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The role of T-cell lymphoma invasion and metastasis 2 (TIAM2) in the barrier function of mammary epithelial cells following *Streptococcus uberis* inoculation

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Abstract:

Mastitis in dairy cows reduces milk yield, milk quality, and overall profits throughout the dairy industry. Mastitis frequently results from an intramammary infection of bacteria, commonly *Streptococcus uberis*, and is typically treated with antibiotic therapy. However, further exploration of genes associated with mastitis recovery and alternatives to antibiotic treatment of mastitis should be considered. In a recent study, novel phenotypes were generated from somatic cell counts following a *S. uberis* experimental challenge on Holstein dairy cows. Significant single nucleotide polymorphisms (SNPs) across the novel phenotypes were then identified. Several SNPs were located in genes associated with cell migration, cell signaling, and apoptosis. One such gene was T-cell lymphoma invasion and metastasis 2 (TIAM2), a Rac guanine nucleotide exchange factor connected to cell migration, barrier function, and production of antimicrobial peptides. Our study aims to develop a successful genotyping technique of the TIAM2 gene and analyze the barrier function of primary bovine mammary epithelial cell lines with differing TIAM2 genetic backgrounds. A successful method of genotyping the TIAM2 gene was developed using polymerase chain reaction and restriction fragment length polymorphisms. This method was utilized to identify two primary cell lines with the heterozygous genotype and one primary cell line and the MacT cell line with the homozygous genotype. The primary cell lines and MacT cell lines were cultured on inserts, and transepithelial resistance was measured daily using a portable ohmmeter and electrodes. The cultures were then inoculated with fluorescently labeled *S. uberis* and the quantity of bacteria that passed through the epithelial layer was measured at 24 hours. Fluorescent images also were taken to analyze cellular morphology and bacterial adherence at the epithelial surface. Results indicate a correlation between transepithelial resistance and the quantity of bacteria that passed through the epithelial

barrier ($r = -0.7573686$) with quantity of bacteria increasing as resistance measures decrease. In addition, the homozygous primary cell line generated greater resistance measurements throughout the experiment as (AVG=2.85k Ω cm²) compared to the heterozygous primary cell lines (AVG=1.21 k Ω cm², 1.23 k Ω cm²). Lower resistance measurements and fluorescent microscopy suggest variations in TIAM2 genotypic backgrounds influence cell barrier functions and ability for bacteria to migrate into tissue. By further determining TIAM2's direct connection to bovine epithelial barrier function, future use of the gene as a genetic marker and target for mastitis recovery may be explored.

Introduction:

Mastitis is defined as inflammation of the mammary gland. This disease plagues the dairy industry as it effects milk quality, milk yield, and overall herd health. Data shows that mastitis can cost upwards of \$2 billion annually and can effect 20% to 50% of a herd as it increases cow morbidity and mortality. Furthermore, in attempt to treat and prevent mastitis, this disease is the number one cause for antibiotic use throughout the dairy industry (Bradley *et. al* 2007, Østerås *et al.* 2006, Piepers *et al.* 2007, Wilson *et al.* 1997). Due to the overwhelming impacts of bovine mastitis, new methods of mastitis prevention and treatment, stretching beyond antibiotics, continues to be a major focus throughout the dairy industry.

Mastitis originates with udder tissue reaction to chemical, thermal, or physical injury or, most commonly, microbial infection. Mastitis is typically monitored by somatic cell count (SCC), udder health, and milk appearance. The microbes most commonly associated with mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli* (Amer *et al.* 2018). Mastitis can be defined as either a subclinical or clinical infection. Subclinical infections are typically asymptomatic and

are characterized by a SCC between 150,000 and 200,000. Clinical mastitis infections are associated with an elevated SCC above 200,000 as well as symptoms ranging from altered milk appearance and udder inflammation to fever and loss of appetite (Leigh *et al.* 1999). SCC measures the number of bodily cells, mainly mammary epithelial cells and leukocytes, found in the milk (Siebert *et al.* 2018). SCC is elevated at the time of microbial infection due to the innate immune response of the mammary gland to intramammary pathogens. Once a pathogen enters the mammary gland, toll-like receptors (TLRs) on macrophages and other immune cells, residing in the mammary tissue, recognize pathogen-associated molecular patterns (PAMPs). These PAMPs include cell wall structures of different bacteria classes and other unique microbial components (i.e. lipopolysaccharide, peptidoglycan, and lipoteichoic acid). The activation of TLRs through PAMP recognition results in the production of various cytokines and the initiation of an innate immune response. Furthermore, following bacterial adhesion or bacterial toxin interaction with mammary epithelial cells, these epithelial cells begin to synthesize cytokines, including tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and interleukin-8 (IL-8). These cytokines amplify the innate immune response and, in combination with macrophage cytokine production, initiate neutrophil recruitment to the site of infection (Oviedo-Boyso *et al.* 2007). Through these signals, neutrophils migrate across the endothelial barrier, between mammary epithelial cells, and into the milk. As a result, intramammary infection increases the number of neutrophils present in the milk and, therefore, increases the milk's SCC (Leigh *et al.* 1999).

Due to the role of microbial interaction with host leukocytes and mammary epithelial cells during infection, one important factor involved in the magnitude of mastitis infection is the barrier function of mammary epithelial cells. Epithelial cells form the barrier between the apical

and basolateral surface within the mammary gland. Tight junctions form the seal between these cells and are dependent on interactions between tight junction protein complexes and cellular cytoskeleton. One of the key proteins involved in tight junction formation is E-cadherin.

