Combined effects of intrinsic host gene (*TRAPPC9*) and extrinsic nutrient (folate) on resistance against *S. aureus* induced bovine mastitis

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Abstract

Background: Drug-resistance and immunological escape of *Staphylococcus aureus* and its “superbug”, methicillin-resistant *S. aureus* (MRSA), have become one of main causes of bacterial infection in both human and animals. In dairy cattle, elimination of bovine mastitis induced by *S. aureus* is of importance because *S. aureus*-infected cows normally are culled passively.

Methods: Here, we investigated the beneficial effects of bovine trafficking protein particle complex 9 (*TRAPPC9*) gene and folic acid supplementation in the control of mastitis induced by *S. aureus* or MRSA by a series of *in vivo* and *in vitro* experiments.

Results: The data showed that the genetic mutations and DNA methylation of *TRAPPC9* were highly linked with the mastitis resistance of dairy cows. Additionally, knockdown of bovine *TRAPPC9* was significantly involved in the mRNA expression levels of interleukin's genes (increased *IL-1 β* and *IL-6*), and down-regulated the protein level of NF- κ B-P65 in the mastitis cell model induced by MRSA. Meanwhile, dose-dependent folic acid addition can inhibit the invasion of MRSA into Mac-T cells and improve *TRAPPC9* expression in dairy cows.

Conclusions: Altogether, our data suggest that an appropriate dose of folic acid can significantly reduce the inflammation caused by MRSA partially through *TRAPPC9* mediated NF- κ B pathway. These findings provide new insights to control the drug-resistant pathogens and to restrict the overuse of antibiotics through combined effects of the intrinsic host gene and extrinsic nutrient.

1. Introduction

Hosts possess innate and adaptive immune systems that are tailored to counter pathogen challenges effectively. Under general bacterial infections, the nuclear factor- κ B (NF- κ B) pathway can be activated in response to stimuli [1–3]. However, *Staphylococcus aureus*, one of the first well-characterized microbes, can modulate or avoid host immune systems and acquire strong resistance to most antibiotics and drugs [4]. Moreover, the “superbug” strain, methicillin-resistant *S. aureus* (MRSA) is still the common causes of infections and mortality in hospitals [5, 6]. Aside from hospital-associated MRSA and community-acquired MRSA, livestock-associated MRSA infection has been rising in frequency since the 1970s [7–10]. The earliest isolation of livestock-associated MRSA strain was reported in lactation cows with mastitis [7]. Since *S. aureus*- or MRSA-infected cows are associated with the increased use of antimicrobials and rising drug resistance, the cows infected by these bacteria are normally culled as soon as they were detected. Therefore, developing useful new methods to control and reduce the infection of *S. aureus* and MRSA in dairy farms is imperative.

Bovine milk is the most popular animal food by humans of all ages. Thus, improving bovine udder health is critical to protect public health. Our previous work observed that the bovine trafficking protein particle complex 9 (*TRAPPC9*) gene is significantly associated with milk somatic cell score (SCS, [11]), a usual indicator of the degree of bovine mastitis. The nuclear factor NF- κ B (NF- κ B) pathway has long been

proved as a prototypical proinflammatory signaling pathway. Considering that *TRAPPC9* plays an important role in the NF- κ B signaling pathway and membrane traffic of Golgi [12, 13], our previous study proved that it can serve as a candidate biomarker for *S. aureus* and MRSA infections in dairy cows and bovine mammary epithelial cell lines (Mac-T) [14, 15]. Aside from providing hosts with genetic resistance to pathogens, folic acid (a synthetic form of folate), a methyl donor, also alleviates inflammation through reducing the expression of pro-inflammatory factors [16–18]. We thus hypothesized that host *TRAPPC9* and supplementation of folic acid improve host defense against *S. aureus* or MRSA infection in dairy cattle and bovine mammary epithelial cells.

In the present study, we designed a series of experiments to investigate the effects of genetic mutations, DNA methylation, and expression of the bovine *TRAPPC9* gene on mastitis progress in a new dairy population and to determine the roles of this gene in the defense against *S. aureus* and MRSA infections in bovine Mac-T cells. We also systematically assessed the influences of folic acid supplementation on the incidence of mastitis *in vivo*, the inflammation progress induced by *S. aureus* and MRSA and the invasion abilities of *S. aureus* and MRSA into Mac-T cells. These data provide new insights into the protection of food animals and public health from the inflammatory disease induced by zoonotic bacteria.

2. Materials And Methods

2.1. Samples

2.1.1. Cows

A total of 514 Chinese Holstein cows used for the association study were randomly collected from six farms in the North of China (Figure 1). The parities of the cows ranged from 1 to 4 and were milked three times a day, the lactation days were among 30-400 days, and SCS varied from 0 to 10. All the dairy cows were fed on the same lactation diet according to the recommendations of lactating Chinese Holsteins.

In addition, 123 healthy perinatal Holstein cows (from a single dairy farm in Hebei Province, China) were randomly selected for the folic acid supplementation experiment. The parities of the cows were 1 or 2, the estimated body weight of each cow was shown in S1 Table, and the expected date of calving of the cows were within 10 days. Accordingly, they were grouped into three experimental groups (groups A, B, and C) supplemented with different doses of folic acid (Group A: 0 mg/day; Group B: body weight (kg) $\times$ 0.24 mg/kg/day; Group C: body weight (kg) $\times$ 0.48 mg/kg/day). In this study, folic acid was coated by Oriental Kingherb Company (Beijing, China). The coated folic acid was fed to groups B and C for 14 days before calving and 7 days after calving.

Milk samples were aseptically collected and immediately sent to the official Dairy Center of China (Beijing, China) for the detection of somatic cell count (SCC). Peripheral blood samples were collected from the cows' tail vein by caudal venipuncture and divided into two tubes. One tube containing coagulant and the other containing potassium ethylenediaminetetraacetic acid (EDTA K3E 15%, 0.12mL;

Becton, Dickinson and Company) were used for DNA and RNA extraction respectively. Sera were isolated from the peripheral blood and sent to Beijing Huaying Biotechnology Research Institute for cytokine (NIBP, NF- κ B, TNF- α , IL17, IL4, IFN- γ , IL6, and IL10) tests detected by radioimmune outfit, which was performed by R-911-automatic radioimmunoassay count instrument.

