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Targeting Nicotinic Acetylcholine Receptor Subunits to Modulate *Brucella*-Induced Inflammation in Murine Macrophages

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Abstract

Objective and design: To determine whether nicotinic acetylcholine receptor (nAChR) subunits modulate *Brucella*-induced macrophage inflammation, we performed an in vitro pharmacological study of infected murine macrophages.

Material or subjects: RAW264.7 murine macrophages infected with *Brucella melitensis* M5 were examined.

Treatment: After infection, cells were treated with the $\alpha 7$ nAChR agonists PNU-282987 (50 μ M) or GTS-21 (50 μ M), or the antagonist methyllycaconitine (5 nM).

Methods: nAChR subunits, NLRP3 inflammasome-associated proteins, cytokines, and signaling proteins were

assessed by RT-qPCR, Western blotting, ELISA, and immunofluorescence; multiple-group comparisons used one-way ANOVA with Dunnett's correction.

Results: *Brucella* infection increased NLRP3, caspase-1, IL-1 β , and IL-18 responses, decreased α 7 nAChR, and increased α 9 nAChR. PNU-282987 and GTS-21 attenuated inflammasome-associated inflammatory responses. PNU-282987 increased JAK2/STAT3 and ERK1/2 phosphorylation and reduced NF- κ B p65 activation.

Conclusions: α 7 nAChR activation limits *Brucella*-induced inflammatory signaling in RAW264.7 macrophages and represents a potential immunomodulatory target that warrants further mechanistic and in vivo validation.

Key words: *Brucella*; nicotinic acetylcholine receptor; α 7 nAChR; NLRP3 inflammasome; macrophage; inflammation

Introduction

Brucellosis is a globally important zoonotic disease caused by *Brucella* species and imposes substantial burdens on public health and livestock production. Infection can induce marked inflammatory responses and can cause major clinical and reproductive manifestations, including abortion in livestock and orchitis in males [1]. Both innate and adaptive immune mechanisms contribute to host defense, with macrophages functioning as key innate immune effector cells through phagocytosis, intracellular pathogen restriction, and inflammatory signaling [2]. The NLRP3 inflammasome is a key intracellular innate immune signaling complex that contributes to host responses during *Brucella* infection [3,4].

Macrophages possess an intrinsic cholinergic system that includes choline acetyltransferase (ChAT), acetylcholinesterase (AChE), and nicotinic acetylcholine receptors (nAChRs) [5]. Previous studies have shown that *Brucella* infection can disrupt cholinergic homeostasis and exacerbate inflammatory and oxidative responses in host cells [6]. These observations implicate neuroimmune signaling in the regulation of *Brucella*-induced inflammation.

Among nAChR subunits, $\alpha 7$ nAChR is a well-established mediator of the cholinergic anti-inflammatory pathway. Activation of $\alpha 7$ nAChR can suppress NLRP3 inflammasome activation and limit mitochondrial DNA release in macrophages [7,8]. $\alpha 7$ nAChR signaling has also been associated with Toll-like receptor 4 (TLR4)/MyD88/NF- κ B signaling [9,10] and to β -arrestin-1-dependent regulation of inflammasome activity in monocytes, macrophages, dendritic cells, and microglia [11,12]. However, the relationship between NLRP3 inflammasome-associated inflammation and the expression dynamics of individual nAChR subunits during *Brucella* infection remains incompletely defined.

Pharmacological evidence further supports the potential of $\alpha 7$ nAChR as an anti-inflammatory target. The selective $\alpha 7$ nAChR agonist PNU-282987 has been reported to attenuate oxidative stress and neuroinflammation through $\alpha 7$ nAChR/Nrf2/HO-1 signaling [13]. Similarly, GTS-21 can improve macrophage function, enhance bacterial clearance, and reduce the production of pro-inflammatory cytokines such as TNF- α and IL-1 β in experimental models [14,15]. Nevertheless, the effects of $\alpha 7$ nAChR agonism on *Brucella*-induced macrophage inflammation require further investigation.

The anti-inflammatory effects of $\alpha 7$ nAChR involve several intracellular signaling mechanisms. These mechanisms include inhibition of NF- κ B activation and nuclear translocation, JAK2-dependent STAT3 phosphorylation, and induction of anti-inflammatory transcriptional programs [16,17]. MAPK signaling, including ERK1/2, and other cholinergic second-messenger pathways have also been implicated [18,19]. Defining these mechanisms in *Brucella*-infected macrophages may help identify pharmacologically tractable targets for limiting excessive inflammatory signaling.

Accordingly, this study aimed to characterize the temporal expression profiles of nAChR subunits during *Brucella*-induced inflammation in murine macrophages, identify receptor subunits associated with the infection-induced inflammatory phenotype, and evaluate the effects of $\alpha 7$ nAChR-targeting ligands. We further examined

how $\alpha 7$ nAChR activation relates to NLRP3 inflammasome-associated mediators and the JAK2/STAT3, NF- κ B, and ERK1/2 signaling pathways.

Materials and Methods

Materials

PNU-282987, GTS-21, and MLA were purchased from MCE Bioscience (China). Dulbecco's Modified Eagle Medium (DMEM), penicillin–streptomycin, phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were obtained from Gibco Biotech (Shanghai, China). RNA extraction, reverse-transcription, and PCR reagents, including the FastPure Cell/Tissue Total RNA Isolation Kit, HiScript II RT SuperMix, 2 $\times$ Taq Plus Master Mix II, and ChamQ Universal SYBR qPCR Master Mix, were obtained from Colin Biotech (Jiangsu, China). Commercial IL-18 and IL-1 β ELISA kits were purchased from JiangLai Scientific (Sichuan, China), and the CCK-8 kit was obtained from Solarbio (Beijing, China). Antibodies against JAK2, p-JAK2, STAT3, p-STAT3, ERK1/2, p-ERK1/2, NF- κ B p65, p-NF- κ B p65, β -actin, NLRP3, caspase-1, and $\alpha 7$ nAChR were obtained from Beyotime Biotechnology (Jiangsu, China), Proteintech (Wuhan, China), and Abcam (USA).

Bacterial strain and culture conditions

Brucella melitensis strain M5 (*Brucella* M5), maintained in our laboratory at the College of Animal Science and Technology, Shihezi University (Shihezi, China), was cultured in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco, MI, USA) at 37 °C in a 5% CO₂ atmosphere. All experimental procedures involving *Brucella* were performed in a biosafety level 3 (BSL-3) laboratory.

Cell culture, infection, and pharmacological treatment

RAW264.7 murine macrophages (BNCC354753) were obtained from BeNa Culture Collection (BNCC, Henan Province, China), a commercial cell repository. The present study used only this established murine macrophage cell line; no live animals, primary animal tissues, human participants, or human materials were used. Accordingly,

animal- or human-research ethics approval was not required for these cell-line experiments. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a 5% CO₂ atmosphere. *Brucella melitensis* M5 during exponential growth was prepared in TSB and diluted in DMEM containing 1% FBS. Cells were infected at a multiplicity of infection (MOI) of 100:1 [27]. After 1 h of infection, gentamicin (50 µg/mL) was added for 1 h to eliminate extracellular bacteria (gentamicin-protection step). Cells were then washed three times with PBS and cultured in fresh DMEM containing 10% FBS, with or without PNU-282987 (50 µM), GTS-21 (50 µM), or MLA (5 nM), according to the experimental design.

