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TGF-β1 promoted the infection of bovine mammary epithelial cells by *Staphylococcus aureus* through increasing expression of cells' fibronectin and integrin β1

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ABSTRACT

Mastitis is a disease that affects dairy cattle and causes a decline in milk quality as well as economic loss worldwide. TGF-β1 levels are usually increased during mastitis; however, it is unknown whether TGF-β1 is involved in bovine mastitis. Therefore, this study evaluated the effects of TGF-β1 on the susceptibility of bovine mammary epithelial cells (BMECs) to *Staphylococcus aureus* (*S. aureus*). The results revealed that *S. aureus* adhesion to and invasion of BMECs was significantly increased after cells were treated with TGF-β1. Adhesion of *S. aureus* to BMECs was increased dramatically by upregulation of fibronectin (Fn) and integrin β1 (ITGB1), while the increase in the susceptibility of BMECs to *S. aureus* was blocked by specific antibodies against either Fn or ITGB1. These results indicated that adhesion and invasion were increased by TGF-β1-induced upregulation of both Fn and ITGB1. Furthermore, TGF-β1 treatment prior to *S. aureus* infection significantly increased *S. aureus* colonization as well as Fn and ITGB1 expression in the mammary glands of mice. These results suggest that TGF-β1 promoted the expression of Fn and ITGB1 on the surface of BMECs and contributed to mammary gland infection in vitro and in vivo. The results of this study imply that Fn and ITGB1 may be useful therapeutic targets for the treatment of mastitis in dairy cows.

1. Introduction

In the dairy industry, bovine mastitis is a major disease that causes economic loss worldwide (Lahouassa et al., 2007). *Staphylococcus aureus* (*S. aureus*) is postulated to be the most important etiological organism responsible for bovine mastitis, causing clinical and subclinical mammary infections (Wu et al., 2016). *S. aureus* was originally considered an extracellular pathogen (Di Grazia et al., 2014); however, evidence shows that it enters host cells to avoid immune attack and cellular barriers (Edwards et al., 2011). Consequently, prompt detection of pathogens by the innate immune system is of prime importance. Bovine mammary epithelial cells (BMECs) are the main, front-line functional cells in mammary gland tissue, and they are important sentinels (Gilbert et al., 2013). Therefore, it is important to explore the mechanisms of bacterial infection in bovine mammary glands to prevent and control this disease.

In recent years, it has been reported that some cytokines are associated with many diseases (Nie et al., 2008; Zarzynska, 2014). There is evidence that IL-27 can modulate susceptibility to *S. aureus* pneumonia after influenza infection (Robinson et al., 2015); IFN-γ increases the susceptibility of HEI-OC1 cells to TNF-α cytotoxicity via JAK1/2-STAT1 signaling (Sordillo, 2016). TGF-β1 is a multifunctional cytokine that has broad-spectrum biological activities in epithelial cells and derived carcinoma cell populations both in vitro and in vivo (Zarzynska, 2014). Early studies revealed that TGF-β1 promotes coronary artery disease by upregulating sphingosine kinase 1 (SPHK1) and further upregulating its downstream effector TIMP-1 (Wang et al., 2018). *Mycobacterium avium* upregulates TGF-β1 expression, which can increase the susceptibility of

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specific tissues to many other bacterial pathogens (Aung et al., 2002).

TGF-β1 is essentially required for normal growth and development of the mammary gland, especially during involution of the mammary gland (Moses and Barcellos-Hoff, 2011). It is known that the primary function of TGF-β1 is to regulate the production of extracellular matrix (ECM) components, including Fn, collagen, laminin and multiple integrin subunits (Sugiyama et al., 2013). Furthermore, when the breasts of dairy cows were infected with bacteria, TGF-β1 levels were increased in the serum and milk of the cows (Bannerman et al., 2008; Chockalingam et al., 2005). It has been reported that *S. aureus* can increase the production of TGF-β1 during intramammary infection, and TGF-β1 further regulates the corresponding immune response (Andreotti et al., 2014; Jensen et al., 2013). Moreover, Wu et al. found that *S. aureus* upregulated the secretion of TGF-β1 by activating NF-κB and AP-1 in BMECs (Wu et al., 2016). Our team also demonstrated that a corn straw-based diet increased the secretion of TGF-β1 in peripheral blood mononuclear cells of dairy cows (Che et al., 2018). Therefore, we formulated the general hypothesis that higher TGF-β1 levels in the body may influence the incidence of dairy mastitis. Breast tissue is mainly composed of mammary fibroblasts and BMECs, and the effect of TGF-β1 on the susceptibility of these two types of cells to bacteria should be monitored. Previously, we revealed that TGF-β1 promoted the adhesion of *S. aureus* to bovine mammary fibroblasts through the ERK signaling pathway (Zhao et al., 2017), but the effect of TGF-β1 on the susceptibility of BMECs to *S. aureus* remains unknown.

In this study, we established an infection model to identify the susceptibility of BMECs to *S. aureus* and clarify the role of TGF-β1 in this infection process. Two genes associated with TGF-β1-mediated upregulation in *S. aureus* infection were screened by RT-PCR. In addition, the roles of the two corresponding proteins during *S. aureus* adhesion to BMECs were evaluated. Finally, we assessed the effect of TGF-β1 on mastitis caused by *S. aureus* in mice.