2.1.2. Bacterial strains

S. aureus (strain 90-1) was originally isolated from a subclinical case of bovine mastitis by our labs in Beijing, China. MRSA (strain W18), provided by Prof. Xin Wang (Northwest A&F University, Yangling, Shanxi, China), was isolated from a clinical case of bovine mastitis. *S. aureus* and MRSA (50 μ L) were inoculated into 5 mL of tryptone soya broth (Beijing Land Bridged Technology Ltd.), and allowed to grow overnight for 24 h at 37 °C, 200 rpm/min. The concentrations of *S. aureus* and MRSA were determined by serial dilution and standard plate counting. In Brief, *S. aureus* and MRSA were diluted six gradients successively with DPBS. Then, 100 μ L of the diluent was transferred into plate count agar (PCA, Beijing Land Bridged Technology Ltd) and spread with a glass spreader. Subsequently, the agar plates were incubated at 37 °C for 18–24 h. After the PCA plate culture of the *S. aureus* and MRSA diluents, the numbers of *S. aureus* and MRSA colonies were counted. Each diluent PCA was conducted in triplicate. Finally, the bacteria were diluted in DMEM to obtain 1×10^8 CFU/mL.

2.1.3. Cell culture

The bovine mammary epithelial cell line (Mac-T) was provided by Zhejiang University. Mac-T cells were re-suspended in the warm growth medium of DMEM with Glutamax (Gibco) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and streptomycin (100 mg/mL, Gibco). Mac-T cells were seeded in a 25 cm² tissue culture flask at 37 °C in a 5% CO₂ humidified incubator, and the medium was changed once every 24 h. After 48 h, cells at 85% confluence were split by adding 1 mL of 0.25% trypsin/EDTA (Gibco) after washing with 2 mL of DPBS (Gibco). Mac-T cells were cultured up to a maximum of three passages to reduce the risk of aberrant expression caused by extended culturing. These cells were centrifuged in 5 mL of DMEM growth medium for 5 min at 1000 rpm/min, seeded at 5×10^5 cells in a 6-well cell culture plate (Corning), and then allowed to grow in a growth medium at 37 °C in 5% CO₂ humidified incubator.

2.2. Total RNA extraction, reverse transcription and quantitative Real-Time PCR

Buffly coat of fresh blood and collected Mac-T cells were placed directly in 1 mL of Trizol reagent (Invitrogen), and total RNA was extracted following the manufacturer's instructions. The mRNA was converted to cDNA with the PrimeScriptTM RT reagent kit (Takara). RT-qPCR was performed using the SYBR Green I Master kit (Roche Diagnostics GmbH) on the LightCycler® 480 II (Roche Diagnostics Ltd) following the manufacturer's instructions. RT-qPCR reaction was performed using the following program: 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. At the end of each run, a dissociation melt curve was determined. All melt curves showed a single peak and were

consistent with the presence of a single amplicon. Bovine *GAPDH* was used as the house-keeping gene to normalize the target genes' expression levels. Each cDNA sample was analyzed in duplicate. The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are as follows (obtained from Tsingke): *TRAPPC9*-forward: CTGCTCCGCTCGGTGAATGAC, *TRAPPC9*-Reverse: GCTTTACCGCCAGTTCCACCA; *IL-1 β* -Forward: CTGTCGGACCCATATGAGC, *IL-1 β* -Reverse: GCTCATGGAGAATATCACTTGTTG; *IL-6*-Forward: TCCTGAGAAACCTTGAGAAT, *IL-6*-Reverse: ATAAGTTGTGTGCCAGTGG; *IL-8*-Forward: TGAAGCTGCAGTTCTGTCAAG, *IL-8*-Reverse: TTCTGCACCCACTTTTCCTTG; *GAPDH*-Forward: GGTGCTGAGTATGTGGTGGGA, *GAPDH*-Reverse: GGCATTGCTGACAATCTTGA.

2.3. DNA extraction and genotyping of the bovine samples

The genomic DNA was extracted from the whole blood of the dairy cows using the DP-318 Blood DNA kit (Tiangen Biotech Co) following the manufacturer's instructions. DNA was assessed with the NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific) and by 1% agarose gel electrophoresis.

The genotyping of the bovine samples consists of two steps. First, SNPs identification was conducted by DNA pooling and sequencing. Briefly, a total of 30 samples were randomly selected from 514 cows to construct a DNA pool with an equal DNA concentration of 50 ng/ μ L for each sample. PCRs were performed in a 25 μ L volume containing 12.5 μ L of PreMIX (Takara Biotechnology Co), 1 μ L of genomic DNA (50 ng), 9.5 μ L of ddH₂O, and 1 μ L of each primer (10 pmol/ μ L). Cycling reaction conditions were as follows: initial denaturation at 95 °C for 5 min; followed by 34 cycles of 30 s at 95 °C, annealing from 52 °C to 62 °C for 35 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were sequenced using the ABI3730XL DNA analyzer (Applied Biosystems). In total, four SNPs were identified in the new population.

Second, the identified SNPs were genotyped for 514 cows through the multiplex Snapshot method (Applied Biosystems). The primer sequences of the four identified SNPs were as follows (obtained from Tsingke Inc.): *TRAPPC9*-SNP1-Forward: AGGAAAGTGAAGTCGCTCAGT, *TRAPPC9*-SNP1-Reverse: CCTTTTATTGGAGGTCTATGTG; *TRAPPC9*-SNP2-Forward: TTACATTAACCTTCGGCAGGT, *TRAPPC9*-SNP2-Reverse: GACTCCAGGGTCTTGACCACA; *TRAPPC9*-SNP3-Forward: GCTTCAGTCTTCTGGACCCTCT, *TRAPPC9*-SNP3-Reverse: GATACCCCTGGTCCCCTTTAG and *TRAPPC9*-SNP4-Forward: TTTATCCTGACGATGTCTGCC, *TRAPPC9*-SNP4-Reverse: CTGCTGTGAGCCCCAAACTAT.

For SNP1, SNP2, SNP3 and SNP4, 492, 511, 492 and 501 samples were detected successfully (S2 Table). And Haploview (Version 4.1) was used for linkage disequilibrium analysis of identified SNPs.

2.4. DNA bisulfite treatment and hot-start PCR

The EZ DNA Methylation Golden kit was used for the sodium bisulfite conversion of genomic DNA following the manufacturer's instructions (ZYMO Research). The PCR and sequencing primers of the *TRAPPC9* gene used for DNA methylation detection were designed with Oligo 6.0 software and PSQ

Assay Design software (Qiagen). Hot start PCR was carried out in a 40 μ L system including 20 μ L of hot-start PCR premix (ZYMO Research), 1 μ L of forward primer (10 pmol/ μ L), 0.1 μ L of reverse primer with the universal tail (10 pmol/ μ L, [19]), 1 μ L of biotin-labeled universal primer (10 pmol/ μ L), and 4 μ L of bisulfite-treated DNA. The PCR conditions were 95 °C for 15 min, followed 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s for 45 cycles, and 72 °C for 10 min. The PCR products were detected using 2% agarose gel with ethidium bromide [19].