CCK-8 cell viability assay

RAW264.7 cells were seeded in 96-well plates at 1,000 cells/well. After 24 h of culture, cells were treated with the indicated concentrations of PNU-282987, GTS-21, or MLA for an additional 24 h. The medium was then replaced with 100 µL of culture medium containing 1% CCK-8 reagent, and cells were incubated at 37 °C for 1–2 h. Absorbance at 450 nm was measured using a SpectraMax M2 microplate reader. Cell viability was expressed relative to the corresponding untreated control on the basis of absorbance values.

$$\text{Relative cell viability (\%)} = \frac{\text{OD of treatment group} - \text{OD of control group}}{\text{OD of blank group} - \text{OD of control group}}$$

RT-qPCR and ELISA

Total RNA was extracted from treated RAW264.7 cells using the FastPure Cell/Tissue Total RNA Isolation Kit, and cDNA was synthesized using HiScript II RT SuperMix. RT-qPCR was performed using ChamQ Universal SYBR qPCR Master Mix, with GAPDH used as the endogenous reference gene. Primer sequences are listed in Table 1. The amplification conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 57 °C for 20 s, and 72 °C for 30 s, with a final extension at 65 °C for 10 min. Relative transcript abundance was calculated using the 2^{−ΔΔCt} method.

For cytokine quantification, cell-culture supernatants were collected and centrifuged at 12,000 rpm for 10 min

at 4 °C. IL-18 and IL-1 β concentrations were quantified using the corresponding commercial ELISA kits according to the manufacturer's instructions, and absorbance was measured at 450 nm.

Western blot analysis

Cells were lysed in RIPA buffer containing phosphatase inhibitors, and total protein concentrations were determined using a BCA assay. Equal amounts of protein (30 μ g) were separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated overnight at 4 °C with the indicated primary antibodies, washed, and then incubated with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using the West-Pico ECL detection system. Western-blot panels in the main figures were cropped for clarity of presentation, and individual target blots are shown as separate strips with clear spacing. Original source blot images corresponding to Figs. 1E, 2D, 4E, and 5A are presented in Supplementary Figs. S3-S6, respectively, with membrane edges and molecular-weight references visible where available.

Immunofluorescence staining

RAW264.7 cells were fixed with 4% paraformaldehyde and blocked with 5% bovine serum albumin (BSA). Cells were incubated overnight at 4 °C with an anti- α 7 nAChR primary antibody, followed by incubation with the appropriate Alexa Fluor-conjugated secondary antibody. Nuclei were counterstained with DAPI, and fluorescence images were acquired using a Leica fluorescence microscope. α 7 nAChR immunoreactivity was quantified as the fluorescence-positive area relative to the total field of view.

Statistical analysis

All experiments were performed independently at least three times. Data are expressed as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Multiple-group comparisons were performed using one-way analysis of variance (ANOVA) followed, where appropriate, by Dunnett's multiple-comparisons test. A two-sided P value < 0.05 was considered statistically

133 significant. Significance is indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.

134 **Table 1** Primer sequences used for RT-qPCR

Gene	Forward/reverse primer (5'→3')	Annealing temperature (°C)
GAPDH	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA	57
Caspase-1	F: ACAAGGCACGGGACCTATG R: TCCCAGTCAGTCCTGGAAATG	57
NLRP3	F: ATTACCCGCCCCGAGAAAGG R: TCGCAGCAAAGATCCACACAG	57
IL-18	F: GACTCTTGCGTCAACTTCAAGG R: CAGGCTGTCTTTGTCAACGA	57
IL-1β	F: GCAACTGTTCTGAACTCAACT R: ATCTTTTGGGGTCCGTCAACT	57
α4 nAChR	F: CATCTCGGTGCTGCTTTCTC R: GGTGGTGTACATTGAGCACG	55
α5 nAChR	F: ATCCTCTGCTGCAAAACATGA R: TCCACGTCCACTAACTGAGAT	55
α6 nAChR	F: TAAAGGCAGTACAGGCTGTGA R: AAAATGCACCGTGACGGGAT	55
α7 nAChR	F: ATGCCACATTCCACACCAACG R: AGGACCAGGACCCAAACTTCAG	55
α9 nAChR	F: GCCGTGAAGAACGTCATCTC	55

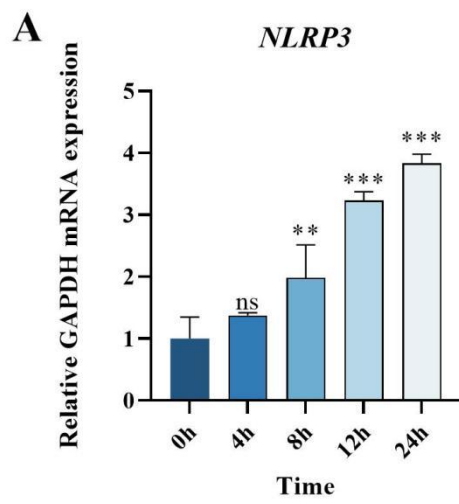
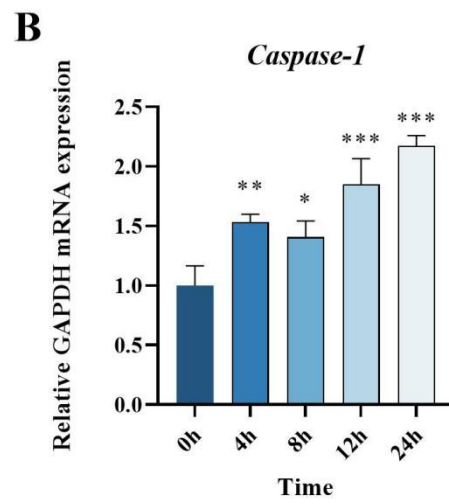
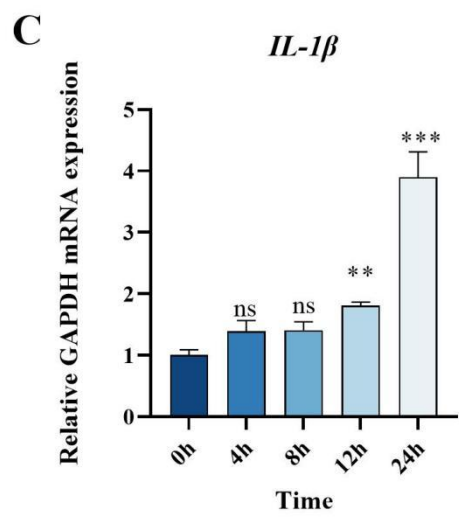
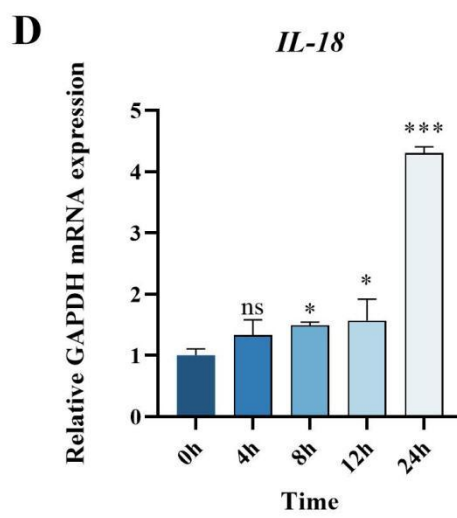
	R: GCGGAGCGAGGAACGATATG	
	F: GGTGACTGTACAGCTCATGG	
β2 nAChR		55
	R: GCTTAGAAGGGAGTCGGACT	
	F: CAGTGCCACTCTCTCAGGTTC	
β3 nAChR		55
	R: GGGCGGACACATTTCTGATAAC	
	F: CTCCTCCCAGCTCATCTCCATC	
β4 nAChR		55
	R: AGGCGGTAGTCAGTCCATTCC	

