TLR2 signaling pathway combats *Streptococcus uberis* infection by inducing production of mitochondrial reactive oxygen species

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Abstract

Mastitis caused by *Streptococcus uberis* is a hazardous clinical disease in dairy animals. In this study, the role of Toll-like receptors (TLRs) and TLR-mediated signaling pathways in mastitis caused by *S. uberis* was investigated using mouse models and mammary epithelial cells (MECs). We used *S. uberis* to infect mammary glands of wild type, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice and quantified the adaptor molecules in TLR signaling pathways, proinflammatory cytokines, tissue damage and bacterial count in mammary glands. When compared with TLR4 deficiency, TLR2 deficiency induced more severe pathological changes through myeloid differentiation primary

response 88 (MyD88)-mediated signaling pathways during *S. uberis* infection. In MECs, TLR2 detected *S. uberis* infection and induced mitochondrial reactive oxygen species (mROS) to assist host control of secretion of inflammatory factors and elimination of intracellular *S. uberis*. Our results demonstrate that TLR2-mediated mROS have a significant effect on *S. uberis*-induced host defense responses in mammary glands as well as MECs.

Author summary

S. uberis contributes significantly to global mastitis and remains a major obstacle for inflammation elimination due to its ability to form persistent infection in mammary tissue. The Toll-like receptor (TLR) family plays a significant role in identifying infections of intracellular bacteria and further triggering inflammatory reactions in immune cells. However, the detailed molecular mechanism by which TLR is regulated, and whether MECs, as the main cells in mammary gland, are tightly involved in these processes is poorly understood. Here, we used *S. uberis* to infect mammary glands of wild type, TLR2$^{-/-}$, TLR4$^{-/-}$ mice and MECs to assess pathogenesis, proinflammatory cytokines, ROS as well as mROS levels during infection. We found that during *S.uberis* infection, it is TLR2 deficiency that induced more severe pathological changes through MyD88-mediated signaling pathways. In addition, our work demonstrates that mROS mediated by TLR2 has an important role in host defense response to combat *S. uberis* infection in mammary glands as well as MECs.

Introduction

Mastitis is a type of inflammation mainly caused by intramammary infection and causes great harm to the dairy industry [1]. *Streptococcus uberis* is an environmental pathogen that is emerging as the most important mastitis-causing agent in some regions [2]. Previous studies in our laboratory have demonstrated that persistent inflammation including swelling, secretory epithelial cell degeneration, and polymorphonuclear neutrophilic leukocyte (PMN) infiltration occurs in mammary tissue following injection with *S. uberis* [3]. The inflammatory responce caused by *S. uberis* is lighter than that caused by *E. coli* [3].These pathological response connected with intracellular infection of *S. uberis* as it escaped the elimination of immune cells and formed persistent infection.

Activation of pattern recognition receptors (PRRs) to produce natural inflammatory immune response is important to control the intracellular infection induced by bacteria like *S. uberis* [4]. TLR family plays a critical role in these processes. Upon activation by microbes, the MyD88-dependent pathway triggers production of inflammatory cytokines through activation of nuclear factor (NF)- κ B and mitogen-activated protein kinases, and/or a TIR-domain-containing adapter-inducing interferon (IFN)-β (TRIF)-dependent pathway associated with induction of IFNs and stimulation of T cell responses [5]. Previous research has found that PRRs are not only expressed by immune cells, but also in conventional non-immune cells, for example, endothelial and epithelial cells, which also contribute to immune regulation [6].

Strandberg et al. demonstrated for the first time in 2005 that TLRs and their downstream molecules are expressed on bovine MECs [6]. Ibeagha-Awemu et al. further demonstrated that expression of TLR4, MyD88, NF-κB, TIR domain-containing adapter molecule 2 (TICAM2) and IFN-regulatory factor 3 increased in bovine MECs challenged by lipopolysaccharide [7]. These studies announced that MECs might have a pivotal role in host defense with TLRs for their huge number in the mammary gland. Our laboratory did a lot of work on the function of TLRs and MECs in eliminating *S. uberis* infection *in vivo* and *in vitro* models. We found that TLRs, mainly TLR2 but no not excluding TLR4, initiated a complex signaling network characterized by NF-κB and nuclear factor of activated T cells. In addition, they activated the secretion of cytokines and chemokines accompanied with their self-regulation pathways in response to *S. uberis* challenge.

Reactive oxygen species (ROS) are free radicals that contain oxygen atoms, including hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}) and hydroxyl radical (OH^-) [8]. They are produced intracellularly through multiple mechanisms depending on the cell and tissue types. However, the two major sources in mammalian cells are membrane-associated NADPH oxidase-induced and the mitochondria [9]. It has been reported that, in most tissues, mROS from the respiratory chain are important [10]. Mitochondria function as a defense against bacterial infection in innate immunity, mainly through mROS, which is demonstrated by the fact that mROS modulate several signaling pathways, including NF-κB, C-Jun N-terminal kinase and the caspase-1 inflammasome [11]. Previous studies have shown that restriction of

89 pathogen-induced mROS impairs NF-κB activation, suggesting that mROS positively
90 control the NF-κB signaling pathway [12, 13]. In addition, the production of mROS in
91 immune cells (e.g. macrophages) involves recruitment of tumor necrosis factor (TNF)
92 receptor-associated factor (TRAF)6 to mitochondria, which also acts as an adaptor of
93 the TLR signaling pathway [14]. MECs are the main cells for lactation in mammary
94 tissue. In recent years, they have also been found to play a non-negligible role in the
95 regulation of infection. Pathogens invading mammary tissue and epithelial cells can
96 stimulate MECs to produce proinflammatory cytokines, anti-inflammatory factors
97 and chemokines such as TNF-α, interleukin (IL)-1β, IL-4, IL-6, IL-8 and IL-10 [15]. It is
98 possible that MECs are also involved in the generation of ROS in infection. However,
99 few studies have investigated the interaction between TLRs and mROS against
100 *S. uberis* infection *in vitro and in vivo*. Therefore, we investigated whether the
101 process of TLR induction of mROS production plays an important role against
102 *S. uberis* infection in host and MECs.
103

104 ## Results

105 **TLR2 mediates tissue damage and anti-*S. uberis* infection in mammary glands**

106 *S. uberis* belongs to gram-positive bacteria which is mainly recognized by TLR2.
107 However, previous research had demonstrated that the role of TLR4 could not be
108 ignored in *S. uberis* infection for their close relationship, similar structure and
109 function [16, 17]. In this work, we explored the role of TLR2 and TLR4 in *S. uberis*
110 infection in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice to understand further the molecular defence

111 mechanisms in *S. uberis* mastitis. No histological changes were observed in WT-B6 or
112 WT-B10 mammary glands of control mice, whereas, there was some suspicion of
113 tissue damage in TLR2$^{-/-}$ and TLR4$^{-/-}$ control mice (Fig 1A). Inflammation and tissue
114 damage appeared in mammary tissue after infection with *S. uberis* in all challenged
115 groups. This response was characterized by PMN infiltration, increased bleeding and
116 epithelial cell degeneration, and excess adipose tissue. Compared with WT-B6 mice,
117 TLR2 deficiency induced more severe pathological damage. A higher score was
118 present for the three indexes mentioned above and there were significant increases
119 in bleeding and degeneration, and excess adipose tissue ($P < 0.05$; Fig 1B). However,
120 TLR4 deficiency caused no inflammation and tissue damage during *S. uberis*
121 challenge (Fig 1A and 1C).

122 NAGase, a marker enzyme of MECs and mammary gland damage, was significantly
123 elevated in TLR2$^{-/-}$ mice, but not in TLR4$^{-/-}$ mice when compared with WT mice at 24
124 h post-challenge ($P < 0.05$; Fig 1D). Similarly, the bacterial numbers were higher in
125 the mammary tissue of TLR2$^{-/-}$ mice than in WT-B6 mice ($P < 0.05$) but there was no
126 significant difference between TLR4$^{-/-}$ and WT-B10 mice. We conclude that TLR2
127 primarily mediated the tissue damage and antibacterial effect in mammary glands
128 during *S. uberis* infection.

129

130 **TLR2 and TLR4 deficiency affect secretion of cytokines in *S.uberis* infection**

131 The secretion of proinflammatory cytokines in mammary glands which better
132 reflects the level of inflammation have been reported previously [18, 19]. However,

133 the massive release of cytokines can cause irreversible damage to tissues. Here, we
134 investigated the role of TNF-α, IL-1β and IL-6 in response to *S. uberis* infection in
135 TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (Fig 2A and 2B). *S. uberis* challenge increased TNF-α level
136 significantly in WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice ($P < 0.05$). Compared with
137 corresponding WT mice, TNF-α and IL-1β in TLR2$^{-/-}$ mice and TNF-α in TLR4$^{-/-}$ mice
138 significantly decreased ($P < 0.05$). These results indicate that TLR2 and TLR4
139 deficiency affected secretion of cytokines in *S.uberis* infection.
140
141 **MyD88-dependent pathway predominates in *S. uberis* infection**
142 MyD88 dependent and independent pathways are vital in host's response after TLRs
143 activated [20, 21]. We assessed the expression of MyD88 and TRIF respectively by
144 immunohistochemistry in mammary glands, which are the key molecules of the
145 MyD88-dependent or independent signaling pathways. There was a significant
146 increase in MyD88 but not TRIF expression in WT, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice ($P < 0.05$).
147 Lower levels of MyD88 were observed in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice compared with
148 WT mice ($P < 0.05$; Fig 3A and 3B). As the main functional cells in mammary glands,
149 our former study had established that MECs played a key role in anti-infection
150 response in mammary glands. We also detected that interference of TLR2 and/or
151 TLR4 by specific siRNA significantly decreased MyD88 expression in *S. uberis*
152 infection ($P < 0.05$; Fig 3C, 3D and 3E). These data suggest that the
153 MyD88-dependent pathway predominates in *S. uberis* infection in MECs and in
154 mammary glands following TLR activation.

