N6-Methyladenosine-Modified circRNA in the Bovine Mammary Epithelial Cells Injured by Staphylococcus aureus and Escherichia coli

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Mastitis is a common disease that hinders the development of dairy industry and animal husbandry. It leads to the abuse of antibiotics and the emergence of super drug-resistant bacteria, and poses a great threat to human food health and safety. Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) are the most common pathogens of mastitis in dairy cows and usually cause subclinical or clinical mastitis. CircRNAs and N6-methyladenosine (m6A) play important roles in immunological diseases. However, the mechanisms by which m6A modifies circRNA in bovine mammary epithelial cells remain poorly understood. The aim of our study was to investigate m6A-modified circRNAs in bovine mammary epithelial cells (MAC-T cells) injured by S. aureus and E. coli. The profile of m6A-modified circRNA showed a total of 1,599 m6A peaks within 1,035 circRNAs in the control group, 35 peaks within 32 circRNAs in the S. aureus group, and 1,016 peaks within 728 circRNAs in the E. coli group. Compared with the control group, 67 peaks within 63 circRNAs were significantly different in the S. aureus group, and 192 peaks within 137 circRNAs were significantly different in the E. coli group. Furthermore, we found the source genes of these differentially m6A-modified circRNAs in the S. aureus and E. coli groups with similar functions according to GO and KEGG analyses, which were mainly associated with cell injury, such as inflammation, apoptosis, and autophagy. CircRNA–miRNA–mRNA interaction networks predicted the potential circRNA regulation mechanism in S. aureus- and E. coli-induced cell injury. We found that the mRNAs in the networks, such as BCL2, MIF, and TNFAIP8L2, greatly participated in the MAPK, WNT, and inflammation pathways. This is the first report on m6A-modified circRNA regulation of cells under S. aureus and E. coli treatment, and sheds new light on potential mechanisms and targets from the perspective of epigenetic modification in mastitis and other inflammatory diseases.

Keywords: N6-methyladenosine, circRNA, epithelial cells, cell injury, inflammation, S. aureus, E. coli
INTRODUCTION

Mastitis is currently the most widely investigated disease in dairy cows and is usually caused by infection with specific pathogenic microorganisms (1). Currently, treatment with antibiotic causes the abuse of antibiotics and the emergence of super drug-resistant bacteria. It is a great threat to human health. For instance, methicillin-resistant Staphylococcus aureus (MRSA) from bovines is now the most serious acute infectious disease in hospitals, neonatal delivery rooms, and communities (2). S. aureus and Escherichia coli are the most common pathogens that cause mastitis, and the infection has variable clinical symptoms ranging from different pathogens (3). The characteristics of subclinical mastitis caused by S. aureus are chronic or invisible, which makes disease detection and therapy difficult (4). Conversely, mastitis caused by E. coli and some Gram-negative bacteria usually passes through acutely and is associated with a list of severe symptoms (5). Bovine mammary epithelial cells, as the first layer, have an essential defense function, and the tight junctions of the cells provide a barrier to enhance natural mammary immunity. The cells secrete inflammatory factors to fight bacterial infections when activated (6). Moreover, it will express a set of cytokine/chemokine factors and directly resist pathogens, such as bactericidal β-defensins (7), complement factors, and acute phase proteins (8, 9). This response occurs through an inflammatory-related pathway (10–13). Some studies have confirmed the immune response of mastitis in S. aureus and E. coli infection (14–18). However, the regulatory function of noncoding RNA and epigenetic modification is ignored.

CircRNA (circRNA) has attracted considerable critical attention in recent years. As an indispensable noncoding RNA, circRNA is constituted by a highly stable and conserved covalent closed loop structure without a 5’ cap and 5’ polyA structure in organisms. Depending on the source gene of circRNA, different studies reported that the circRNA could be separated into different categories (19–21), such as exonic circRNA, intergenic circRNA, intronic circRNA, antisense circRNA, and sense overlapping circRNA. CircRNAs have been reported to bind to miRNAs as sponges and regulate target gene expression by combining with miRNAs (22). With the development of sequencing technology, many researchers have found that circRNAs widely exist in immune cells (23–25). Additionally, KEGG and GO analyses indicated that circRNAs played an essential role in many pathways associated with inflammation and immunity, such as the NF-κB, NLR, and TNF pathways (26). Therefore, circRNA has been considered a breakthrough in the innovative treatment of inflammatory disease.