2. Methods

2.1. Bacterial strains and culture conditions

The *S. aureus* strain ATCC35556 was obtained from the clinical veterinary medicine department laboratory. Bacteria were grown at 37°C in Mueller-Hinton (MH, Hopebio, China) medium in a shaker for approximately 10 h to an OD600 of 1.6, equivalent to 5.3×10^6 cfu/ml. Bacteria were then washed three times with PBS prior to the adhesion and invasion assays.

2.2. Cell culture

A primary culture cell separation technique was used to isolate BMECs, as previously described (Hu et al., 2009). BMECs were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS, Clark, Australia), 100 mg/ml streptomycin, and 100 U/ml penicillin and were incubated at 37°C in a humidified atmosphere containing 5% (v:v) carbon dioxide.

2.3. S. aureus adhesion to BMECs

Approximately 10^5 BMECs in complete medium were plated in 24-well plates and incubated for 24 h; cells were then treated with varying concentrations of TGF-β1 (0 ng/ml, 0.1 ng/ml, 1 ng/ml, 5 ng/ml, and 10 ng/ml) for 24 h. Next, *S. aureus* was added to cells at a multiplicity of infection (MOI) of 100:1 and incubated at 37°C in a humidified incubator with 5% CO_2 for 30 min. The supernatant was removed, and 100 μl of lysis buffer (containing 50 μl of 0.25% Triton X-100 and 0.05% trypsin) were added to lyse cells. The lysates were diluted 10^2-10^4, and 100 μl of each dilution was applied to LB solid medium and cultured overnight for colony counting.

(In addition, we calculated the number of bacteria that were internalized into the cells. The number of bacteria that were internalized into the cells was extremely small and could be ignored.)

2.4. S. aureus invasion of BMECs

BMEC cultures were established as described above and incubated with *S. aureus* at an MOI of 100:1 at 37°C in a humidified incubator with 5% CO_2 for 4 h. The supernatant was then removed, and gentamycin was added (100 μg/ml) for 2 h to kill any residual *S. aureus* bacteria on the cell surface. The cell layer was washed three times with PBS, and 100 μl of lysis buffer was added to lyse cells. The lysates were diluted 10^2-10^4, and 100 μl of each dilution was applied to LB solid medium and cultured overnight for colony counting.

To determine the mechanism by which TGF-β1 promotes *S. aureus* adhesion to and invasion of BMECs, we divided cells into four groups for the experiments. The first group of normal BMECs did not receive any treatment. The second group of BMECs were treated with TGF-β1 (5 ng/ml). In addition, after we treated BMECs with TGF-β1 (5 ng/ml) for 24 h, the supernatant was removed, and a new basal medium was added. Cells subjected to this treatment were our third experimental group. Moreover, the removed supernatant (after TGF-β1 treatment for 24 h) was transferred to the untreated cells. Cells subjected to this treatment were our fourth group. After 24 h of culture, adhesion and invasion assays were performed.

Further, to determine the effect of Fn and ITGB1 on BMEC adhesion and invasion by *S. aureus*, cells were treated with rabbit anti-Fn antibody (diluted 1:500, Affinity Biosciences, AF5335) and rabbit anti-ITGB1 antibody (diluted 1:500, Affinity Biosciences, BF0336) for 45 min. Then, BMECs were treated with TGF-β1 for 24 h, and adhesion and invasion assays were performed.

2.5. Real-time (RT)-PCR for measuring target gene mRNA levels

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. After each group of RNA was adjusted to 1 μg, reverse transcription was performed. cDNA was synthesized using reverse transcriptase (Fermentas, Burlington, USA). During the reverse transcription process, the reverse transcription reagents that we use can first clear the genomic DNA. All PCR amplicons were sequenced to ensure correct transcript quantification (data not shown). The relative transcript levels of the target genes were determined using the 2-△△CT method. We used GAPDH as a housekeeping gene for quantitative analyses. Primers were adopted from previous works (Table 1). TGF-β1 was applied at a concentration of 5 ng/ml for 24 h.

The reaction conditions were as follows: 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min, followed by a melting curve.

Table 1
Summary of target genes evaluated by RT-PCR in the present study.

Gene name	Amplicon size (bp)	Primer sequences: F and R
Fn	118	F-5′-CCTCCCACTGATTTGCGATTT-3′ R-5′-CATTTTTCACAGGCGAGTAGCG-3′
ICAM1	131	F-5′-GGATGGAGGAAAATGAGGAGGG-3′ R-5′-AGGAGGACTCAGGGTCAGGGTG-3′
ITGB1	157	F-5′-TTCCACGGATGCTGGGTTTCAC-3′ R-5′-TTCGCTTAGTTTCTGGACAAGGTGAG-3′
EFNA1	178	F-5′-GCAAGGATTTCAAAGAGGGACA-3′ R-5′-AGAACCTGCACCTCCGGGTCAT-3′
TLR4	104	F-5′-AGCTCTGCCTTCACTACAGGG-3′ R-5′-GGACACCACGACAATAACCTTAC-3′
GAPDH	176	F-5′-GGGTCATCATCTCTGCACCT-3′ R-5′-GGTCATAAGTCCCTCCACGA-3′