2.5. Quantitative DNA methylation analyses by pyrosequencing

The pyrosequencing assays were used to analyze the promoter methylation level of the *TRAPPC9* gene quantitatively. Six CpG sites located in the promoter region of the bovine *TRAPPC9* gene were tested to analyze the DNA methylation level. The Pyro Q-CpG system (Qiagen) was used to analyze DNA methylation following the manufacturer protocol. In brief, bisulfite PCR products were bound with *Streptavidin Sepharose* High Performance (GE Healthcare). The *Sepharose* beads grasping the biotin-labeled PCR products were purified in 70% ethanol for 5 s, denatured in a denaturing buffer for 5 s, and then washed in washing buffer for 15 s using the Pyrosequencing Vacuum Prep Tool (Qiagen). Subsequently, a 0.5 μ M pyrosequencing primer was annealed to the purified single-stranded PCR product with annealing buffer (Qiagen). The levels of CpG methylation were expressed as the percentage $^mC/ (^mC+C)$, and non-CpG cytosine residues were used as internal controls to verify bisulfite conversion. The primers measuring the methylation levels of bovine *TRAPPC9* are as follows (obtained from Tsingke): R1 Region-Forward: GGGAAGAGTATAGATAATAGTTAGATAGT, R1 Region-Reverse: GGGACACCGCTGATCGTTTAAACAATCCACTCCAATTACTATTACC, and R1 Region-Sequencing: TTTTAAAGGAAAGGAAAGTG. R2 Region-Forward: GAGTTTGGAGTGGTTTTTTTAGG, R2 Region-Reverse: GGGACACCGCTGATCGTTTAAAATAAATTCCCCTTTTACTATC, and R2 Region-Sequencing: GTTTGGAGTGGTTTTTTTAGG.

2.6. Invasion assay of MRSA and *S. aureus*

The gentamicin protection assay was performed to analyze the invasion ability of MRSA and S. aureus into Mac-T cells as described previously [20]. In brief, Mac-T cells at 85% confluence in 6-well plates were cultured in modified growth medium at 37 °C in 5% CO₂. The monolayers were stimulated with MRSA and S. aureus (1×10⁸ CFU/mL) for 3, 6, 8 and 10 hr at a multiplicity of infections of 10 bacteria to 1 Mac-T cell (MOI = 10:1). Then, the Mac-T cells were washed three times with PBS and incubated in DMEM supplemented with 100 μ g/mL gentamycin for 2 h in 5% CO₂ at 37 °C without serum. Each experimental treatment was conducted in triplicate. These cells were washed three times in PBS and collected at different time points post-inoculation using 0.25% trypsin/EDTA for RNA extraction. The number of CFU/mL was determined by the qPCR technique.

2.7. Challenging Mac-T cells with *S. aureus* and MRSA strains

Mac-T cells at 85% confluence in 6-well plates were cultured in modified growth medium at 37 °C in 5% CO₂. The monolayers were stimulated with *S. aureus* (90-1) or MRSA (W18) (1×10⁸ CFU/mL) for 3, 6, 12 and 24 h at a multiplicity of infections of 10 bacteria to 1 Mac-T cell (MOI = 10:1). The Mac-T cells infected with bacteria alone and cultured in the same volume of DMEM growth media without any treatment served as controls. Each experimental treatment was conducted in triplicate. These cells were washed three times in PBS and collected at different time points post-inoculation for RNA extraction.

2.8. Cell viability assay

Cell viability was measured using the One Solution Cell Proliferation assay (Promega Corporation) to investigate the effect of folic acid on cell growth. In brief, the Mac-T cells were seeded at 5×10⁴ cells/mL in 96-well plates at 37 °C in 5% CO₂ for 24 h, and the cell medium included different concentrations of folic acid (0, 5, 10, 20, and 40 µg/mL). After 24 h, the cells were treated with 20 µL of MTS solutions/well at 37 °C with 5% CO₂ for 4 hr. The Mac-T cells cultured in the medium alone and the medium without cells in sextuplicate served as zero adjustment controls. Optical density was measured at 490 nm on a microplate spectrophotometer (Tecan).

2.9. Mac-T cells treated with folic acid and followed bacterial infection

Mac-T cells at 85% confluence in 6-well plates were cultured in modified growth medium at 37 °C in 5% CO₂. The monolayers were treated with folic acid at different doses (5, 10, 20, and 40 µg/mL) for 2 hr, and then stimulated with *S. aureus* (90-1) or MRSA (W18) (1×10⁸ CFU/mL) for 6 hr at a multiplicity of infections of 10 bacteria to 1 Mac-T cell (MOI = 10:1). The Mac-T cells infected with *S. aureus* or MRSA alone and cultured in the same volume of DMEM growth media without any treatment served as controls. Each experimental treatment was conducted in triplicate. These cells were washed three times in PBS and collected at 6 hr post-inoculation for RNA extraction.

2.10. Cell transfection and RNA interference (RNAi)

For cell-based functional assays, cell transfections were conducted with Lipofectamine 2000 (Invitrogen) on the RNA interference method following the manufacturer's manual. In brief, the Mac-T cells were seeded at 5×10⁵ cells/mL in 6-well plates for 24 hr, and then treated with DMEM containing extra folic acid (5 µg/mL) (treated group) or only DMEM (control group) for 2 h. For RNAi, cells were co-transfected with synthetic small interfering RNA (siRNA, 100 pmol), Lipofectamine 2000 (5 µL) supplemented with warm DMEM containing 10% FBS in a humidified atmosphere with 5% CO₂ at 37 °C at 2 h post-inoculation. The Mac-T cells were treated with folic acid and then RNAi (*TRAPPC9*), because the purpose of this study was to investigate the protective effect of folic acid on the expression of inflammatory cytokines (indicators of inflammation). The cells were stimulated with *S. aureus* or MRSA for 6 hr with MOI = 10:1 at 48 h post RNAi transfection. Each experimental treatment was performed in triplicate. The Mac-T cells were washed three times in PBS and collected at 6 hr post-inoculation for RNA extraction. The primers used for bovine RNAi are as follows (designed and synthesized by GenePharma): *TRAPPC9*

siRNA-Forward: GCAUGGAAGCGUCGGAUUTT, and *TRAPPC9* siRNA-Reverse: AAUCCGACGCUUCCAUGCTT. Negative control siRNA-Forward: UUCUCCGAACGUGUCACGUTT, and Negative control siRNA-Reverse: ACGUGACACGUUCGGAGAATT.

2.11. Total protein extraction and Western Blot analysis

Mac-T cells were stimulated for 6 hr with *S. aureus* at 72 hr post-transfection and 2 hr pre-treated with folic acid (5 µg/mL). Finally, these cells were harvested with 500 µL of 0.25% trypsin/EDTA and washed twice in PBS for protein extraction. Total proteins from the cells were extracted by mammalian protein extraction reagent. The cellular proteins were separated by 10% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, USA). The membrane was blocked for 2 hr with 5% non-fat dry milk in Tris-buffered saline-Tween (TBST) at room temperature on a rotary shaker and then washed three times (10 min each) with TBST. The membrane was blotted with 1:500 specific primary anti-bovine antibodies followed by 1:5000 secondary antibody conjugated with horseradish peroxidase at room temperature for 1 hr. The membrane was again washed three times (10 min each), and blots were generated with the ECL Plus Western Blotting Detection System (Amersham Life Science). Rabbit anti-GAPDH polyclonal antibody (Good Here) was used as the loading control.