m6A is a common epitranscriptome modification of RNA that is widely distributed in mammary epithelial cells (27). m6A has been found in diverse ribonucleic acids (28–31). By regulating RNA splicing (32), degradation (33), stability (34), and translation (35), the final manifestation is the impact on various pathological or physiological processes, such as the cell cycle (36) and immune function (37). Interestingly, circRNA could be regulated by m6A modification, showing a different m6A pattern from mRNA (38). Recent studies have shown that m6A-modified circRNAs have a certain impact on immune liver metastasis (39). Nevertheless, there is no study of the relationship between m6A-modified circRNAs and mastitis in dairy bovines. To identify the potential function of m6A modification in regulating circRNA, we used RNA immunoprecipitation and high-throughput sequencing (MeRIP-seq) for the first time to profile the circRNA regulated by m6A modification in mammary epithelial cells (MAC-T) injured by inactivated S. aureus and E. coli. The features of m6A-modified circRNAs were identified first, and the potential functions were also predicted by GO enrichment analysis and KEGG pathway analysis. Moreover, a circRNA–miRNA–mRNA interaction network was constructed to predict competitive endogenous RNAs (ceRNAs). For further investigation, conjoint analysis of circRNA-seq and MeRIP-seq was performed. Together, our findings demonstrated the potential molecular function of circRNAs regulated by m6A modification caused by S. aureus and E. coli-induced cell injury, which suggested that S. aureus and E. coli may affect the cell inflammation process through m6A-modified circRNAs, and provided us with possible diagnosis and treatment targets in mastitis.

MATERIALS AND METHODS

Cell Lines and Bacteria

MAC-T cells (an immortalized bovine mammary epithelial cell line) were donated by Professor Zhou Rui and Professor Wang Xiangru of Huazhong Agricultural University. MAC-T cells were cultured by adapting the procedure used by Li et al. (40). S. aureus (ATCC 29213) and E. coli (ATCC 25922) were donated by Professor Zhou Rui and Professor Wang Xiangru of Huazhong Agricultural University. S. aureus and E. coli were resuscitated, single colony purified, cultured, and heat-inactivated by adapting the procedure used by Li et al. (40).

MAC-T Cell Injury Induced by Inactivated S. aureus and E. coli

MAC-T cells were seeded in 6-well plates (Corning, US) at a density of 2×10⁵ cells/well. After 12 h of culture, inactivated bacteria were added at an MOI of 10:1. Inactivated bacteria and cells were cocultured for 24 h. Cells in each well were washed 3 times with cold PBS (HyClone, China) before TRIzol (Invitrogen, US) was added. There were 3 replicate wells for each treatment and 3 replicate wells for the control, for a total of 9 samples.

Extraction of Total RNA and RT-qPCR

Total RNA of the above 9 samples was extracted according to the instructions of the commercial reagent manufacturer and the RNA isolation steps. A NanoDrop ND-1000 instrument (Agilent Inc. USA) was used to measure RNA concentration and purity, and the quality control index of RNA purity was based on the OD260/OD280 value between 1.8 and 2.0. Denaturing agarose gel electrophoresis was used to measure RNA integrity and gDNA contamination. The sample was stored at −80°C for later use.
Flow Cytometry
After MAC-T cells were digested, collected, and washed three times with PBS, the cells were resuspended in 100 µl of Binding Buffer. Then, 5 µl each of FITC and PI dyes (Vazyme, China) was added and left for 10 min at room temperature in the dark. After adding 400 µl of Binding Buffer, the cells were detected using CytoFLEX-LX (Beckman Coulter, Indianapolis, IN, USA).

Transmission Electron Microscopy
MAC-T cells were treated with S. aureus and E. coli and fixed with 2% glutaraldehyde fixation buffer. The cells were used for TEM slide preparation. Sections were observed via a 100 KV H7650 transmission electron microscope (HITACHI, Japan).

Establishment of circRNA Library
Nine samples under different treatments were sent to Cloud-Seq Biotech (Shanghai, China) for MeRIP-CircRNA sequencing. The operation was briefly described as follows: (1) To enrich circRNA, a circRNA Enrichment Kit (Cloud-seq, USA) was used according to the supplier’s instructions. (2) An NEBNext Ultra II Directional Library Prep Kit (New England Biolabs, Inc., Massachusetts, USA) was used to pretreat RNA, and a sequencing library was constructed by immunoprecipitation. (3) Quality control and quantification of the library were performed with the BioAnalyzer 2100 system (Agilent Technologies, USA), and the Illumina HiSeq instrument was used to perform sequencing, the downstream mRNAs were predicted using miRBase (http://miRNA.org/index.shtml).

