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RESEARCH ARTICLE

The α7 Nicotinic Acetylcholine Receptor Maintains Uterine Immune Tolerance During *Escherichia coli*-Induced Bovine Endometritis

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ABSTRACT

The uterus has a strong self-regulatory capacity that maintains immune homeostasis and self-tolerance by balancing antimicrobial defense with protection against excessive inflammation. *Escherichia coli* (*E. coli*) is the major etiological agent of bovine endometritis, which severely impairs reproductive performance and causes substantial economic losses. The α7 nicotinic acetylcholine receptor (α7nAChR) has been implicated in the regulation of infectious and inflammatory diseases; however, its role in *E. coli*-induced bovine endometritis remains incompletely understood. In this study, *E. coli*-infected bovine neutrophils and endometrial tissue models were established to investigate the potential regulatory role of α7nAChR in infection-induced uterine inflammation. α7nAChR was highly expressed in normal bovine neutrophils and endometrial tissues, but was markedly reduced following *E. coli* infection. *E. coli* markedly increased inflammatory cytokine levels, whereas pretreatment with PNU-282987 (an α7nAChR agonist) reduced these levels, while pretreatment with MLA (an α7nAChR antagonist) further enhanced them. Western blot analysis showed that α7nAChR activation was associated with increased phosphorylation of JAK2 and STAT3 and reduced phosphorylation of ERK, p38, and p65. In addition, activation of α7nAChR alleviated *E. coli*-induced bovine endometrial injury, whereas its blockade aggravated inflammatory damage. Collectively, these findings suggest that α7nAChR may regulate inflammatory responses and exert protective effects in *E. coli*-induced bovine endometritis, possibly through the JAK2/STAT3, MAPK, and NF-κB pathways.

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INTRODUCTION

The endometrium is essential for embryo implantation and early development, as it provides an optimal environment that supports fertility in both humans and animals (Ma *et al*., 2017). Under physiological conditions, the uterine endometrium exhibits a remarkable self-regulatory capacity that maintains immune homeostasis and self-tolerance by balancing antimicrobial defense with protection against excessive inflammation (Zhang *et al*., 2018). This balance is achieved through the coordinated actions of epithelial cells, immune cells, and regulatory cytokines that sustain immune quiescence while permitting defense against invading pathogens (Chao *et al*., 2024). Uterine epithelial and stromal cells constitutively express pattern recognition receptors, which are essential for detecting microbial components and initiating tightly regulated innate immune responses (Song *et al*., 2007; Gao *et al*., 2025).

Among uterine immune cells, neutrophils play a crucial dual role in this self-regulatory network. Under physiological conditions, they promote host defense and tissue remodeling through the regulated release of antimicrobial peptides, matrix metalloproteinases (MMPs), and reactive oxygen species (ROS) (Gupta and Kaplan, 2016; Salvo and Sandoval, 2022). Once pathogens are cleared, apoptotic neutrophils are efficiently removed by macrophages via efferocytosis. This process triggers IL-10 and TGF-β release, which helps restore local immune tolerance (Yang *et al*., 2025). Subversion of resolution, characterized by prolonged neutrophil activation and apoptotic evasion, precipitates excessive cytokine cascades, culminating in epithelial damage and immune collapse (Lee *et al*., 2020; Liu *et al*., 2021). Macrophages and regulatory T cells (Tregs) cooperatively sustain uterine integrity and enable successful reproduction (Özcan and Boyman, 2022; Dubrovskyi *et al*., 2025). Cellular interactions between macrophages and regulatory T cells constitute a dynamic regulatory network essential for uterine integrity and reproductive success (Zhang *et al*., 2024).

Disruption of this delicate immune balance underlies the pathogenesis of endometritis, a common uterine inflammatory disorder. Bovine endometritis frequently develops in the postpartum period, when the cervix remains open, and the uterus is highly susceptible to microbial invasion (Shen *et al*., 2022; Wang *et al*., 2022). Among the causative agents, *E. coli* is the predominant opportunistic pathogen in the uterine microenvironment. It acts as the primary etiological agent of postpartum endometritis and critically compromises the reproductive longevity of dairy cows (Singh *et al*., 2008; Basbas *et al*., 2022). Infection with *E. coli* disrupts uterine immune homeostasis, triggering excessive neutrophil infiltration and hyperactivation of pro-inflammatory cytokines. This inflammatory cascade subsequently impairs the endometrium's intrinsic capacity for self-regulation (Davies *et al*., 2008; Wankhade *et al*., 2017). This immune imbalance disrupts epithelial barrier function and endometrial receptivity, leading to infertility and imposing substantial economic losses on the dairy industry (Jiang *et al*., 2021; Cai, 2024). Despite being the primary therapeutic approach, the extensive use of antibiotics promotes bacterial resistance and leaves detectable drug residues in animal-derived foods, which threatens public health and food safety (Jiang *et al*., 2021). Therefore, developing therapeutic strategies that restore uterine immune regulation and tolerance rather than relying solely on antibiotics has become an urgent need.

The cholinergic anti-inflammatory pathway (CAP) has been identified in recent investigations as a pivotal neuroimmune mechanism crucial for regulating inflammatory responses and maintaining tissue homeostasis (Su *et al*., 2022). Within the CAP, α7nAChR acts as a crucial molecular conduit and a potent immunomodulatory brake. It suppresses the systemic release of pro-inflammatory cytokines by recalibrating downstream intracellular signaling cascades (Wu *et al*., 2021). Activation of α7nAChR restores immune balance in various inflammatory models by enhancing JAK2/STAT3 signaling while suppressing the ERK/p38 MAPK/NF-κB pathways (Zhao *et al*., 2023; Wang *et al*., 2025). In the reproductive system, α7nAChR-mediated signaling reduces inflammatory lesion formation and restores local immune tolerance in uterine disorders like endometriosis and adenomyosis (Xu *et al*., 2016; Yamada-Nomoto *et al*., 2016). These results imply that α7nAChR may serve as a crucial molecular mediator linking neuroimmune regulation to uterine immune self-regulation.

However, the role of α7nAChR in uterine immune homeostasis and tolerance during *E. coli*-induced bovine endometritis is unknown. Our research, therefore, investigated the contribution of α7nAChR to uterine immune regulation under infectious stress. To elucidate its function, we used in vitro models of *E. coli*-infected bovine neutrophils and endometrial tissue to evaluate its impact on inflammatory cytokine profiles and potential uterine injury.

MATERIALS AND METHODS

Collection of bovine endometrial tissues and blood: Healthy uteri and corresponding blood samples (n=18) were obtained from 3–5-year-old Holstein cattle weighing 500–600 kg immediately after slaughter at a local abattoir. Collected samples were immediately placed on ice and transported to the laboratory under aseptic conditions. The endometrial tissues were carefully isolated and washed sequentially with PBS (Biosharp, Beijing, China) to preserve their histological integrity. The animal ethics approval number for this study is NND2025030.

Bacterial strains: *Escherichia coli* (*E. coli,* strain certification number: SYS110017) was isolated and identified in our laboratory, where it is now preserved. The strain was revived and cultured in LB broth at 37°C with shaking. The bacterial culture achieved the logarithmic growth phase when the optical density at 600nm (OD_{600}) approximated 0.75. To ascertain the concentration, the culture underwent serial dilution and plating onto LB agar, followed by 12h of incubation at 37°C. The final bacterial load was calculated as 1.6×10^9 CFU/mL using the standard colony counting technique. Reproducibility was ensured by conducting all experiments independently in at least eight replicates, where CFU measurements exhibited high consistency.