2.6. Western blot analysis for protein expression

For extraction of total protein, cells were washed with cold PBS and lysed in 40 μl of RIPA buffer. The protein concentration was determined using the BCA (Thermo, USA) method, and the protein samples were mixed with 5 × SDS sample buffer and subjected to 12% SDS–PAGE. After an electrophoresis unit was used to transfer the proteins to a polyvinylidene fluoride membrane, the membrane was submerged in 5% (w/v) nonfat milk in TBST for blocking for 4 h and was then incubated with the primary antibodies—anti-Fn (diluted 1:1000) and anti-ITGB1 (diluted 1:1000)—at 4℃ overnight (Peng et al., 2016). The next day, the samples were incubated with HRP-conjugated secondary antibody (diluted 1:6,000, Life Science, China) for 45 min at room temperature. The relative levels of the target proteins were estimated using densitometry, and β-actin was used as the internal control. TGF-β1 was applied at a concentration of 5 ng/ml for 24 h.

2.7. Immunofluorescence analysis

BMEC cultures were established as described above, and cells were treated with DMEM/F12 medium containing 5 ng/ml TGF-β1 for 24 h (normal cells not stimulated with TGF-β1 or bacteria were used as the control group). Next, *S. aureus* was added to cells at an MOI of 100:1 and incubated at 37℃ in a humidified incubator with 5% CO_2 for 1 h. Next, the supernatant was removed, and 4% paraformaldehyde was added to fix cells. Cells were treated with goat serum for 30 min after the addition of 0.5% Triton-X-100 and were incubated with primary antibodies—anti-Fn (diluted 1:100), anti-ITGB1 (diluted 1:100) and anti-Protein A (anti-SPA, diluted 1:100, Abcam, USA)—at 4 ˚C overnight (Su et al., 2015). The next day, the cells were incubated at 37 ˚C for 45 min and with fluorescent secondary antibody (diluted 1:1000, Affinity, USA) for 2 h at room temperature in the dark (Odell and Cook, 2013). After Hoechst was added to the cells, they were washed three times with PBS to remove the excess Hoechst. PBS was then added to each well, and images were acquired using a fluorescence microscope (Olympus, FV300).

2.8. Animal experiments

A total of 32 pregnant female ICR mice weighing 40 g were purchased from the Center of Experimental Animals at Baiqiuen Medical College of Jilin University (Jilin, China). The animals were maintained on a 12 h light/dark cycle with free access to food and water. The mice were randomly divided into 4 groups (A, B, C and D). Groups B and D were subcutaneously injected with 100 μl of TGF-β1 (200 ng/ml) in the fourth pair of mammary fat pads. In addition, groups A and C were injected with saline as a control via the same method. After 24 h, groups C and D were injected with 100 μl of *S. aureus* suspension (10^8 cfu/ml), and groups A and B were injected with saline as a control. Group A was the control group, and groups B, C, and D were the experimental groups. Before sacrifice of the mice by cervical dislocation at 24 h post injection, blood was harvested. Then, the mammary glands and spleens were harvested on ice in sterile PBS. The blood and spleen suspensions were diluted 10^2-10^4, and 100 μl was applied to LB solid medium and cultured overnight for colony counting. The fourth pair of mammary glands was dissected and used for colony counting and for histopathological and immunohistochemical analyses. The mammary gland tissues were fixed for 3 days in 4% formaldehyde. Next, 5-μm sections were cut for histopathological and immunohistochemical analyses. After rehydration, antigen retrieval was performed by microwaving the slides in 10 mM sodium citrate (pH 6) for 20 min. Then, the slides were sequentially incubated in 3% H_2O_2 to block endogenous peroxidase activity and in 5% bovine serum albumin (BSA, Sigma-Aldrich) to block nonspecific binding. Primary antibodies, which were diluted 1:100 in 5% BSA, against Fn and ITGB1 were used at 4 ˚C overnight in a humidified chamber. HRP-labeled goat anti-rabbit IgG was the secondary antibody (Proteintech, USA). The primary antibody was replaced with the same dose of PBS in the negative control group, and the other steps were identical. Immunohistochemical staining was examined under a microscope and analyzed using ImageJ. The differences among groups were analyzed with SPSS19. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee, and all animals were cared for in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.9. Statistical analysis

Data were statistically analyzed using SPSS software (ver. 19 for Windows; SPSS Inc., Chicago, IL, USA). The differences between the mean values of normally distributed data were assessed by one-way ANOVA (Dunnett's test) and a two-tailed Student's t-test. A P-value of less than 0.05 was considered statistically significant. All graphs of statistical analysis results were generated in GraphPad Prism 6.0 software.