RESULTS
Inflammation, Apoptosis, and Autophagy in MAC-T Cells Treated With Inactivated S. aureus and E. coli
To determine whether the stimulation of inactivated bacteria on MAC-T cells can cause cell damage, we added bacteria to stimulate the cells for 24 h. The results showed that IL-1β, IL-6, and TNF-α were significantly upregulated in the S. aureus group and E. coli group, and the expression of these three genes in the E. coli group was significantly higher than that in the S. aureus group (Figures 1A–C). Additionally, cell apoptosis was significantly increased (Figures 1D–F). It showed almost 5 times the apoptosis rate in the S. aureus group and E. coli group compared to the control group. In contrast to the trend in mRNA expression associated with inflammation, flow cytometry illustrated a similar apoptosis rate when cells were treated with S. aureus and E. coli (Figure 1G).

To more intuitively observe the cell injury in S. aureus treatment and E. coli treatment, transmission electron microscopy was used (Figures 1H–M). Interestingly, we found the nuclear membrane disappearance, chromatin condensation (Figure 1K), and autophagosomes (Figure 1L) in MAC-T cells injured by S. aureus (Figure 1I), which indicated minor damage in these cells. However, cells with acute cytosolic voids (Figure 1J) and apoptotic bodies (Figure 1M) were found in MAC-T cells injured by E. coli.

Identification of m6A-Modified circRNAs in MAC-T Cells Treated With Inactivated S. aureus and E. coli
To investigate the circRNA profile in MAC-T cells injured by heat-inactivated S. aureus or E. coli, purified cellular RNA was subjected to circRNA-seq and MeRIP-seq. The sequencing raw reads were generated from the control group, S. aureus group, and E. coli group. Bioinformatic approaches, including Cutadapt and Bowtie2 software, were performed for data filtering and quality control. We obtained clean reads and the proportions of
net reads were between 99.122% and 99.995% with stringent quality control (Table S2).

To clarify the relationship between m^6^A modification and normal circRNA, we first analyzed the circRNA profile from circRNA-seq and found that most circRNAs were less than 2,000 bp and derived from exons (Figures S1A, B). Moreover, genes were mainly distributed on chromosome 3 (Figure S1E). When MAC-T cells were injured by S. aureus and E. coli, the source and length of the differential circRNA showed similar patterns (Figures S1C, D, F). Most circRNAs were located on chromosome 3 in the S. aureus group, while most circRNAs were located on chromosome 8 in the E. coli group (Figures S1F, G).

Further analysis of the MeRIP-seq data was performed, and the m^6^A methylation peaks were compared and analyzed based on the sequencing data. There were 1,599 m^6^A methylation
peaks within 1,035 circRNAs in the control group, 35 peaks within 32 circRNAs in the S. aureus group, and 1,016 within 728 circRNAs in the E. coli group (Figures 2A, B).

Characteristics of m^6^A-Modified circRNAs in MAC-T Cells Treated With Inactivated S. aureus and E. coli

A total of 17 m^6^A peaks and 18 circRNA transcripts were observed in all three groups, whereby 939 methylation peaks and 687 circRNAs occurred in both the E. coli group and the control group. The number of m^6^A methylation peaks was obviously different among the three groups. There were 18 m^6^A peaks in the S. aureus group instead of the control group and 83 m^6^A peaks in the E. coli group instead of the control group.

Further analysis was performed to assess the features of m^6^A-modified circRNAs. The number of m^6^A methylation peaks in each circRNA was highly similar in the control group, S. aureus group, and E. coli group (Figure 2C). Similar to recent studies, we have found that most circRNAs have one m^6^A methylation peak, but there are also a large number of circRNAs with more than one m^6^A methylation peak, which indicates that m^6^A modification sites are not unique in circRNAs. Moreover, the lengths of m^6^A-modified circRNAs in each group were analyzed. The length of most m^6^A-modified circRNAs was less than 5,000 bp, and similar characteristics of length were shown in all three

![Figure 2](https://example.com/figure2.png)

