine and H₂S. (G) Western blot of CBS expression in uninfected (UI) and *S. aureus*-infected EpH4-Ev cells and MAC-T cells. (H) Mice mastitis model establishment, and protein expression of CBS in uninfected mice (UI) and those infected with *S. aureus* confirmed by qPCR (I) and immunohistochemistry (J) (n=6). Data are presented as means ± SEMs (n=3, unless otherwise indicated).

*(P<0.05)=significantly different.

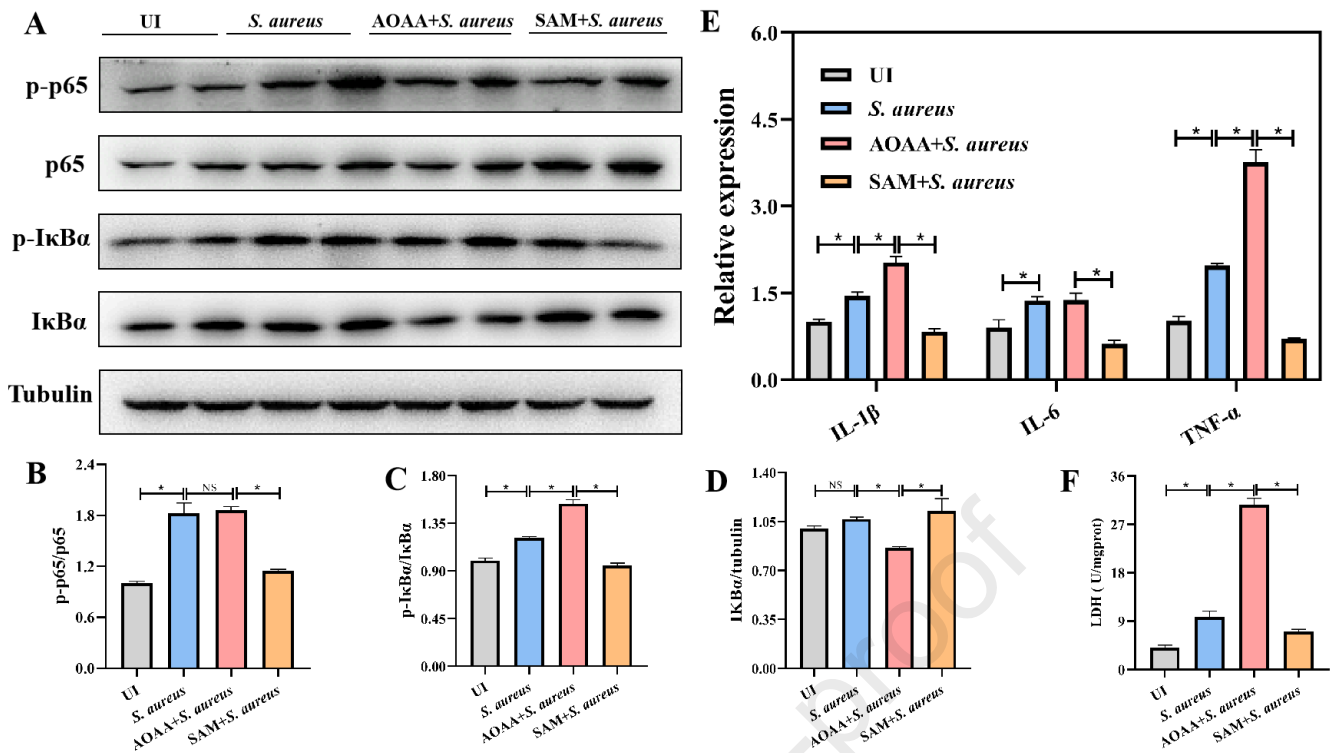


Figure 2. The degree of *S. aureus*-induced inflammatory injury is related to CBS expression. (A-D) Western blot showing expression of (p)IκBα, (p)65 in uninfected and *S. aureus*-infected EpH4-Ev cells, with or without AOAA and SAM. (E) Gene expression of pro-inflammatory factors. (F) The release of lactate dehydrogenase (LDH) in the culture supernatant. Data are presented as means $\pm$ SEMs (n=3).

*($P < 0.05$)=significantly different.

