Remimazolam protects against LPS-induced endotoxicity improving survival of endotoxemia mice

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Research

Abstract

Background: Remimazolam is a new benzodiazepine of sedative drugs with an ultra-short-acting anesthetic effect, being commonly used for critically ill patients (especially septic patients) in intensive care units (ICUs). Although some anesthetics have been reported to show certain anti-inflammatory effects, the role of remimazolam in inflammation is still remained unknown.

Methods: In the present study, LPS-induced endotoxemia mice were used to observe the effect of remimazolam in vivo. LPS-stimulated bone marrow cells and Raw 264.7 cells were used to evaluate the influence of remimazolam on macrophage in vitro.

Results: Compared with LPS treatment group, remimazolam remarkably improved survival rate of endotoxemia mice and decreased the release of LPS-induced inflammatory mediators, such as TNF-a, IL-6 and IL-1b. Remimazolam not only inhibited the activation of MyD88 and PI3K signal pathway at 15min after LPS treatment but also disturb Rab5a related TLR4 expression at cell surface in response to LPS at later time.

Conclusions: Remimazolam inhibit LPS-induced inflammatory responses of macrophages and obviously improve the survival rate of endotoxemia mice.

Background

Sepsis is a severe inflammatory response to infection and remains the primary cause of morbidity and mortality in intensive care unit (ICU) patients worldwide.(1) Sepsis usually triggers cell dysfunction and subsequent organ failure, leading to death. Inflammatory factors such as tumor necrosis factor-alpha (TNF-a), interleukin-6 (IL-6), and interleukin-1 (IL-1) act as key regulators of the immune response and play a critical role in the complex pathophysiological mechanisms of sepsis.(2, 3) Excessive release of these inflammatory cytokines can lead to dysfunction of the immune system and, consequently, damage to multiple tissues.(3) Therefore, coordinating the expression of these inflammatory cytokine genes will be benefit in the treatment of sepsis.

Remimazolam is a new ultra-short acting benzodiazepine that is currently under development for intravenous use in procedural sedation and general anesthesia.(4, 5) Remimazolam possess a fast offset of sedation and faster recovery than seen with currently available drugs of the same class (6–9). In ICU, remimazolam is used to manage critically ill patients who require sedation and analgesia for increasing their comfort and collaboration,(10) and long-term infusion or higher doses of remimazolam do not accumulate and prolong effects.(4) Although ramimazolam has been gradually used in the clinic, it is not clear whether it has anti-inflammatory effect, and no literature has been reported at present. The anti-inflammatory effects of remimazolam have not been reported, though remimazolam have been applied gradually in clinic practice.

Here, we studied the effect of remimazolam on the inflammatory response induced by LPS. Our results demonstrated that remimazolam remarkably improved survival rate of LPS-induced endotoxemia mice. We also displayed that the release of LPS-induced inflammatory mediators (such as TNF-a, IL-6 and IL-1b) were restricted by remimazolam in vivo. Furthermore, in vitro, we found that remimazolam disturb LPS-induced macrophages overactivation process, including inflammatory signal transduction and the cell surface translocation of TLR4. Such evidence suggests that remimazolam might be beneficial to septic patients who are suffering uncontrolled inflammatory responses.

Materials And Methods

Animals

C57BL/6 (WT) mice were purchased from SeBiona Bio-Tech (Guangdong, China). All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Guangdong Medical University. Eight-week-old C57BL/6 mice with a mean body weight of 25g were used.

LPS Induced Endotoxemia

Mouse endotoxemia was induced by intraperitoneal injection of LPS (25 mg/kg) (LPS group). To assess the effects of remimazolam, mice were administered with remimazolam intraperitoneally (16 mg/kg) 30 min before and subcutaneously (8 mg/kg) 30 min after the injection of LPS (Re + LPS group). Mice received only remimazolam without LPS were regarded as the Re group. All reagents above were dissolved in normal saline at the indicated concentrations. NS was used as the blank vehicle in the mice of the control group. The mice were killed 12 h after the LPS injection. Serum and organ samples were harvested at the time of execution.

Histological Analysis

Liver and lung tissue were fixed in 4% paraformaldehyde overnight, embedded in paraffin and cut into 4 mm sections. The sections were stained with hematoxylin and eosin.

Measurement of TNF-a, IL-6 and IL-1b

TNF-a, IL-6 and IL-1b protein were measured with a mouse TNF-a, IL-6 and IL-1b ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The measurements were standardized with cell numbers.