**FIGURE 2** | Overview of m^6^A-modified circRNAs in MAC-T cells injured by S. aureus and E. coli. (A) Venn diagram and histogram displaying the specific m^6^A peaks between the control group, S. aureus group, and E. coli group. (B) Venn diagram and histogram showing the specific circRNA with m^6^A modification between the three groups. (C) The number of m^6^A peaks per circRNA in the three groups. (D) The length of m^6^A-modified circRNAs in the three groups. (E) The source of m^6^A-modified circRNAs in the three groups. (F) Chromosome distribution.
groups (Figure 2D). In our studies, the sources of the circRNA in the three groups were subsequently analyzed. CircRNAs in the control group and E. coli group derived from exons were the most abundant. However, in the S. aureus group, there was a certain amount of m⁶A-modified circRNA derived from antisense and sense overlapping (Figure 2E). Finally, chromosome distribution also revealed that m⁶A-methylated circRNA is more likely to be present on chromosome 3 (Figure 2F).

Comparison of m⁶A-Modified circRNAs in MAC-T Cells Injured by S. aureus and E. coli

To identify the role of m⁶A-modified circRNA in regulating MAC-T cell injury induced by S. aureus and E. coli, the various m⁶A modification levels between S. aureus/E. coli and the control group were analyzed (fold change > 2, p-value < 0.05). Compared to the control group, a total of 259 differential m⁶A modification peaks within 200 circRNAs were found. Further analysis confirmed that 67 m⁶A methylation peaks within 63 circRNAs were significantly different between the S. aureus group and the control group, of which 26 hypermethylated peaks were within 25 circRNAs (e.g., circRNA_OPA3), and 41 hypomethylated peaks were within 38 circRNAs (e.g., circRNA_AKT3). Meanwhile, 192 m⁶A methylation peaks within 137 circRNAs were significantly different between the E. coli group and the control group, of which 75 hypermethylated peaks were within 47 circRNAs (e.g., circRNA_GRB10), and 117 hypomethylated peaks were within 90 circRNAs (e.g., circRNA_SMAD1). Data visualization analysis was performed by IGV to show the differential m⁶A peaks between the control group and treatment group (Figure 3A). The top 20 differentially methylated circRNAs with hypermethylation or hypomethylation in the S. aureus and E. coli group compared to the control group are shown (Tables 1, 2).

Additionally, the feature of the differentially m⁶A-methylated circRNA in the S. aureus/E. coli group compared to the control group is also shown. Instead of being a major part of short m⁶A-modified circRNA derived from exon (Figure 2D), it is longer and derived more from sense overlapping when MAC-T cells are injured by S. aureus and E. coli (Figures 3B, C). Additionally, the differential circRNA with methylation showed totally reverse patterns in the S. aureus group compared to the E. coli group, which were mainly hypomethylation in the S. aureus group and hypermethylation in the E. coli group. The most differentially methylated circRNA in the S. aureus group was found on chromosome 10, while in the E. coli group, it was on chromosome 19 (Figure 3D).

GO and KEGG Pathway Enrichment Analyses of m⁶A-Modified circRNAs in MAC-T Cells Treated With S. aureus and E. coli

Recent studies have shown that circRNAs can affect the expression of its cis genes (42). Therefore, to clarify the essential role of m⁶A-modified circRNAs in MAC-T cell injury induced by S. aureus and E. coli, GO and KEGG pathway enrichment analyses for the m⁶A-modified circRNA source genes were performed (p-value < 0.05) (Tables S3, S4). In the S. aureus group, the GO annotation of circRNA with hypermethylation illustrated that it was mainly enriched in transmembrane, cell part morphogenesis, plasma membrane-bound cell projection organization, P-body, and protein tyrosine kinase activator activity (Figure 4A). Furthermore, the KEGG analyses indicated that they were enriched in endocytosis, antigen processing and presentation, and ubiquitin-mediated proteolysis (Figure 4A). Likewise, the GO analysis of circRNA with hypomethylation showed that it was closely related to clathrin, SMAD binding, integrin binding, and PI3K (Figure 4B). KEGG analysis of hypomethylated circRNAs revealed enrichment in focal adhesion, the RAP1 signaling pathway, and regulation of the actin cytoskeleton (Figure 4B).

In the E. coli group, the GO analysis predicted that the functions of circRNAs with hypermethylation were significantly enriched in cytoskeleton, and actin filament (Figure 4C). The KEGG pathway analysis indicated that circRNAs with hypermethylation were significantly related to the Ras signaling pathway, endocytosis, and PI3K-Akt signaling pathway (Figure 4C). Correspondingly, the GO analysis of differential m⁶A-modified circRNA with hypomethylation illustrated that the meaningful terms may be related to regulation of the retinoic acid receptor signaling pathway, SMAD binding, Ras GTPase binding, and small GTPase binding (Figure 4D). The KEGG analysis also revealed enrichment in focal adhesion, regulation of actin cytoskeleton, bacterial invasion of epithelial cells, and Rap1 signaling pathway (Figure 4D).