2.12. Statistical analysis

The number (n) of individual animals used per group is described in each figure panel or figure legend. The replicates of experiments are described in each figure legend. The data were analyzed using SAS 9.2. For a single comparison between two groups, t-test was used, and for multiple groups comparison, ANOVA followed by Tukey's studentized range multiple tests (Tukey) was used. Graphs show mean ± SEM. The upper threshold for statistical significance for all experiments was set at $P < 0.05$. In summary, ANOVA followed by Tukey's studentized range multiple tests (Tukey) were applied in the data of Figures 1A, 1B, 4E, 5B, 5C, 5E and S1C; and t-tests were applied in Figures 1C (left panel), 1F, 2C, 3B, 3D, 3E, 4A, 4C, 4D, S1A.

Association study of these four SNPs in *TRAPPC9* with milk somatic cell score (SCS) and the concentration of NF-κB, IL-17, IL-6, IL-4, TNF-α and IFN-γ were analyzed using general linear model (SAS 9.0):

$$y = \mu + hys + p + m + h + g + e$$

where y is the phenotype value, μ is the overall mean, hys is the effect of herd-year-season, p is the effect of parity, m is the effect of lactation stages, h is the effect of healthy status, g is the effect of SNP, and e is random residuals.

Healthy status was divided into three grades: Grade 1, health ($SCC \leq 20 \times 10^4/\text{mL}$), Grade 2, subclinical mastitis ($20 \times 10^4/\text{mL} < SCC < 50 \times 10^4/\text{mL}$), and Grade 3, clinical mastitis ($SCC > 50 \times 10^4/\text{mL}$). Healthy

status was not considered in the model when conducting association analyses between the SCS and SNPs of *TRAPPC9*.

3. Results

3.1. Remarkable genetic and epigenetic effects of bovine *TRAPPC9* on mastitis resistance

We had assessed that the genetic effect of mutations in the bovine *TRAPPC9* gene was significantly associated with bovine mastitis resistance (three significant SNPs, SNP2, SNP3 and SNP4 within *TRAPPC9* were identified, at P values 1.24E-10, 3.29E-08 and 3.64E-08, respectively, Fig. 1D) on the genome-wide level with Illumina Bovine SNP50 BeadChip ($n = 2093$, [11]). In the present study, a new Holstein population was hired to screen and validate the genetic effects of the gene on mastitis resistance.

First, to dissect the influence of *TRAPPC9* on mastitis resistance, the 514 experimental cows were divided into three categories (health, subclinical mastitis, and clinical mastitis, Fig. 1A). We observed that the transcriptional levels of the gene in peripheral lymphocyte cells decreased along with the degree of mastitis (left panel, $P = 0.003$, Fig. 1A). Similar trends were observed for the concentration of *TRAPPC9* coding protein (NIBP) (middle panel, Fig. 1A, $P = 0.008$) and NIBP-regulated protein NF- κ B in serum (right panel, Fig. 1A, $P = 0.012$).

Next, four screened SNPs in bovine *TRAPPC9* gene with pooled DNA samples ($n = 30$) were genotyped in the new dairy cattle population ($n = 514$, S2 Table), of which SNP2, SNP3 and SNP4 were detected in our initial work [11], and SNP1 was a novel mutation. We observed that SNP1 was associated with SCS (a widely used mastitis-related trait) at P -value of 0.06, SNP2 was significantly associated with the concentrations of TNF- α and IFN- γ in the serum ($P < 0.05$), while SNP3 and SNP4 were not significantly associated with any mastitis-related traits (S3 Table). In particular, the investigation of the influence of the four SNPs on the gene's transcriptional levels also clarified that only SNP2 had a significant effect on the expression level of the gene ($P = 0.016$, $n = 37$, Fig. 1B). The results indicated the importance of the SNP2 at the *TRAPPC9* gene on dairy mastitis resistance.

On the bovine genome, copy number variation (CNV) is another type of genetic variation. A previous study observed that human *TRAPPC9* is a multi-copy gene and its CNVs are associated with intellectual disability [21]. We further measured the CNVs of bovine *TRAPPC9* (2 ~ 8 copies) and assessed their association with the traits of mastitis resistance. The data revealed that the CNVs were remarkably associated with the incidence of mastitis, i.e. the subclinical/clinical mastitis cows ($SCC > 20 \times 10^4/\text{mL}$) had significantly higher CNVs than the healthy ones ($SCC < 20 \times 10^4/\text{mL}$) (left panel, $P = 0.006$, Fig. 1C). The population-specific median copy numbers of the gene (five-copies) can serve as a switch point (red arrow, Fig. 1C) for mastitis incidence, because the cows possessing less than or more than five-copies had significantly lower or higher risks of mastitis (right panel, Fig. 1C).

Aside from genetics, epigenetic modification is also critical for disease prevention. A previous study reported that the change of average DNA methylation in *TRAPPC9* is related to the regrowth of the Michigan Cancer Foundation-7 (MCF-7) breast cancer cell line [22]. In the present study, we analyzed the connection between the promoter methylation level of bovine *TRAPPC9* and four immune-related traits in the dairy cattle population (Fig. 1F and S1A Figure). On the promoter region of bovine *TRAPPC9*, two CpG regions (R1 and R2) were chosen to examine the methylation levels quantitatively by bisulfite sequencing and analyze their relationship with different immune-related traits separately (Figs. 1D-1F). Data showed that the methylation levels at the CpG 1–1 and CpG 1–2 sites in the R1 region were 100% in all the examined cattle (Fig. 1E). Using DNA sequencing, we found that the cytosine in the CpG 1–3 is on the site of SNP3 (C to T mutation). Therefore, the non-methylation of the CpG on this site could be attributed to the C→T SNP. Notably, the methylation status of the three CpG sites in the R2 region can be divided into two statuses. One is non-methylation (0%) and another is methylation (3.10% ~ 15.16%) for the tested cows (Fig. 1F). In addition, a significant association was found between the methylation status and the concentration of NF-κB in serum for the CpG 2–2 site ($P = 0.006$, Fig. 1F). Moreover, the three CpG sites in the R2 region were remarkably related to the concentrations of serum TNF-α, IL-17, and IFN-γ (S1A Figure).

The above results explained that bovine mastitis-related status traits were closely linked to the transcriptional levels, CNVs, and SNPs at the *TRAPPC9* gene and that the promoter methylation levels of the gene could significantly influence the immune responses of the dairy cows.