Bone marrow derived macrophage (BMDM) isolation and culture

Bone marrow was flushed out with pre-chilled Dulbecco's Modified Eagle Medium (DMEM) from femurs and tibias, which were harvested from C57BL/6 WT mice following the methods as described previously. (11)

Flow cytometry analysis

Macrophages collected from peritoneal lavage were labeled with F4/80 at 4°C for 15min. For measuring cell surface expression of TLR4, macrophages were stained with PE conjugated anti-mouse CD284 (TLR4) antibody (eBioscience affymetrix, San Diego, CA, USA) for 30min. For detecting cell death, cells were incubated with PI and Annexin V (BD PharmingenTM, San Jose, CA, USA) for 15min at room temperature, and the double-stained cells were counted as dead cells. Cells were analyzed by flow cytometry, and acquisition was performed on 30,000 events using a FACScalibur cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo-V10 software (Tree Star, Ashland, OR, USA).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from BMDM or RAW264.7 cells using TRIzol RNA Isolation Reagents (Life Technologies, Pittsburgh, PA, USA). Total RNA (1 μg) was used to synthesize cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Expression of mRNA was determined by quantitative real-time PCR (RT-PCR) using the TB Green Premix Ex Taq II (TaKaRa, Japan), respectively. RT-PCR was performed on the ABI 7500 system (Applied Biosystems, United States). The gene-specific primers were listed as below: TNF-a Forward Primer: CCCTCACACTCAGATCATCTTCT; Reverse Primer: GCTACGACGTGGGCTACAG. IL-6 Forward Primer: TAGTCCTTCCTACCCCAATTTCC; Reverse Primer: TTGGTCCTTAGCCACTCCTTC. IL-1b Forward Primer: GCAACTGTTCCTGAACTCAACT; Reverse Primer: ATCTTTTGGGGTCCGTCAACT. The results of the analyses were calculated and expressed according to an Eq. ($2^{-\Delta\Delta Ct}$) which provides the amount of the target, normalized to an internal reference. Ct is a threshold cycle for target amplification. Each biological sample was tested in triplicate.

Immunofluorescence

Raw264.7 cells were plated in 35mm Glass Bottom Dish (NEST, Wuxi, China) and then fixed in 4% paraformaldehyde, and permeabilized with 0.5% TritonX-100, followed by blocked with 1% BSA for 30min at room temperature. Next, the primary antibody was added and incubated with cells at 4°C overnight. After washing the cells three times with PBS appropriate fluorescent secondary antibodies were added and incubated with cells for 1h at RT in the dark. Cells were washed three times again, counterstained with DAPI and then observed under a fluorescence microscope.

Gene knockdown

Raw264.7 cells (4 × 10^5 cells) were cultured in 35mm dishes for 12h before transfection. 100pmol per dish of mouse PBR siRNA or non-specific siRNA (RiboBio, Guangzhou, China) was transfected using Lipofectamine RNAiMAX Transfection Reagent (13778150 Invitrogen Life Technologies) following the manufacturer's instructions. At 48h after transfection, the efficiency of the corresponding gene knockdown was confirmed with Western blot.

Antibodies and Reagents

Antibodies

AKT (4691), p-AKT (4060), P38 (8690), p-P38 (4511), ERK1/2 (4695), p-ERK1/2 (4370), IkBa (4814), p-IkBa (2859), p-IKKa/b (2697), NF-kB (8242), Rab5a (46449), horse anti-mouse IgG (H&L) (7076) and goat anti-rabbit IgG (H&L) (7074) were obtained from Cell Signaling Technology (Danvers, MA, USA). IKKβ (ab124957), PBR (ab109497) was from Abcam (CA, USA). Alexa Fluor 488 goat anti-rabbit (A0423) and Alexa Fluor 647 goat anti-mouse (A0473) secondary antibodies were obtained from Beyotime Biotechnology (Shanghai, China). TLR4 (66350-1-Ig), GAPDH (60004-1-Ig) and b-Tubulin (10068-1-AP) were obtained from Proteintech (Wuhan, Hubei, China). Lipopolysaccharides(L4391) was from Sigma (Louis, MO, USA), Remimazolam Tosilate for Injection (Remimazolam)were obtained from Hengrui (Jiangsu, China).

Statistical analysis

Results are presented as mean ± SD. Differences between two groups were analyzed by a Student t test. For multi-group comparisions, One-way ANOVA was performed. Survival studies were analyzed by Chisquare test. $p < 0.05$ was considered statistically significant. Graphs and figures were made with Graphpad Prism 6 (GraphPad software, CA, USA).