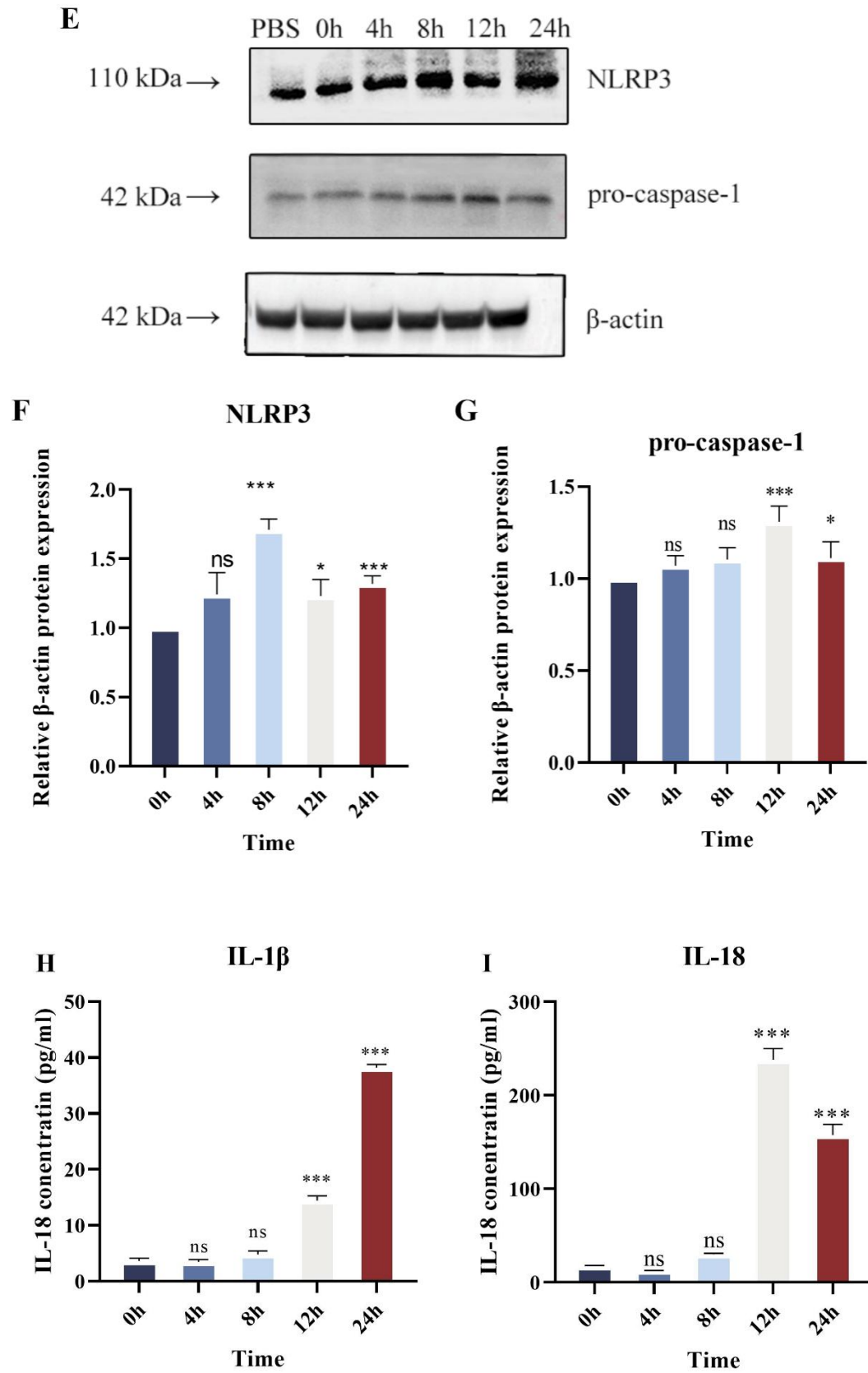
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136 Results

137 *Brucella* infection induces an inflammatory response in murine macrophages

138 To characterize the inflammatory response to *Brucella* infection, we measured NLRP3 inflammasome-
139 associated mediators in RAW264.7 cells at 0, 4, 8, 12, and 24 h post-infection. RT-qPCR showed that NLRP3,
140 caspase-1, IL-1β, and IL-18 transcript levels generally increased over time, with the most pronounced changes at
141 12 and 24 h (Fig. 1A–D). NLRP3 and IL-18 transcript abundance began to increase at 8 h and were significantly
142 elevated at 12 and 24 h (Fig. 1A,D). IL-1β transcript levels remained relatively unchanged at 4 and 8 h but
143 increased significantly at 12 and 24 h (Fig. 1C). Western blotting further demonstrated time-dependent changes in
144 NLRP3 and pro-caspase-1 protein abundance (Fig. 1E–G), while ELISA demonstrated increased concentrations
145 of secreted IL-1β and IL-18 in culture supernatants (Fig. 1H,I). Collectively, these findings indicate that *Brucella*
146 infection induces a time-dependent inflammatory response associated with NLRP3 inflammasome signaling in
147 RAW264.7 macrophages in vitro.