3.2. Expression of *TRAPPC9* and interleukin genes of bovine mammary epithelial cells were modulated by *S. aureus* and MRSA

Given the condition that genetic mutations and DNA methylations of bovine *TRAPPC9* are related to the inflammatory progression of the bovine mammary gland, we asked this question “whether the gene contributes to the inflammation pathway of the mammary gland epithelial cells or not”. To answer this question, we established a bovine mammary epithelial cell model with the Mac-T cell line induced by *S. aureus*, a major cause of bovine mastitis. Similarly, the MRSA-induced mastitis cell model was also established. Both strains of *S. aureus* (90 – 1) and MRSA (W18) used in the current study were separated from fresh bovine milk (Fig. 2A and S2 Figure).

We measured the transcriptional expression patterns of *TRAPPC9* and interleukin genes (*IL-1β*, *IL-6*, and *IL-8*) that are involved in the NF-κB signaling pathway in the *S. aureus*- and MRSA-induced mastitis cell models (Figs. 2B and 2C). We observed that the mRNA expression levels of *TRAPPC9* gradually reduced from 3 h to 24 h after the infection of *S. aureus* or MRSA (Fig. 2C). The expression of the interleukin genes initially increased and then decreased after *S. aureus*/MRSA infection (Fig. 2C), which are similar to other results [23]. Similar expression trends in Mac-T cells were observed for the four genes after *S. aureus* or MRSA infection. Remarkably, compared with uninfected cells (control), *TRAPPC9* significantly decreased at 6 h post-infection, whereas interleukin genes *IL-1β*, *IL-6*, and *IL-8* significantly increased at this time point. On the other hand, a western blot revealed that the protein level of *TRAPPC9* (NIBP) at 6 h

post-*S. aureus* infection is consistent with the variation in mRNA expression levels; moreover, the level of NF- κ B-p65 slightly decreased in the *S. aureus* infected cells compared with the control cells (Fig. 2D).

We further carried out the knockdown of *TRAPPC9* in the cell model and followed by 6 h infection of *S. aureus* or MRSA. Figure 3A shows the procedures of the RNAi assay of *TRAPPC9* and bacterial infection. The results of RT-qPCR and Western blot indicated that the RNAi targeting *TRAPPC9* was effective (Figs. 3B and 3C). The data showed that knockdown of bovine *TRAPPC9* without bacterial treatment (Control) correlated with the significant upregulation *IL-6* expression ($P < 0.001$) (the left histograms, Fig. 3D). In the cell model, compared with the *TRAPPC9*-non-knockdown cells (NC), knockdown of the gene followed by *S. aureus* infection can only significantly elevate the expression of *IL-1 β* (the middle histograms, Fig. 3D, $P < 0.05$), whereas knockdown of the gene followed by the infection of the MRSA strain (W18) can significantly up-regulate the expression of *IL-1 β* ($P < 0.05$) (the right histograms, Fig. 3D). In addition, comparing with the control cells without bacterial treatment (the white histograms in Control, upper panel, Fig. 3D), we observed that the expression of *IL-1 β* in the Lipofectamine 2000-treated Mac-T cells followed by *S. aureus* infection was significantly down-regulated ($P < 0.05$). It was contradictory with the results in Fig. 2C (upper right panel) and may be caused by the interaction between a possible toxic side effect of transfection reagent of RNAi (Lipofectamine 2000) [24, 25] and *S. aureus* infection.

Furthermore, we assessed the influence of *TRAPPC9* knockdown on the invasion abilities of *S. aureus* and MRSA into Mac-T cells. The invasion capacities of the MRSA strain (W18), as well as the *S. aureus* strain (90 – 1) into the Mac-T cells were first measured. Results showed that *S. aureus* and MRSA had different invasion trends and peaked at 6 h and 8 h (upper panel, Fig. 3E), respectively. These two time points were individually chosen for the following functional validation of *TRAPPC9* for the two types of bacteria. The knockdown of *TRAPPC9* did not influence the invasion number of *S. aureus* strain (middle panel, Fig. 3E) but slightly decreased that of MRSA strain (lower panel, Fig. 3E).

Collectively, these data suggest that the expression of *TRAPPC9*, NIBP, interleukins genes and NF- κ B-p65 can be modulated by both *S. aureus* and MRSA strains. Knockdown of *TRAPPC9* can promote the progress of inflammation *in vitro* by upregulating the expression of pro-inflammatory genes *IL-1 β* and *IL-6*.

3.3. The appropriate dose of folic acid increases *TRAPPC9* expression and decreases the invasion of the MRSA strain *in vitro*

The extent of inflammation can be reduced by dose-dependent folic acid through regulating the expression of key genes [16–18, 26]. Thus, to determine whether folic acid can effectively influence the expression of *TRAPPC9* and interleukin genes (*IL-1 β* , *IL-6* and *IL-8*), we investigated the effects of folic acid at five doses on the expression of the four genes. The invasion abilities of the two strains of *S. aureus* (90 – 1) and MRSA (W18) into Mac-T cells were also measured at the effective dose of folic acid.

We first evaluated the viability of the Mac-T cells under different doses of folic acid (0, 5, 10, 20, and 40 μ g/mL FA) with MTS assay. Results showed that the viability of the folate-treated cells was not

affected compared with the untreated cells (0 µg/mL, Fig. 4A). Thus, these five doses of folic acid were used for the next assays (Fig. 4B). We assessed the mRNA expression levels of *TRAPPC9* and the three interleukin genes via RT-qPCR. Folic acid treatment at 5 µg/mL remarkably increased the mRNA expression level of *TRAPPC9* and reduced the expression of the interleukin genes in the strains of *S. aureus* (90 – 1) or MRSA (W18) infected Mac-T cells (Fig. 4C, $P < 0.05$). Thus, 5 µg/mL folic acid was the suitable dose for restricting the infection of *S. aureus* and MRSA at least by increasing the expression of *TRAPPC9* and reducing the expression of *IL-1β*, *IL-6* and *IL-8*.

Folic acid treatment of Mac-T cells followed by *TRAPPC9* knockdown was performed to determine whether folic acid can mediate the expression of the interleukins by regulating *TRAPPC9*. In the Mac-T cells added with 5 µg/mL folic acid, knockdown of *TRAPPC9* can significantly reduce the mRNA expression of *IL-6* and *IL-1β* after the MRSA strain (W18) infection (upper and middle panels in Fig. 4D), and moderately decreased that of *IL-8* (lower panel in Fig. 4D). No apparent changes were observed in the *S. aureus* (strain 90 – 1)-infected cells for the three genes (Fig. 4D). This finding suggests that either *TRAPPC9* or folic acid can down-regulate the inflammation factors of MRSA-induced cells.

Finally, the influence of folic acid in the invasion abilities of the two strains of *S. aureus* and MRSA was investigated. Results showed that 5 µg/mL folic acid can significantly decrease the invasion number of MRSA (W18) into Mac-T cells (right panel, Fig. 4E, $P < 0.05$) but did not affect the invasion of *S. aureus* (left panel, Fig. 4E). Hence, the above results imply that a suitable dose of folic acid has a positive impact on defense against MRSA-induced mastitis.