3. Results

3.1. TGF-β1-induced S. aureus adhesion to and invasion of BMECs

To identify whether TGF-β1 affected *S. aureus* adhesion to and invasion of BMECs, BMECs were treated with varying concentrations of TGF-β1 (0, 0.1, 1, 5 and 10 ng/ml) for 24 h, and adhesion and invasion assays were then performed. Treatment with 5 ng/ml TGF-β1 significantly increased *S. aureus* adhesion to and invasion of BMECs ($P < 0.05$) (Fig. 1A and B). Furthermore, the enhanced susceptibility

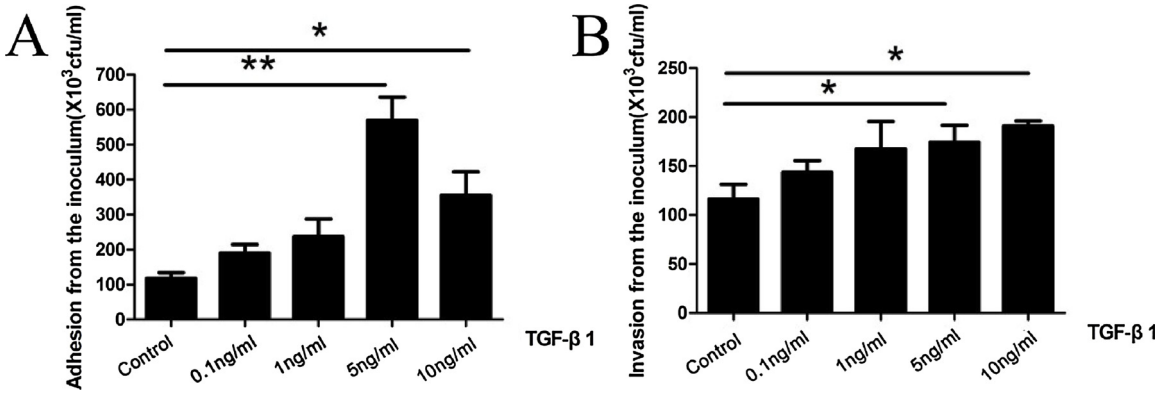

Fig. 1. *S. aureus* adhesion to and invasion of BMECs. *S. aureus* adhesion to and invasion of BMECs was significantly increased after treatment with 0 ng/ml, 0.1 ng/ml, 1 ng/ml, and 5 ng/ml TGF-β1 for 24 h. The results are expressed as the mean cfu/ml ± SD from experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$.

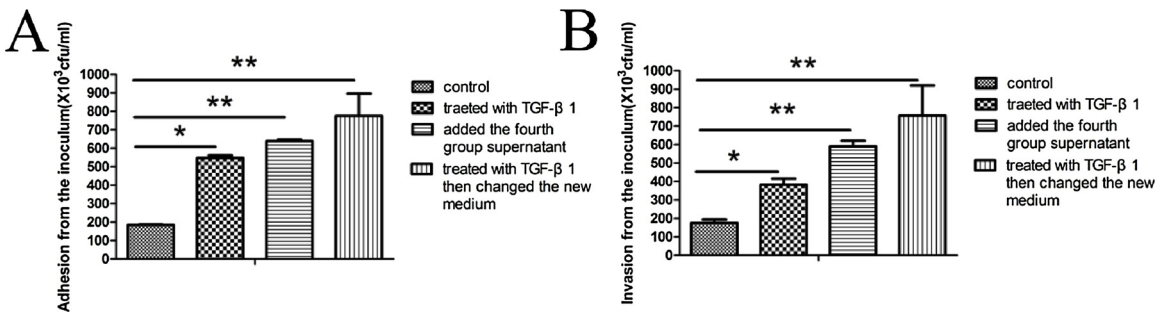

Fig. 2. TGF-β1 promoted *S. aureus* adhesion to and invasion of BMECs via receptors on the cell surface. After BMECs were cultured with TGF-β1 (5 ng/ml) for 24 h, adhesion and invasion assays were performed. *, $P < 0.05$; **, $P < 0.01$.

induced by TGF-β1 was dose-dependent in a range from 0 to 5 ng/ml.

3.2. TGF-β1 promoted the infection of BMSCs by S. aureus by altering the cell membrane receptors

TGF-β1 treatment of BMECs at different stages significantly increased *S. aureus* adhesion to and invasion of cells (Fig. 2A and B). *S. aureus* adhesion and invasion were enhanced in cells treated with TGF-β1 for 24 h compared with control cells. In addition, after BMECs were treated with TGF-β1 for 24 h, the supernatant was removed, and a new basal medium was added. With this treatment, *S. aureus* adhesion to and invasion of BMECs was also increased. In addition, after TGF-β1 was added to the cells for 24 h, cell culture supernatant was used to culture the untreated cells, and *S. aureus* adhesion and invasion were still significantly enhanced. These results showed that TGF-β1 increased *S. aureus* adhesion to and invasion of BMECs by promoting alterations of the cell membrane receptors. This effect may be due to TGF-β1 stimulation in BMECs, causing cells to secrete certain matrix components.

3.3. The expression of Fn and ITGB1 was upregulated by TGF-β1

Several cellular components may be associated with TGF-β1-mediated upregulation during *S. aureus* infection (Goldmann et al., 2016; Ivarsson et al., 2013). Therefore, we screened five genes, namely, Fn, ITGB1, hepatic E1 (EFNA1), intercellular cell adhesion molecule-1 (ICAM1) and Toll-like receptor 4 (TLR4). We used RT-PCR to measure the expression of these genes in the different groups. The results showed that the expression of Fn and ITGB1 was significantly upregulated in TGF-β1-treated BMECs (Fig. 3A). Fn and ITGB1 protein expression was assessed via western blot analysis (Fig. 3B). TGF-β1 significantly promoted Fn and ITGB1 expression (Fig. 3A and B).