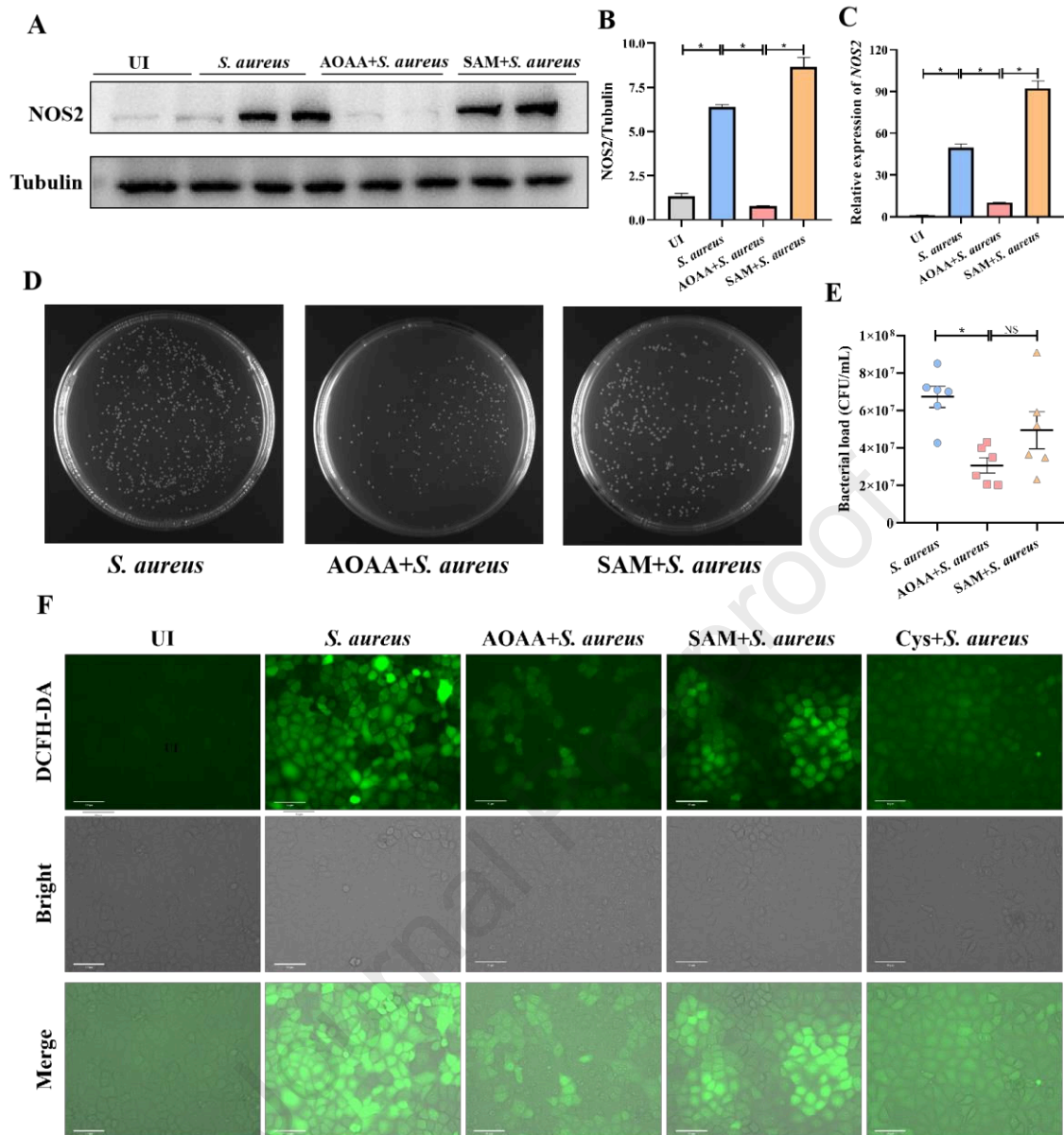


Figure 3. CBS agonists lower bacteria by utilizing a noninflammatory pathway.

(A-B) Western blot showing expression of NOS2 in uninfected and *S. aureus*-infected EpH4-Ev cells, with or without AOAA and SAM, and validated by qPCR (C). (D-E) Intracellular growth profile of *S. aureus*. (D) ROS content of EpH4-Ev cells following the use of AOAA and SAM, with cysteine positive control. Data are presented as the means±SEMs (n=3). *($P<0.05$)=significantly different.