Also, some representative genes closely related to apoptosis and inflammation in the KEGG pathways analysis were shown, such as BOLA, AKT3, MAP2K4, and JAK2 (Figures 5A-D). Given the above, we found that the functions of differentially expressed m⁶A-modified circRNAs were highly similar when MAC-T cell injury was induced by either S. aureus or E. coli. These findings may suggest that these m⁶A-modified circRNAs regulated immunity, cell junctions, growth metabolism, and resistance to bacterial invasion through a similar pathway in both induced groups.

CircRNA–miRNA–mRNA Interaction Network Identified Similar Enrichment of mRNA in the S. aureus and E. coli Groups

CircRNAs are able to regulate the expression of target genes as sponges for miRNAs based on complementary base pairing. By predicting the target miRNA between circRNA and mRNA, we constructed a circRNA–miRNA–mRNA interaction network (Table S5). In this study, 10 circRNAs (5 hypermethylation and 5 hypomethylation), 10 miRNAs, and 22 mRNAs were included in the S. aureus group (Figure 6A), and 10 circRNAs (5 hypermethylation and 5 hypomethylation), 8 miRNAs, and 19 mRNAs were included in the E. coli group (Figure 6B).

In the interactive network of the S. aureus group, many inflammation-related miRNAs were predicted. For example,
FIGURE 3 | Distribution of significantly differentially expressed m^6^A-modified circRNAs in the S. aureus/E. coli group compared to the control group. (A) Data visualization analysis was performed by IGV, showing the location of differential m^6^A peaks in the source gene of circRNA (circRNA_OPA3, circRNA_AKT3, circRNA_GRB10, and circRNA_SMAD1) between the control group and treatment group. (B) The length, (C) source, and (D) chromosome distribution of differentially expressed m^6^A-modified circRNAs in the S. aureus and E. coli groups (circRNAs were named by their host genes).
TABLE 1 | The top 20 differential m6A methylation peaks in the S. aureus group.

| Chromosome | txStart | txEnd | tcName | Log2 (fold change) | Log10 (p-value) | Regulation | Gene Name |
|------------|---------|-------|--------|-------------------|----------------|-----------|-----------|
| NC_037337.1 | 46247281 | 46247520 | NC_037337.1 | 5.935459749 | -5.034591042 | Up | HERC1 |
| NC_037330.1 | 868628301 | 868628540 | NC_037330.1 | 5.935459749 | -5.028296479 | Up | FGFRY |
| NC_037333.1 | 59952041 | 59962420 | NC_037333.1 | 5.92599941 | -5.069792883 | Up | ABPB2 |
| NC_037350.1 | 27893981 | 27894220 | NC_037350.1 | 5.754867502 | -5.204708591 | Up | BoLA |
| NC_037332.1 | 111626281 | 111626680 | NC_037332.1 | 5.296864998 | -5.083292528 | Up | TNP66B |
| NC_037334.1 | 47628661 | 47629060 | NC_037334.1 | 4.161166243 | -5.101862075 | Up | ROR1 |
| NC_037333.1 | 50191621 | 50192140 | NC_037333.1 | 5.646936522 | -5.128146679 | Up | ITGB6 |
| NC_037322.1 | 47863661 | 47864140 | NC_037322.1 | 3.85029113 | -5.070148314 | Down | HMG2A |
| NC_037328.1 | 837385801 | 837386800 | NC_037328.1 | 6.324302999 | -5.067635523 | Down | TUBG1 |
| NC_037332.1 | 111626281 | 111626680 | NC_037332.1 | 3.646936522 | -5.128146679 | Down | FN1 |
| NC_037340.1 | 17844281 | 17844540 | NC_037340.1 | 7.109127227 | -5.205686668 | Down | AB1 |
| NC_037346.1 | 48234961 | 48236200 | NC_037346.1 | 8.727920345 | -8.428020111 | Down | LOCB16254 |
| NC_037341.1 | 60850021 | 60850740 | NC_037341.1 | 6.350929113 | -6.070148314 | Down | HMG2A |
| NC_037346.1 | 42680281 | 42680900 | NC_037346.1 | 3.243029999 | -5.067635523 | Down | TUBG1 |
| NC_037332.1 | 103364341 | 103364858 | NC_037332.1 | 3.646936522 | -5.128146679 | Down | FN1 |
| NC_037340.1 | 73166041 | 73166600 | NC_037340.1 | 2.831843942 | -5.069444449 | Down | SLC38A6 |