155

156 **TRAF6 and ECSIT participate in signal sensing from TLRs in *S. uberis* infection**

157 We next evaluated the level of TRAF6 and ECSIT, which are downstream targets of
158 the MyD88 signaling pathway, using immunohistochemistry in mammary glands of
159 mice. The adaptors TRAF6 and ECSIT increased dramatically in all mice after *S. uberis*
160 infection ($P < 0.05$), although TLR2 or TLR4 deletion weakened expression of ECSIT in
161 WT compared with TLR2$^{-/-}$ and TLR4$^{-/-}$ mice ($P < 0.05$; Fig 4A and 4B). In MECs,
162 interference of TLR2 or TLR4 significantly reduced expression of TRAF6 and ECSIT
163 after *S. uberis* infection ($P < 0.05$; Fig 4C, 4D and 4E). TRAF6 and ECSIT expression did
164 not differ between TLR2$^{-/-}$ and TLR4$^{-/-}$ mice. The results confirm that TRAF6 and
165 ECSIT downstream of the MYD88 pathway mediate the anti-*S. uberis* response in
166 mice and MECs.

167

168 **TLRs mediate redox status of mammary glands during *S. uberis* infection**

169 TRAF6 activated by TLRs transfers from the cytoplasm to mitochondria, where it
170 engages ECSIT to produce mROS, which induces cellular anti-bacterial response [22].
171 Since the level of ROS in tissue cannot be detected well, we analyzed T-AOC, SOD,
172 MDA and UCP2 in mammary glands to reflect indirectly the antioxidant levels. The
173 levels of MDA and UCP2 were significantly increased due to the infection of *S. uberis*
174 in WT and TLR2$^{-/-}$ and TLR4$^{-/-}$ ($P < 0.05$; Fig 5A and 5B). There was no obvious
175 distinction between TLR2$^{-/-}$, TLR4$^{-/-}$ and WT mice ($P > 0.05$). T-AOC was significantly
176 lower in all groups of mice after *S. uberis* challenge ($P < 0.05$). Deletion of TLR4

177 rather than TLR2 significantly decreased SOD activity after *S. uberis* infection ($P <$
178 0.05). These results indicate that the host's oxidation level does change after
179 *S. uberis* infection and these changes are related to the TLR signaling pathway.

180 We aimed to clarify whether MECs were involved in the change in redox status
181 and had a crucial role in *S. uberis* infection after activation of the TLR signaling
182 pathway. We interfered with the expression of TLR2 and/or TLR4 in EpH_4-Ev cells and
183 detected ROS, mROS and UCP2 levels. *S. uberis* infection caused a significant increase
184 in ROS and mROS (Fig 5C and 5D). Special targeting of siRNA to TLR2 significantly
185 reduced the level of ROS and mROS ($P < 0.05$). SiTLR4 decreased their levels to some
186 extent, but no significant difference was observed ($P > 0.05$). Expression of UCP2
187 decreased, and interference with siTLR2 reversed this change ($P < 0.05$). Taken
188 together, these results demonstrate that infection with *S. uberis* changed the redox
189 status of mammary glands and MECs, and TLR2 played an essential role in this
190 process, especially in MECs.

191

192 **mROS play an important role against *S. uberis* infection in MECs**

193 GKT137831, a specific inhibitor of NADPH oxidase 1 (NOX1) and NOX4; and NG25, an
194 inhibitor of TAK1 [23, 24], were used to suppress ROS generation from NOX
195 complexes and to down-regulate production of proinflammatory cytokines,
196 respectively. GKT137831 and NG25, simultaneously or separately, reduced the
197 generation of ROS but not mROS after challenge with *S. uberis* ($P < 0.05$; Fig 6A). The
198 bacterial counts of *S. uberis* in MECs were significantly higher in the inhibitor-treated

199 groups ($P < 0.05$) (Fig 6B). We inhibited production of mROS by siECSIT to establish
200 mROS role in regulating inflammation and anti-*S. uberis* activity. ROS and mROS
201 levels decreased significantly after using siECSIT ($P < 0.05$; Fig 6D). Similar results
202 were observed for TNF-α, IL-1β and IL-6 expression; their levels were up-regulated
203 after *S. uberis* infection while siECSIT reduced them (Fig 6E). The bacterial counts of
204 *S. uberis* in MECs were significantly higher in the siECSIT treatment group (Fig 6F).
205 These results demonstrate that mROS does play an important role against *S. uberis*
206 infection in MECs.
207

208 ## Discussion

209 The intrusion signal (from molecules broadly shared by pathogens that could be
210 recognized by the immune system) of intracellular bacteria captured by PRRs is
211 crucial for host control of inflammation and pathogen proliferation [25]. TLRs are
212 one of the most ancient, conserved components of the immune system, and have
213 been established by our laboratory to sense and respond to *S. uberis* [3]. *S. uberis* is a
214 kind of Gram-positive bacterium and TLR2 is the principal receptor that can sense its
215 invasion [26]. However, TLR2 and TLR4 share the same delivery system, and current
216 studies have not yet distinguished their exact roles in defending against *S. uberis*
217 infection. We used TLR2$^{-/-}$ and TLR4$^{-/-}$ mice to investigate, for the first time
218 thoroughly, the roles of these two high-correlation receptors in *S. uberis* infection.
219 Deficiency of TLR2, but not TLR4, induced a more severe inflammatory response and
220 tissues damage in mammary gland and bacterial viability was higher. These results

confirmed that TLR2 detected *S. uberis* infection, initiated the antibacterial immunological reaction and controlled the inflammatory status in mammary glands.

Proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, are secreted following activation of TLRs and their respective downstream signaling pathways mainly in immune cells [27]. They are involved in upregulation of inflammatory reactions and play a role in regulating host defense against pathogens mediating the innate immune response. In this study, TNF-α, the initiating factor in the cytokine storm, increased dramatically after *S. uberis* challenging in variant mice. This change was only seen in TLR2$^{-/-}$ mice for IL-1β. No changes were observed for IL-6. These findings were consistent with previous reports that expression of TNF-α, IL-1β and IL-6 had a chronological order, and for the samples detected here, were only expressed at 24 h post-infection [28]. Compared with WT mice, in TLR2$^{-/-}$ mice, TNF-α and IL-1β were obviously decreased. This further demonstrated the important role of TLR2 in the interaction of *S. uberis* infection with the host. TNF-α levels in TLR4$^{-/-}$ mice had similar changes. This could be explained by the fact that inflammatory response networks are complex after infection. Positive and negative inflammatory factor feedback loops both exist in *S. uberis*-infected mammary glands. The secondary inflammation induced by initial inflammatory factors might result partly from activation of TLR4. Hence, down-regulation of TNF-α caused by deficiency of TLR4 was not the same as that losing TLR2, which could not neutralize inflammatory response challenged by *S. uberis*.

Two distinct signaling pathways, the MyD88-dependent and TRIF-dependent

243 pathways, are triggered by dimerized and activated TLRs [29]. Our experiments *in*
244 *vivo* found that MyD88 instead of TRIF was affected significantly in *S. uberis* infection,
245 and thus confirmed that the MyD88-dependent pathway predominated in this
246 process. This phenomenon also existed in other bacterial infections. For example,
247 Wiersinga et al. reported it in *Burkholderia pseudomallei* infection [30]. In the
248 MyD88-dependent pathway, MyD88 recruits IL-1 receptor-associated kinases and
249 then phosphorylates and activates TRAF6, which in turn polyubiquinates TAK1, and
250 induces secretion of inflammatory cytokines in our research on *S. uberis* infection *in*
251 *vivo* and *in vitro* [3, 31]. Recently, it have been suggested that activated TRAF6
252 translocates to the mitochondria, which leads to ECSIT ubiquitination, resulting in
253 increased mROS generation [32]. This signaling pathway plays an important role in
254 the innate immune response against intracellular bacteria. A recent study has also
255 shown that ECSIT- and TRAF6-depleted macrophages have decreased levels of
256 TLR-induced ROS and are significantly impaired in their ability to kill intracellular
257 bacteria [13]. Sonoda et al. similarly found that estrogen-related receptor α and
258 PPAR gamma Coactivator-1 β (PGC-1β) act together as key effectors of IFN-γ-induced
259 mitochondrial ROS production and host defense [33]. Our study emphasizes the
260 importance of mROS in killing bacteria. Since accumulating ROS in tissues is difficult,
261 previous research has always detected the presence of members of the antioxidant
262 system, such as T-AOC, SOD, MDA and UCP2, in organs to reflect production of ROS
263 indirectly [34, 35]. Our results showed that *S. uberis* challenge caused changes in
264 redox status of mammary glands. This indicates that the

TLRs/MyD88/TRAF6/ECSIT/mROS axis participated in the defense response to *S. uberis* infection.