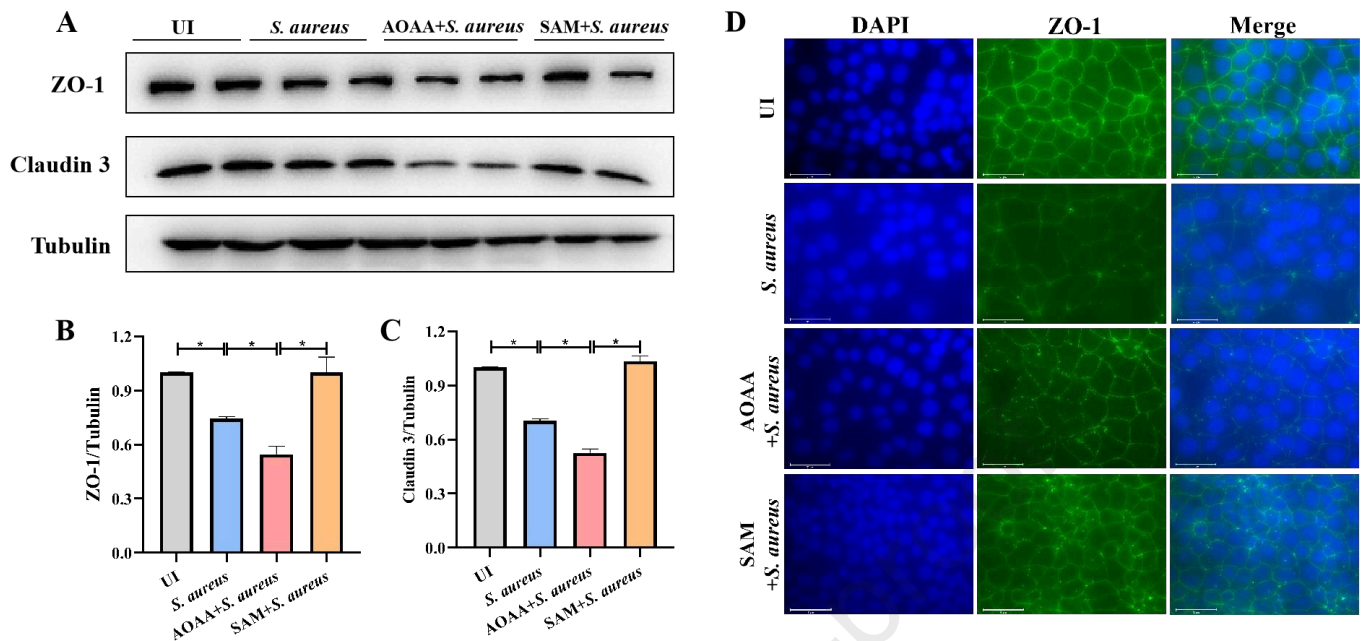


Figure 4. CBS plays a role in preserving the integrity of the BMB. (A-C) The expression of ZO-1 and Claudin 3 in Eph4-Ev cells, both uninfected and infected with *S. aureus*, was analyzed using western blot. Additionally, the effect of AOAA and SAM were also studied. The results were confirmed by immunofluorescence (D). Data are presented as the means $\pm$ SEMs (n=3). *($P<0.05$)=significantly different.