Isolation and culture of bovine neutrophils: Eutrophils were isolated from bovine peripheral blood using a commercially available Bovine Peripheral Blood Neutrophil Isolation Kit (Haoyang Biotechnology, Tianjin, China). Briefly, 3mL of anticoagulated bovine peripheral blood was carefully layered onto the kit-provided separation medium and centrifuged at 2000rpm for 25min at room temperature. The white interfacial layer was collected and further purified using a second separation step under identical centrifugation conditions. After discarding the supernatant, red blood cells were lysed for 2min and neutralized with PBS. The harvested cells were pelleted by centrifugation, resuspended in RPMI-1640 supplemented with 10% fetal bovine serum, and cultured in six-well plates under standard conditions (37°C, 5% CO_2).

In vitro culture of endometrial tissue: The endometrial tissue was carefully dissected into small pieces measuring approximately 2mm×1mm. Approximately 40mg of tissue explants was randomly placed into each well of a 6-well culture plate. Each well contained 3mL of DMEM/F12, with 20% FBS and 2% penicillin-streptomycin (both from Hyclone, Logan, UT, USA). After overnight incubation at 37°C in 5% CO_2, the medium was replaced with DMEM/F12 supplemented with 20% FBS.

Experimental infection and treatment in-vitro: To clarify the regulatory role of α7nAChR in *E. coli*-induced inflammation, bovine neutrophils and endometrial tissues were subjected to different treatments, including MLA (Liu *et al*., 2025), PNU-282987 (Ji *et al*., 2026), and *E. coli* infection, either alone or in combination. Cells were pretreated with MLA (1μM; MedChemExpress, Monmouth Junction, NJ, USA) or PNU-282987 (1μM; MedChemExpress) for 15min before *E. coli* exposure. For endometrial tissue cultures, MLA (10μM) or PNU-282987 (1μM) was added under the same pretreatment conditions. Following 1h of infection, tobramycin (0.1mg/mL, MedChemExpress) was added to remove extracellular bacteria. Neutrophils were subsequently incubated for 11h, and tissue blocks were cultured for a further 8h. At the designated time points, both the supernatants and the samples were collected for analysis.

Cell counting kit-8: Cells were exposed to MLA or PNU-282987 (0.1-100μM) for 12h. Viability was quantified via the CCK-8 assay, following the supplier's instructions. After adding the CCK-8 solution, plates were incubated, and absorbance (450nm) was recorded using a microplate reader.

ELISA assay: After treatment, we collected the culture supernatants from bovine neutrophils and endometrial tissue. The levels of IL-1β (Kingfisher Biotech, Saint Paul, MN, USA), IL-6 and TNF-α (R&D Systems, Minneapolis, MN, USA) were subsequently determined using commercially available ELISA kits, following the manufacturers' recommended protocols.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis: Total RNA isolation was accomplished utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by an immediate treatment with DNase I to eradicate any contaminating genomic DNA. Reverse transcription and gene quantification were carried out via real-time PCR on an iCycler iQ5 system. All primer sequences employed are detailed in Table 1. Cycling conditions encompassed an initial 10 min denaturation at 95°C, followed by 40 cycles of 15s at 95°C and 60s at 60°C. Relative mRNA expression was computed using the $2^{-\Delta\Delta Ct}$ method.

Western blotting assay: Total proteins were extracted from bovine neutrophils and endometrial tissue. Protein quantification was conducted using the BCA Protein Assay Kit (Thermo Scientific). Defined amounts of protein (10μg from cells or 15μg from tissues) underwent separation via SDS-PAGE before transfer onto PVDF membranes (Millipore, Carrigtwohill, Ireland). The membranes were subjected to a 4h blocking step with 3% BSA (Amresco, Solon, OH, USA) in TBST at room temperature. Primary antibody incubation involved overnight treatment at 4°C with: p65, p38, ERK, STAT3, JAK2, p-ERK, p-p38, p- p65 p-STAT3, and p-JAK2 (1:1000; Cell Signaling Technology, Beverly, MA, USA), α7nAChR (1:500; Novus Biological, Cambridge, UK), or GAPDH (1:10,000; Abcam, Cambridge, UK). After washing, secondary detection was performed by incubating the membranes with HRP-conjugated antibody. Protein signals were rendered visible using Affinity ECL Reagent (FG-level) and quantified employing ImageJ.

Histopathology and immunohistochemical analysis: The harvested bovine endometrial blocks were initially placed in 4% paraformaldehyde for fixation at ambient temperature over a 48h period. Tissue processing involved sequential dehydration through an ethanol gradient, followed by xylene clearing and final paraffin embedding. Five-μm-thick sections from paraffin-embedded bovine endometrial tissues were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Resultant histological micrographs were acquired with an Axio Scan.Z1 digital slide scanner (Carl Zeiss, Oberkochen, Germany). Histopathological scoring was performed based on the extent of hemorrhage/edema, tissue damage, and inflammatory cell infiltration (Hu *et al*., 2018). The scoring criteria are detailed in Table 2.

Table 1: Primers used for gene amplification, sequencing, and accession no.

Gene symbol	Accession No.	Primer sequence
B-actin	XM_070780256.1	Forward 5'-CCAAGGCCAACCGTGAGAAGAT -3'
IL-1β	EU276067.1	Reverse 5'-CCACGTTCCGTGAGGATCTTCA -3'
IL-6	EU276071.1	Forward 5'-AAAATCCCTGGTGCTGGCTA -3'
TNF-α	JN635499.1	Reverse 5'-AGCTCATGCAGAACACCACT -3'
		Forward 5'-ATGCTTCCAATCTGGGTTC -3'
		Reverse 5'-TGAGGATAATCTTTGCGTTC -3'
		Forward 5'-CTCCTTCCTCCTGGTTGCAG -3'
		Reverse 5'-CACCTGGGGACTGCTCTTC -3'

Table 2: Histopathologic scoring criteria.

Feature	Description	Score
Hyperemia/edema	Normal	0
	Mild	1
	Moderate	2
	Severe	3
Infiltration with neutrophil	0–1	0
	2–5	1
	6–10	2
	11–15	3
	16–20	4
	>20	5

For immunohistochemistry, sections were processed similarly, followed by antigen retrieval and blocking of nonspecific binding. Sections were incubated overnight at 4°C with anti-α7nAChR primary antibody (1:100), followed by incubation with horseradish peroxidase-conjugated secondary antibody and DAB visualization. Images were acquired under a microscope, and semi-quantitative analysis was performed based on relative staining intensity, with the uninfected group normalized to 1.

Immunofluorescence: Frozen uterine sections, 6μM in thickness, were harvested and subjected to 10 min fixation in chilled acetone. Following washing with PBST, the sections were blocked for one hour at room temperature using 5% BSA. The sections were then probed with primary antibodies HMGB1 (1:100; Novus Biologicals) and HABP2 (1:50; Abcam) with overnight incubation at 4°C. After three rinses in PBST, immunoreactivity was visualized by incubating the sections with a secondary antibody (1:1000; Abcam). This procedure was conducted strictly in the dark to prevent photobleaching of the fluorophore. Fluorescence intensity was quantified by capturing images using a Carl Zeiss LSM 800 confocal microscope under a 100× objective lens. For quantification, one field of view was randomly chosen from the three captured fields per sample.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 8. Data were analyzed by one-way ANOVA followed by the appropriate multiple comparisons test. Dunnett's test was used for comparisons versus the control group, whereas Tukey's test was used for pairwise comparisons among multiple groups. Data are presented as the mean ± SD, and $P<0.05$ was considered statistically significant.