The inflammatory phenomena from mammary glands involve integrated responses of all kinds of mammary cells including macrophages, PMNs, lymphocytes, MECs and even matrix cells [36]. In the past decade, we paid more attention to the defensive ability of MECs because they are the most numerous cells in the udder, and we have detected TLRs-mediated signaling pathways and secretion of more than 40 cytokines [28]. In addition, we showed that *S. uberis* adhered and internalized in MECs, which establishes that MECs are one of the main target cells of *S. uberis* (data not published). Intriguingly, MECs are not real immune cells and have a distinctive response to bacterial infection. For example, we have found that the PI3K/Akt/mTOR pathway in MECs generates a positive contribution to inflammation following viable *S. uberis* challenge, which is not consistent with the usual situation in some immune cells [28]. Thus, we treated MECs with specific siRNAs targeting to TLR2 and/or TLR4 and then evaluated the effect of *S. uberis* challenge on the expression of key adaptor proteins. The data confirmed that the MyD88-dependent pathway predominated in *S. uberis*-infected MECs after TLR2 activation. A similar signal transfer process was reported in macrophages infected with *Mycobacterium tuberculosis,* another Gram-positive bacterium [37]. In the present study, we were interested in whether the TLR2/MyD88/TRAF6/ECSIT axis regulated production of ROS. Suppression of TLR2, but not TLR4, reduced the level of ROS and mROS in MECs after *S. uberis* challenge. This result was further explained by the detection of UCP2, which

287 separates oxidative phosphorylation from ATP synthesis and thus improves the
288 production of ROS and mROS [38].

289 Initially, mROS was considered to be a by-product of bio-oxidation, and its
290 synthesis cannot be regulated. A large body of researches have established that
291 oxidative phosphorylation in mitochondria is the main pathway for mROS production
292 and is the main source of ROS [39]. To express catalase in mitochondria could
293 effectively reduce the production of mROS, thereby reducing the killing effect of
294 macrophages on pathogens, indicating that mROS is a key driver in the process of
295 antibacterial activity [40]. Our current study showed that TLR2 regulates the
296 generation of ROS, including mROS, during *S. uberis* infection both *in vivo* and *in vitro*
297 [3]. We suggest that TLR2-mediated mROS are involved in *S. uberis* infection. To
298 confirm our hypothesis, GKT137831 and NG25 were used alone or simultaneously to
299 suppress ROS from NOX complexes and production of proinflammatory cytokines.
300 We found that the antibacterial activity of MECs was restrained to some extent, and
301 this established that ROS from NOX complexes and cytokines were involved in the
302 host defensive reaction. This is consistent with our previous study [28]. Furthermore,
303 we inhibited the synthesis of mROS by siECSIT and investigated the changes in
304 inflammation and the effect of reducing mROS on bacterial viability. The bacterial
305 counts of *S. uberis* were significantly higher in the siECSIT treatment group. These
306 results demonstrate that TLR2-mediated mROS are a key factor against *S. uberis*
307 infection in MECs.

308 In conclusion, mROS participate in the host response against *S. uberis* infection,

and TLR2 is involved in sensing *S. uberis* invasion and controlling mROS production by regulating expression of TRAF6 and ECSIT. Additionally, the function of mROS against *S. uberis* infection probably relies on their ability to regulate cytokine levels, thereby controlling the level of inflammation. This study increased our understanding of the molecular defense mechanisms in *S. uberis* mastitis, and provides theoretical support for the development of prophylactic strategies for this critical disease.

Materials and methods

Bacterial strain, cell culture and treatment

S. uberis 0140J (American Type Culture Collection, Manassas, VA, USA) was inoculated into Todd–Hewitt broth (THB) supplemented with 2% fetal bovine serum (FBS; Gibco, USA) at 37°C in an orbital shaker to mid-log phase (OD_{600} 0.4–0.6). MECs (American Type Culture Collection) were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and plated at 80% confluence in 6-well cell cultrue cluster. After culture in serum-free DMEM for 4 h, the monolayer was treated with 40 nM NG25 (inhibitor of TGFβ-activated kinase 1; TAK1: Invitrogen, Carlsbad, CA, USA) for 24 h; 4 μm MK2206 (inhibitor of NADPHase: SellecK Chemicals, Houston, TX, USA) for 24 h; or transfected with 50 nM siTLR2 or/and siTLR4 for 72 h. SiECSIT with 20 nM were performed for 48 h using Lipofectamine 3000 reagent (Invitrogen) . The sequences of siRNA were designed and listed as follows. siTLR2: GTCCAGCAGAATCAATACA; siTLR4: CAATCTGACGAACCTAGTA; siECSIT:

GGTTCACCCGATTCAAGAA. The treated cells were infected with *S. uberis* at a multiplicity of infection (MOI) of 10 for 2 or 3 h at 37°C. The supernatant and cells were collected separately and stored at −80 °C until use.

Mice and treatment

Mice, including wild-type C57BL/6 (WT-B6), wild-type C57BL/10 (WT-B10), TLR2$^{-/-}$ (C57BL/6) and TLR4$^{-/-}$ (C57BL/10), aged 6–8 weeks were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and bred under specific pathogen-free conditions in the Nanjing Agricultural University Laboratory Animal Center. All experimental protocols were approved by the Regional Animal Ethics Committee and in compliance with Animal Welfare Act regulations as well as the Guide for the Care and Use of Laboratory Animals.

Seventy-two hours after parturition, all experimental groups of female mice were infused with 50 µL *S. uberis* according to the number of complex infections (MOI = 10) into the left 4 (L4) and right 4 (R4) teats. The animals in the control groups were infused with same volume of phosphate-buffered saline (PBS). The offspring were weaned 1 h prior to experimental infusion. All mice were killed 24 h post-infusion. The mammary glands and serum were aseptically collected and stored at −80°C.

Mammary gland was fixed in 10% neutral buffered formalin. Sections of 5 µm thickness were stained with hematoxylin and eosin. Mammary gland tissues were weighed and homogenized with sterile PBS (1:5, W/V) on ice. After centrifuged at 500 *g* at 4°C for 40 min, the supernatant was centrifuged again. The second

353 supernatant was collected and stored at −80°C until assayed.

354

355 **Histological observation and immunohistochemistry**

356 The mammary tissue fixed in 10% neutral buffered formalin was trimmed and
357 flushed in water for at least for 4 h, and then dehydrated in alcohol solutions ranging
358 from 75% to 100%, with 5% increase at 1 h intervals. After soaking in xylene, the
359 tissues were embedded in wax for 3 h at 60°C. Slices (5 μm thick) were cut and
360 stained with hematoxylin and eosin. The histological changes including PMN
361 infiltration, bleeding and degeneration, and adipose tissue loss were analyzed by
362 light microscopy (BH2; Olympus, Tokyo, Japan) at a magnification of 40×. Four
363 sections of mammary tissue were quantified for each animal. Ten fields were
364 randomly selected per tissue section and assigned a score of 1, 2 or 3 based on the
365 degree of damage.

366 Immunohistochemical staining was performed as follows. Tissue sections were
367 washed with PBS, then covered with 3% H_2O_2 for 15 minutes at 37°C to inhibit
368 further endogenous peroxidase activity. Tissue slices were blocked with 5% bovine
369 serum albumin and incubated with antibodies against MyD88, TRAF6, ECSIT and TRIF
370 (Cell Signaling Technology, Danvers, MA, USA), at 4°C in a humidified chamber.
371 Overnight, biotinylated anti-rabbit IgG (Boster Bio-Technology, Wuhan, China) was
372 incubated for 30 min at 37°C. After rehydration, the sections were incubated with
373 avidin–biotin peroxidase complex for 40 min at 37°C. Finally, the sections were
374 washed and bound conjugates were revealed by diaminobenzidine staining (Boster

375 Bio-Technology).

376

377 **RNA extraction and quantitative real-time polymerase chain reaction (PCR)**

378 PCR was carried out as previously described [41]. Total RNA was extracted by TRIzol
379 reagent (TaKaRa, Dalian, China). Corresponding cDNA was obtained using reverse
380 transcriptase and Oligo (dT) 18 primer (TaKaRa). An aliquot of the cDNA was mixed
381 with 25 µL SYBR® Green PCR Master Mix (TaKaRa) and 10 pmol of each specific
382 forward and reverse primer. All mixed systems were analyzed in an ABI Prism 7300
383 Sequence Detection System (Applied Biosystems, Waltham, MA, USA). Fold changes
384 were calculated as $2^{-\Delta\Delta Ct}$. All primer sequences (Table S1) were synthesized by
385 Invitrogen Biological Company (Shanghai, China).