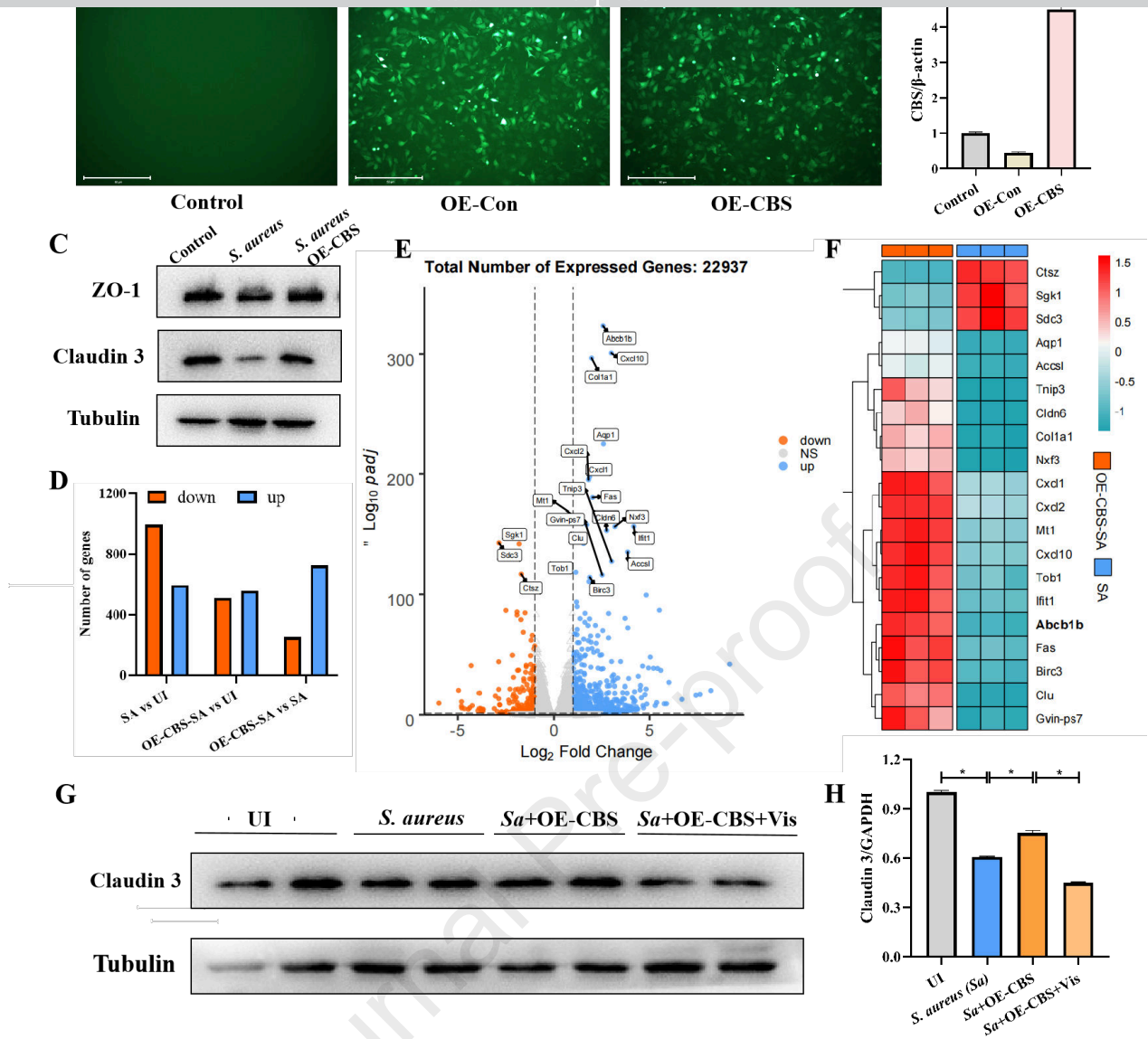


Figure 5. The induction of *Abcb1b* expression by CBS enhances the integrity of the BMB. (A-B) Fluorescence indicates that the plasmid was successfully transfected into Eph4-Ev cells. qPCR verifies the results. (C) The expression of ZO-1 and Claudin 3 in Eph4-Ev cells and OE-CBS Eph4-Ev cells during *S. aureus* infection. (D) Up- and down-regulation of differential genes. (E-F) Volcano plot and heatmap of differential genes. (G-F) The expression of Claudin 3 in Eph4-Ev cells and OE-CBS Eph4-Ev cells with or without Vismodegib during *S. aureus* infection. Data are presented as the means $\pm$ SEMs (n=3).

*($P < 0.05$)=significantly different.