miR-2305 and miR-1777a, which have been considered to have an effect on immunity (43), were also shown to enrich for transcripts related to inflammation and apoptosis in our study (including key genes such as MAPK8IP2 and WNT10A). Additionally, we predicted that some miRNAs may be regulated by multiple circRNAs at the same time. For instance, miR-11988 was predicted to be regulated by circ_FAM214A, circ_NEDD4 L, and circ_SETD2 simultaneously.

Similarly, in the E. coli group, a large amount of mRNA was also predicted to be associated with inflammation, mainly in the MAPK signaling pathway and the WNT signaling pathway. In addition, BCL2, which is the key molecule in apoptosis, was predicted to be regulated by miR-2305. Some natural immune-related miRNAs such as TNFAIP8L2, FCER1G, and MSR1B were present in the network. It is predicted that the miRNAs may regulate multiple miRNAs. For example, miR-2305 could be able to interact with BCL2, FCER1G, and MAX. Interestingly, in both groups, we found a large number of similar miRNAs, suggesting that circRNAs modified with different m6A modifications may play a similar role in S. aureus- and E. coli-induced cell injury.

Conjoint Analysis of circRNA-seq and MeRIP-seq

To further explore the potential function of circRNAs with m6A modification in S. aureus- and E. coli-induced cell injury, a conjoint analysis of circRNA-seq and MeRIP-seq was performed.

In addition, the differential expression of circRNAs with significant changes in the S. aureus and the E. coli groups compared to the control group was analyzed (Figures 7A-D).
We found 3,914 novel circRNAs, of which 1,532 were upregulated and 11 were downregulated in the \textit{S. aureus} group, and 536 were upregulated and 11 were downregulated in the \textit{E. coli} group. The top 20 most significantly differentially expressed circRNAs in the \textit{S. aureus} group and \textit{E. coli} group are listed separately (Tables S6, S7).

To predict the especially important circRNA in MAC-T injury and reveal the relationship between m$^6$A modification and expression, a conjoint analysis was performed. Finally, 3 significantly upregulated circRNAs with hypermethylation and 4 upregulated circRNAs with hypomethylation were found in the \textit{S. aureus} group (Figure 7E). Two upregulated circRNA with hypomethylation were found in the \textit{E. coli} group (Figure 7F).

**DISCUSSION**

Bovine mastitis is a common disease that seriously harms the dairy industry (44). Currently, antibiotics are still the major treatment for mastitis, which also causes some serious
FIGURE 5 | KEGG enrichment analysis. (A) The top 10 KEGG pathway enriched for the source genes of differential circRNA with hypermethylation and (B) hypomethylation in the S. aureus group. (C) The top 10 KEGG pathway enriched for the source genes of differential circRNA with hypermethylation and (D) hypomethylation in the E. coli group (the predicted KEGG pathways in the blue box and representative genes in the red box were related to inflammation, apoptosis, and autophagy).
FIGURE 6 | CircRNA–miRNA–mRNA ceRNA interaction network. The box size represents the strength of the binding. The color represents that the miRNA has a binding site with mRNA and circRNA, and if the color is gray, it represents two or more target binding sites. (A) The S. aureus group. (B) The E. coli group (circRNAs were named by their host genes or chromosome location).
Conjoint analysis of circRNA with or without m6A modification. Volcano plot showing the differentially expressed circRNAs in the (A) *S. aureus* group and (B) *E. coli* group. Heatmap illustrating the differentially expressed circRNAs in the (C) *S. aureus* group and (D) *E. coli* group. Conjoint analysis between the expression level and m6A modification in the (E) *S. aureus* group and (F) *E. coli* group.
problems, such as drug resistance and threatening side effects. Recent studies have confirmed that circRNA plays an important role in diseases (45), and it can be modified by N^6^-methyladenosine and plays a more complex regulatory role (46). Nevertheless, the mechanism of m^6^A-modified circRNA in bovine mastitis is still undiscovered. This study is the first to reveal the relationship between m^6^A-modified circRNA and cell injury induced by S. aureus and E. coli, and it reveals that circRNA with m^6^A modification may be related to cell injury by S. aureus and E. coli treatment (Figure 8).