386

387 **Total protein extraction and western blotting**

388 Cells were washed twice in 2 mL ice-cold PBS and collected in an Eppendorf tube
389 (gently scraped by a rubber policeman) after being lysed on ice for 20 min in lysis
390 buffer (Beyotime, Nantong, China). Extracts with equal amounts of proteins were
391 solubilized by SDS sample buffer (BioRad, Califonia, USA), separated by SDS-PAGE,
392 and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA,
393 USA). The membranes were incubated with corresponding polyclonal antibodies:
394 anti-MyD88, anti-TRAF6, anti-ECSIT anti-TRIF, and anti-GAPDH. The signals were
395 detected by an ECL western blot analysis system (Tanon, Shanghai, China). Analysis of
396 bands was quantified with Image J software (NIH, Bethesda, MD, USA).

397

398 **Measurement of ROS and mROS**

399 EpH4-Ev cells were incubated in dichloro-dihydrofluorescein diacetate (10 μM, 30
400 min) (Beyotime, Nantong, China) or MitoSOX (5 μM, 20 min) (Thermo, Waltham, MA,
401 USA) at 37°C, washed three times in PBS and detached. The cells were centrifuged at
402 400 *g* for 5 min, resuspended in PBS, and immediately analyzed by flow cytometry
403 using FACSCanto (BD, New Jersey, USA). Ten thousand cells per sample were
404 analyzed using CellQuest Pro acquisition and analysis software.

405

406 **Assay of TNF-α, IL-1 , and IL-6 by ELISA**

407 The levels of TNF-α, IL-1β, and IL-6 in mammary glands and EpH_4-Ev cells were
408 measured by ELISA (Rigor Bioscience, Beijing, China). Prepared standards (50 μL),
409 and antibodies (40 μL) labeled with enzyme (10 μL) were reacted for 60 min at 37°C
410 and the plate was washed five times. Chromogen solutions A (50 μL) and B (50 μL)
411 were added and incubated for 10 min at 37°C. Stop solution (50 μL) was added and
412 optical density value was measured at 450 nm within 10 min. Qualitative differences
413 or similarities between the control and experimental groups were consistent
414 throughout the study.

415

416 **Detection of NAGase, T-AOC, SOD, MDA and UCP2**

417 The activities or levels of *N*-acetyl-β-D-glucosaminidase (NAGase), total antioxidant
418 capacity (T-AOC), superoxide dismutase (SOD), malondialdehyde (MDA) and

419 uncoupling protein 2 (UCP2) were determined using commercial kits purchased from
420 Nanjing Jiancheng Bioengineering Institute (China).
421
422 **Viable bacterial count assay**
423 Viable bacteria were enumerated as colony-forming units (CFU) on THB agar. The
424 mammary glands were aseptically homogenized with sterile PBS (1:5, W/V). The
425 supernatants were spread on plates. CFUs were counted by the spread plate method
426 after incubation for 12 h at 37°C.
427 MECs and MECs with siECSIT were incubated in DMEM with 10% FBS and plated at
428 80% confluence in 6-well plates. After culture in serum-free DMEM for 4 h, at
429 mid-exponential phase (OD_{600} 0.4–0.6), *S. uberis*-infected cells were washed 3 times
430 with PBS containing 100 mg/mL gentamicin, followed by gentamicin-free PBS. Cells
431 were pelleted at 1.4 *g* for 10 min. The same number of cells were lysed with sterile
432 triple distilled water, and CFUs were counted by the spread plate method after
433 incubation for 12 h at 37°C.
434
435 **Statistical analysis**
436 Results were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc.,
437 La Jolla, CA, USA). Data were expressed as means standard error of the mean (SEM).
438 Differences were evaluated by one-way analysis of variance followed by post-hoc
439 tests. Significant differences were considered at $P < 0.05$.
440 ## Supporting information

441 **S1 Table. Oligonucleotide sequences used for RT-qPCR.**

442 **S1 Fig. TLR2/4 mediates the NAGase activity after challenge with *S. uberis* in the**
443 **supernatant of MECs.**

444 **S2 Fig. TLR2/4 mediates the inflammatory response after challenge with *S. uberis* in**
445 **MECs.**

446 **S3 Fig. The suppression of mROS reduces the inflammation factors after challenge**
447 **with *S. uberis* in the supernatant of MECs.**

448 **S4 Fig. The protein expression of ECSIT were determined by Western blot after**
449 **using siECSIT in MECs.**

450

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458

459 ## Author Contributions

460 Conceived and designed the experiments: Bin Li, Zhixin Wan. Performed the
461 experiments: Bin Li, Zhixin Wan, Zhenglei Wang. Analyzed the data: Bin Li, Zhixin
462 Wan, Jiakun Zuo, Yuanyuan Xu. Contributed reagents/materials/analysis tools: Xiangan

463 Han, Vanhnaseng Phouthapane, Jinfeng Miao. Wrote the paper: Bin Li, Zhixin Wan,

464 Jinfeng Miao.

465

466 Competing interests

467 The authors have declared that no competing interests exist.

468

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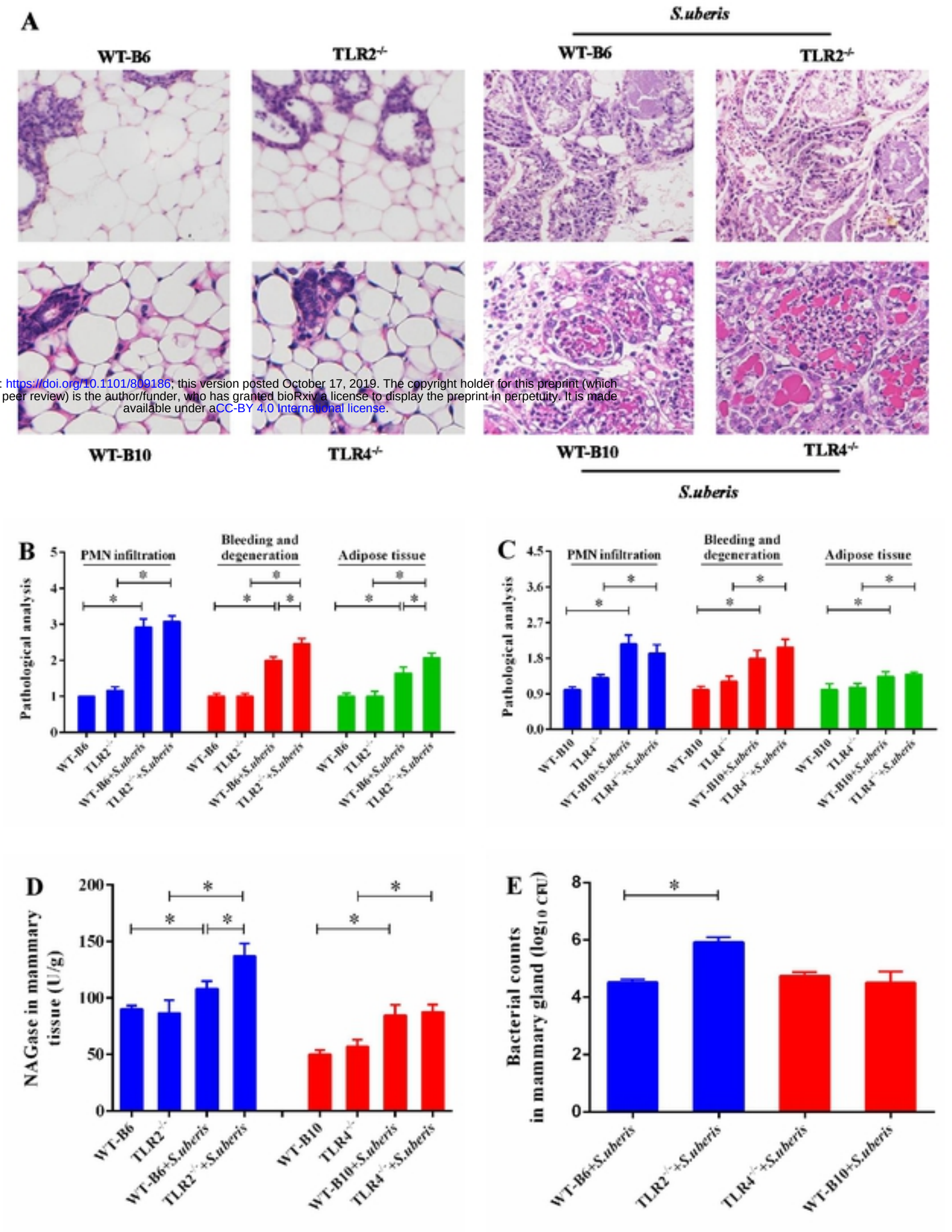


Fig 1. TLR2 mediates tissue damage and anti-*S. uberis* infection in mammary gland. (A, B, C) Mammary glands were stained through hematoxylin and eosin, and the bleeding and degeneration, PMN infiltration, adipose tissue were analyzed by light microscopic. (D) NAGase activity was analyzed in mammary glands. (E) Viable bacteria was counted via the plate with THB agar medium. Data are presented as the means ± SEM (n=6). *($P< 0.05$) = significantly different between the indicated groups.