3.4. Fn and ITGB1 contributed to S. aureus adhesion to and invasion of BMECs

TGF-β1 contributed to *S. aureus* infection of BMECs by enhancing Fn and ITGB1 expression. Immunofluorescence was used to detect the expression of Fn and ITGB1 proteins (red). *S. aureus* adhesion to BMECs was identified with SPA, a specific antibody against *S. aureus*. TGF-β1 significantly promoted Fn and ITGB1 expression as well as the adhesion of *S. aureus* to BMECs (Fig. 4A and B). After blocking Fn and ITGB1 with anti-Fn and anti-ITGB1 antibodies, respectively, the ability of *S. aureus* to adhere to and invade BMECs was significantly inhibited (Fig. 4C and D). The effect of the blocking antibodies was inhibiting bacterial interaction with Fn or ITGB1. TGF-β1 increased *S. aureus* adhesion to and invasion of BMECs by promoting the expression of Fn and ITGB1 on the cell membrane.

3.5. TGF-β1 promoted S. aureus infection in mouse mammary glands

The mouse model of *S. aureus* infection was established to assess the effect of TGF-β1 on *S. aureus* colonization and determine whether *S. aureus* could spread into the bloodstream. The *S. aureus* colonization rate in the mammary glands and blood of the TGF-β1-treated group was significantly higher than that in the untreated group (Fig. 5A and B). This result indicated that TGF-β1 also promoted the speed of *S. aureus* transfer into the blood. However, the amount of *S. aureus* in the spleen was not significantly affected (Fig. 5C). *S. aureus* destroyed the integrity of breast tissue and caused damage to breast tissue; in addition, TGF-β1 pretreatment enhanced the degree of inflammatory cell infiltration and acinar damage caused by *S. aureus*. Therefore, we concluded that TGF-β1 treatment could lead to the typical symptoms of mastitis (Fig. 5D). On the other hand, we found that TGF-β1 promoted the expression of Fn and ITGB1 in damaged acini and the surrounding connective tissue (Fig. 5E, 5 F, 5 G). Taken together, these results revealed that TGF-β1 enhanced mammary gland damage caused by *S. aureus* by promoting the expression of Fn and ITGB1 in vivo.

4. Discussion

Cytokines are usually regulated by diet or nutrition, and dysfunction of cytokines also influences immunity in host cells and tissues (Smidowicz and Regula, 2015). It has been reported that TGF-β1 is elevated in the serum and milk of cows fed with corn straw as roughage and was positively related with mastitis (Che et al., 2018). In this study, we found that TGF-β1 significantly enhanced *S. aureus* adhesion to and invasion of cells by increasing the expression of Fn and ITGB1 in BMECs and markedly exacerbated mastitis symptoms and mammary gland lesions in infected mice. This finding implicated TGF-β1 as an "accomplice" of mastitis—i.e., increased TGF-β1 secretion in cows elevated the susceptibility of breast tissue to pathogens; therefore, mastitis was caused not only by pathogens but also by nutritional factors. This finding provides us with a new approach for controlling mastitis.

TGF-β1 has a dual role—maintaining health or inducing disease. Studies have shown that TGF-β1 can cause blood-brain barrier disorders by promoting *E. coli* K1 adhesion to and invasion of human brain microtubule endothelial cells (Zhang et al., 2002) and promotes of Group A *Streptococcus* (GAS) invasion of Hep-2 cells (Wang et al., 2006). TGF-β1 is mainly required during the physical development of mammary gland tissue in cows, but it also induces the proliferation of fibroblasts, leading to fibrosis, and promotes *S. aureus* adhesion to and invasion of fibroblasts by promoting the secretion of large amounts of type I collagen and α-SMA (Zhao et al., 2017). Our results showed that TGF-β1 induced the expression of Fn and ITGB1 on the surface of BMECs and systematically confirmed that Fn and ITGB1 promoted *S. aureus* adhesion to and invasion of BMECs in vitro and in vivo. These results indicate that TGF-β1, as a host factor, is an important molecule related to the pathogenicity of *S. aureus* to the host.

Mastitis is chiefly attributed to breast infection by extrinsic bacteria, such as *S. aureus*, *E. coli* and *Streptococcus*; thus, pathogens have been regarded as research targets. In the past 20 years, the relationship between animal nutrition and immunity has received increasing attention.