RESULTS

Effect of *E. coli* on expression of α7nAChR in bovine endometrial tissues: To identify the peak inflammatory response, a time-course analysis was performed. TNF-α expression in bovine endometrial tissues reached its highest level at 9 h after *E. coli* infection ($P<0.001$) (Fig. 1A). Based on this result, α7nAChR expression was further examined at 9 h post-infection. Immunohistochemical staining showed that α7nAChR immunoreactivity was reduced in the *E. coli*-infected group compared with the uninfected group, as indicated by weaker brown-yellow positive staining (Fig. 1B-C). This finding was consistent with the Western blot result, which showed that α7nAChR protein expression was significantly downregulated after *E. coli* infection (Fig. 1D). In addition, the optimal concentrations of MLA (an α7nAChR antagonist) and PNU-282987 (an α7nAChR agonist) were determined for subsequent experiments. MLA at 10μM ($P<0.001$) and PNU-282987 at 1μM ($P<0.001$) showed the most pronounced effects and were therefore selected for subsequent pretreatment experiments (Fig. 1E-F).

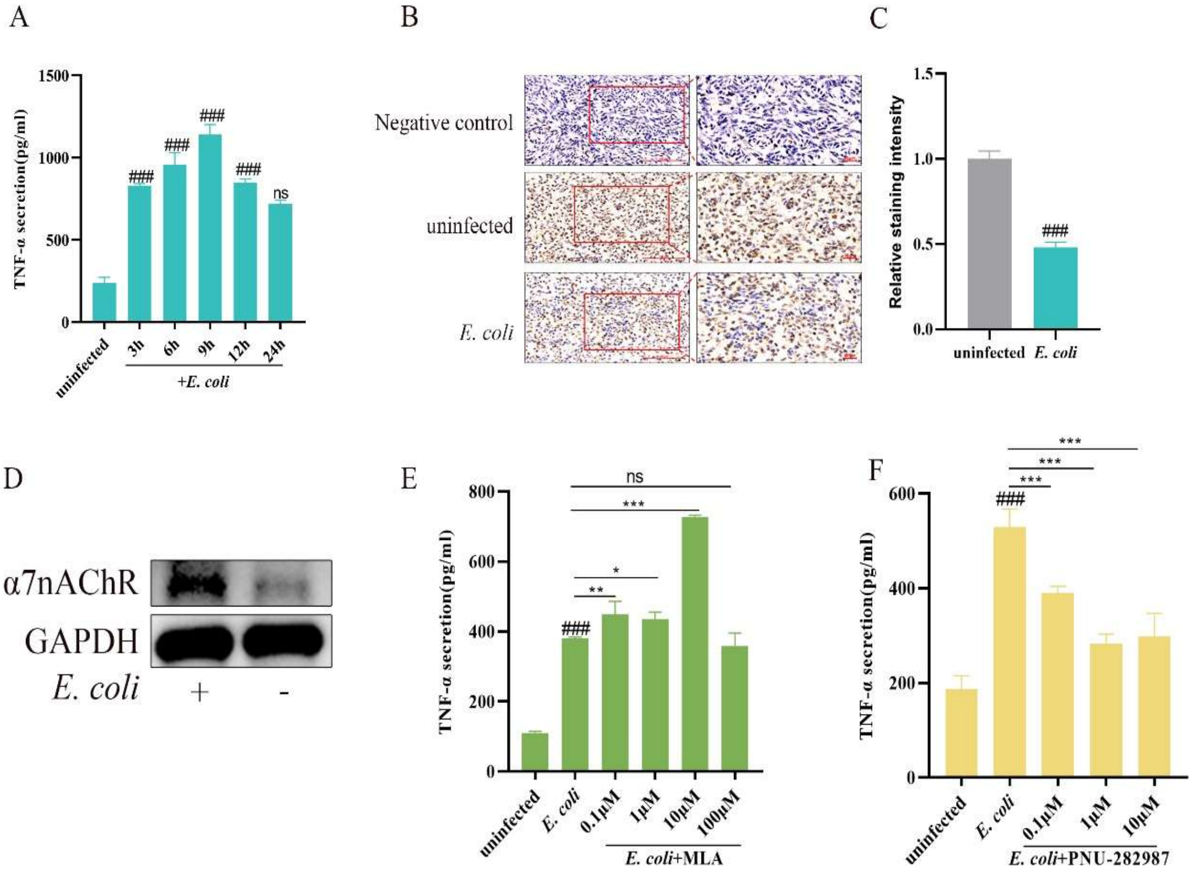

Fig. 1: (A) Secretion levels of TNF-α in bovine endometrial tissues at 3 h, 6 h, 9 h, 12 h, and 24 h after treatment with *E. coli* were measured by ELISA. (B) Representative images of PBS negative control staining and α7nAChR immunohistochemical staining in the uninfected and *E. coli*-infected groups. Brown-yellow staining indicates positive expression of α7nAChR. The right panels show enlarged views of the boxed areas. Scale bars = 100μm (left) and 20μm (right). (C) Semi-quantitative analysis of α7nAChR-positive staining. The staining intensity was normalized to the uninfected group, which was set to 1. (D) α7nAChR protein expression in bovine endometrial tissues with or without *E. coli* treatment was detected by Western blot. (E-F) TNF-α secretion in bovine endometrial tissues pretreated with MLA or PNU-282987 (0.1, 1, 10, and 100 μM) during *E. coli* infection was measured by ELISA. Data are presented as mean ± SD from three independent experiments. Panel A was analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Panel C was analyzed by an unpaired two-tailed t-test. Panels E and F were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ versus the uninfected group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ indicate statistically significant differences between the indicated groups.

Effect of α7nAChR on bovine endometrial tissue tolerance during *E. coli* infection: Histopathological changes were evaluated by H&E staining 9 h after *E. coli* infection (Fig. 2A&B). The uterine tissue of bovines treated with MLA or PNU-282987 alone exhibited normal histological morphology comparable to the untreated control group, with intact endometrial architecture and well-organized epithelial and glandular structures. In contrast, *E. coli* infection caused glandular disruption and mild epithelial swelling. Treatment with PNU-282987 markedly improved tissue tolerance to infection, as evidenced by alleviated epithelial shedding and reduced glandular destruction. Conversely, MLA treatment significantly reduced bovine endometrial tissue tolerance to *E. coli* infection, characterized by pronounced epithelial swelling, cytoplasmic pallor, and large vacuolated (balloon-like) regions within the glands.

Effect of α7nAChR on Bovine Endometrial Tissue Injury During *E. coli* Infection: Immunofluorescence analysis of bovine endometrial tissue injury was conducted 9 h after infection with *E. coli*. The results showed no significant difference in HMGB1 and HABP2 expression between the MLA or PNU-282987 treatment groups and the uninfected control group (Fig. 3A-B). However, the expression levels of HMGB1 and HABP2 were found to be substantially upregulated in the *E. coli*-infected bovine endometrial tissue, diverging significantly from the baseline values observed in the control group (P<0.001, Fig. 3C). Notably, treatment with PNU-282987 reversed the *E. coli*-induced upregulation of HMGB1 and HABP2 (P<0.001, Fig. 3D), whereas MLA treatment further enhanced their expression.