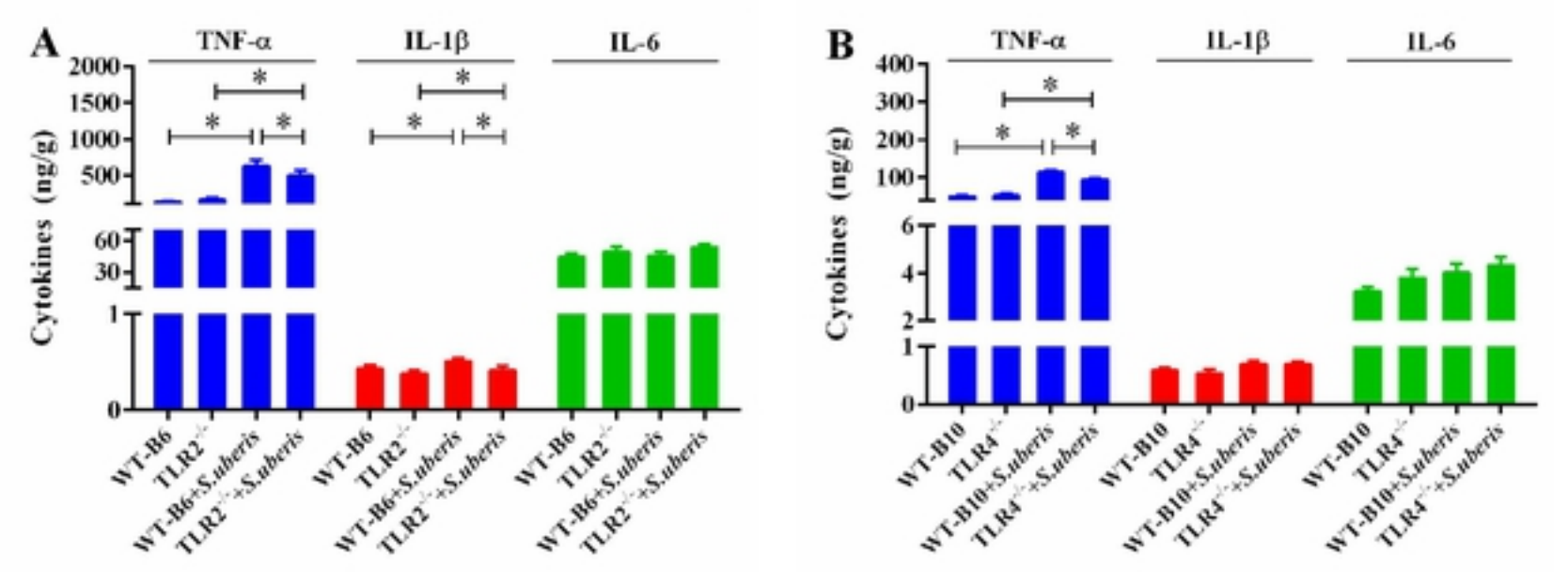
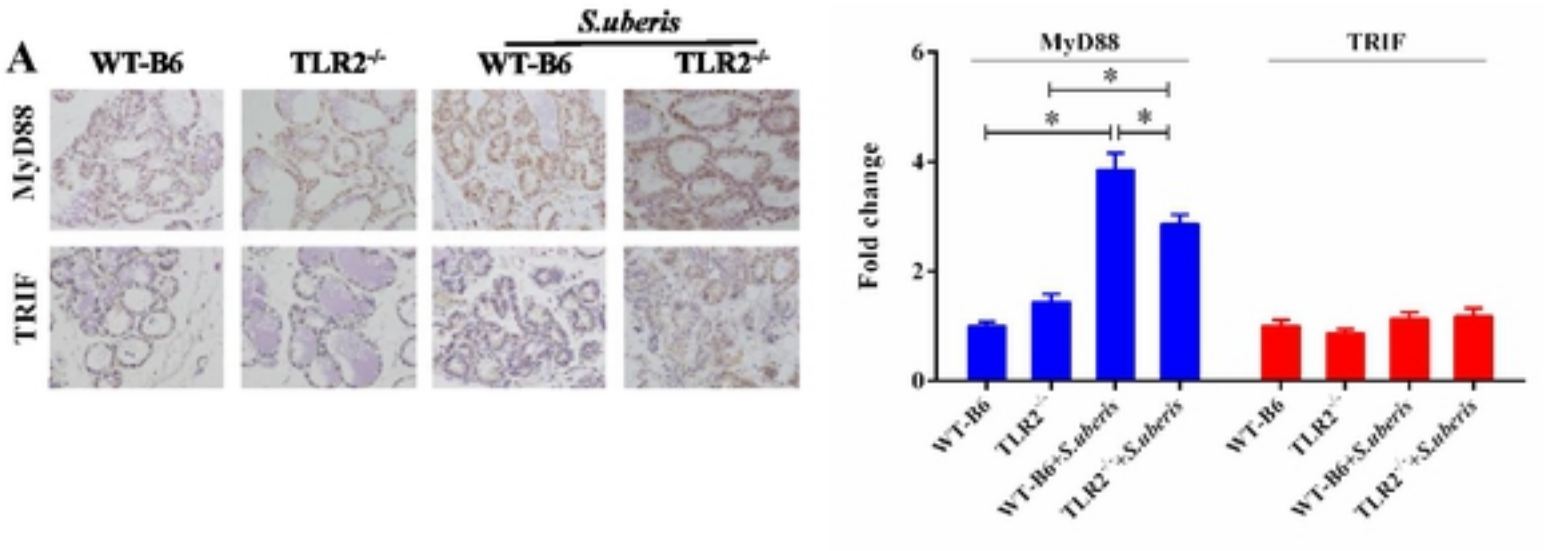
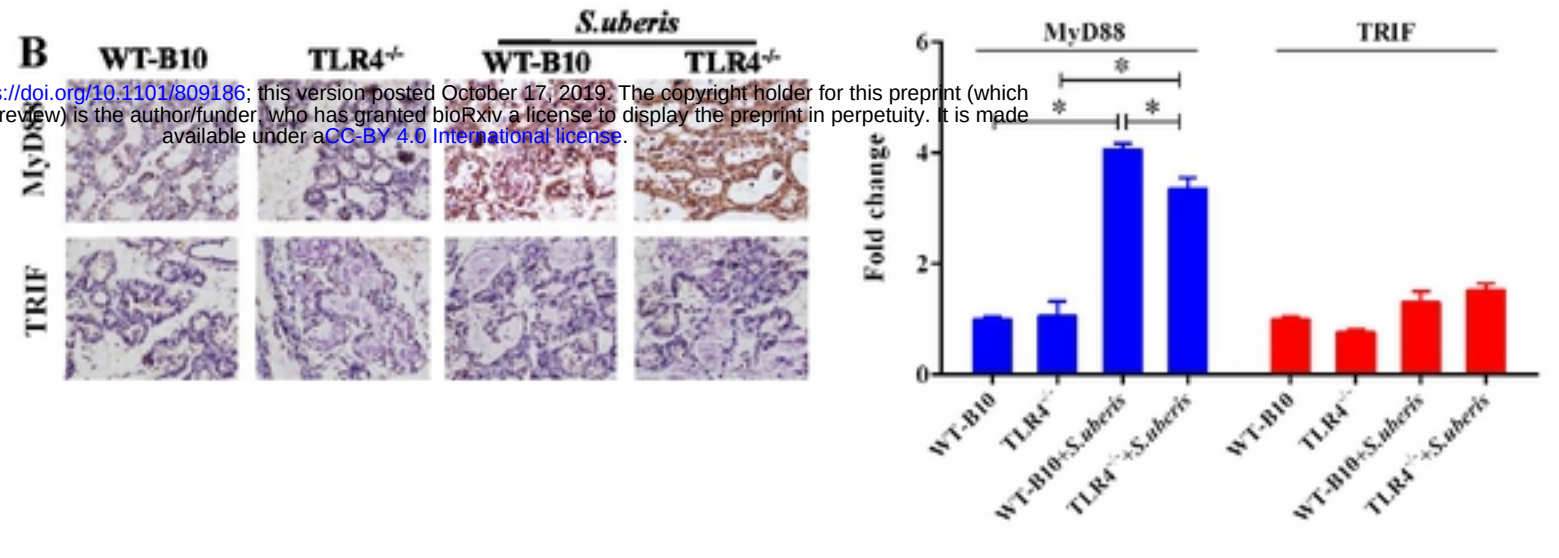
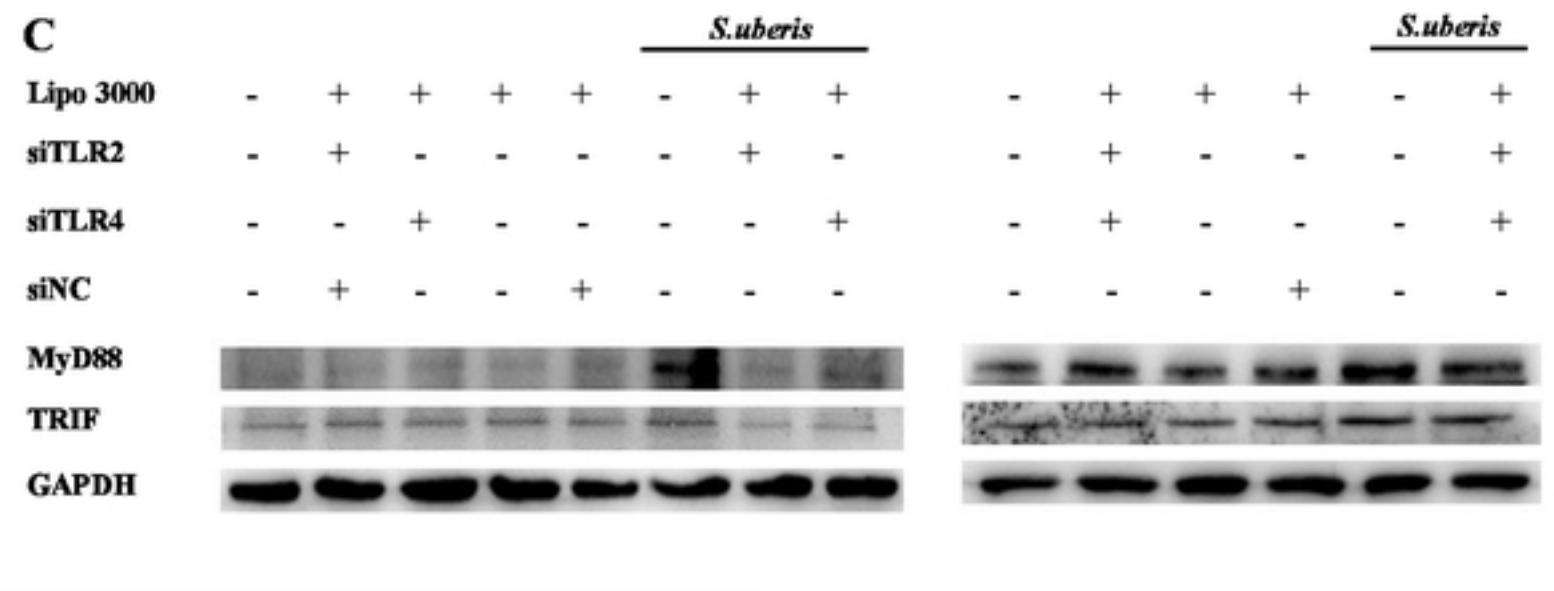