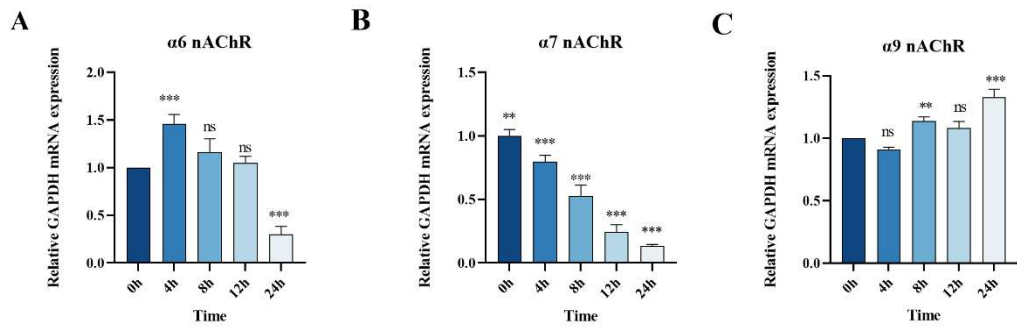
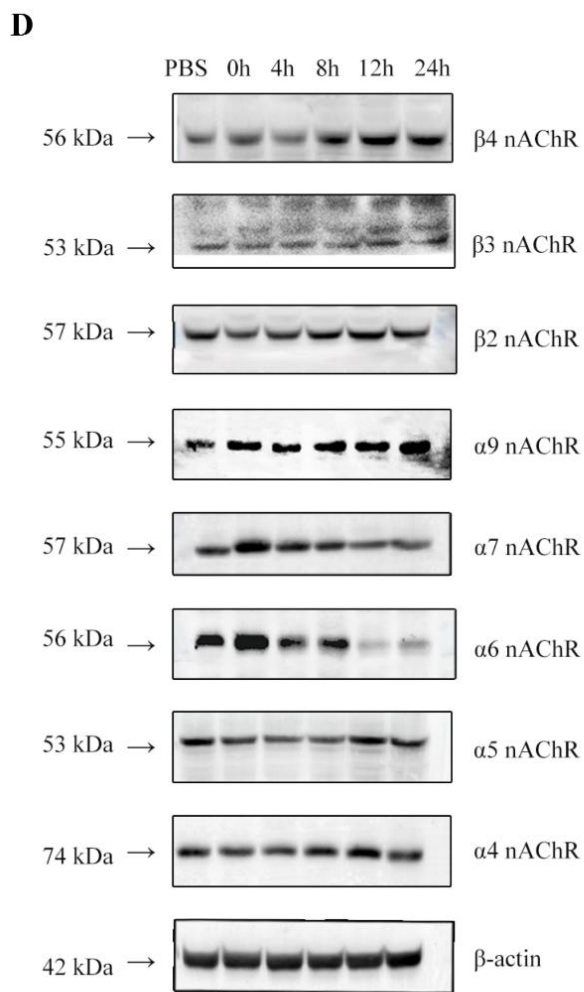
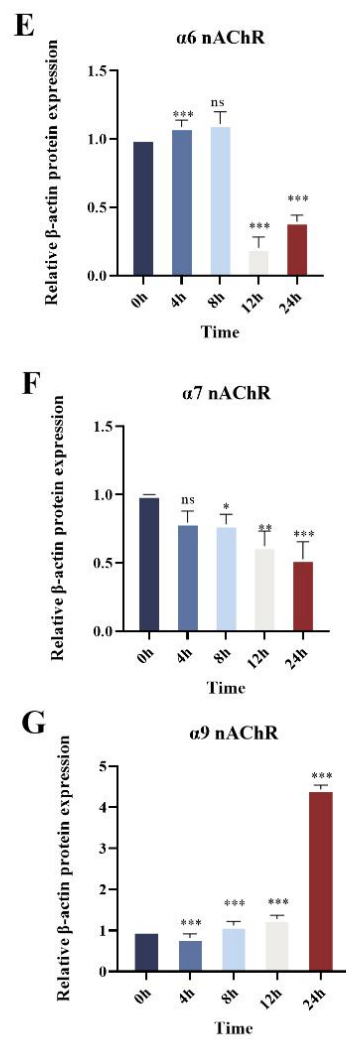
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Fig. 1 Time-dependent inflammatory responses in *Brucella*-infected RAW264.7 macrophages. Cells were analyzed at 0, 4, 8, 12, and 24 h post-infection. The transcript levels of NLRP3, caspase-1, IL-1 β , and IL-18 were quantified by RT-qPCR (A–D). NLRP3 and pro-caspase-1 protein abundance were assessed by Western blotting and densitometric analysis (E–G). IL-1 β and IL-18 concentrations in culture supernatants were measured by ELISA (H,I). Statistical comparisons were made between each infection time point and the 0 h control group. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The Western-blot images in panel E were cropped for presentation. Original blots are presented in Supplementary Fig. S3.

Differential expression of nicotinic acetylcholine receptor subunits in *Brucella*-infected murine macrophages

Multiple nAChR subunits are expressed in immune cells, and $\alpha 7$ nAChR is a key mediator of cholinergic anti-inflammatory signaling in macrophages [20]. To determine whether *Brucella* infection alters the nAChR subunit expression profile, we examined eight nAChR subunits ($\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 9$, $\beta 2$, $\beta 3$, and $\beta 4$) by RT-qPCR and Western blotting. The mRNA levels of $\alpha 4$, $\alpha 5$, $\beta 2$, $\beta 3$, and $\beta 4$ nAChR subunits showed a broadly similar pattern, with an initial increase followed by a decline as infection progressed (Fig. S1). In contrast, $\alpha 9$ nAChR transcript abundance increased progressively, whereas $\alpha 6$ and $\alpha 7$ nAChR transcript abundance decreased. $\alpha 7$ nAChR transcript abundance was significantly lower at 12 and 24 h than at 0 h (Fig. 2A–C). At the protein level, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, and $\beta 4$ nAChR subunits also showed time-dependent changes (Fig. S2). $\alpha 7$ nAChR protein abundance decreased significantly at 12 and 24 h, whereas $\alpha 9$ nAChR protein abundance increased with infection duration (Fig. 2D–G). Immunofluorescence analysis further confirmed a progressive reduction in $\alpha 7$ nAChR staining during *Brucella* infection (Fig. 2H, I). On the basis of these reciprocal expression changes, $\alpha 7$ nAChR was selected for subsequent functional investigation.



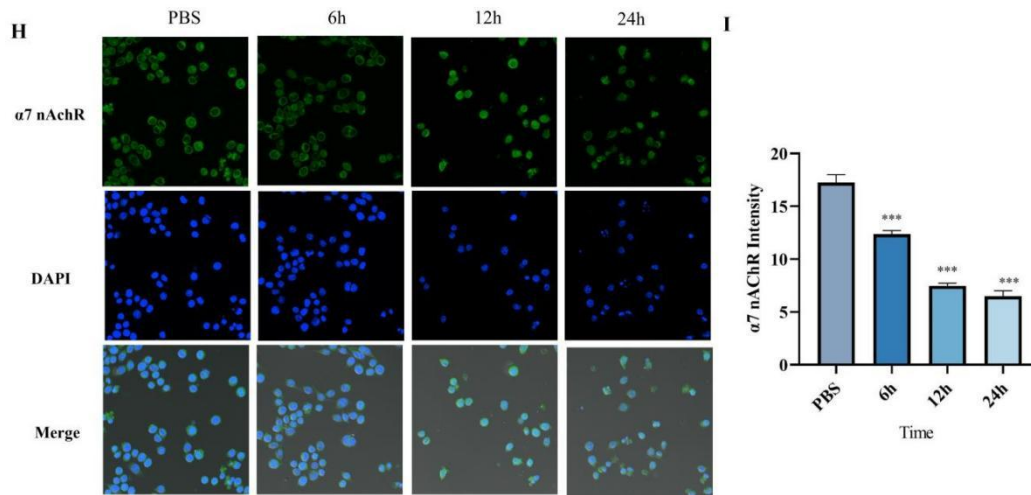
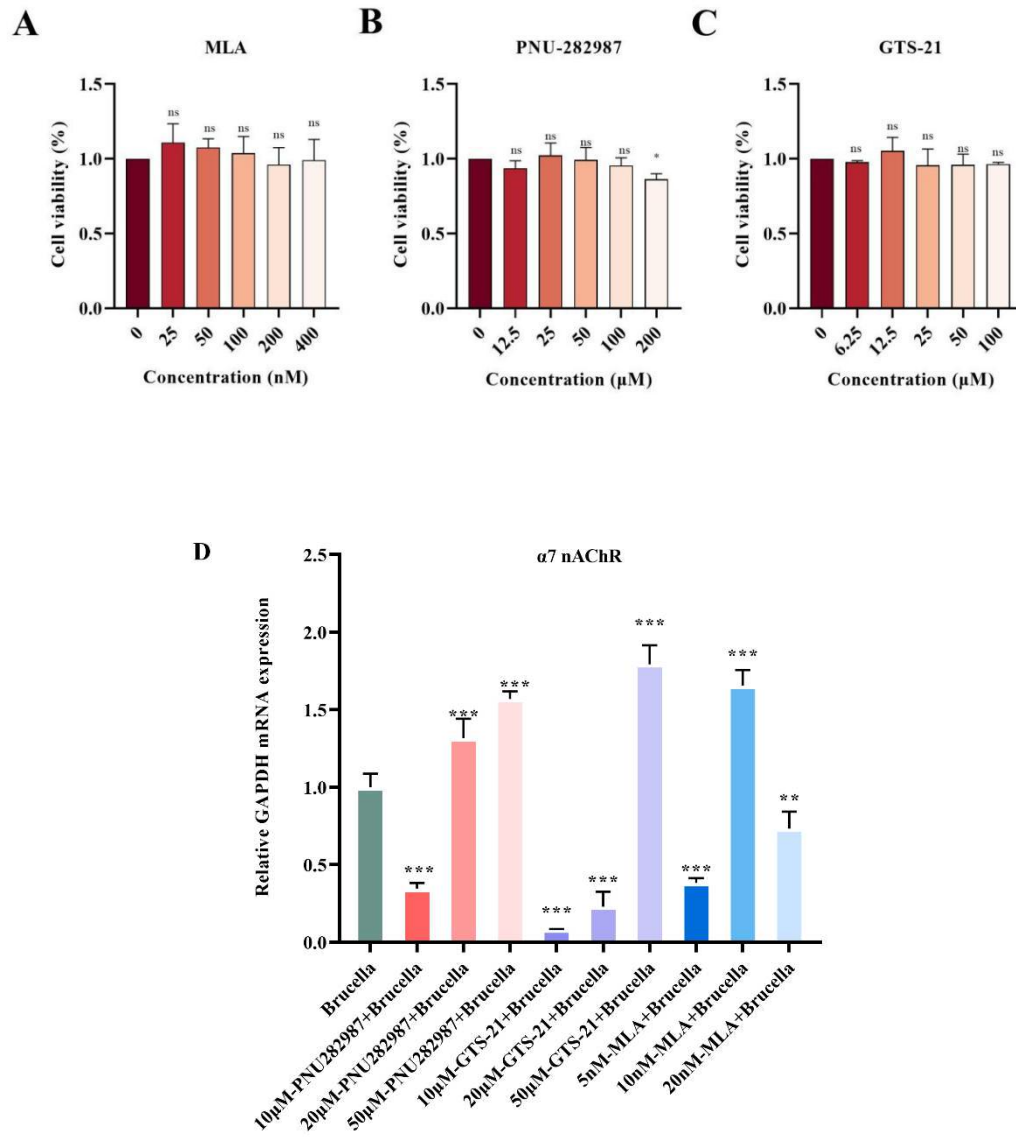