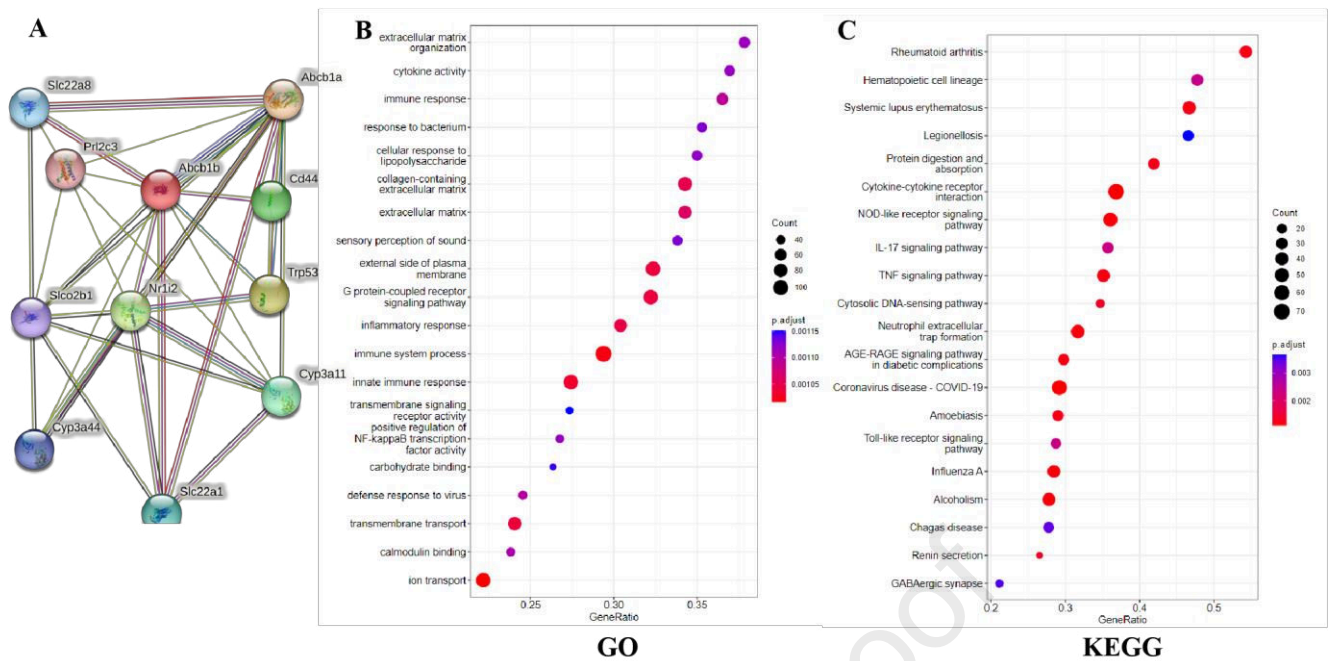


Figure 6 CBS increases gene expression linked to the natural defense system of the body, and pathways connected to communication between cells. (A) Protein-to-protein interaction network analyzed using STRING. GO enrichment analysis (B) and KEGG enrichment analysis (C). Size of the bubble represents the number of genes enriched in the GO terms and KEGG pathways. The color of the bubble corresponds to the FDR p value.