Fig 2. TLR2 and TLR4 deficiency affect the secretion of cytokines in *S.uberis* infection. (A, B) The protein expression of TNF-α, IL-1β and IL-6 were determined by ELISA in mammary glands. Data are presented as the means ± SEM (n=6). *($P< 0.05$) = significantly different between the indicated groups.







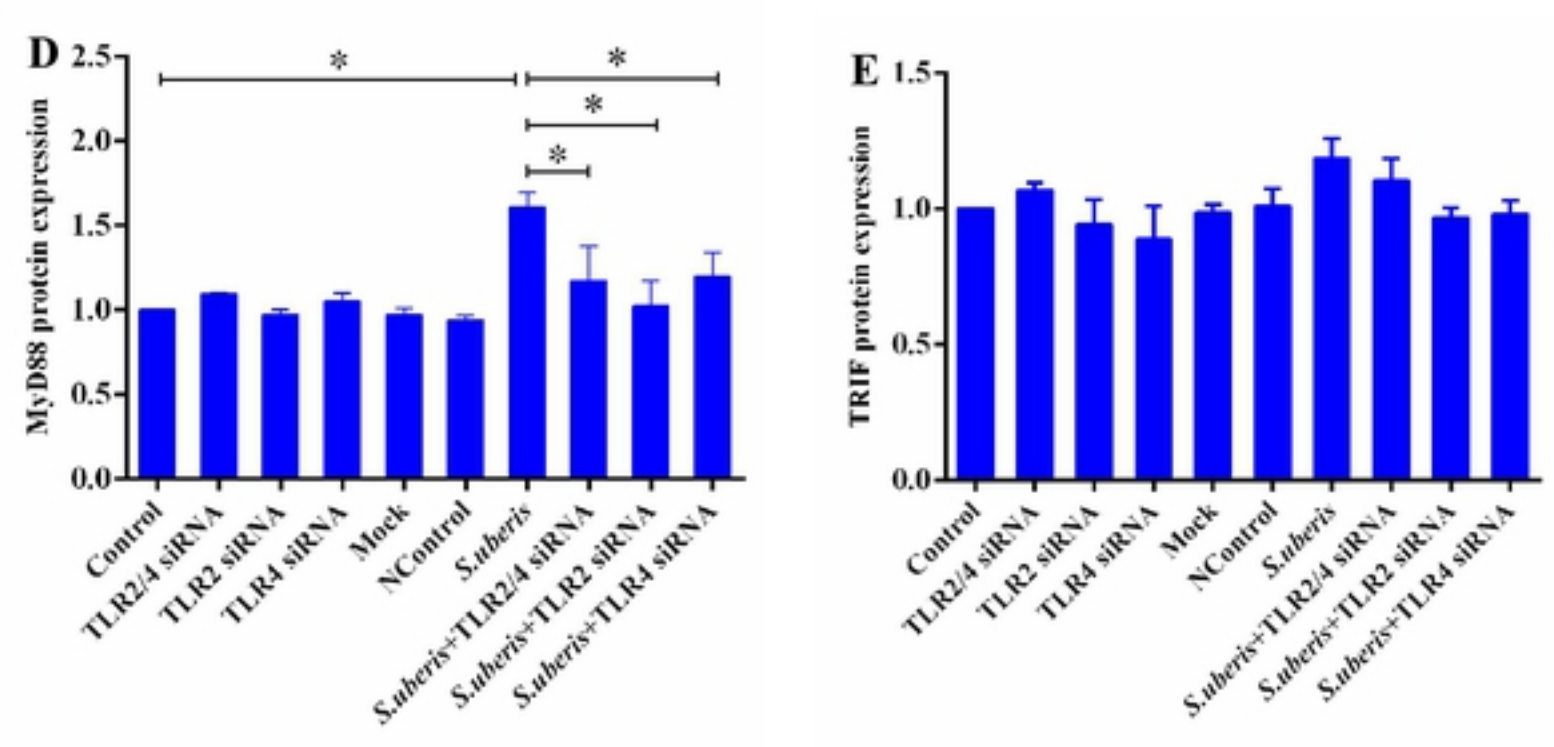
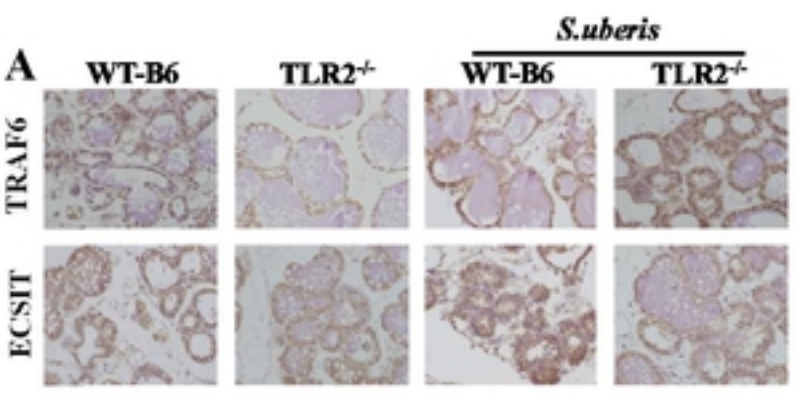
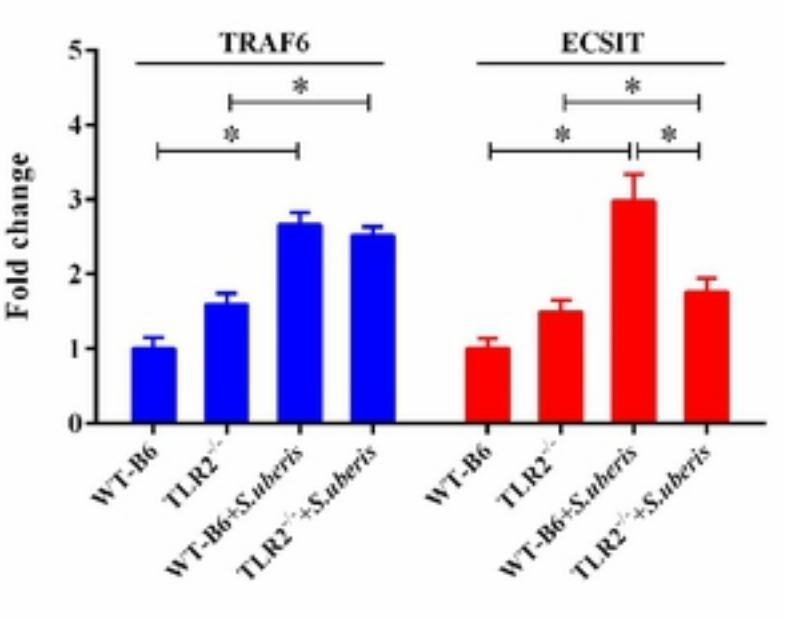
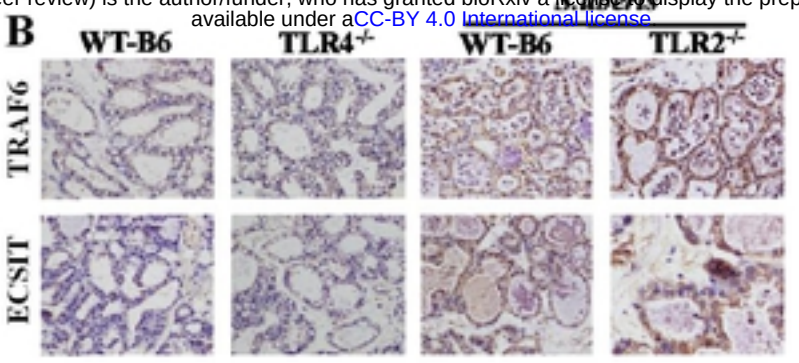
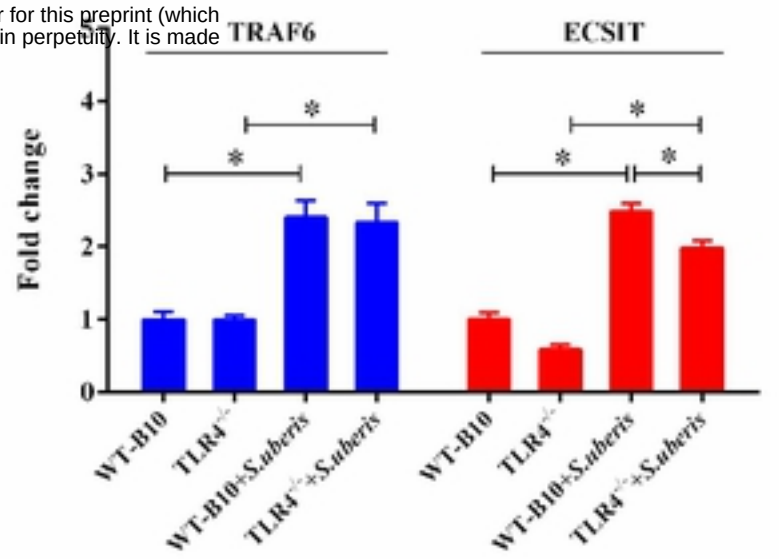