Fig. 2 Expression profiles of nicotinic acetylcholine receptor (nAChR) subunits in *Brucella*-infected RAW264.7 macrophages. Cells were examined at 0, 4, 8, 12, and 24 h post-infection. The transcript levels of $\alpha 6$, $\alpha 7$, and $\alpha 9$ nAChR were quantified by RT-qPCR (A-C). Protein levels of $\alpha 4$ - $\alpha 7$, $\alpha 9$, and $\beta 2$ - $\beta 4$ nAChR subunits were assessed by Western blotting (D-G). $\alpha 7$ nAChR expression was additionally evaluated by immunofluorescence, with representative images and quantitative fluorescence analysis shown in H and I. Statistical comparisons were made with the 0 h control group or PBS control group, as appropriate. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The Western-blot images in panel D were cropped for presentation. Original blots are presented in Supplementary Fig. S4.

$\alpha 7$ nAChR agonists increase $\alpha 7$ nAChR expression in *Brucella*-infected murine macrophages

A CCK-8 assay was first used to evaluate the effects of the $\alpha 7$ nAChR agonists PNU-282987 and GTS-21 and the antagonist MLA on RAW264.7 cell viability. Cell viability was not substantially altered across most tested concentrations, whereas PNU-282987 at 200 μ M significantly reduced viability (Fig. 3A-C). We next examined the effects of these ligands on $\alpha 7$ nAChR transcript abundance in *Brucella*-infected macrophages. $\alpha 7$ nAChR transcript abundance increased with increasing concentrations of PNU-282987 and GTS-21, with the highest transcript abundance observed at 50 μ M for both agonists (Fig. 3D). In contrast, $\alpha 7$ nAChR transcript abundance showed a biphasic response to MLA and was lowest at the selected MLA concentration. Immunofluorescence

analysis corroborated these findings: PNU-282987 and GTS-21 increased $\alpha 7$ nAChR staining, whereas MLA reduced fluorescence intensity (Fig. 3E,F). Based on these experiments, 50 μ M PNU-282987, 50 μ M GTS-21, and 5 nM MLA were selected for subsequent experiments.



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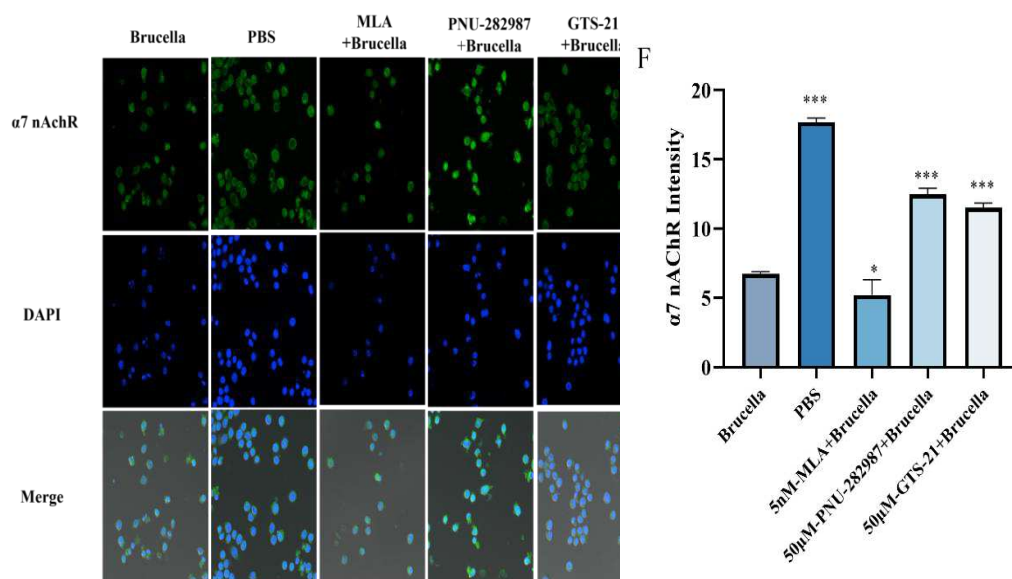
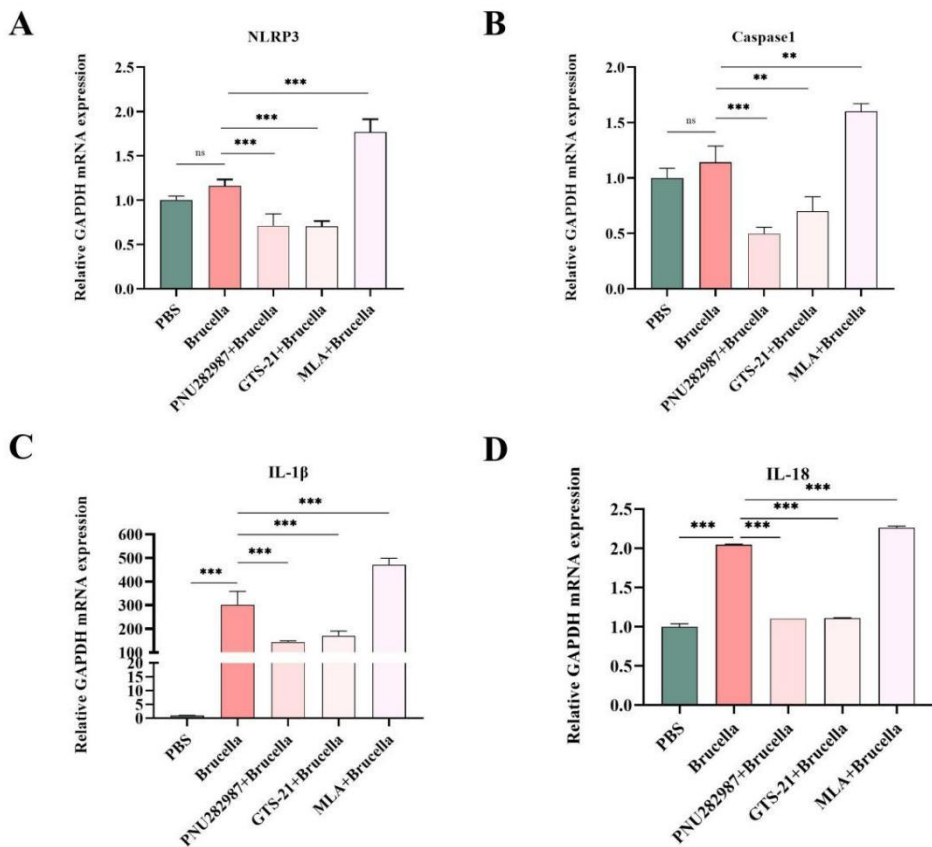