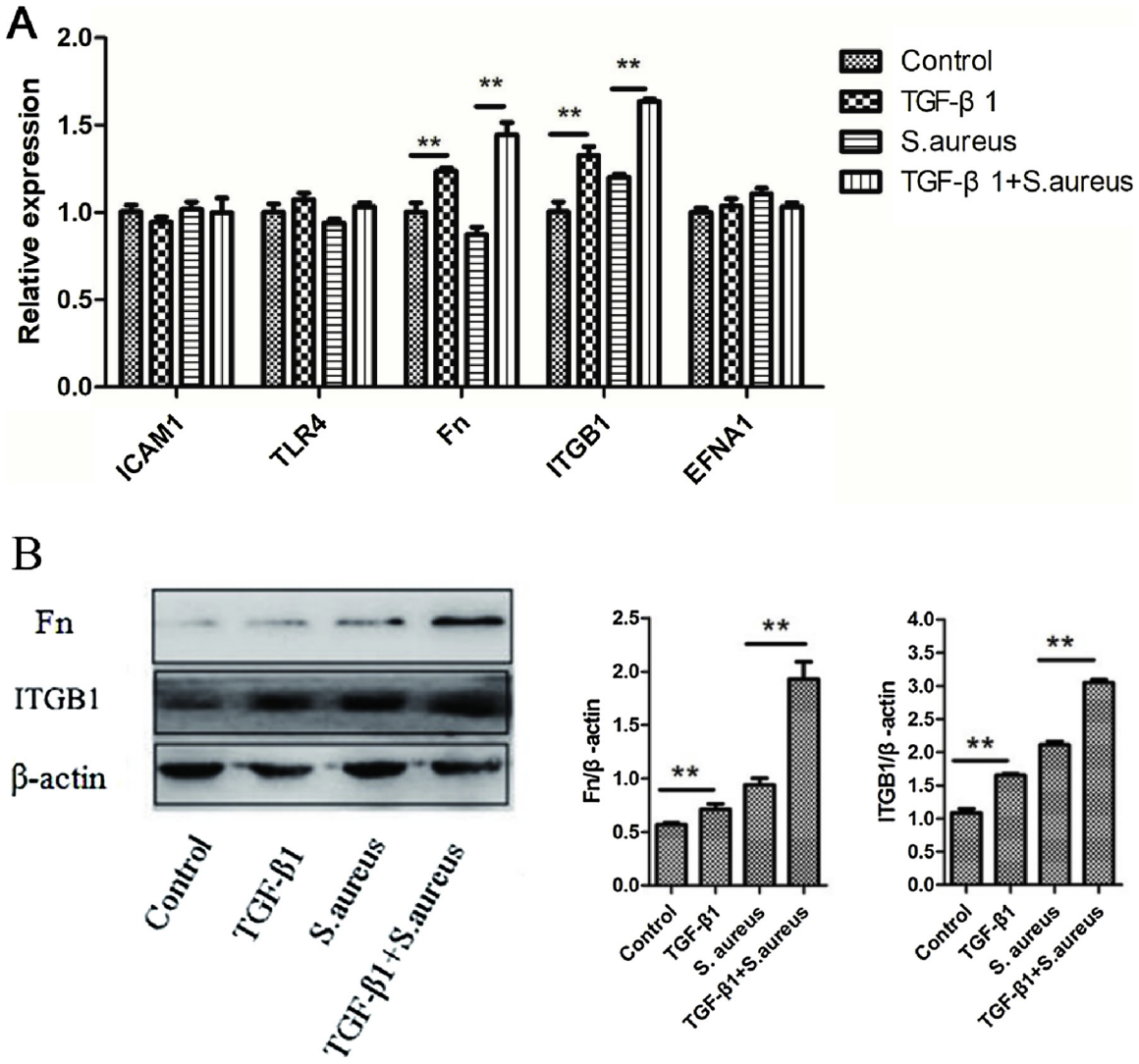

Fig. 3. BMEC surface receptors were assessed using RT-PCR and western blot analyses. A. The genes associated with TGF-β1-mediated upregulation during *S. aureus* infection. B. The Fn and ITGB1 protein levels were significantly increased with TGF-β1 treatment. *, $P < 0.05$; **, $P < 0.01$.

Studies have shown that feeding of different nutrient fodder to animals caused changes in certain cytokine levels in the body and further affected the body's immune status (Sordillo, 2016). For example, improvement of the diet of pigs regulated the secretion of mucin and its immune spectrum in the intestine (Ferrandis Vila et al., 2018); dietary supplementation with wheat germ reduced the levels of IL-1β, IL-6, IFN-γ and TNF-α in the serum (Ojo et al., 2019); and addition of soluble plantain non-starch polysaccharide attenuated the adhesion of *Salmonella typhimurium* to the intestine (Parsons et al., 2014). Hence, cytokines are becoming a research focus as one of the causes of some diseases. In a previous study by our group, corn stalk feed enhanced the TGF-β1 level in animal blood (Che et al., 2018) and further affected the lactation ability and proliferation ability of BMECs (Xia et al., 2016). In the current study, we indicated that TGF-β1 had a significant promotive effect on *S. aureus* adhesion to and invasion of BMECs. Our findings extend the factors responsible for the pathogenesis of dairy mastitis from microbes to immunopathologic factors and establish a bridge linking nutrition and disease, animal husbandry and veterinary medicine.

The extracellular matrix is a macromolecular substance that plays an important role in bacterial adhesion to and invasion of cells (Hammerschmidt et al., 2019). Although Fn and ITGB1 have been reported to be involved in bacterial adhesion to and invasion of cells, the results in different kinds of cells were not consistent. It has been reported that *Mycobacterium tuberculosis* Rv3717 bound to Fn in a dose-dependent manner (Li et al., 2018), that *Yersinia enterocolitica* invaded eukaryotic cells by binding to host β1 integrin (Zeitouni et al., 2016), and that *Campylobacter jejuni* infected host epithelial cells via a fibronectin-integrin Beta1-FAK-DOCK180/Tiam-1 signaling cascade (Boehm et al., 2011). However, it has also been reported that TGF-β1 enhanced the expression of Fn and α5 integrin but ultimately inhibited the invasion of PK-15 cells by *H. parasuis* (Li et al., 2016). The current study adequately verified that TGF-β1 led to high expression levels of Fn and ITGB1 and further increased *S. aureus* adhesion to and invasion of BMECs, which caused severe breast infection. Fn and ITGB1 are involved in *S. aureus* adhesion to and invasion of cells but may not be suitable for adhesion by other pathogens. The mechanism of bacterial invasion is worthy of future research.