Different bacteria types have been shown to effect these tight junctions and the overall epithelial barrier function. One such study showed that group A *Streptococcus* (GAS), upon adherence to host epithelial cells, disrupts epithelial cell tight junctions by the loss of E-cadherin (Balkovetz *et al.* 2003). This loss allows for the migration of GAS across the epithelial barrier and a decrease in trans-epithelial electrical resistance. Furthermore, group B *Streptococcus* (GBS) have also been shown to migrate across epithelial barriers. However, these bacteria migrate through epithelial cell tight junctions by modifying the junctional complexes. These complexes then reform, and the epithelial cells do not show a change in trans-epithelial electrical resistance following bacterial exposure (Soriano *et al.* 2006). Furthermore, the initial trans-epithelial electrical resistance may impact the bacteria's ability to disrupt tight junctions and begin transmigration across the epithelial barrier. By better understanding the factors that are associated with mammary epithelial barrier function as well as mastitis pathogens interaction with the epithelial barrier, the role of mammary epithelial cells in mastitis infection can be further understood.

A recent study found a correlation between specific bovine genes associated with barrier function and mastitis cure rates. The study generated novel phenotypes from milk SCC following a *S. uberis* experimental challenge of Holstein dairy cows. Significant single nucleotide polymorphisms (SNPs) across the novel phenotypes were then identified and linked to different cure rates. One such significant SNP associated with differing mastitis cure rates is located within an intron of the T-cell lymphoma invasion and metastasis 2 gene (TIAM2). The

homozygous TIAM2 genotype, CC, correlated with mastitis cure rates of less than 21 days while those with the homozygous TIAM2 genotype, TT, correlated with mastitis cure rates of greater than 28 days (Siebert *et al.* 2018). Studies have identified TIAM2 as a gene that codes for a Rac guanine nucleotide exchange factor involved in cellular processes related to tight junction formation. Furthermore, the human TIAM2 gene has been linked to cell migration, cell invasion, and barrier function in human non-small cell lung cancer and other cancer types (Zhao *et al.* 2013). However, the exact role of TIAM2 in bovine mammary epithelial cell barrier function is unknown.

This study aimed to develop a simple genotyping technique for the bovine TIAM2 gene so that the barrier function of bovine primary mammary epithelial cell lines with differing TIAM2 genetic backgrounds could be evaluated. These cell lines were then cultured and challenged with *S. uberis* in order to quantify each cell line's initial barrier function. Due to the results derived in the previous study, we hypothesized that the cell lines with the homozygous CC genotype, as compared to those with heterozygous CT genotype, will show the greatest barrier function as indicated by a higher initial resistance and less translocated *S. uberis* across the epithelial barrier following *S. uberis* inoculation. By understanding the role of TIAM2 in bovine mammary epithelial cells, potential use of this gene for mastitis prevention and treatment can be explored.

Materials and Methods:

Bovine TIAM2 SNP Genotyping of Primary Bovine Mammary Epithelial Cell Lines

Fourteen primary bovine mammary epithelial cell lines (3648, 4041, 4078, 4089, 4088, 4097, 4112, 4114, 3839, 4015, 4015, 4038, Bm, MacT) collected from Holstein cows in a previous study were selected for bovine TIAM2 SNP rs109484182 genotyping. Genotyping was

conducted through use of polymerase chain reaction (PCR), a digestion reaction that utilized restriction fragment length polymorphism (RFLP), and gel electrophoresis. Initially, the 14 primary bovine mammary epithelial cell lines were removed from liquid nitrogen storage and thawed. DNA was extracted from these samples using a DNA extraction kit. The DNA samples were then diluted 1:5.

In preparation for polymerase chain reaction (PCR), a solution was developed using the following ratios:

Distilled H₂O: 29 μ L

GoTaq 5X buffer (Promega, Madison WI): 10 μ L

25 μ M MgCl₂ (Promega): 4 μ L

10 μ M dNTPs (Promega): 1 μ L

376-377 primer (IDT, Coralville, IA): 6 μ L

GoTaq Flexi DNA Polymerase (Promega): 0.25 μ L

Following preparation of the above solution, 2 μ L of each sample of diluted DNA was added to 18 μ L of the PCR mix. The samples were placed in a thermocycler, and the PCR was run under the following conditions.

Step 1: 94°C for 2 minutes

Step 2: 94°C for 15 seconds

Step 3: 53°C for 15 seconds

Step 4: 72°C for 1 min

Step 5: Repeat steps 2-4 30 times

Step 6: 72°C for 10 minutes

Step 7: 4°C hold

After completion of the PCR, in preparation for the digestion reaction, a solution was developed using the following ratios:

10X Cutsmart buffer (New England Biolabs, Ipswich, MA): 1 μ L

AatII enzyme (Thermo Fisher Scientific, Waltham, MA): 0.1 μ L

Distilled H₂O: 3.9 μ L

Following preparation of the solution, 5 μ L of the post-PCR samples was added to 5 μ L of the digestion solution. The samples were placed in a thermocycler and incubated at 37°C for 72 hours then 80°C for 20 minutes.