It was reported that cell injury plays an important role in mastitis induced by S. aureus and E. coli (14). Perhaps the most compelling finding was that IL-1Bet, IL-6, and TNF-α were significantly upregulated, which indicated inflammatory changes. Additionally, apoptosis and autophagy are common cell injuries during inflammation (47). Inflammatory mRNA expression levels and TEM illustrations both demonstrated that E. coli induced more accelerated cell injury.

It is known that m^6^A modification of circRNA usually changes the development of disease. In our research, we found some novel m^6^A peaks first, and also identified the differential m^6^A-modified circRNA profile in the S. aureus group and E. coli group. This result suggested that this circRNA with different levels of m^6^A modification may play a vital role in S. aureus- and E. coli-induced cell injury. Most circRNAs upstream of mRNAs are able to regulate gene expression (48), so the altered m^6^A-modified circRNA could be a potential treatment target in mastitis. Furthermore, the unique structure of the conserved covalent closed loop allowed circRNA to remain stable, which makes it a better target than mRNA or other linear RNAs.

CircRNAs as a result of the diverse splicing of mRNAs, and a large number of studies have identified that the major source of circRNAs is derived from exons (49–51), which was also proven in our research. Some researchers have found that circRNAs are produced through contranscription and competition with conventional splicing (52). As a result, biogenesis of circRNA leads to a decrease in mRNA synthesis from the same location. Here, circRNA production acts as an RNA trap for mRNA production. However, we found that the features of m^6^A-modified circRNAs changed when MAC-T cells were injured by S. aureus and E. coli. Most of the differential m^6^A-modified circRNAs are longer and come from sense overlapping regions, which means that these differential and long m^6^A-modified circRNAs derived from sense overlapping regions play a more important function and provide us with a new direction in the m^6^A-modified circRNA regulatory mechanism of mastitis. In addition, the m^6^A-modified circRNA in both groups showed reverse density patterns of length, which may suggest a difference in cell injury in S. aureus and E. coli treatment. In fact, there are currently few studies on the m^6^A methylation of circRNA, and whether m^6^A modification can regulate mastitis in dairy cows needs further confirmation.

Since the function of circRNA in dairy cow mastitis is not fully understood, we performed GO/KEGG analysis to predict the function of altered m^6^A methylated circRNA. Previous studies have confirmed that PI3K-AKT and Ras-MAPK are important signaling pathways of natural immunity (53, 54). Li et al. found that ulinastatin reduced LPS-induced inflammation in mouse macrophage cells by activating the PI3K/Akt/Nrf2 pathway (55). Dong et al. found that Astragalus polysaccharides alleviate LPS-induced inflammation via the NF-κB/MAPK signaling pathway (56). When epithelial cells are injured by bacteria, the tight junctions between cells are compromised through focal adhesion and actin cytoskeletal signaling pathways (57). In our research, we predicted that the differentially expressed circRNAs with m^6^A modification were enriched in the Ras-MAPK, focal adhesion, and PI3K-AKT signaling pathways in MAC-T cells induced by S. aureus and E. coli, which are closely related to cell apoptosis and inflammation. The pathways and GO annotations of differentially expressed m^6^A-modified circRNAs in the S. aureus and E. coli groups were highly similar.

Many studies have shown that circRNA can be used as a molecular sponge to interact with miRNA to regulate mRNA (22). Recent studies have proven that specific circRNA–miRNA–mRNA axes cause apoptosis and inflammation in bovine mammary epithelial cells (58). In our ceRNA network, we found that 20 circRNAs and 26 mRNAs had a total of 10 miRNA binding sites, which were obviously related to apoptosis and inflammation. MAX, an important apoptosis gene, was upregulated by direct binding to the miR-181a promoter to increase MYCT1 expression, leading to apoptosis (59). In our study, we found that miR-2305 was able to regulate MAX and BCL2 in both ceRNA networks, and multiple circRNAs had binding sites with miR-2305 (60). In addition, some researchers have shown that downregulation could block the AKT pathway, inhibiting cell survival and proliferation, which means that MIF may be an important gene working in cell proliferation (61).