Regulation of inflammatory mediator expression in bovine endometrial tissues by α7nAChR during *E. coli* infection: To assess the local inflammatory response, the expression levels of uterine inflammatory mediator proteins and their corresponding mRNA were measured in cultured bovine endometrial tissues using ELISA and quantitative real-time PCR (qRT-PCR), respectively, at 9 h post-*E. coli* challenge. Inoculation with *E. coli* profoundly upregulated the uterine expression of IL-1β, IL-6, and TNF-α at expression levels, reinforcing the severity of the localized infection. (Fig. 4 A-F). However, PNU-282987 reduced the expression of these inflammatory cytokines in cultured bovine endometrial tissue, while MLA treatment significantly increased their expression (P<0.001).

α7nAChR modulates signaling pathway activation in bovine endometrial tissue during *E. coli* infection: To identify the regulatory nodes governing the inflammatory response, we assessed the phosphorylation of key proteins in the JAK2/STAT3, MAPK, and NF-κB pathways by Western blot after *E. coli* challenge. *E. coli* challenge significantly increased the phosphorylation of key signaling mediators, including JAK2, STAT3, ERK, p38, and the p65 subunit of NF-κB. Activation of α7nAChR by PNU-282987 markedly suppressed *E. coli*-induced phosphorylation of ERK, p38, and p65, as well as the ratios of p-ERK/ERK, p-p38/p38, and p-p65/p65 (Fig. 5B, E–G), but enhanced the phosphorylation of JAK2 and STAT3 and increased the p-JAK2/JAK2 and p-STAT3/STAT3 ratios (Fig. 5A, C–D). In contrast, MLA intervention amplified the *E. coli*-induced phosphorylation of ERK, p38, and p65, while paradoxically diminishing the activation of JAK2 and STAT3 under the same conditions.

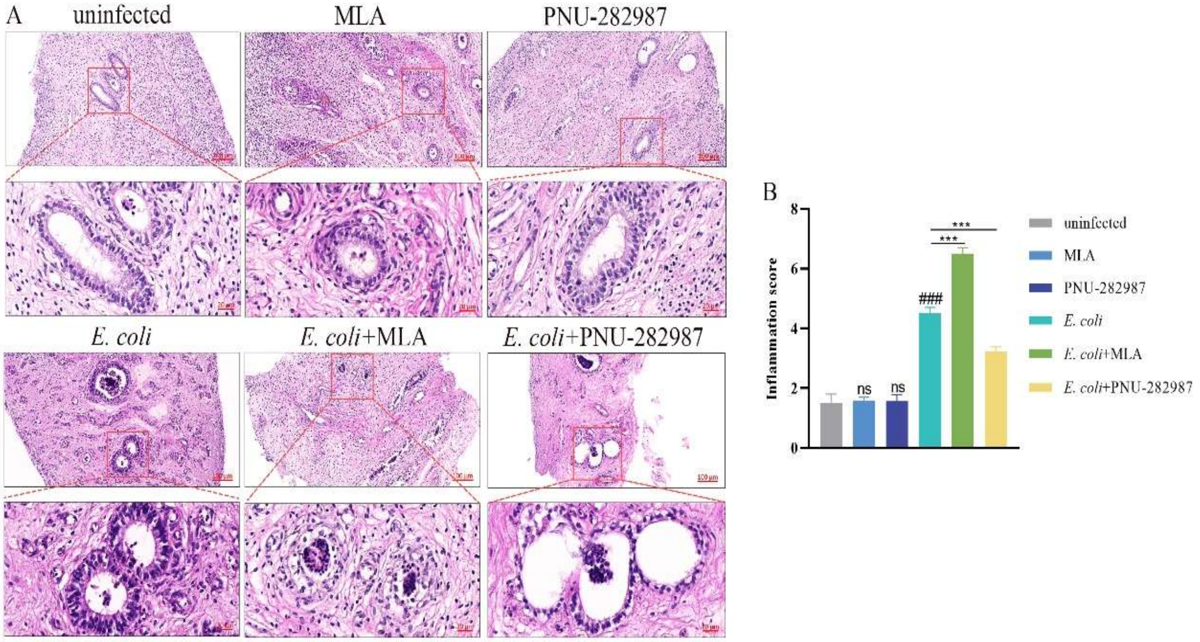

Fig. 2: (A) The effect of α7nAChR on the uterus tissue pathologic changes during *E. coli*-induced endometritis. Endometrial tissues were pretreated with MLA (10μM) or (1μM) PNU-282987 for 15 min, followed by infection with *E. coli* at an MOI of 5:1 for 9 h. Tissue sections were prepared from paraffin-embedded samples, stained with hematoxylin and eosin (H&E), and observed under a light microscope to assess histopathological changes. (B) Inflammation score of the uterus tissue. The histopathologic scoring was a cumulative score of hyperemia and PMN infiltration. Data are presented as mean ± SD from three independent experiments. Panel B was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}$P<0.05, $^{\#\#}$P<0.01, and $^{\#\#\#}$P<0.001 versus the uninfected group; *P<0.05, **P<0.01, and ***P<0.001 indicate statistically significant differences between the indicated groups.