At the completion of the digestion reaction, a 1% agarose gel was created using 1X tris-acetate-EDTA (TAE), agarose powder (Phenix Research Products, Buncombe County, NC), and Ethidium Bromide. 10 μ L of the Invitrogen standard DNA ladder (Thermo Fisher Scientific, Waltham, MA) was mixed with loading dye and loaded into one lane of the prepared gel. 9 μ L of the post-digestion products were also mixed with loading dye and loaded into individual lanes of the gel. The gel was run at 90V for ~25 minutes. The gel was then observed using the ChemiDoc Imaging System (Bio-Rad Laboratories, Hercules, CA).

Cell Culturing of Primary Bovine Mammary Epithelial Cell Lines

Following completion of genotyping, all 14 bovine primary mammary epithelial cell lines were cultured in 6-well plates using a medium composed of Dulbecco's Modified Eagle Medium F12 (DMEM/F-12, Life Technologies, Grand Island, NY), 10% fetal bovine serum (FBS, Mediatech Inc., Manassas, VA), and 1% penicillin-streptomycin solution. The cultures were incubated at 37°C and 5% CO₂. After 24 hours, the cell cultures were examined under a phase-contrast inverted microscope and analyzed for cobblestone appearance and the absence of fibroblasts. Following initial culturing, only 4 cell lines (3839, 4114, 4038, and MacT) showed

viable growth and a consistent cobblestone appearance. The cell cultures of these primary cell lines were then transferred from the 6-well plate into a 75mL flask using the warm trypsin method to lift the cells. In this method, 4mL of warmed Trypsin-EDTA (1X, Life Technologies, Grand Island, NY) was added to each flask. The flasks were incubated at 37°C and 5% CO₂ for 8-15 minutes until 95% of cells had lifted from the flask. The lifted cells were then re-suspended in fresh medium and transferred to a new 75mL flask. Following 100% confluency in the 75 mL flask, the cell cultures were then transferred to a 175mL flask by again using the warm trypsin method. With a change of medium occurring every 2 days, the cultures were maintained in the 175mL flasks until the cultures reached 80% confluency.

Insert Seeding with Primary Bovine Mammary Epithelial Cell Lines

In preparation for the *S. uberis* challenge to analyze each cell line's barrier function, each primary cell line was transferred to a set of Millicell inserts (3.0µm, Merck Millipore Ltd., Darmstadt, Germany) placed in the wells of a 24-well plate. The transfer occurred when the primary cell lines showed 80% confluency in the 175mL flasks. Once 80% confluency was reached, all medium was removed from the flask and the cells were washed with 10mL of warmed phosphate buffered saline (PBS). The cells were trypsinized using the warm trypsin method by adding 4mL of warmed Trypsin-EDTA (1X, Life Technologies, Grand Island, NY) to each flask. The flasks were incubated at 37°C and 5% CO₂ for 8-15 minutes until 95% of cells had lifted from the flask. The detached cells were re-suspended in 10mL of fresh medium, and the number of cells present was determined using a hemocytometer. Following cell counting, the cells were re-suspended at a final concentration of 7.1×10^5 cell/mL. Next, 0.35mL of each primary cell line's cell suspension was added to 16 inserts placed in the wells of a 24-well plate. In addition, 0.55mL of fresh medium was added to every well in each of the four 24-well plates.

Each plate also contained a blank insert with no cells for use as a negative control. The plates were incubated at 37°C and 5% CO₂. Starting 96 hours after seeding, the media in the wells was changed every 2 days, and the cultures' transepithelial electrical resistance was measured every 24 hours using Millicell ERS Probes (EMD Millipore Corporation, Burlington, MA) and an epithelial/volt ohm reader (World Precision Instruments, Sarasota, FL). These measurements continued for 7 days post-seeding.

S. uberis Challenge of Mammary Epithelial Barrier

Fluorescently labeled *S. uberis* was added to at least four cultured inserts for each cell line 7 days post-seeding to analyze the barrier function of each bovine primary epithelial cell line. To develop the *S. uberis* culture for fluorescent labeling, 10µL of frozen *S. uberis* stock was added to 10mL of warmed Todd Hewitt Broth (THB). The tube was incubated for 16 hours at 37°C and 125rpm. Following incubation, 1 mL of the prepared broth was added to 49mL of fresh THB. The diluted culture was then incubated for 2 hours and 30 minutes at 37°C and 125rpm. The incubated *S. uberis* culture was then spun down and re-suspended in 12.5mL of medium composed of phenol red free DMEM/F12 (GE Healthcare Life Sciences, Logan, UT) and 10% FBS (Mediatech Inc., Manassas, VA). The *S. uberis* was then fluorescently labeled by adding SYTO 64 red fluorescent nucleic acid stain (5mM, Thermo Fisher Scientific, Waltham, MA) at a 10µM concentration to the *S. uberis* culture.

All existing medium was removed from both the insert and the wells in the 24-well plates containing the primary cell line cultures. Following removal of all medium, 0.35mL of the labeled *S. uberis* solution at a concentration of 6.8×10^7 CFU/mL was added to 8 of the cultured inserts in each plate and the blank insert. 0.35mL of fresh phenol red free medium was added to

the remaining 8 inserts, and 0.55mL of fresh phenol red free medium was added to the bottom of every well in each plate. The plates were incubated for 24 hours at 37°C and 5% CO₂.

Assessing *S. uberis* Migration through Mammary Epithelial Cells

Following the 24 hour incubation period, migration of *S. uberis* across the mammary epithelial barriers was assessed using bacteria plating. In order to dislodge any loosely attached bacteria from the insert, 0.25mL of medium in the insert was pipetted up and down. This same method was also performed on each well. The inserts were then transferred to a fresh 24-well plate that contained pre-warmed Hank's Balanced Salt Solution (HBSS). These plates were then stored at 4°C for later use. The plates without inserts were also stored at 4°C to prevent further bacteria growth in the wells while dilutions for plating were prepared.