3.4. Folic acid dose-dependently prevents mastitis and increases TRAPPC9 expression in vivo

A dairy cattle experiment was designed to investigate the effective dose of folic acid to prevent mastitis. A total of 123 Holstein cows during the perinatal period were divided into three groups (Figs. 5A). After coated folic acid supplementation for 14 days before calving and 7 days after calving, the cows supplemented with body weight (kg) × 0.24 mg/day of folic acid (group B) showed apparently higher expression of *TRAPPC9* than the controls ($P > 0.05$) (Fig. 5B, left panel). Especially, after folic acid supplementation, the expression levels of *TRAPPC9* in the group B cows were significantly increased compared with before supplementation ($P < 0.05$) (Fig. 5B, right panel). Meanwhile, the cows fed with body weight (kg) × 0.24 mg/day (group B) of folic acid exhibited significantly higher expression of *IL-6* than the controls (Fig. 5C, $P < 0.01$). After delivery, we recorded the incidence of subclinical mastitis of the dairy cattle. The body weight (kg) × 0.24 mg/day of folic acid supplementation can effectively decrease the incidence of bovine subclinical mastitis (Fig. 5D). Moreover, the cows in groups B and C added with folic acid showed moderately lower SCCs than the control cows (group A) at the second, third and fourth months after folic acid supplementation (Fig. 5E).

The above data indicate that folic acid can dose-dependently increase the expression of bovine *TRAPPC9* and prevent bovine mastitis.

4. Discussion

S. aureus and its “superbug” strain MRSA, have strong abilities of immunodepression and antibiotic-resistance [27]. In the present study, we designed a series of experiments to clarify the favorable roles of bovine *TRAPPC9*, a candidate gene of mastitis resistance, and folic acid in the prevention of mastitis and resistance to *S. aureus* and MRSA infections in dairy cows. The consequences of these examinations condensed three major findings.

The first is that the candidate gene *TRAPPC9* can be considered as a biomarker for bovine mastitis because all the investigated factors, including mRNA levels, protein expressions, SNP, CNVs, and DNA methylation of the gene, were apparently related to mastitis progress in the dairy population. *TRAPPC9* was disclosed as a bovine mastitis candidate gene in our previous GWAS study [11], which encodes the novel protein called NIK- and IKK β -binding protein (NIBP) in the NF- κ B pathway [12]. A new Holstein population was hired to investigate the effects of the gene on bovine mastitis-related traits. Not only the mRNA expression of the gene but also its protein (NIBP) level in serum were significantly correlated with mastitis progression. In specific, higher expression of the gene indicated stronger mastitis resistance of the cows. Notably, linkage disequilibrium analysis did not show close linkage disequilibrium among the identified four SNPs (S1B Figure). Among the previously significant SNPs (SNP2, SNP3 and SNP4, designed in the Illumina 50K SNP-chip), only SNP2 was associated with mastitis-related traits (serum TNF- α and IFN- γ) in the present study. The others (SNP3 and SNP4) and the novel detected SNP (SNP1) did not show a significant effect on the seven studied traits (S3 Table). This finding reminds us that for low-heritability traits (such as bovine mastitis), SNPs effects among different population may vary, thus searching for the causal mutations is necessary. In addition, abnormal CNV of human *TRAPPC9* is correlated with hereditary breast cancer [28]. Our results showed that the cows possessing five or more copies of *TRAPPC9* had a higher incidence of mastitis (Fig. 1C). However, no significant differences were found between the CNVs and the mRNA expression of *TRAPPC9* in the cows (S1C Figure), which are similar to the results of Kaufman’s studies in human [29]. The underlying reason could be that the samples chosen in the expression assay were peripheral blood tissue, while the expression level of the gene varied largely among different types of tissues [12]. Thus, choosing more types of tissues or specific blood cell types may guarantee clearer results in the future.

Genetic mutants and epigenetic modifications regulate immune gene expression [30]. Kuhmann et al. (2011) reported that the methylation changes of *TRAPPC9* are related to the regrowth of MCF-7 (human breast adenocarcinoma cell line) cells [22]. In the present study, we provided evidence that the unmethylated *TRAPPC9* promoter in region R2 is significantly correlated with higher concentration of NF- κ B and cytokines in cow serum compared to the methylated cows. *TRAPPC9* has been reported to be associated with some human diseases (liver disease and breast/colon cancer) [31]. Based on our results *in vivo* and *in vitro*, mRNA expression and methylation status of *TRAPPC9* can be used to measure the healthy status of bovine udder, and CNVs and SNP2 in this gene can be regarded as the genetic basis of marker assistant selection for bovine mastitis resistance.

The second major finding is that *in vitro* and *in vivo* data indicated the preventive effect of folic acid on inflammation induced by *S. aureus* and MRSA. Decreasing the effectiveness of antibiotic drugs for

zoonotic *S. aureus* and MRSA is a critical issue facing modern medical treatment. Increasing evidence indicates that the epidemiology of livestock-associated MRSA has raised concerns about its existence in food animals [32]. Up to date, the transmission of livestock-associated MRSA from farms to human is reported in several countries [9, 33, 34]. Folic acid is an essential water-soluble B vitamin with multiple biological functions, such as antibacterial, antibiotic, anti-apoptotic, anticancer, and anti-inflammatory [35–39]. However, the effect of folic acid on inflammation induced by *S. aureus* or MRSA was rarely reported. In the current study, *S. aureus*- and MRSA-induced mastitis cell models were formulated to investigate the effects of folic acid on mastitis resistance and prevention. In our results, the expression patterns of the interleukin genes (*IL-1 β* , *IL-6*, and *IL-8*) in Mac-T cells are similar to other results [40, 41], i.e., the mRNA expression of *IL-1 β* , *IL-6*, and *IL-8* initially increased and then decreased (Fig. 2C), which may be related to immunosuppression mediated by *S. aureus* [28, 42]. These results indicated the success of the formulated *S. aureus*- and MRSA-induced mastitis cell models. Folic acid plays a positive role in preventing inflammation, oxidative stress, and some diseases possibly by mediating the expression of cytokines (*IL-1 β* , *IL-6*, TNF- α and others) [17, 19, 43, 44]. During female pregnancy and lactation, appropriate folate intake reduced the expression of *IL-6* and TNF- α of dams and their offspring [45]. Our *in vitro* experiments showed similar results that appropriately supplementary folic acid (5 μ g/mL) reduced the inflammation of the mammary epithelial cells induced by either *S. aureus* or MRSA may through influencing the expression of interleukins genes (*IL-1 β* , *IL-6*, and *IL-8*). Lower incidence of mastitis was also observed in the cows with folic acid supplementation compared with the control cows. It is worth noting that contradiction exists between the *in vivo* and *in vitro* studies regarding the effect of folic acid on *IL-6*. This may be due to the two different experimental materials *in vivo* (peripheral blood) and *in vitro* (Mac-T cells), and context-dependent pro- and anti-inflammatory properties of *IL-6* [46]. In the future, effect of folic acid on the *IL-6* expression level *in vivo* and *in vitro* will be further investigated.