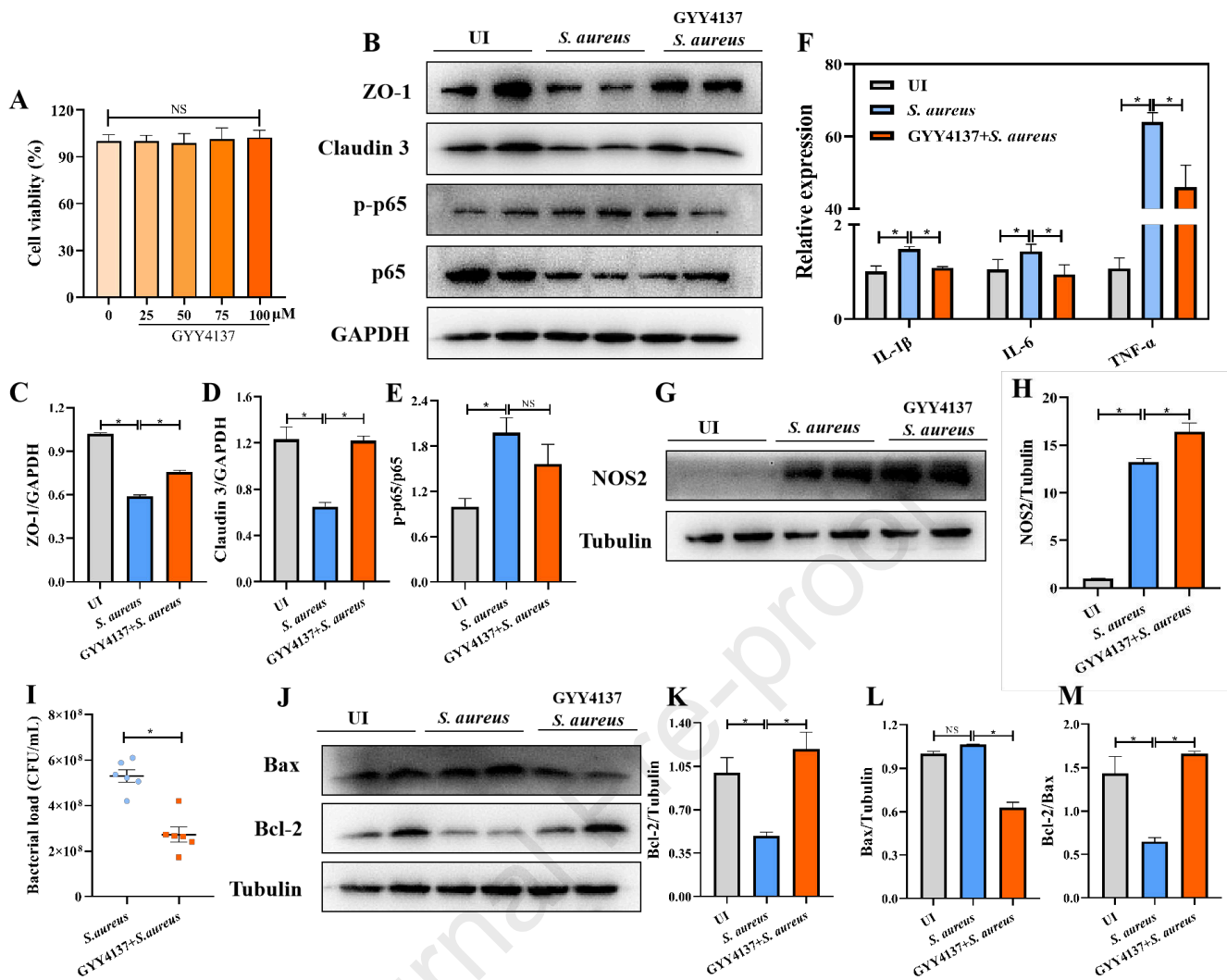


Figure 7. GYY4137 shows potential in reducing inflammation and cell damage

caused by *S. aureus*. (A) Cytotoxicity of GYY4137 on EpH4-Ev cells was

determined using a CCK8 kit. (B-E) Western blot showing expression of ZO-1,

Claudin 3 and (p)65 in uninfected and *S. aureus*-infected EpH4-Ev cells after being

preprocessed with GYY4137. (F) Gene expression of pro-inflammatory factors. (G-H)

NOS2 protein level. (I) Intracellular growth of *S. aureus*. (J-M) Bax and Bcl-2 protein

levels. Data are presented as the means $\pm$ SEMs (n=3). *($P < 0.05$)=significantly

different.