In order to quantify the number of translocated *S. uberis*, the medium in each well that contained an insert challenged with *S. uberis* underwent a series of 10 fold dilutions through a final dilution of 10⁶. Then, 100µL of each dilution was plated on a Todd Hewitt Broth agar plate. The plates were incubated at 37°C and 5% CO₂ for 24 hours. Following incubation, the number of colony forming units (CFU) were counted on each plate to identify the quantity of translocated *S. uberis* across the mammary epithelial barriers.

Fluorescent Staining of *S. uberis* Challenged Primary Bovine Mammary Epithelial Cell Lines

In order to visualize the morphology and unique properties of each cell line, as well as any bacteria adhered to the mammary epithelial barrier, fluorescent staining and microscopy was utilized. To prepare for staining, the cells in the inserts required fixation and permeabilization. The plates containing the transferred inserts were removed from 4°C storage, and both the inserts and wells were rinsed twice with pre-warmed HBSS. The HBSS was removed from the insert, but not the well, and 0.15mL of 4% paraformaldehyde was added to each insert and allowed to

incubate in the dark for 15 minutes at room temperature. The inserts and wells were then washed twice with HBSS. Following washing, the HBSS was removed from the insert and 0.15mL of 0.1% Tween-20 in PBS were added to each insert. The plates were again incubated in the dark for 15 minutes at room temperature. Following incubation, the membranes containing the cultured cells were removed from the inserts and transferred to ColorFrost Plus Slides (Fisher Scientific, Pittsburg, PA).

To perform fluorescent staining, 3 slides from each primary mammary epithelial cell line were selected. One slide was labeled with pan-cytokeratin AE1/AE13 mouse monoclonal IgG₁ (Santa Cruz Biotechnology, Dallas, TX) to analyze the expression of cytokeratin in each cell line. One slide was labeled with TIAM2 (c-5) mouse monoclonal IgM (Santa Cruz Biotechnology, Dallas, TX) to analyze TIAM2 expression in each cell line, and the final slide was labeled with a combination of mouse IgG₁ purified antibody (Life Technologies, Carlsbad, CA) and mouse IgM purified antibody (Caltag Laboratories, Burlingame, CA) to determine the presence of any non-specific antibody binding. In addition, all slides were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen Molecular Probes, Eugene, OR) to visual the nuclei. The primary antibodies were first diluted in phosphate-buffered saline to the following concentrations:

Pan-cytokeratin AE1/AE13 mouse monoclonal IgG₁- 1:30

TIAM2 (c-5) mouse monoclonal IgM- 1:50

Mouse IgG₁ purified antibody - 1:30

Mouse IgM purified antibody-1:50

The desired primary antibody was then added to each slide at a volume of 50μL and allowed to incubate for one hour in the dark at room temperature in a humidified container while shaking.

Following incubation, the slides were washed with cold PBS 3 times. The secondary antibodies were then diluted in PBS to the following concentrations:

DyLight 488 Anti-Mouse IgG horse antibody- 1:2000

Goat Anti-Mouse IgM FITC- 1:1000

The appropriate secondary antibody was then added to each slide at a volume of 100 μ L and allowed to incubate for one hour in the dark at room temperature in a humidified container while shaking. Following incubation, the slides were washed 3 times with cold PBS.

Finally, DAPI stain was added to every slide at a concentration of 300nM. The slides were incubated for 5 minutes in the dark at room temperature in a humidified container while shaking. The slides were again washed 3 times with cold PBS. A drop of anti-fade mounting medium and a coverslip were added to each slide. Each slide was then visualized using fluorescent microscopy.

Results:

Bovine TIAM2 SNP Genotyping of Primary Bovine Mammary Epithelial Cell Lines

Fourteen primary bovine mammary epithelial cell lines (3648, 4041, 4078, 4089, 4088, 4097, 4112, 4114, 3839, 4015, 4015, 4038, Bm, MacT) were genotyped for the bovine TIAM2 SNP rs109484182. Genotyping was conducted through PCR, RFLP, and gel electrophoresis. The genotypes were identified by banding patterns following gel electrophoresis (Figure 1). Of the cell lines genotyped, 7 of the lines were identified as CC (3648, 4078, 3839, 4015, 4016, Bm) and the other 7 were identified as CT (4041, 4089, 4088, 4097, 4112, 4114, 4038). No cell lines were identified as having the TT genotype, so no TT primary cell lines were included in this study.

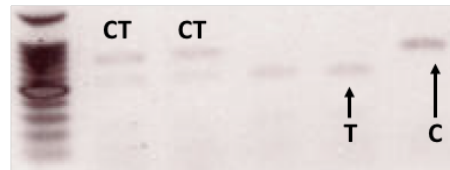


FIGURE 1 Banding patterns for TIAM2 SNP rs109484182 genotyping. The image shows the results of all 3 potential TIAM2 genotypes (CC, CT, and TT). CC corresponds to a single band with a greater number of base pairs and therefore shorter migration down the gel. TT is indicated by a single band with a shorter number of base pairs and therefore further migration down the gel. CT is indicated by the presence of both the C and T bands.