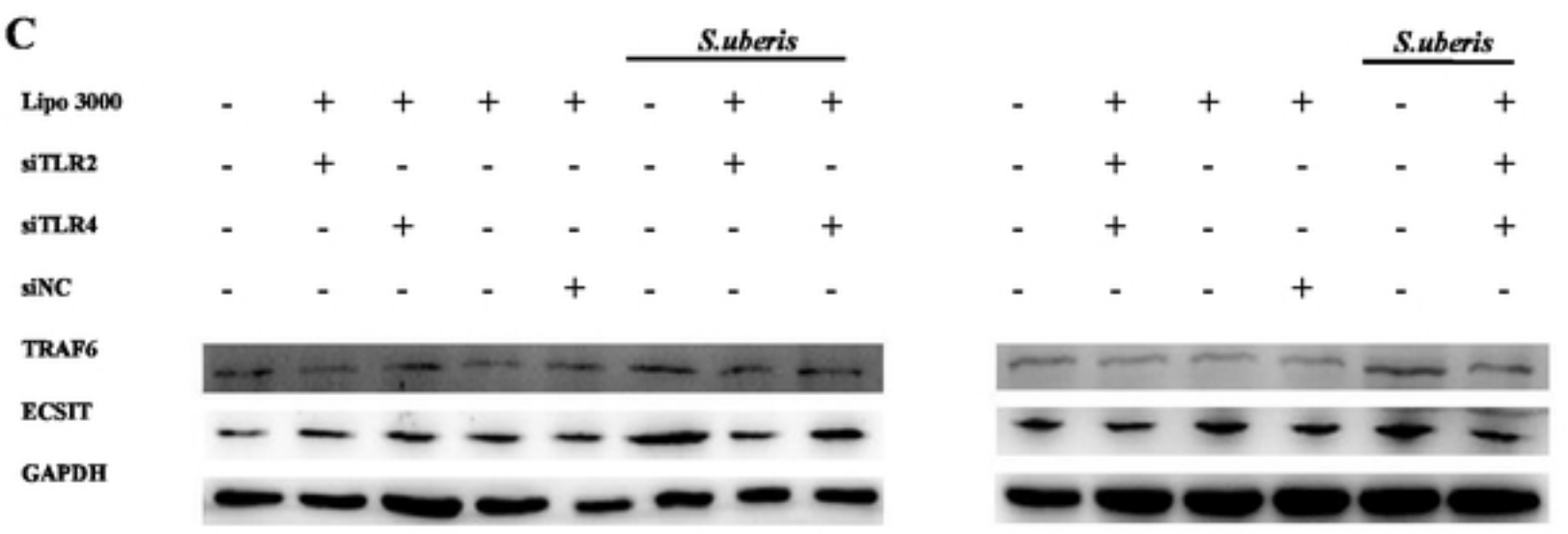
Fig 3. MyD88 dependent pathway predominates in *S. uberis* infection. (A, B) Immunohistochemistry was used to analyze the expression of MyD88 and TRIF in mammary glands. Data are presented as the means ± SEM (n=6). *($P< 0.05$) = significantly different between the indicated groups. (C, D, E) The protein expression of MyD88 and TRIF were determined by Western blot in MECs. Data are presented as the means ± SEM (n=3). *($P< 0.05$) = significantly different between the indicated groups.

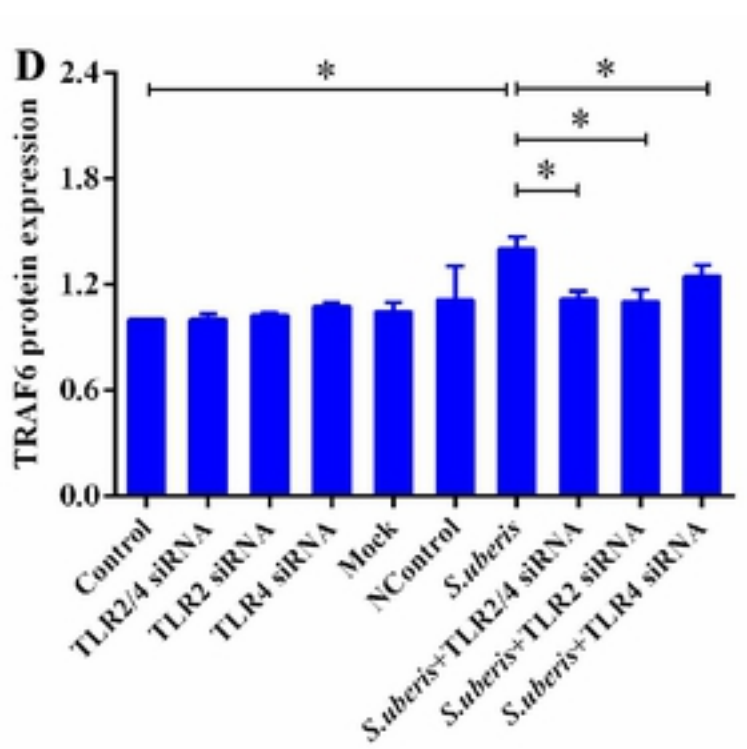












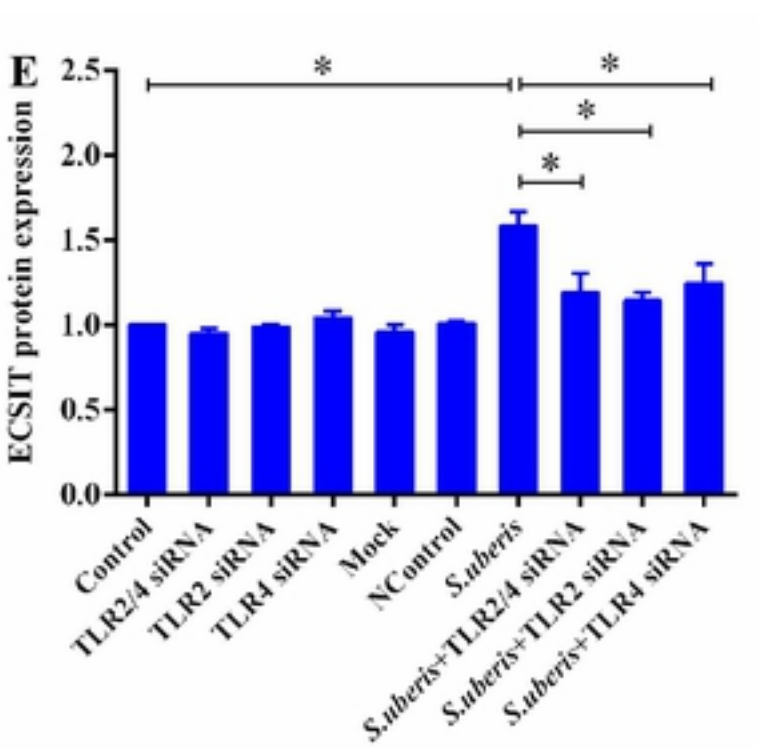


Fig 4. TRAF6 and ECSIT participate in senseing signal from TLRs in *S. uberis* infection. (A, B) Immunohistochemistry was used to analyze the expression of TRAF6 and ECSIT in mammary glands. Data are presented as the means ± SEM (n=6). *($P< 0.05$) = significantly different between the indicated groups. (C, D, E) The protein expression of TRAF6 and ECSIT were determined by Western blot in MECs. Data are presented as the means ± SEM (n=3). *($P< 0.05$) = significantly different between the indicated groups.