In conclusion, this study links cytokines, BMECs and the susceptibility of BMECs to *S. aureus* infection, providing a theoretical basis for controlling mastitis via feed nutrition in practice.

Declaration of Competing Interest

The authors declare no conflicts of interest.

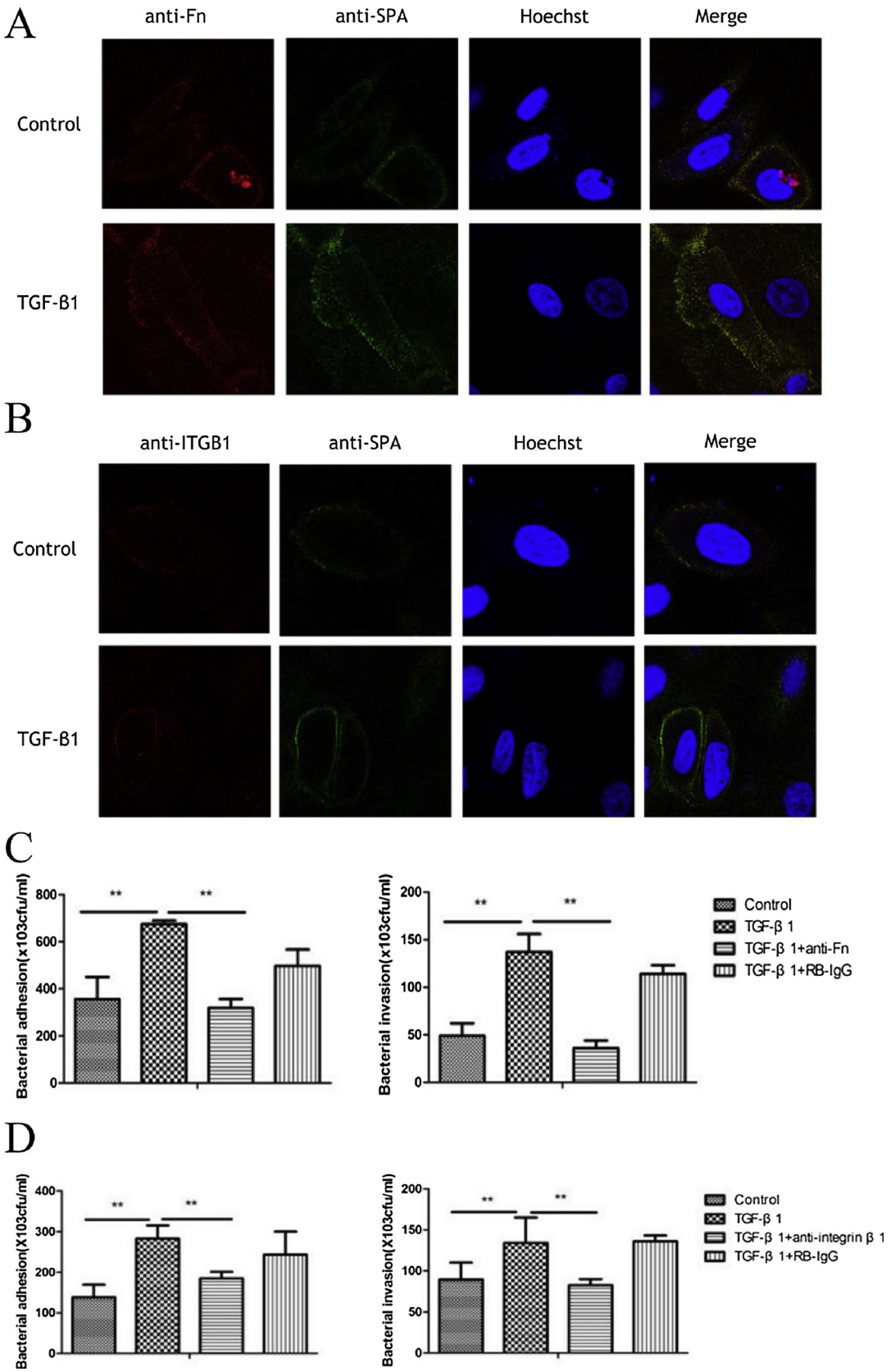

Fig. 4. The relationship between Fn and ITGB1 expression and *S. aureus* adhesion to BMECs treated with TGF-β1. A. TGF-β1 significantly promoted Fn expression as well as *S. aureus* adhesion to BMECs. B. TGF-β1 significantly promoted ITGB1 expression as well as *S. aureus* adhesion to BMECs. SPA is a surface protein of *S. aureus* that binds to IgG molecules by their Fc region. C, D. BMECs were treated first with 5 ng/ml TGF-β1 for 24 h and then with anti-Fn and anti-ITGB1 antibodies for 45 min before adhesion and invasion assays were performed as previously described. *, $P < 0.05$; **, $P < 0.01$.