When MAC-T cells were treated with S. aureus and E. coli, we found that circ_RCOR1, circ_NEDD4 L, circ_MBB21D2, and circ_PAN3 regulate MIF through miR-2881 in the S. aureus group, and circ_AKAP13, circ_ABCCI, circ_TUBG1, and circ_LOC104975415 regulate MIF through miR-2881 in the E. coli group. This ultimately had a potential effect on MAC-T cell proliferation. RAC3 is an NF-κB coactivator closely related to inflammation, whose expression is regulated by TNF, in addition to being a negative regulator of autophagy (62–64). In our research, miR-11972 was predicted to have binding sites for RAC3. Strangely, the expression of IL-1Bet, IL-6, and TNFα was different in the cell injury model, while the levels of apoptosis in the two groups were similar (Figures 1A–F). Through our ceRNA networks, we conjectured that the m^6^A-circRNA regulated the mRNA enriched in some similar pathways of apoptosis, but there may be more complicated mechanisms that were related to inflammation. Overall, circRNAs with different m^6^A modification levels regulated mRNAs enriched in the MAPK, WNT, and natural immune signaling pathways through circRNA–miRNA–mRNA axes and ultimately affected S. aureus- and E. coli-induced mastitis. In our research, ceRNA networks showed that mRNAs were enriched in MAPK, WNT, and natural immuno logic signaling pathways in both the S. aureus- and E. coli-induced mastitis.
FIGURE 8 | Proposed model of $m^6$A-modified circRNAs that regulate MAC-T cells injured by inactivated *S. aureus* and *E. coli*. $m^6$A-modified circRNAs regulated MAC-T cell apoptosis, inflammation, and autophagy.
aureus and E. coli groups, which may be the reason for the similar levels of apoptosis.

To further reveal the relationship between circRNA and m⁶A modification, we performed an analysis of circRNA-seq. We found a total of 3,914 novel circRNAs with expression differences. The length and source of altered circRNAs in the S. aureus and E. coli groups are consistent with the circRNA characteristics of previous studies, and most of them are shorter and exon-derived circRNAs. In the joint analysis of MeRIP-seq, we found that a total of 9 circRNAs showed a significant association between expression and m⁶A modification. To change circRNA expression, m⁶A modification has many functions, such as regulated circRNA splicing and degradation (45). For instance, Li et al. confirmed that circMETTL3 is upregulated in a m⁶A-dependent manner and promotes breast cancer progression (65).

This study is the first report to clarify the profile of m⁶A-modified circRNAs in bovine mammary epithelial cells injured by S. aureus and E. coli. Additionally, we predicted similar pathways and target genes in both groups. This finding provided us with new insights for exploring the cellular injury mechanism of mastitis. However, we did have some limitations. For the purpose of exploring the innate immune response, we used heat-inactivated bacteria instead of live bacteria, which may differ from real mastitis disorders in bovines. In our circRNA networks, only the circRNAs with different m⁶A levels are shown, without a detailed description of the “up-down” or “down-up-down” model of differential expression. Therefore, the mechanism of cell injury needs to be revealed by further experiments, and animal models are needed to clarify the complex function of m⁶A-modified circRNAs in vivo.

CONCLUSION

Mastitis is a common disease with a complicated pathological mechanism in bovines. m⁶A-modified circRNA plays an important role in regulating disease, but its function in mastitis remains unknown. Our study clearly profiled the m⁶A methylation of circRNA in MAC-T cell injury induced by S. aureus and E. coli and made predictions on differential m⁶A-methylated circRNA firstly. We provided novel insight into MAC-T cell injury induced by S. aureus or E. coli via m⁶A-modified circRNAs, which may represent a new research direction for future studies in mastitis.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196736.

AUTHOR CONTRIBUTIONS

HX and CL: Conceptualization, Validation, Formal analysis, Investigation, Resources, Data curation, Writing—original draft, Visualization, Writing—review and editing, and Supervision. YZ and TL: Validation, Formal analysis, and Investigation. JY and SC: Writing—review and editing. CH, JC, XC, YC, and AG: Project administration and Funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.873330/full#supplementary-material

Supplementary Figure 1 | Overview of the circRNA profile. (A) Novel circRNAs, mainly exonic, intronic, intergenic, sense overlapping, and antisense circRNAs. (B) The length of the novel circRNA found by circRNA-seq. (C, D) Pie charts showing the length of differentially expressed circRNAs in the S. aureus and E. coli groups. (E) The chromosome distribution of the novel circRNAs. (F) Histogram showing the source of differentially expressed circRNAs in the S. aureus and E. coli groups. (G) The chromosome distribution of differential circRNAs in the S. aureus and E. coli groups.
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