The third major finding is that folic acid prevents the inflammation progresses induced by MRSA partially through a *TRAPPC9* mediated NF- κ B pathway. Folic acid can alleviate inflammation by regulating some inflammation-related genes (e.g., interleukin genes) in the NF- κ B pathway [17, 47, 48]. NF- κ B is a ubiquitous transcriptional factor and plays a pivotal role in many pathophysiological processes including inflammation, immune response, aberrant cell growth or apoptosis [49–51]. NF- κ B activation is widely proved to be the central initiating cellular affair of host responses to invasion by bacteria. Under normal conditions, NF- κ B remains inactive by the inhibitor of NF- κ B (I κ B), and it can be activated by I κ B kinase (IKK) or NF- κ B inducing kinase (NIK) [1]. In the current study, NF- κ B-P65 and NIK- and IKK β -binding protein NIBP (coded by *TRAPPC9* gene) decreased at 6 h post-stimulation with *S. aureus* (Fig. 2C and 2D). Opposite expression trends between *TRAPPC9* and interleukin genes (*IL-1 β* and *IL-6*) in Mac-T cells infected with *S. aureus* or MRSA were verified in RNAi assay (Fig. 2C and 3D). These data are consistent with the previous report that high expression of *IL-1 β* corresponds to the peak SCC in cows with mastitis [52]. Based on our results, *IL-1 β* and *IL-6* in these three cytokines are more likely to be regulated by *TRAPPC9*. The importance of NF- κ B in the process of bovine mastitis has been widely reported [53, 54], thus as the upstream regulatory factor of NF- κ B, *TRAPPC9* coding NIBP may possess greater potential to

promote bovine mastitis resistance and can be regarded as a more valuable biomarker for bovine mastitis. Thus, a deeper study of this potential effect is warranted to be conducted.

Furthermore, in dairy cattle, the expression levels of *TRAPPC9* and NF-κB reduced with the increased inflammation of mammary glands (Fig. 1A). However, the expression level of *TRAPPC9* was only moderately increased in the cows after feeding extra folic acid during the periparturient period, which may be due to the existence of the above-mentioned dosage effect of folic acid or insufficient feeding time at the individual level. The transcriptional expression levels of *RELA* and *RELB* in the NF-κB family and *MAP3K14* and *IKBKB* in the NF-κB pathway were positively correlated with that of *TRAPPC9* in a healthy dairy population post folic acid supplementation (Figure S3, $P < 0.05$). It is reported that infections caused by methicillin-resistant strains of *S. aureus* (MRSA) have a higher mortality rate than infections caused by methicillin-susceptible strains [55]. Some researchers verified that MRSA has a high capacity to destroy the function of innate immune cells and complement, evade neutrophil killing and survive after phagocytosis [56]. Consistently, our study showed that MRSA led to more severe mastitis inflammation responses than *S. aureus* in dairy mammary epithelial cells, which could adhere and invade quickly and strongly to Mac-T cells. Moreover, folic acid can markedly restrict the invasion of MRSA into bovine Mac-T cells, and reduce the inflammation induced by MRSA. However, the invasion of *S. aureus* cannot be decreased. More doses and time points of folic acid addition for the prevention of *S. aureus* infection should be explored later. Overall, combined above data with previous studies results, we propose that folic acid can prevent MRSA-induced mastitis partially through a *TRAPPC9*-mediated NF-κB pathway (Fig. 6).

5. Conclusion

In summary, we have revealed that folic acid has a benignant effect on bovine MRSA mastitis by up-regulating the expression of *TRAPPC9* in a dose-dependent manner in Mac-T cells (5 µg/mL) and in dairy cows (body weight (kg) × 0.24 mg/day). Our study highlighted the importance of internal bovine *TRAPPC9* mediated NF-κB pathway and external folic acid supplementation in treating and preventing zoonotic *S. aureus* and MRSA infections, which may reduce the overuse of antibiotics in livestock effectively and improve public health.

Abbreviations

<i>TRAPPC9</i>	trafficking protein particle complex 9
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
MRSA	methicillin-resistant <i>S. aureus</i>
<i>IL-1β</i>	interleukine 1β
<i>IL-6</i>	interleukine 6

<i>IL-8</i>	interleukine 8
IL-17	interleukine 17
Mac-T	bovine mammary epithelial cell lines/bovine mammary alveolar cells
NF-κB	nuclear factor-kappa B
SCC	somatic cell count
SCS	somatic cell score
EDTA	ethylenediaminetetraacetic acid
PCA	plate count agar
CFU	colony-forming units
qPCR	quantitative real-time PCR
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase gene
MOI	multiplicity of infection
PBS	phosphate buffer saline
DMEM	Dulbecco's modification of Eagle's medium Dulbecco
RNAi	RNA interference
SDS-PAGE	sodium dodecyl sulfate -polyacrylamide gel electrophoresis
TBST	Tris-buffered saline-Tween
ANOVA analysis of variance	
TNF-α	tumor necrosis factor alpha
IFN-γ	Interferon-gamma
NIBP	<i>TRAPPC9</i> coding protein
CNV	copy number variation
MCF	Michigan Cancer Foundation-7 breast cancer cell line
W18	MRSA strain

90-1	<i>S. aureus</i> strain
IκB	inhibitor of NF-κB
IKK	IκB kinase
NIK	NF-κB inducing kinase

Declarations

Ethics approval and consent to participate:

All animal experiments were approved by the Animal Care Committee, China Agricultural University.

Consent for publication

Not applicable.

Availability of data and material

The raw data for the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Conceptualization: Y.Y.; Data curation: S.M. and L.S.; Formal analysis: S.M.; Funding acquisition: Y.Y.; Investigation: M.S., Y.D., Z.Z., L.F. and L.S.; Methodology: S.M.; Resources: L.F.; Supervision: Y.Y.; Validation: S.M, M.S. and Y.D.; Writing – original draft: S.M.; Writing – review & editing: S.M. and Y.Y.