Cell Culturing and Insert Seeding of Primary Bovine Mammary Epithelial Cell Lines

All 14 of the genotyped primary cell lines were cultured to assess culture viability and morphology. As stated above, of the 14 cell lines, only 4 lines (3839, 4114, 4038, MacT) showed continued *in vitro* growth and culture viability. In addition, these 4 cell lines demonstrated a typical mammary epithelial cell cobblestone appearance, but the cell lines did differ slightly in fibroblast development throughout the culturing procedures. Morphology characteristics were consistent in both Trial 1 and Trial 2. At 80% confluency prior to insert seeding, 3839 showed the greatest fibroblast development with an 80:20 cobblestone to fibroblast ratio. At 80% confluency prior to insert seeding, 4114 demonstrated a 90:10 cobblestone to fibroblast ratio, and 4038 averaged a 95:5 cobblestone to fibroblast ratio. In contrast, MacT developed no fibroblasts throughout the culturing procedures. Ultimately, due to culture viability and morphology, 3839 and MacT were used to represent the CC TIAM2 genotype, and 4114 and 4038 were used to represent the CT TIAM2 genotype for the *S. uberis* challenge of mammary epithelial barrier function.

Primary Bovine Mammary Epithelial Cell Lines Transepithelial Electrical Resistance

Beginning 96 hours after insert seeding, the transepithelial electrical resistance of each primary bovine mammary epithelial barrier was measured every 24 hours for 3 days. Each seeded insert's individual transepithelial electrical resistance was determined, and then the

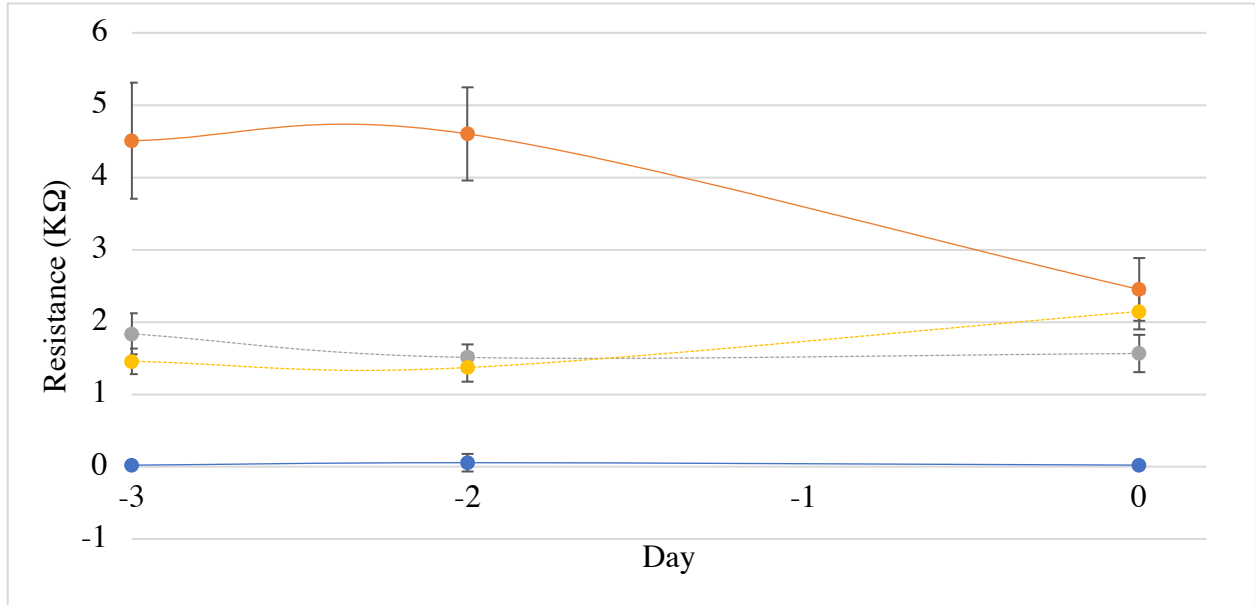
individual insert resistances for each cell line were averaged to determine the cell lines transepithelial resistance each day. These measurements were conducted in Trial 1 and Trial 2.

In Trial 1, MacT showed the lowest resistance and the least variation between inserts with an average resistance close to $0\text{K}\Omega$ for all three days and almost no change in resistance between days. In contrast, 3839 showed the greatest resistance for all 3 days. However, 3839 did show the greatest variation between insert measurements as indicated by a larger standard deviation. Line 3839 also demonstrated the greatest change in resistance over the 3 days as shown by the large decrease in resistance between the first and last day of resistance measurements with the final resistance measurement being $2.451\text{K}\Omega$. 4114 also showed a decrease in resistance over the 3 days with a final resistance measurement of $1.565\text{K}\Omega$. In contrast, 4038 showed both a decrease and increase in resistance over the 3 days with a final resistance measurement of $2.147\text{K}\Omega$. Ultimately, 4038 showed a greater final resistance than 4114, but both 4038 and 4114 ended with lower resistance measurements than 3839. However, the difference between 3839 and 4038 final resistances may not be statistically significant (Figure 2a).