Fig. 3 Selection of $\alpha 7$ nAChR ligand concentrations and their effects on $\alpha 7$ nAChR expression. RAW264.7 macrophages were treated with different concentrations of the antagonist MLA (A), the agonist PNU-282987 (B), or the agonist GTS-21 (C), and cell viability was assessed using the CCK-8 assay. The tested concentration series were 0, 25, 50, 100, 200, and 400 nM for MLA; 0, 12.5, 25, 50, 100, and 200 μ M for PNU-282987; and 0, 6.25, 12.5, 25, 50, and 100 μ M for GTS-21. $\alpha 7$ nAChR mRNA expression was measured by RT-qPCR after ligand treatment in the *Brucella*-induced inflammation model (D). $\alpha 7$ nAChR immunofluorescence and quantitative fluorescence intensity are shown in E and F. Statistical comparisons were made between the indicated drug-treated groups and the relevant controls. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

$\alpha 7$ nAChR activation attenuates *Brucella*-induced inflammatory signaling

To evaluate the functional role of $\alpha 7$ nAChR in *Brucella*-induced inflammation, we quantified NLRP3 inflammasome-associated mediators following pharmacological modulation. Compared with the *Brucella*-infected group, treatment with the $\alpha 7$ nAChR agonists PNU-282987 and GTS-21 significantly reduced the mRNA levels of NLRP3, caspase-1, IL-1 β , and IL-18, whereas MLA generally increased their transcript levels (Fig. 4A–D). Western blotting showed that PNU-282987 and GTS-21 increased $\alpha 7$ nAChR protein abundance in *Brucella*-

214 infected cells, whereas MLA did not produce a comparable increase (Fig. 4E–I). Consistent with the infection
 215 time-course experiment, $\alpha 7$ nAChR expression was lower in *Brucella*-infected cells than in the PBS control group
 216 (Fig. 4H), whereas $\alpha 4$ nAChR showed comparatively modest treatment-associated changes (Fig. 4G). NLRP3
 217 protein abundance and the cleaved-caspase-1/pro-caspase-1 ratio were increased in the *Brucella*-infected group
 218 relative to the PBS control and were reduced by PNU-282987 and GTS-21 (Fig. 4F, I). ELISA further showed that
 219 IL-18 and IL-1 β concentrations were elevated after *Brucella* infection and were reduced after agonist treatment
 220 (Fig. 4J, K). The response to MLA was not uniform across all readouts, indicating that antagonist-associated effects
 221 in this model may depend on the measured endpoint.



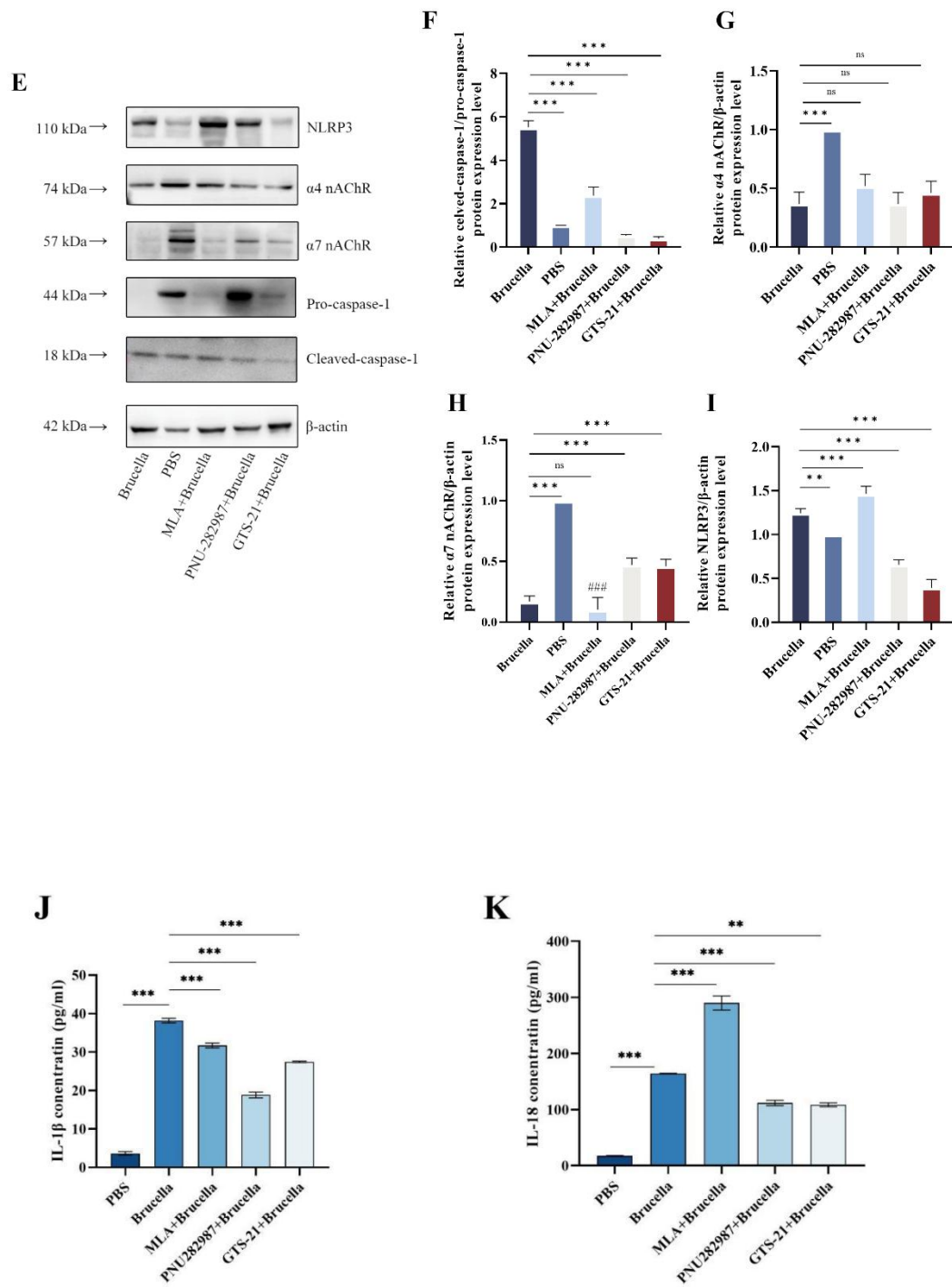
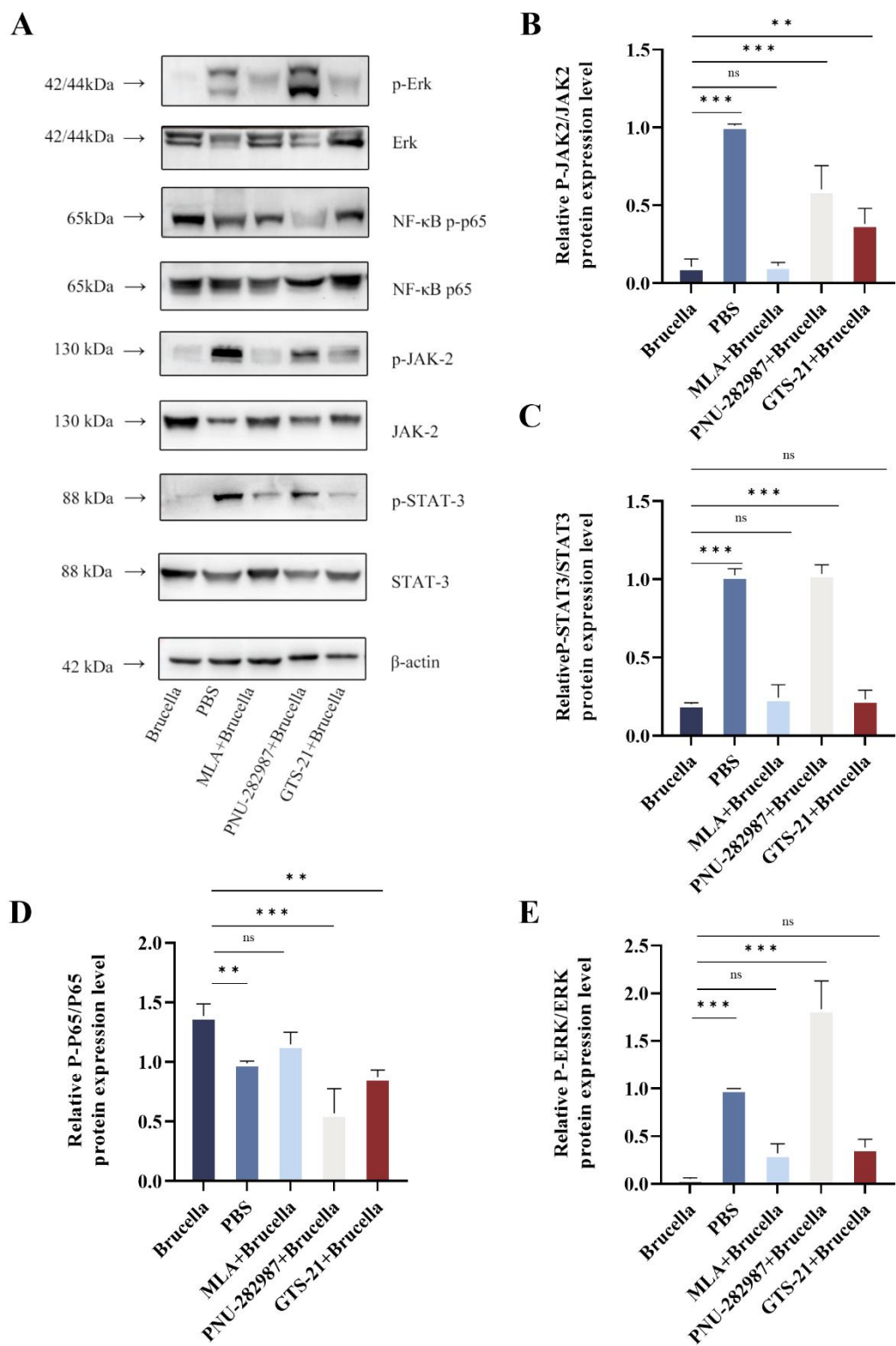