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Figures

Figure 1

Systematic validation of the genetic and epigenetic effects of the TRAPPC9 gene on bovine mastitis resistance (A) The relative mRNA expression of TRAPPC9, the concentration of NIBP and concentration of NF-κB in the healthy cows (HC), subclinical mastitis cows (SC), and clinical mastitis cows (CC) ($n = 12/\text{group}$). HC refers to cows with $\text{SCC} \leq 20 \times 10^4/\text{mL}$; SC means $20 \times 10^4/\text{mL} < \text{SCC} < 50 \times 10^4/\text{mL}$, and CC was diagnosed by a veterinarian. (B) Relative mRNA expression of TRAPPC9 in different

genotypes of SNP2. (C) Distribution and difference of bovine TRAPPC9 gene copy numbers in HC (n = 62) and SC or CC (n = 51). (D) Sketch map of SNPs about the bovine TRAPPC9 gene. Blue boxes represent the exon in the gene. Red boxes represent SNPs identified and analyzed in this study. Orange ellipses represent two CpG regions (R1 and R2) in the promoter of the bovine TRAPPC9 gene; the number in brackets represents the locations of these SNPs on the chromosome. The P values on the right of SNP2, SNP3 and SNP4 were derived from our previous GWAS study. (E) Methylation level at the CpG sites in R1 (n = 166). (F) Relationship between the methylation status at the CpG sites in R2 and the concentration of NF- κ B (n = 146). Data are means $\pm$ SEM. *P < 0.05, **P < 0.01.

Figure 2

The expression level of TRAPPC9 and interleukin genes and bacterial invasion abilities in the mastitis cell model. (A) *S. aureus* (left panel) and MRSA (right panel) strains used in this experiment. (B) Procedures of measuring the expression level of host genes and the invasion abilities of *S. aureus* and MRSA. (C) Relative mRNA expression levels of TRAPPC9, IL-1 β , IL-6, and IL-8 in Mac-T cells infected with *S. aureus* or MRSA respectively, from 0 to 24 hr. Control means uninfected Mac-T cells. *, **, *** (MRSA) or #, ##,

(*S. aureus*) indicate a P-value less than 0.05, 0.01, and 0.001 determined by t-test respectively. (D) The protein level of TRAPPC9 and NF-κB-P65 in uninfected Mac-T cells and Mac-T cells at 6 hr post-infection. C: control; S: *S. aureus* infection.

Figure 3

Influence of TRAPPC9 on the invasion abilities and progress of inflammation induced by *S. aureus* or MRSA. (A) Procedures of RNAi and bacterial infection. (B) Relative mRNA expression of TRAPPC9 in Mac-

T cells with negative control of siRNA NC or TRAPPC9 siRNA with (right panel) or without (left panel) *S. aureus* infection. Bovine GAPDH was used as the house-keeping gene. (C) Western blot results of NIBP in Mac-T cells with NC-siRNA or TRAPPC9 siRNA. (D) Relative mRNA expression of interleukin genes (IL-1 β , IL-6, and IL-8) in Mac-T cells with NC-siRNA/TRAPPC9 siRNA (Control) or with NC-siRNA/TRAPPC9 siRNA and followed by *S. aureus* or MRSA infection. (E) The upper panel shows the number of *S. aureus* and MRSA invading into the Mac-T cells over time, the middle panel shows the number of *S. aureus* invading the Mac-T cells at 8 hr after *S. aureus* infection in NC siRNA and TRAPPC9 siRNA, and the lower panel shows the number of MRSA invading the Mac-T cells at 6 hr post MRSA infection in NC siRNA or TRAPPC9 siRNA. S: *S. aureus* infection; M: MRSA infection; N: Negative control of siRNA; T: TRAPPC9 siRNA; MRSA: Methicillin-resistant *S. aureus*. Data were collected from three independent infections (three wells infected Mac-T cells). Data are shown as mean $\pm$ SEM. *, ** indicate a P-value less than 0.05, 0.01 determined by t-test, respectively.

Figure 4

Functional analysis of folic acid in *S. aureus*- and MRSA-induced mastitis cell models in vitro. (A) Effects of folic acid on the viability of Mac-T cells determined by MTS assay. (B) Procedures of folic acid supplement, RNAi and bacterial infection. (C) Relative mRNA expression levels of TRAPPC9, IL-1 β , IL-6, and IL-8 in Mac-T cells with folic acid at different doses for 2 h, followed by stimulating with *S. aureus* or MRSA (1×10^8 CFU/mL) for 6 h at a multiplicity of infection of 10 bacteria to 1 Mac-T cell (MOI = 10:1).

Control means uninfected Mac-T cells. # shows the comparison between the control cells and the cells (infected with *S. aureus* or MRSA) treated with folic acid at 0 $\mu\text{g/mL}$. * shows the comparisons between the Mac-T cells (infected with *S. aureus* or MRSA) treated with 5, 10, 20, and 40 $\mu\text{g/mL}$ folic acid and the cells treated with 0 $\mu\text{g/mL}$ folic acid. (D) Relative mRNA expression of the interleukin genes (IL-1 β , IL-6, and IL-8) in Mac-T cells with or without folic acid supplementation (5 $\mu\text{g/mL}$), knockdown of TRAPPC9, and followed with *S. aureus* or MRSA infection. (E) The number of *S. aureus* or MRSA invading the Mac-T cells at 8 h after *S. aureus* or MRSA infection with prior folate supplement (5 $\mu\text{g/mL}$) and TRAPPC9 knockdown. S: *S. aureus* infection; M: MRSA infection; T: TRAPPC9 siRNA; F: supplement of folic acid; N: negative control. Data were collected from three independent infections (three wells infected Mac-T cells). The data are shown as mean $\pm$ SEM. *, ** or #, ## indicate a P-value less than 0.05 and 0.01 determined by t-test, respectively.

Figure 5

Influence of folate supplement in vivo. (A) Folate supplement for Holstein cows during the perinatal period. Group A: 0 mg/day feeding of folate, group B: (body weight (kg) $\times$ 0.24mg/day, and group C: (body weight (kg) $\times$ 0.48mg/day). (B) Relative mRNA expression of TRAPPC9 in the three groups after 21

days of folate supplementation. (C) The concentration of IL-6 in the serum in the three groups after 21 days of folic acid supplementation. (D) Incidence of bovine subclinical mastitis during this experiment within one month after folate supplementation. The red number in brackets means the number of cows with subclinical mastitis, and black number means the total number of cows used in each group. Fisher's exact test was used to analyze this data ($P > 0.05$). (E) SCC in the three groups at second, third, and fourth months after calving.

Figure 6

Proposed mechanisms about the prevention of MRSA-induced mastitis by folic acid pretreatment and TRAPPC9 activation. (A) Healthy Mac-T cells without MRSA infection. NIBP (TRAPPC9) can combine IKK β and then activate the classical pathway of NF- κ B. (B) Mac-T Cells infected with MRSA significantly decreased NIBP and moderately decreased RELA (NF- κ B-P65). In addition, a possibly negative feedback relationship was found between NIBP and pro-inflammatory factors (IL-1 β and IL-6). Folic acid plays a role of prevention in MRSA-induced mastitis at least partially by elevating the expression of TRAPPC9, reducing the expression of IL-1 β and IL-6, and inhibiting the invasion of MRSA.

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