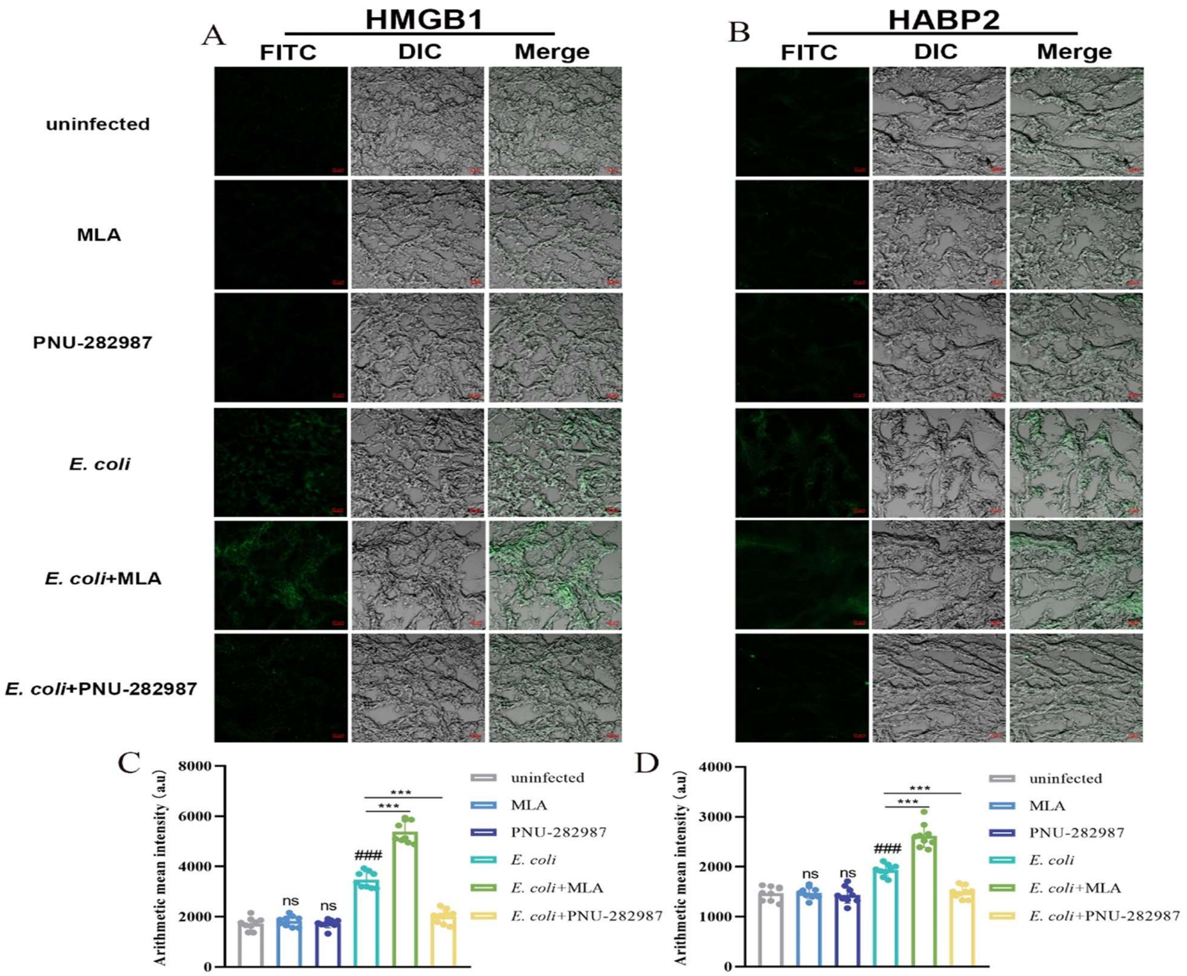

Fig. 3: Bovine endometrial tissues were pretreated with MLA (10µM) or PNU-282987 (1µM) for 15min, followed by infection with *E. coli* at an MOI of 5:1 for 9h. The expression levels of HMGB1 and HABP2 proteins (green) in the endometrium were observed by fluorescence microscopy at 9h post-infection (scale bar = 20µm). The arithmetic mean fluorescence intensity of expression (a.u., arbitrary units) was analyzed using Zen software (Carl Zeiss AG). Data are presented as mean±SD from three independent experiments. Panels C and D were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}$P<0.05, $^{\#\#}$P<0.01, and $^{\#\#\#}$P<0.001 versus the uninfected group; *P<0.05, **P<0.01, and ***P<0.001 indicate statistically significant differences between the indicated groups.

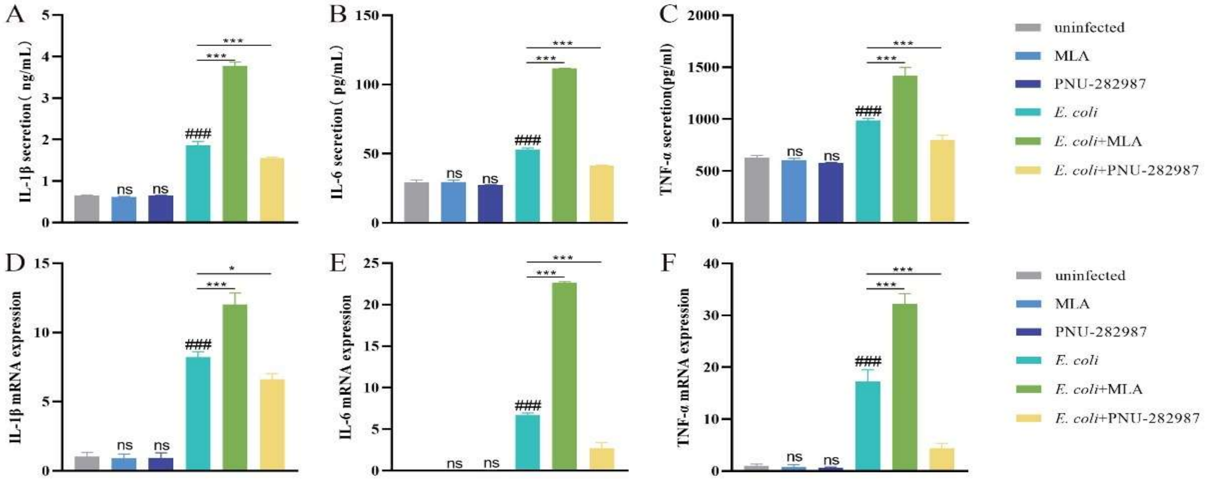

Fig. 4: Bovine endometrial tissues were pretreated with MLA (10µM) or PNU-282987 (1µM) for 15min, followed by infection with *E. coli* at an MOI of 5:1. After 9h of infection, the secretion levels of IL-1β, IL-6, and TNF-α in the tissue culture supernatants were measured by ELISA (A–C). Total RNA was extracted from the tissues, and the mRNA expression levels of IL-1β, IL-6, and TNF-α were determined by qRT-PCR (D–F). Data are presented as mean±SD from three independent experiments. Panels A–F were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}$P<0.05, $^{\#\#}$P<0.01, and $^{\#\#\#}$P<0.001 versus the uninfected group; *P<0.05, **P<0.01, and ***P<0.001 indicate statistically significant differences between the indicated groups.

Effect of *E. coli* infection on the expression of α7nAChR in bovine neutrophils: TNF-α expression in bovine neutrophils was quantified at 3, 6, 9, 12, and 24h post-infection with *E. coli* to determine the peak of the inflammatory response. The results demonstrated that TNF-α expression was highest at 12h post-infection (P<0.001, Fig. 6A). Western blotting showed α7nAChR protein expression was significantly reduced in neutrophils 12 h after *E. coli* infection versus uninfected controls (Fig. 6B). MLA and PNU-282987 each effectively modulated *E. coli*-induced neutrophil inflammatory responses at 1µM, with no cytotoxicity detected (P<0.001; Fig. 6C–F).

α7nAChR regulates inflammatory mediators in neutrophils infected with *E. coli*: To assess the inflammatory profile of neutrophils, we quantified the transcriptional and translational levels of key mediators using qRT-PCR and ELISA, respectively, at 12h post-infection with *E. coli*. There were no significant changes in IL-1β, IL-6, or TNF-α in the MLA and PNU-282987 groups. The *E. coli* challenge induced a robust inflammatory response, which was evident from the concurrent surge in both the transcriptional and translational abundance of these cytokines (P<0.001, Fig. 7A-F). PNU-282987 treatment significantly suppressed IL-1β, IL-6, and TNF-α expression in neutrophils infected with *E. coli*, whereas MLA antagonized this inhibitory effect (P<0.001, Fig. 7A-F).