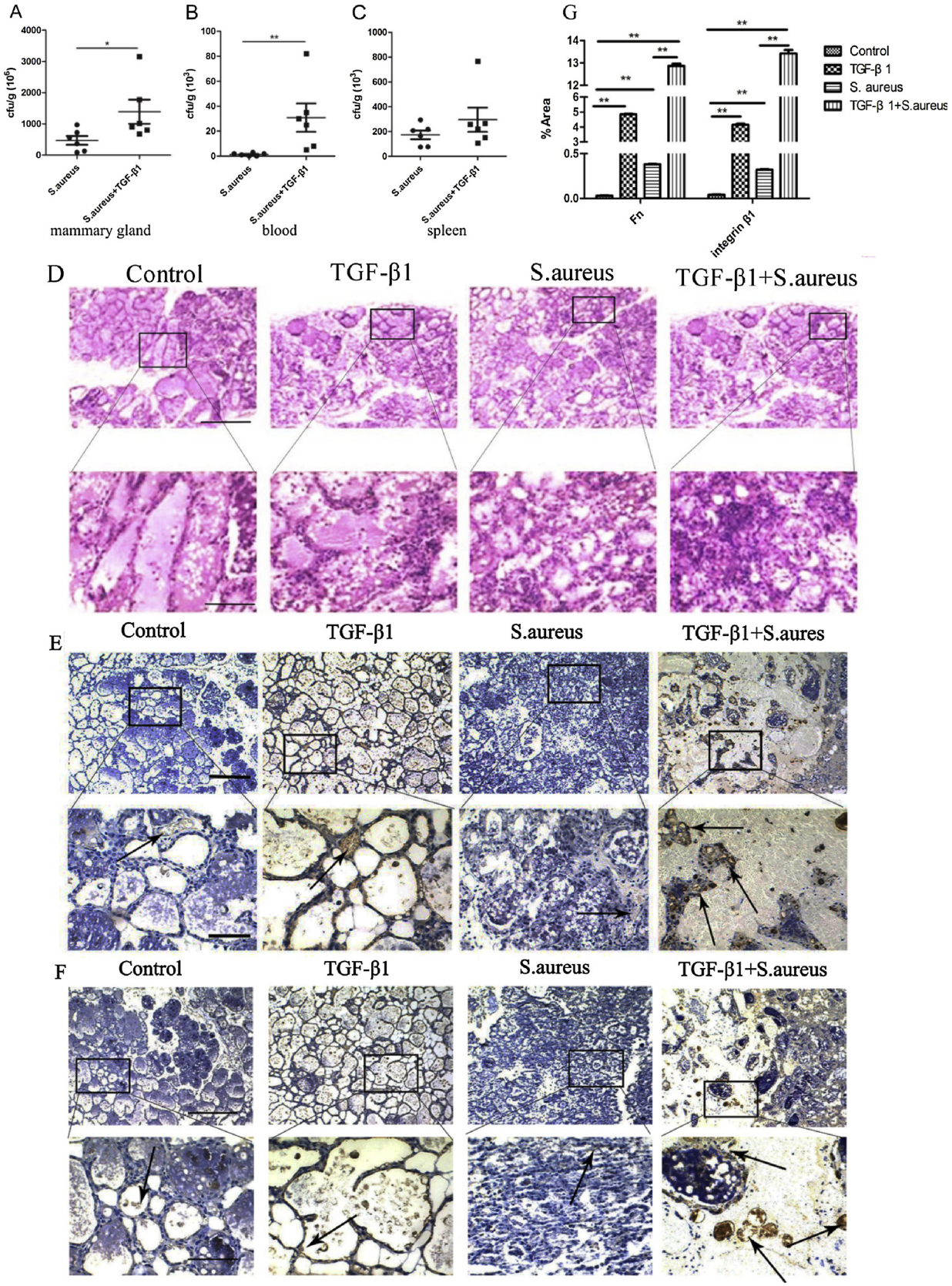

Fig. 5. TGF-β1 contributed to *S. aureus* colonization of mouse mammary glands and transfer into the bloodstream by promoting Fn and ITGB1 expression. A, B. *S. aureus* colonization in mouse mammary glands and blood was significantly higher in the TGF-β1-treatment group than in the untreated group. C. The effect of *S. aureus* colonization on the spleen did not significantly differ between the TGF-β1-treated group and the untreated group. D. TGF-β1 pretreatment aggravated the damage to acini caused by *S. aureus* and caused more inflammatory cell infiltration. E, F. TGF-β1 increased *S. aureus*-mediated damage to the mammary gland by promoting the expression of Fn and ITGB1 in damaged acini and the surrounding connective tissue. Fn and ITGB1, ABC method, counterstained with hematoxylin. G. The percentage of positive staining, as measured by ImageJ. *, $P < 0.05$; **, $P < 0.01$. The outlined areas in the top row indicate the enlarged area shown below. Scale bars, 400 μm (top row of each group) or 100 μm (bottom row of each group). The arrows indicate positive expression.

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