In Trial 2, all average resistance values were lower than those in Trial 1. MacT continued to show the lowest resistance value and the least variation between days with an average resistance close to $0\text{K}\Omega$ for all 3 days. 3839 again showed the highest resistance values, but demonstrated a steady increase in resistance over the 3 days with an average resistance value of $1.482\text{K}\Omega$ on the final day. Unlike Trial 1, 4114 showed a decrease then increase in resistance with a final resistance of $0.865\text{K}\Omega$ on the final day. 4038 showed an increase then decrease in resistance with a final resistance of $0.749\text{K}\Omega$, which was lower than that of 4114. Ultimately, in Trial 2, 3839 again demonstrated consistently higher resistance than that of 4114 and 4038 on the

final day of measurements. However, in Trial 2, the difference was substantial as the final resistance value of 3839 was almost double that of 4114 (Figure 2b).

a)



b)

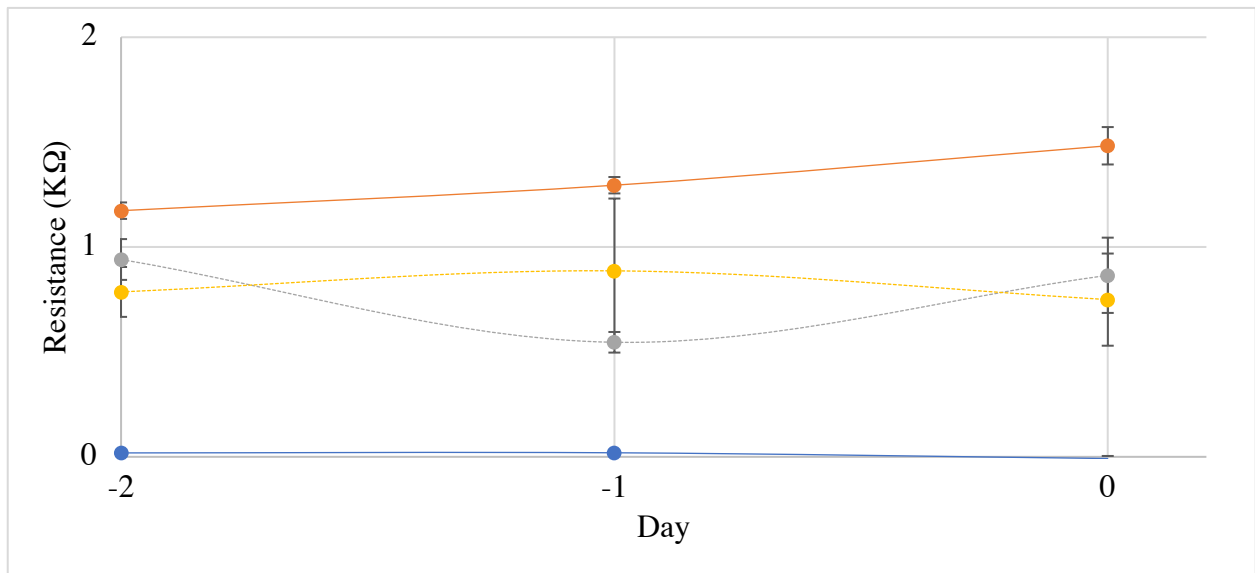


FIGURE 2 Transepithelial electrical resistance of primary bovine mammary epithelial cell lines. The resistance of each seeded insert was taken 96 hours after seeding for 3 days, and the average resistance (KΩ) for each cell line was calculated. a) The graph demonstrates the average resistance values for each cell line at each day in Trial 1. b) The graph demonstrates the average resistance values for each cell line at each day in Trial 2. The orange represents

3839. The blue represent MacT. The gray represent 4114, and the yellow represent 4038. The solid lines indicate cell lines with the CC TIAM2 genotype, and the dashed lines represent cell lines with the CT TIAM2 genotype.

Assessing *S. uberis* Migration through Mammary Epithelial Cells

Following 3 days of transepithelial resistance measurements of each primary bovine mammary epithelial cell line seeded in inserts, the mammary epithelial barrier was challenged by inoculation of the inserts with *S. uberis*. After 24 hours, the quantity of translocated *S. uberis* was determined by bacterial plating. This method was only successful in Trial 1, so all further results pertain to Trial 1 only.

The quantity of translocated *S. uberis* across the epithelial barrier for each insert in each cell line was determined and then averaged to quantify the average number of translocated *S. uberis* in each cell line. Such calculations indicated that MacT had a substantially greater number of translocated *S. uberis* across the epithelial barrier, with an average value of 1.56×10^4 CFU/mL of translocated *S. uberis*, than that of the other cell lines. 3839 had the lowest value of translocated *S. uberis* at 1.13×10^3 CFU/mL. However, in order to standardize the quantity of translocated *S. uberis*, the quantity of translocated *S. uberis* across the blank insert was utilized. By dividing the average number of translocated *S. uberis* in each cell line by the number of translocated *S. uberis* across the blank insert, the percent of translocated *S. uberis* for each cell line was determined. This value indicated that again MacT showed the highest percent of translocated *S. uberis*, but 4038 actually showed the lowest percent of translocated *S. uberis* when utilizing the blank insert values. Line 3839 had a slightly greater percent of translocated *S. uberis* than that of 4038, but 4114 showed a substantially greater percent of translocated *S. uberis* than both 4038 and 3839 (Table 1).

TABLE 1 The average percent of translocated *S. uberis* across the mammary epithelial barrier of each primary bovine mammary epithelial cell line. Seeded inserts were inoculated with *S. uberis*. After 24 hours, the quantity of translocated *S. uberis* was determined through dilutions and bacterial plating. Both the average number of translocated *S. uberis* and the percent of translocated *S. uberis* as compared to the blank inserts was determined for each cell line.