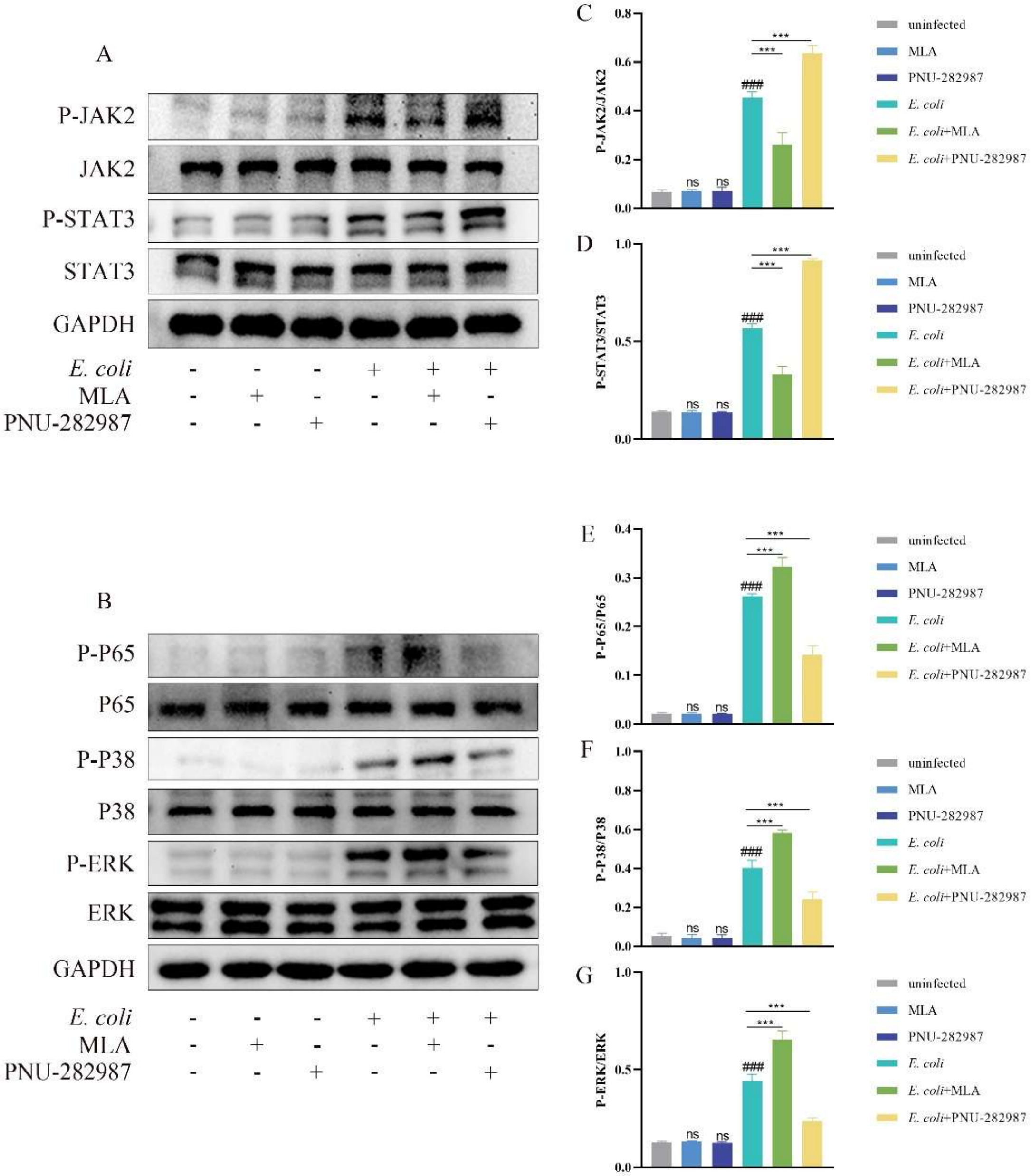

Fig. 5: Bovine endometrial tissues were pretreated with MLA (10µM) or PNU-282987 (1µM) for 15min, followed by infection with *E. coli* at an MOI of 5:1 for 9h. Phosphorylation of JAK2 and STAT3 (A) and MAPK/NF-κB pathway-related proteins (B) was analyzed by Western blot, with GAPDH as the loading control. Quantification of p-JAK2/JAK2 (C), p-STAT3/STAT3 (D), p-p65/p65 (E), p-p38/p38 (F), and p-ERK/ERK (G) is shown. Data are presented as mean ± SD from three independent experiments. Panels C–G were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}$P<0.05, $^{\#\#}$P<0.01, and $^{\#\#\#}$P<0.001 versus the uninfected group; *P<0.05, **P<0.01, and *P<0.001 indicate statistically significant differences between the indicated groups.

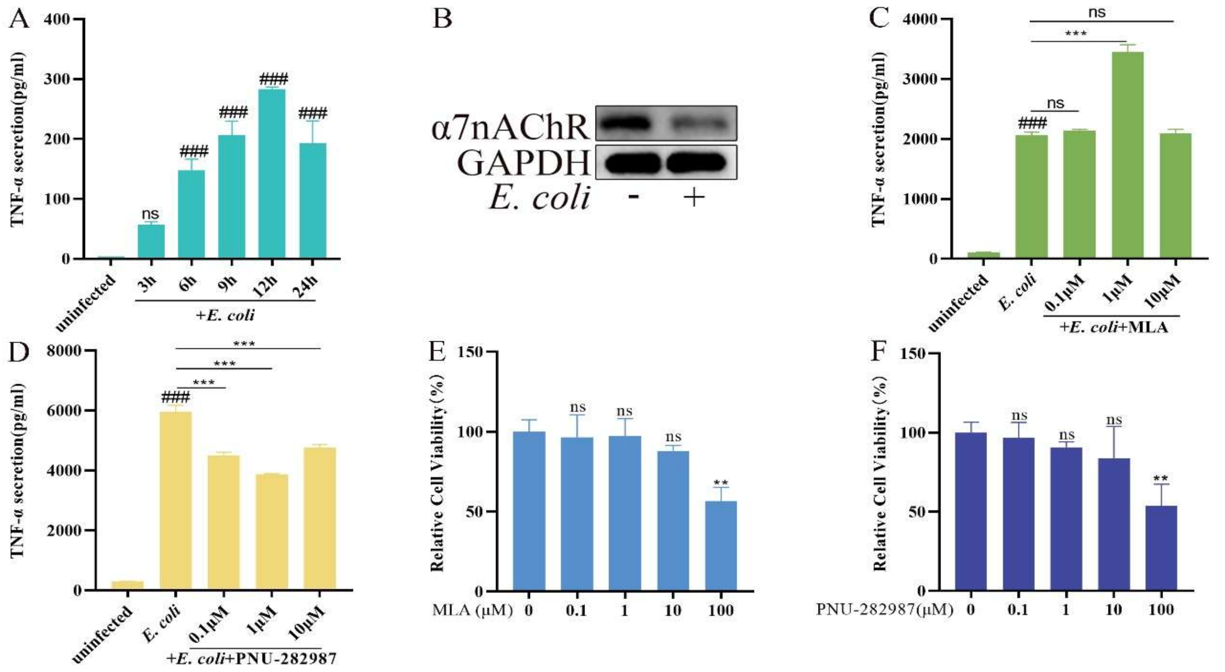

Fig. 6: (A) Secretion of TNF-α from bovine neutrophils treated with *E. coli* for 3 h, 6 h, 9 h, 12h, and 24h was measured by ELISA. (B) Expression of α7nAChR in neutrophils treated with or without *E. coli* was detected by Western blot. (C–D). TNF-α secretion was determined by ELISA in neutrophils pretreated with MLA or PNU-282987 at concentrations of 0.1, 1, 10, and 100μM during *E. coli* infection. (E–F) The cytotoxic effects of different concentrations of MLA and PNU-282987 on neutrophils after 12 h of treatment were assessed using the CCK-8 assay. Data are presented as mean ± SD from three independent experiments. Panels A, E, and F were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test, whereas panels C and D were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ versus the uninfected group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ indicate statistically significant differences between the indicated groups.