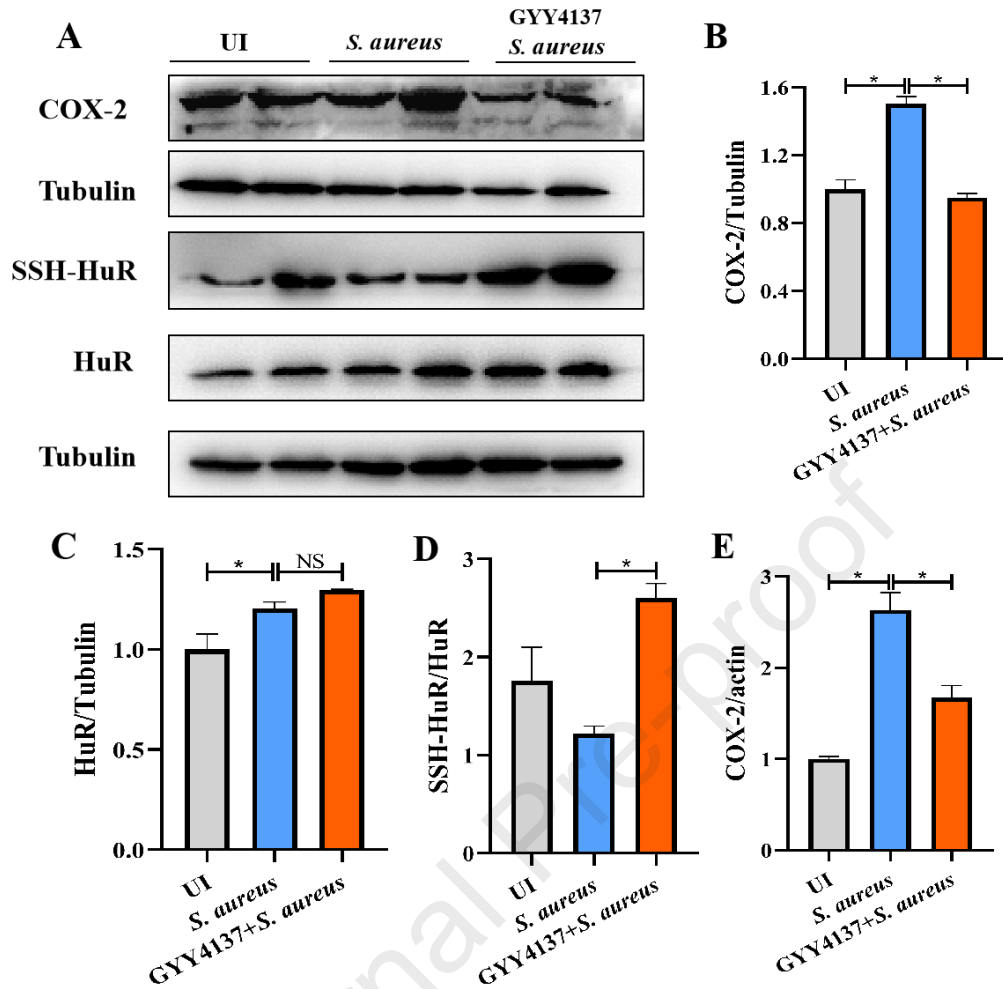


Figure 8 The CBS-H₂S axis decreases expression of COX-2 and stabilizes mRNA through S-sulphydration of HuR. (A) Representative western blot images of COX-2, HuR and SSH-HuR proteins in EpH4-Ev cells with or without GYY4137. (B-D) Relative band intensity. (E) Gene expression of COX-2. Data are presented as the means $\pm$ SEMs (n=3). *($P < 0.05$)=significantly different.