Fig. 4 Effects of $\alpha 7$ nAChR-targeting ligands on NLRP3 inflammasome-associated inflammatory responses in *Brucella*-infected macrophages. NLRP3, caspase-1, IL-1 β , and IL-18 transcript levels were quantified by RT-qPCR (A–D). Representative Western blots and quantitative analyses of NLRP3, caspase-1, $\alpha 7$ nAChR, and $\alpha 4$ nAChR are shown in E–I. The groups in panel E are, from left to right, *Brucella*-infected, PBS control, MLA + *Brucella*, PNU-282987 + *Brucella*, and GTS-21 + *Brucella*. IL-18 and IL-1 β concentrations were measured by ELISA (J,K). Statistical significance is indicated as shown in the figure: ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. #### $P < 0.001$ versus the PBS control group. The Western-blot images in panel E were cropped for presentation. Original blots are presented in Supplementary Fig. S5.

To investigate signaling mechanisms associated with $\alpha 7$ nAChR activation, we quantified the ratios of phosphorylated to total JAK2, STAT3, ERK1/2, and NF- κ B p65 by Western blotting (Fig. 5A–E). Relative to the *Brucella*-infected group, the PBS control showed higher p-JAK2/JAK2, p-STAT3/STAT3, and p-ERK1/2/ERK1/2 ratios and lower p-NF- κ B p65/NF- κ B p65. Treatment with PNU-282987 increased JAK2 and STAT3 phosphorylation and reduced NF- κ B p65 phosphorylation compared with *Brucella* infection alone. PNU-282987 also enhanced ERK1/2 phosphorylation. GTS-21 produced smaller changes in STAT3 and ERK1/2 phosphorylation, and not all comparisons reached statistical significance. Together, these results suggest that $\alpha 7$ nAChR agonists attenuate *Brucella*-induced inflammatory signaling through partially distinct modulation of JAK2/STAT3, NF- κ B, and ERK1/2 signaling.



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Fig. 5 Effects of $\alpha 7$ nAChR agonists on inflammation-associated signaling pathways in *Brucella*-infected macrophages. (A) Representative Western blots for p-JAK2, JAK2, p-STAT3, STAT3, p-NF- κ B p65, NF- κ B p65, p-ERK1/2, and ERK1/2. Groups are *Brucella*-infected, PBS control, MLA + *Brucella*, PNU-282987 + *Brucella*, and GTS-21 + *Brucella*. (B-E) Densitometric analysis of p-JAK2/JAK2, p-STAT3/STAT3, p-NF- κ B p65/NF- κ B p65, and p-ERK1/2/ERK1/2 ratios. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus the indicated comparison or *Brucella*-infected group. The Western-blot images in panel A were cropped for presentation. Original blots are presented in Supplementary Fig. S6.

Discussion

Nicotinic acetylcholine receptors (nAChRs) are widely expressed and participate in diverse physiological and pathophysiological processes, including immune regulation [21,22]. Although $\alpha 7$ nAChR has been extensively studied as a central component of the cholinergic anti-inflammatory pathway, the expression dynamics of individual nAChR subunits during pathogen-induced inflammation and their relationship with NLRP3 inflammasome signaling remain incompletely understood. In this study, we characterized the expression of eight nAChR subunits ($\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 9$, $\beta 2$, $\beta 3$, and $\beta 4$) in *Brucella*-infected RAW264.7 macrophages. The most prominent reciprocal changes were progressive downregulation of $\alpha 7$ nAChR and upregulation of $\alpha 9$ nAChR (Fig. 2F, G). Given the established anti-inflammatory functions of $\alpha 7$ nAChR, including suppression of NLRP3 inflammasome signaling [23,24], its downregulation may attenuate endogenous cholinergic restraint of inflammation during *Brucella* infection. Conversely, the increase in $\alpha 9$ nAChR suggests a potentially distinct immunoregulatory role for this subunit, but its functional contribution in *Brucella* infection remains to be established experimentally.