Cell Line	Genotype	Avg. Resistance on challenge day (KΩ)	Avg. Translocated <i>S. uberis</i> (CFU/mL)	Translocated <i>S. uberis</i> in blank insert (CFU/mL)	% Translocated <i>S. uberis</i> (cell/blank)
MacT	CC	0.021458	1.56E+04	6.31E+06	0.247669%
3839	CC	2.451666	1.13E+03	1.15E+07	0.009784%
4114	CT	1.565208	2.53E+03	3.49E+06	0.072463%
4038	CT	2.147083	5.14E+03	8.88E+07	0.005786%

In order to assess resistance to the quantity of translocated *S. uberis*, a correlation between the percent of translocated *S. uberis* for each insert in each cell line and the average resistance of each insert in each cell line on the day of the challenge was determined. A strong negative correlation was discovered between these two variables ($r = -0.7573686$).

Fluorescent Microscopy of *S. uberis* Challenged Primary Bovine Mammary Epithelial Cell Lines

Following the *S. uberis* challenge of the barrier function of each primary bovine mammary epithelial cell line, membranes from the inserts inoculated with *S. uberis* were stained for cytokeratin expression (green), stained for TIAM2 expression (green), or stained as a control for the monitoring of non-specific antibody binding. All insert membranes were also stained with DAPI (blue) to visualize the nuclei. In addition, the *S. uberis* used in the challenge was stained prior to inoculation with the Syto 64 fluorescent red nucleic acid stain (red).

Cytokeratin expression varied greatly between the two CC genotypes, 3839 and MacT. 3839 showed an extensive network of cytokeratin as indicated by the bright green fibers seen with fluorescent imaging. In contrast, MacT showed little cytokeratin staining. Furthermore, as

shown by DAPI staining, the cellular morphology of MacT and 3839 differed greatly as 3839 having a more uniform cellular pattern and cell morphology (Figure 3).

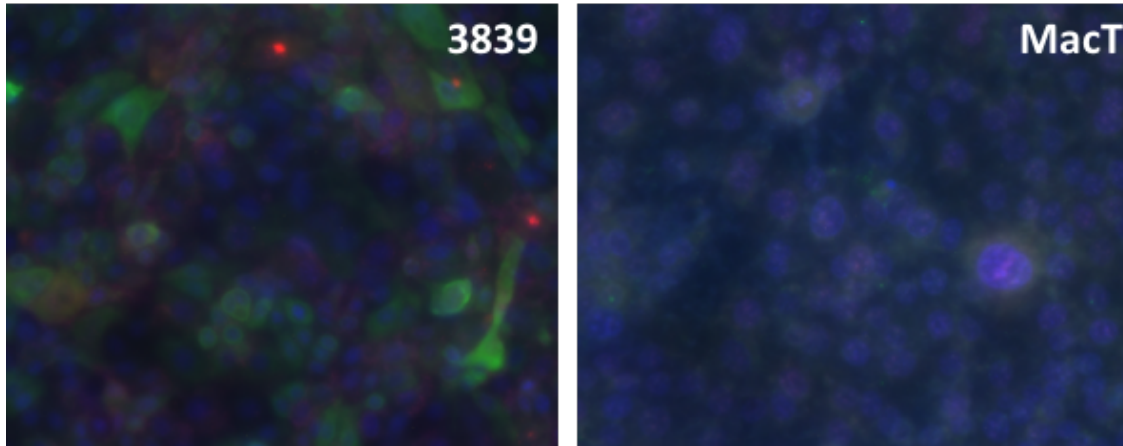


FIGURE 3 Fluorescent cytokeratin staining of 3839 and MacT inserts challenged with *S. uberis*. The membranes of both 3839 and MacT inserts were removed and stained for cytokeratin expression and nuclei visualization. The red indicates the presence of fluorescently labeled *S. uberis*. The green indicates the expression of cytokeratin, and the blue indicates nuclei.

The morphology and cytokeratin expression of 3839, 4114, and 4038 did not vary greatly between the cell lines. However, more adhered *S. uberis* was visualized on the slides containing 3839 and 4038 insert membranes. Unfortunately, little difference was seen between the slides stained with TIAM2 and those used as the control, so the TIAM2 staining was deemed unsuccessful in this experiment.

Discussion:

This study aimed to connect the bovine TIAM2 gene to the barrier function of mammary epithelial cells. By analyzing the initial transepithelial electrical resistance of four primary bovine mammary epithelial cell lines and then challenging those cell lines with *S. uberis*, we aimed to understand the impact of TIAM2 on epithelial cell resistance and translocation of *S. uberis* across a mammary epithelial barrier. Since only one trial was successful at monitoring the translocation of *S. uberis* across the cell barriers, no conclusive results could be drawn from this

study. Therefore, all further interpretation of results are only valid if the trends developed in this study continue.