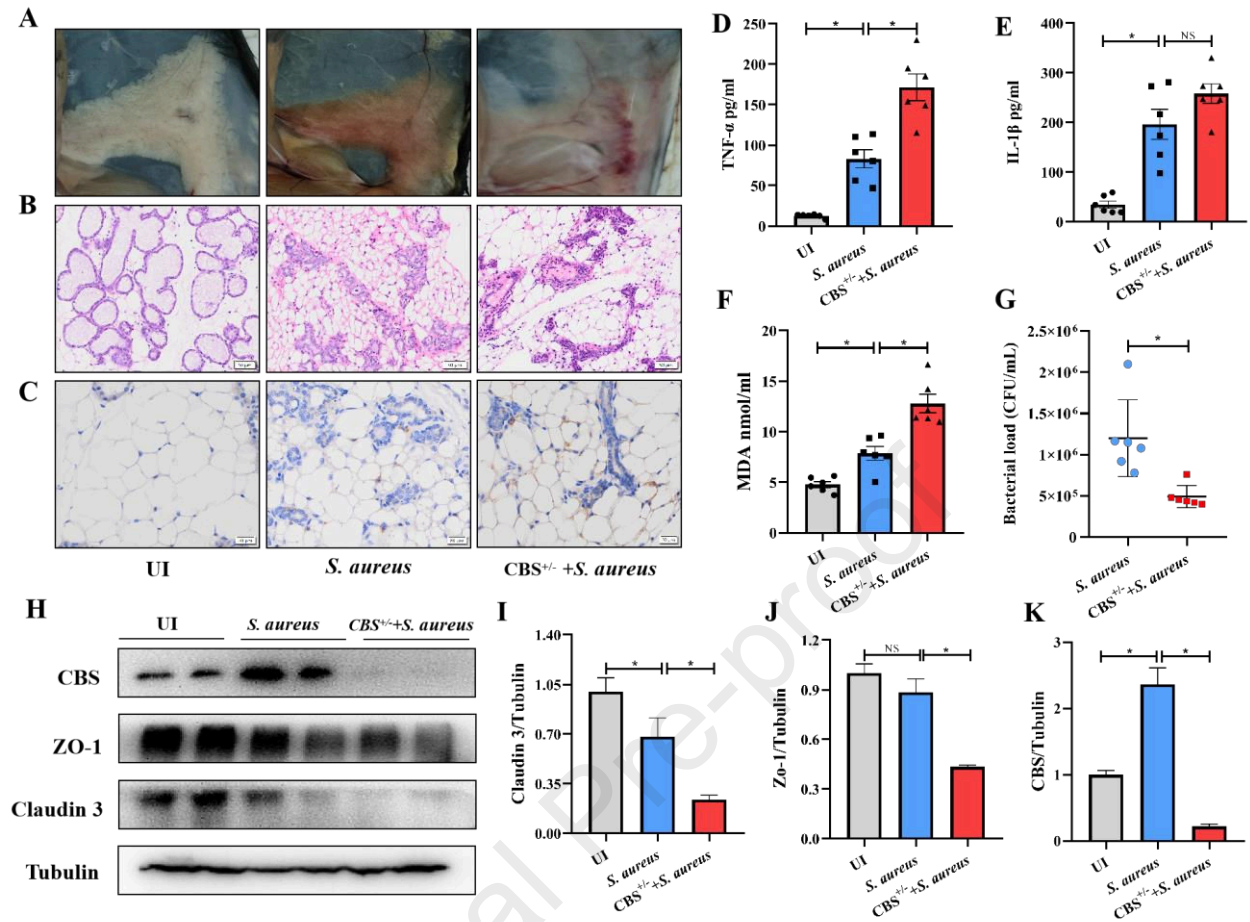


Figure 9 *CBS*^{+/-} mice suffer significant tissue damage in response to *S. aureus* infection. Representative images of visual observation (A) and HE staining (B) of mammary gland of WT and *CBS*^{+/-} mice after *S. aureus* infection. (C) Positive staining for macrophage marker CD68 during *S. aureus* infection. Comparison of serum pro-inflammatory factors including TNF- α (D) and IL-1 β (E) oxidative stress marker MDA (F) as well as bacterial load (G) between WT and *CBS*^{+/-} mice with *S. aureus* infection. (H) Representative western blot images of CBS, ZO-1 and Claudin 3 proteins in WT and *CBS*^{+/-} mice with *S. aureus*. (I-K) Relative band intensity. Data are presented as the means $\pm$ SEMs (n=5/6). *($P < 0.05$)=significantly different.

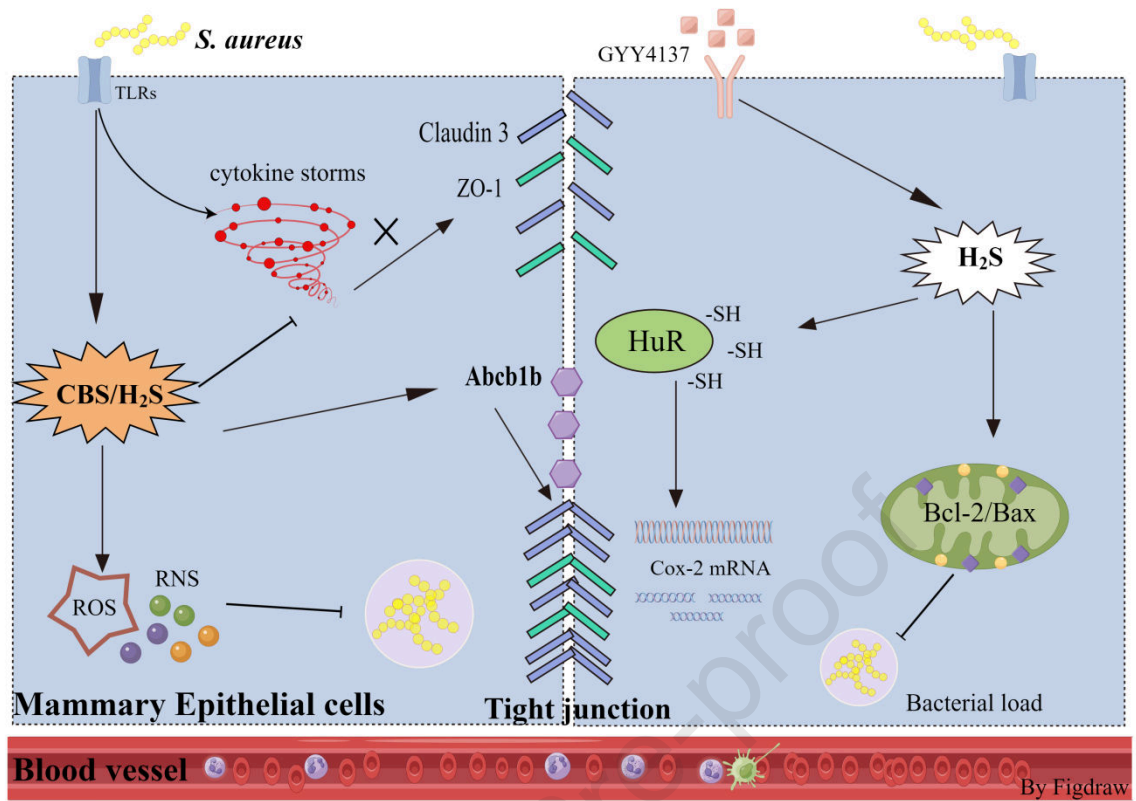


Figure 10. Schematic model showing how CBS attenuates inflammation and maintains the integrity of the BMB during *S. aureus* infection.

Highlights

1. *S. aureus* infection in the udder results in an increase in CBS.
2. CBS plays a role in the negative regulation of inflammation.
3. CBS contribute to maintain BMB integrity via promoting Abcb1b expression

Conflicts of interests

The authors declare no competing interests.



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A critical role for host-derived cystathionine--synthase, in Staphylococcus aureus-induced udder infection

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2024-01-01

Elsevier

<https://doi.org/10.1016/j.freeradbiomed.2023.11.001>

Fu S, Yang B, Gao Y, et al., (2024) A critical role for host-derived cystathionine--synthase, in Staphylococcus aureus-induced udder infection. Free Radical Biology and Medicine, Volume 210, January 2024, pp. 13-24