Pharmacological activation of $\alpha 7$ nAChR with PNU-282987 or GTS-21 partially restored the infection-associated reduction in $\alpha 7$ nAChR expression and attenuated multiple NLRP3 inflammasome-associated inflammatory endpoints (Fig. 4). Both agonists reduced NLRP3, caspase-1, IL-1 β , and IL-18-associated responses,

supporting an anti-inflammatory role for $\alpha 7$ nAChR in *Brucella*-infected macrophages. However, the agonists did not completely restore $\alpha 7$ nAChR expression or normalize all measured inflammatory endpoints, suggesting that additional host signaling pathways and *Brucella*-driven mechanisms contribute to the inflammatory phenotype. The progressive increase in $\alpha 9$ nAChR is therefore of particular interest and warrants direct functional testing using genetic or pharmacological approaches.

At the mechanistic level, $\alpha 7$ nAChR agonist treatment modulated several signaling pathways implicated in inflammatory regulation. PNU-282987 enhanced JAK2/STAT3 signaling, promoted ERK1/2 phosphorylation, and reduced NF- κ B p65 phosphorylation relative to *Brucella* infection alone (Fig. 5). These signaling changes are consistent with the observed attenuation of NLRP3 inflammasome-associated mediators. GTS-21 produced smaller effects on ERK1/2 and STAT3 phosphorylation than PNU-282987, which may reflect differences in pharmacological efficacy, receptor desensitization, or pathway engagement [18]. These findings suggest that the anti-inflammatory effects of $\alpha 7$ nAChR activation depend on both receptor engagement and downstream signaling context.

The NLRP3-associated inflammatory response became most pronounced at 12–24 h post-infection (Fig. 1), indicating a delayed intensification of inflammasome-associated signaling during *Brucella* infection. Delayed inflammasome responses have also been described in other intracellular bacterial infections and may reflect pathogen-specific mechanisms of immune modulation or evasion [25,26]. Because the present experiments were limited to 24 h, extended time-course studies will be required to determine whether nAChR dysregulation persists during prolonged or chronic infection and whether sustained pharmacological modulation influences bacterial persistence.

In summary, our findings support a model in which *Brucella* infection is associated with reciprocal regulation of $\alpha 7$ and $\alpha 9$ nAChR subunits, with reduced $\alpha 7$ nAChR potentially weakening endogenous anti-inflammatory

signaling. Pharmacological activation of $\alpha 7$ nAChR attenuated NLRP3 inflammasome-associated responses and modulated JAK2/STAT3, NF- κ B, and ERK1/2 signaling. Future studies should directly define the function of $\alpha 9$ nAChR, examine longer infection periods, assess possible $\alpha 7/\alpha 9$ receptor interactions, and determine whether combined $\alpha 7$ agonism and $\alpha 9$ inhibition confers additional anti-inflammatory benefit. In vivo validation will also be necessary before therapeutic relevance can be established.

This study shows that *Brucella* infection is associated with substantial changes in nAChR subunit expression in RAW264.7 murine macrophages, particularly downregulation of $\alpha 7$ nAChR and upregulation of $\alpha 9$ nAChR. Pharmacological activation of $\alpha 7$ nAChR with PNU-282987 or GTS-21 attenuated NLRP3 inflammasome-associated inflammatory responses, including reductions in NLRP3, caspase-1, IL-1 β , and IL-18. The attenuation of inflammatory responses was accompanied by modulation of JAK2/STAT3, NF- κ B, and ERK1/2 signaling. These findings support $\alpha 7$ nAChR as a potential immunomodulatory target in *Brucella*-induced macrophage inflammation. Further mechanistic studies, including in vivo validation, are required to determine the translational relevance of $\alpha 7$ nAChR-targeted interventions for brucellosis.

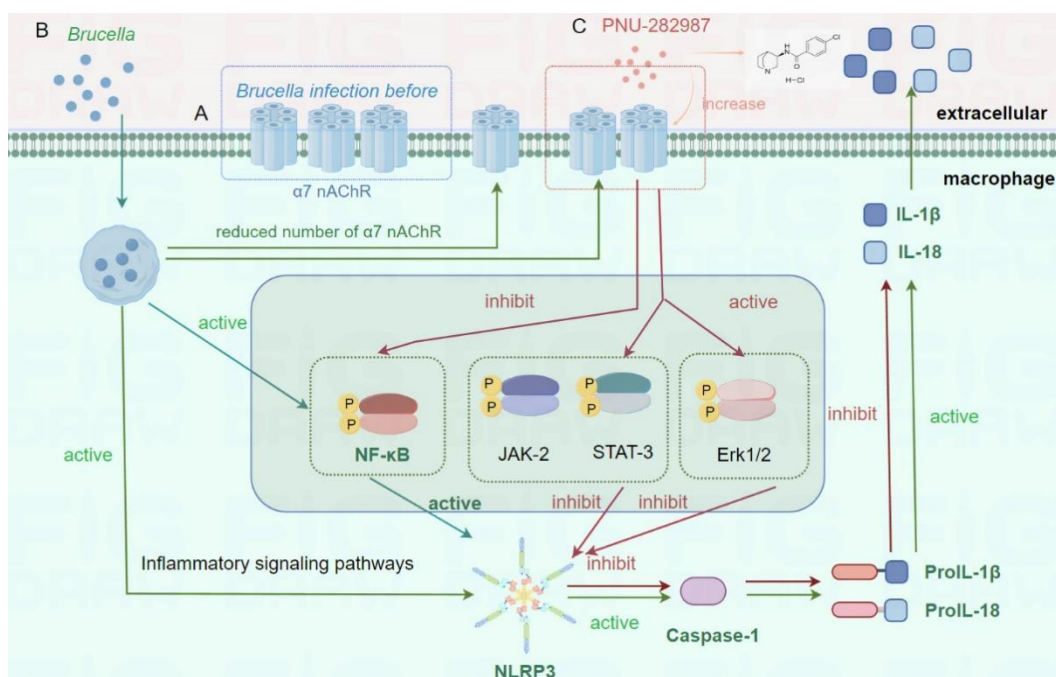


Fig. 6 Proposed model of $\alpha 7$ nAChR-mediated regulation of Brucella-induced inflammatory signaling. (A)

Brucella infection is associated with reduced $\alpha 7$ nAChR expression. (B) Infection increases NLRP3

inflammasome-associated signaling, caspase-1 activation, and the production of IL-1 β and IL-18. (C) Treatment

with the $\alpha 7$ nAChR agonist PNU-282987 increases $\alpha 7$ nAChR expression and suppresses NLRP3-associated

inflammatory signaling. This effect is accompanied by activation of JAK2/STAT3 signaling, enhanced ERK1/2

phosphorylation, and inhibition of NF- κ B activation, resulting in reduced IL-1 β and IL-18 production.

Declarations

Author contributions: C.S., H.L., and Z.S. designed the study. C.S., X.L., H.L. and Y.W. performed the experiments. H.L., H.H., A.A., R.R., and Z.S. wrote the manuscript. C.S., J.G., X.D. and H.Z. reviewed and edited the manuscript. Z.S., H.H. and H.Z. acquired resources, supervised the work, and administered the project. All authors have read and approved the final version of the manuscript.

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Ethics approval: Not applicable. RAW264.7 cells (BNCC354753) were obtained as a commercially available

established murine macrophage cell line from BeNa Culture Collection (BNCC, Henan Province, China). No live animals, primary animal tissues, human participants, or human materials were used in this study; therefore, formal animal- or human-research ethics approval was not required.

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