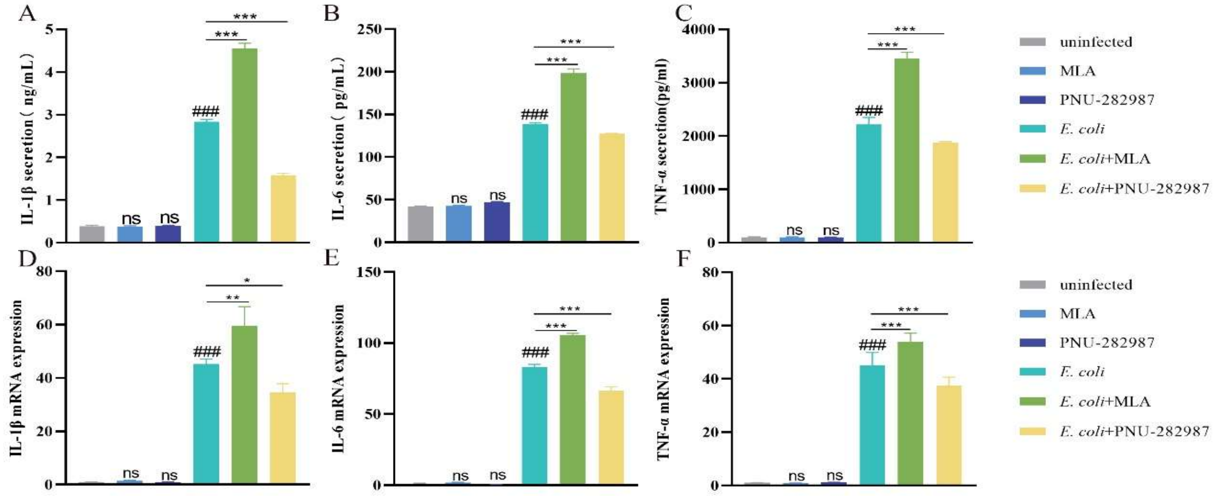

Fig. 7: Bovine neutrophils were pretreated with MLA (1μM) or PNU-282987 (1μM) for 15min, followed by infection with *E. coli* at an MOI of 5:1. After 12h of infection, the secretion levels of IL-1β, IL-6, and TNF-α in the cell culture supernatants were measured by ELISA (A–C). Total RNA was extracted from neutrophils, and the mRNA expression levels of IL-1β, IL-6, and TNF-α were determined by qRT-PCR (D–F). Data are presented as mean ± SD from three independent experiments. Panels A–F were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ versus the uninfected group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ indicate statistically significant differences between the indicated groups.

α7nAChR modulates signaling pathway activation in neutrophils during *E. coli* infection: To elucidate the intracellular signaling mechanisms, immunoblotting was performed to evaluate the phosphorylation status and subsequent activation of the JAK2/STAT3, MAPK, and NF-κB pathways in neutrophils under *E. coli* challenge. *E. coli* challenge notably augmented the phosphorylation status of JAK2/STAT3, MAPK (ERK and p38), and NF-κB (p65) relative to the uninfected control. Treatment with PNU-282987 significantly reduced *E. coli*-induced phosphorylation of p38, ERK, and p65, as well as the ratios of p-ERK/ERK, p-p38/p38, and p-p65/p65 (Fig. 8B, E–G), while enhancing the phosphorylation of JAK2 and STAT3 and increasing the p-JAK2/JAK2 and p-STAT3/STAT3 ratios (Fig. 8A, C–D). In contrast, MLA treatment further increased the phosphorylation of ERK, p38, p65, and reduced that of JAK2 and STAT3 in *E. coli*-infected neutrophils.

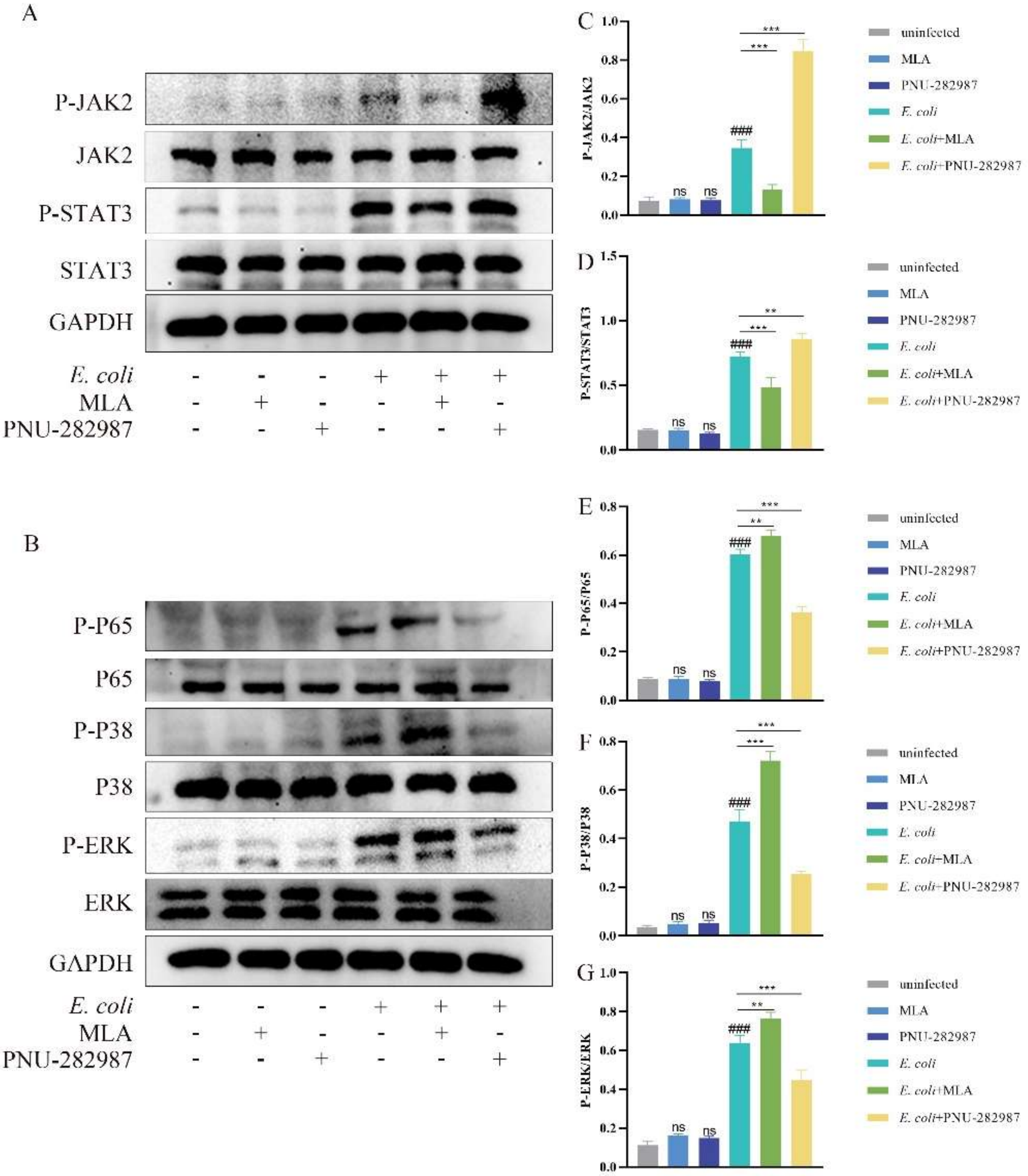

Fig. 8: Bovine neutrophils were pretreated with 1μM MLA or PNU-282987 for 15 min, followed by infection with *E. coli* at an MOI of 5:1 for 12h. Phosphorylation of JAK2 and STAT3 (A) and activation of the MAPK and NF-κB signaling pathways (B) were assessed by Western blot analysis. GAPDH was used as the loading control. Band intensities were quantified using ImageJ software. Data are presented as mean ± SD from three independent experiments. Panels C–G were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ versus the uninfected group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ indicate statistically significant differences between the indicated groups.