Upon analysis of transepithelial electrical resistance, certain trends were seen in both Trial 1 and Trial 2. In both trials, MacT showed almost no resistance for all 3 days of measurements. Since transepithelial electrical resistance typically indicates the formation of tight junctions, the study indicated that the MacT cell line forms little to no tight junctions when cultured *in vitro*. MacT was developed as a transformed cell line from bovine mammary alveolar cells, and the line is commonly used as an epithelial model in bovine mastitis research due to its culture viability, short doubling time, and continuous cobblestone morphology (Huynh *et al.* 1991). However, in this study, the MacT cell line acted substantially different from its TIAM2 genotype counterpart, 3839, and therefore, may not have served as an accurate model for epithelial barrier function. Although both cell lines have the CC genotype, 3839 showed the highest transepithelial electrical resistance of all four cell lines, in both Trial 1 and Trial 2, for all days tested. In Trial 2, 3839 showed consistently elevated resistance across all 3 days with values doubling that of 4114 and 4038. This trial's results indicated that 3839 likely forms more tight junctions than both CT genotype cell lines, 4114 and 4038. Trial 1 also showed high levels of resistance for 3839. However, the values continued to drop throughout the experiment, and the final resistance value was similar to the resistance of the CT genotype cell lines on the final day. Such a decrease in resistance could be contributed to the morphology of the 3839 culture. Line 3839 consistently showed the greatest development of fibroblasts when cultured in flasks (80:20 cobblestone to fibroblast). Fibroblast development typically increases with aging cultures and has been shown to reduce tight junction formation and transepithelial electrical resistance (Roux-Pullen *et al.* 2016). Therefore, further testing would be required to determine if the 3839 cell line

showed consistently, statistically significant differences in transepithelial resistance as compared to the CT cell lines. Furthermore, since the other CC cell line, MacT, analyzed in this experiment did not perform similarly to any other primary bovine mammary epithelial cell line, the inclusion of different CC genotype cell lines should be utilized in future studies to truly understand the role of TIAM2 in transepithelial resistance and tight junction formation.

When analyzing the barrier function of the primary bovine mammary epithelial cell lines by challenging the lines with *S. uberis*, a strong negative correlation between epithelial resistance and translocated *S. uberis* was observed ($r = -0.7573686$). This negative correlation indicated that upon exposure to *S. uberis*, epithelial cells with an initially higher transepithelial electrical resistance resulted in fewer translocated *S. uberis* across the epithelial barrier. A past study connecting human intestinal epithelial electrical resistance to cirrhosis complications showed that a decrease in intestinal epithelial resistance increased the translocation of gut microflora and the patient's risk of sepsis (Wiest *et al.* 2005). Similarly, in this study, the cell lines with lower epithelial resistance resulted in an increase in translocated *S. uberis*, and therefore, epithelial resistance would likely impact the cow's mastitis risk upon *in vivo* bacteria exposure.

However, when connecting barrier function directly to TIAM2 genotype, the results of Trial 1 were not as expected. Both 3839 (CC) and 4038 (CT) showed similar levels of translocated *S. uberis* despite differing in TIAM2 genetic background. Line 4038 performed more like 3839 in the *S. uberis* challenge than like its genetic counterpart, 4114. In addition, 3839 showed no similarities to its genetic counterpart, MacT. The similarities in performance of the 3839 and 4038 cell lines could have occurred as a result of the drop in resistance and potential fibroblast contamination of 3839 as discussed above. The lack of similar performance between 4038 and 4114 could have occurred due to genetic differences between these cell lines

outside their similar TIAM2 genotype. Again, MacT and 3839 likely differed in performance, despite genetic similarities, due to the fact that MacT was a transformed cell line that showed little tight junction formation. To truly connect TIAM2 to barrier function, further testing that included more cell lines with differing TIAM2 backgrounds should be performed. In addition, a direct knockout of the TIAM2 gene in bovine mammary epithelial cells followed by a *S. uberis* challenge would aid in directly identifying the role of TIAM2.

The lack of similarities between 3839 and MacT was further demonstrated by fluorescent staining. Cytokeratin is an intracellular cytoskeletal protein expressed by epithelial cells and is involved in epithelial cell tight junction formation (Sawaf *et al* 1992). When staining for cytokeratin in the 3839 and MacT cell lines, 3839 expressed greater quantities of cytokeratin than MacT, despite their similar TIAM2 genetic background. Such differences in cytokeratin expression, further explains the drastic differences in transepithelial electrical resistance and relates again to MacT inability to form tight junctions. Furthermore, fluorescent staining of the epithelial nuclei demonstrated that MacT and 3839 formed different cellular arrangements as MacT cultures appeared unorganized and dispersed as compared to 3839 cultures. Again, such arrangements further indicated that MacT did not perform similar to its genetic counterpart due to the fact that MacT was a transformed cell line with unique properties. Furthermore, the similarities between 3839, 4114, and 4038 following fluorescent staining did not allow for further interpretation of the role of TIAM2 on epithelial barrier function.

Ultimately, further testing should be conducted to analyze the role of TIAM2 on epithelial barrier function. If studies continue to find that the CC TIAM2 shows higher transepithelial electrical resistance and therefore increased barrier function, then role of TIAM2 on barrier function could be connected to cure rates in mastitis dairy cows. The previous study

that first identified TIAM2 as a significant gene in mastitis cure rates did indicate that the CC genotype had the ability to cure faster than the CT and TT genotype (Siebert *et al.* 2018). Such cure rates could be contributed to the increased resistance and barrier function of the CC cell line and therefore decrease in the number of translocated *S. uberis* across the epithelial barrier. With less translocated *S. uberis*, intramammary bacterial loads would be lower than in the cows with the CT or TT TIAM2 genotype prior to *S. uberis* exposure. The lower bacteria loads would allow for a more rapid elimination of bacterial infection in the CC TIAM2 genotype cows. Although this study did show trends of the CC TIAM2 genotype having higher transepithelial electrical resistance and correlated transepithelial resistance to barrier function, further testing is required to fully understand the role of TIAM2 in epithelial barrier function.

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