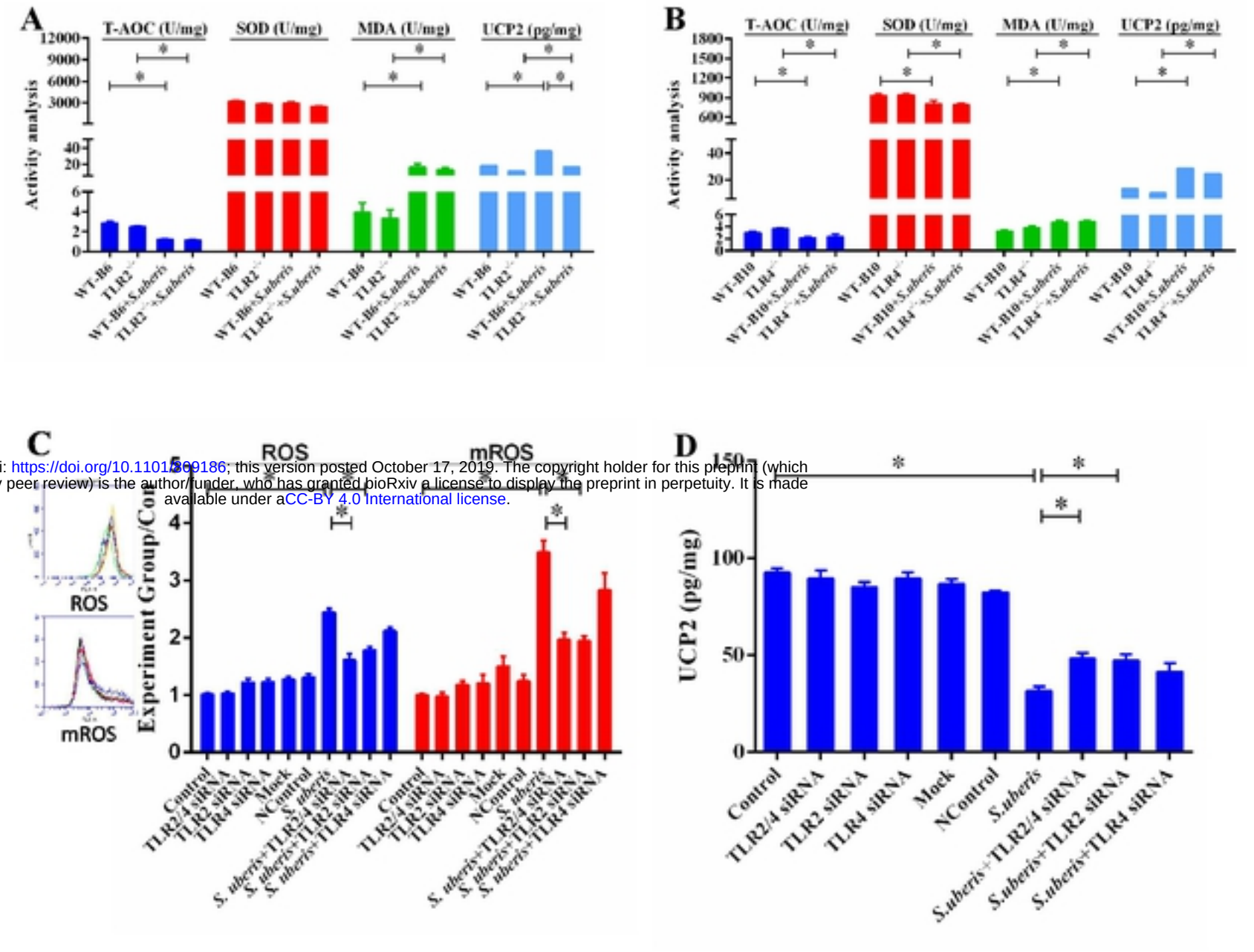


Fig 5. TLRs mediate redox status of mammary glands during *S. uberis* infection. (A, B) The protein expression of T-AOC, SOD, MDA and UCP2 were determined by kits in mammary glands. Data are presented as the means ± SEM (n=6). *($P< 0.05$) = significantly different between the indicated groups. (C) CellQuest Pro acquisition and analysis software analyzed ROS and mROS in MECs. (D) The activity of UCP2 were determined by ELISA in MECs. Data are presented as the means ± SEM (n=3). *($P< 0.05$) = significantly different between the indicated groups.

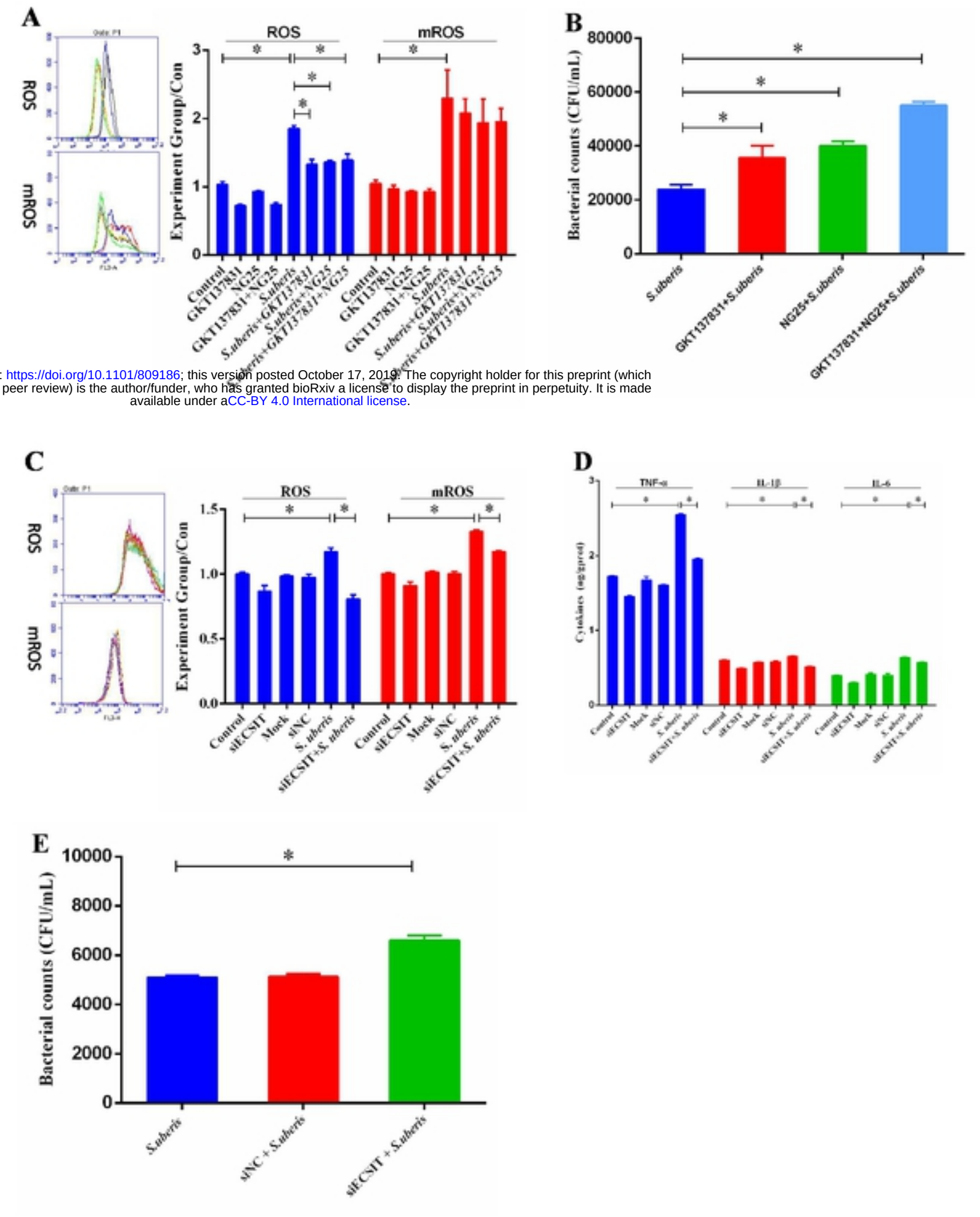


Fig 6. mROS play an important role in anti-*S. uberis* infection in MECs. (A) The expression of ROS and mROS after using GKT137831 and NG25 simultaneously or separately during *S. uberis* infection in MECs. (B) Viable bacteria was counted via the plate with THB agar medium after using GKT137831 and NG25 simultaneously or separately during *S. uberis* infection in MECs. (C) The expression of ROS and mROS after using siECSIT in MECs. (D) The expression of TNF-α, IL-1ß and IL-6 after using siECSIT in MECs. (E) Bacteria counts after using siECSIT in MECs. Data are presented as the means ± SEM (n=3). *($P< 0.05$) = significantly different between the indicated groups.