DISCUSSION

Postpartum endometritis is a major determinant of suboptimal reproductive performance in cattle, frequently resulting in infertility and fetal mortality. It directly diminishes the economic value of bovines and severely impacts the development of the bovine farming industry (Ma, 2021). Therefore, identifying safer and more effective prevention and treatment measures for bovine endometritis holds significant research value. Accumulating evidence identifies α7nAChR as a pivotal molecular transducer within the cholinergic anti-inflammatory pathway (CAP). Its potent immunomodulatory effects are well-documented in diverse pathophysiological contexts, such as mitigating hyperinflammation during acute lung injury and restoring mucosal homeostasis in inflammatory bowel disease (Seyedabadi *et al*., 2018; Huang *et al*., 2024). But the role of α7nAChR in bovine endometritis remains unclear.

Studies indicate increased neutrophil counts in vaginal discharges of bovines with endometritis, suggesting neutrophils are closely correlated with bovine endometritis (Shen *et al*., 2022). Furthermore, bovine endometrial tissue constitutes a critical component of the bovine reproductive system and serves as an excellent model for studying bovine endometritis. Our investigations additionally revealed α7nAChR expression in both neutrophils and bovine endometrial tissue (Fig. 1B and Fig. 6B). Therefore, this study established inflammatory response models using *E. coli*-infected neutrophils and bovine endometrial tissue to investigate the mechanistic role of α7nAChR in endometritis.

As the dominant endotoxic moiety of *E. coli*, Lipopolysaccharide (LPS) is instrumental in orchestrating the inflammatory milieu, leading to an increase in inflammatory cytokine levels (Yuan *et al*., 2019). Thus, this study employed *E. coli* infection to establish inflammatory response models in both bovine neutrophils and endometrial tissue. The α7nAChR is the primary receptor of CAP. Studies in non-bovine models have shown that α7nAChR activation exerts anti-inflammatory effects by reducing pro-inflammatory cytokine production and modulating intracellular signaling pathways. For example, in a mouse model of endotoxemia, electrical vagus nerve stimulation inhibited TNF-α synthesis in wild-type mice, whereas this inhibitory effect was abolished in α7nAChR-deficient mice (Wang *et al*., 2003). Similarly, the study discovered on necrotizing enterocolitis inflammation that administering PNU-282987 (an α7nAChR agonist) reduced IL-1β expression and mitigated intestinal damage in mice with necrotizing enterocolitis via α7nAChR (Shen, 2024). Our study demonstrated that adding the α7nAChR agonist PNU-282987 significantly reduced the secretion levels and mRNA expression of proinflammatory cytokines within both bovine neutrophils and endometrial tissue infected by *E. coli*. This effect contrasted sharply with results from adding the α7nAChR inhibitor MLA (Fig. 4 and Fig. 7). However, the effects of α7nAChR signaling may vary depending on species, tissue type, inflammatory stimulus, and experimental conditions. Therefore, our results suggest that α7nAChR may participate in the regulation of inflammatory responses in bovine endometritis. However, this interpretation should be made with caution, and the specific mechanism requires further investigation.

To further investigate the specific mechanism of α7nAChR in endometritis, we detected relevant proteins in the JAK2/STAT3, MAPK, and NF-κB signaling pathways using Western Blot assays. We found that adding the α7nAChR agonist PNU-282987 promoted phosphorylation of JAK2/STAT3 in both bovine neutrophils and endometrial tissues, and this was associated with an attenuated inflammatory response. Conversely, the addition of MLA, an α7nAChR inhibitor, produced opposite results (Fig. 5A and Fig. 8A). Similarly, the study found that electroacupuncture activated the α7nAChR-mediated JAK2/STAT3 signaling pathway in macrophages, thereby reducing inflammatory cytokine production (Yang *et al*., 2021). The study on acupuncture treatment for atrial fibrillation in rats found that acupuncture alleviates myocardial injury and decreases inflammatory factor secretion levels via the α7nAChR-JAK2/STAT3 pathway (Li *et al*., 2025). These findings align with our experimental results. In contrast to α7nAChR activation promoting JAK2/STAT3 signaling pathway activation, α7nAChR activation significantly suppressed phosphorylation levels of MAPK and NF-κB signaling pathways (Fig. 5B and Fig. 8B). α7nAChR was found to inhibit MAPK and NF-κB activation, thereby mitigating colonic tissue damage (Pu, 2022). In addition, α7nAChR could suppress the TLR4/MAPK/NF-κB pathway, further inhibiting inflammatory cytokine production and alleviating neuroinflammation in PC12 cells (Zhao *et al*., 2023). α7nAChR can also alleviate inflammation by downregulating the MAPK signaling pathway (Chang *et al*., 2018). These experimental results are consistent with our findings on MAPK and NF-κB. However, the present data demonstrate association rather than direct causality, and additional upstream or parallel signaling pathways may also contribute to these effects. Therefore, our findings suggest that α7nAChR may alleviate inflammatory responses in bovine endometritis, at least in part, through modulation of the JAK2/STAT3, MAPK, and NF-κB pathways.

Furthermore, we found that activating α7nAChR was associated with reduced expression of HMGB1 and HABP2 in bovine endometrial tissue (Fig. 3). HMGB1, as an inflammation-damage-associated protein, is widely present in the nucleus and cytoplasm of eukaryotic cells. When cells experience damage or stress, HMGB1 can be passively released extracellularly or actively secreted by immune cells. Functioning as a critical inflammatory mediator, it binds to various cell surface receptors (such as RAGE, TLR2, and TLR4). This process promotes the release of pro-inflammatory cytokines, thereby amplifying inflammatory reactions, and demonstrates an upregulation trend across multiple inflammatory diseases. HABP2, a multifunctional protein belonging to the serine protease inhibitor superfamily, regulates inflammatory cell recruitment and activation during inflammatory reactions by interacting with inflammation-related factors or cell surface receptors, thereby influencing the progression of inflammation. Its expression is closely associated with the inflammatory tissue repair process (Wang *et al*., 2019; Jiang *et al*., 2020; Ren *et al*., 2023). In addition, H&E staining suggested that α7nAChR activation may exert a protective effect against tissue injury in bovine endometrial tissue. Activation of α7nAChR alleviated *E. coli* infection-induced tissue damage (Fig. 2). Nevertheless, the precise relationship between α7nAChR signaling, HMGB1/HABP2 regulation, and tissue protection remains to be clarified. But this study has several limitations. First, it mainly relied on pharmacological activation and inhibition of α7nAChR, and additional loss-of-function approaches, such as receptor knockout, would strengthen the mechanistic interpretation. Second, although changes in the JAK2/STAT3, MAPK, and NF-κB pathways were observed, direct pathway inhibition experiments were not performed to establish causality. Third, the present work was based on in vitro neutrophil and ex vivo endometrial tissue explant models and lacked in vivo validation. Future studies with larger sample sizes, in vivo validation, and more comprehensive mechanistic approaches are needed to confirm and extend these findings.

Conclusions: Collectively, our findings suggest that α7nAChR is involved in the regulation of inflammatory responses in bovine neutrophils and endometrial tissue during *E. coli* infection. Activation of α7nAChR was associated with reduced pro-inflammatory cytokine production, attenuation of tissue injury, and modulation of the JAK2/STAT3, MAPK, and NF-kB pathways. These results support α7nAChR as a potentially relevant target in bovine endometritis, although further studies are required to clarify its precise molecular mechanisms.

Authors contribution: ZFF, RXY, WJN, LJJ, XLL, ZZH, and ZM carried out the laboratory experiments. GRF and LHD designed and supervised the experiments. ZFF wrote the original draft. EAA, RAEM, GRF, and YHD writing – review and editing.

Data Availability Statement: The data for this study are available by contacting the corresponding authors upon reasonable request.

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