Results

1. Remimazolam improved LPS-induced cell death in vitro and vivo

Macrophages are crucial innate immune cells in the body and play an important role in immune homeostasis and inflammation by reason of their immunoregulatory function.(12) Meanwhile, macrophages also play an essential role in the host defense against a variety of pathogens and generation of inflammatory immune response during sepsis.(13) As a result, we will explore the protective effect of remimazolam on excessive inflammatory injury of the body with macrophages as the research object. To investigate the effect of remimazolam on LPS-induced cell death in macrophages, we first use CCK8 to assess cell vitality. As show in Fig. 1A and 1B, different concentrations remimazolam alone will not cause the change of cell vitality. At the same time, cell vitality decrease followed by LPS treatment, while pretreatment of remimazolam recover cell vitality in a concentration-dependent manner. In addition, we also assess of cell death by PI and Annexin V by flow cytometry in BMDM and Raw264.7 cells after LPS treatment for 24h. As shown in Fig. 1C-1F, LPS increased cell death in Raw264.7 cells and BMDM and this was attenuated with remimazolam pretreatment. To recapitulate the in vitro findings in vivo, C57BL/6 mice were administered with remimazolam intraperitoneally (16 mg/kg body weight) 30 min before and subcutaneously (16 mg/kg body weight) 30 min after the injection of LPS. At 12h after LPS treatment, macrophages were collected from peritoneal lavage fluid from the mice and macrophages identified using surface marker F4/80. Cell death was determined by 7-AAD and Annexin V double positive staining. As shown in Fig. 1G and 1H, macrophage death increased from 3.95−28% in response

to LPS, whereas pretreatment with remimazolam significantly prevented LPS-induced macrophage death. These results indicate that remimazolam protests against LPS-induced cell death.

2. Remimazolam inhibits LPS-induced inflammatory response in vitro and vivo

LPS stimulation cause cell inflammatory responses, including increased transcription and expression of inflammatory factors(14). One of the most dramatic consequences of overwhelming acute inflammation is septic shock due to bacterial LPS, the most potent inducer of the inflammatory response. Too much LPS leads to excessive production of pro-inflammatory cytokines including IL-1, TNF-a, and IL-6(15). For this reason, the effect of remimazolam on LPS-induced macrophage inflammatory response were evaluated. First, we used RT-PCR to assess the gene expressions of TNF-a, IL-6 and IL-1b. Within expectation, LPS (1 µg/mL) markedly enhanced the mRNA levels of TNF-a, IL-6 and IL-1b after LPS treatment, and this changes of mRNA levels in pro-inflammatory responses were partially reversed by remimazolam in BMDM and Raw264.7 cells (Figure A-B). Subsequently, ELISA was performed to detect inflammatory cytokine expression, includes TNF-a, IL-6 and IL-1b. As expected, compared with the significantly increased protein levels of inflammatory factors TNF-a, IL-6 and IL-1b in the LPS group, remimazolam pretreatment can significantly inhibit the above inflammatory factor protein expression level, therefore inhibiting the occurrence of inflammation (Fig. 2C-D).

We next investigated a potential therapeutic role for erlotinib in LPS-induced endotoxicity in mice. After 12 hours of LPS processing (25 mg/kg i. p.), serums were collected from mice. Serums inflammatory cytokines (TNF-a, IL-6, IL-1b) production from mice after LPS treatment (LPS group) alone were increased, and remimazolam pretreatment (Re + LPS group) markedly prevented LPS-induced the production of serums inflammatory factors (Fig. 2E). Histological analyses of mouse liver and lung showed that in the control group no inflammation and necrosis were found (Fig. 2F). In liver tissue, Re group resulted in spotty coagulation necrosis in the lobules with mild inflammatory cells infiltration within the necrosis area, consisting of neutrophil and monocyte However, Re plus LPS group exhibited no necrosis and inflammation in the liver tissue although the mice received same dose of LPS. Similarly, in LPS group, the lung tissue displayed serious injury with the features of disrupted alveoli, hemorrhage, thickness of alveolar septum, and infiltration of inflammatory cells. Those pathohistological changes were resolved by treatment with remimazolam. These results revealed that remimazolam prevented the liver and lung injury caused by LPS and protected their function to some extent as well. Meanwhile, the survival rate of control, remimazolam alone, LPS and LPS plus remimazolam group were studied. As shown in Fig. 2I, 75% of mice treated with remimazolam by intraperitoneal injection survived 72 h after LPS administration, much longer than control mice injected with LPS alone. Remimazolam alone was not toxic. Collectively, these results indicate that remimazolam significantly prevented multiple organ injury caused by LPS and thus raise survival of endotoxemia mice. In addition, these results give us a hint that remimazolam, not just macrophage, may play a potential role to prevent or treat inflammatory diseases in other immune cells.

3. Suppressive effects of remimazolam on LPS induced activation of the MAPK and PI3K signaling pathway in macrophage.

MAPK and PI3K signaling pathway, as an upstream signaling pathway of NF-kB, is activated by LPS and is involved in the production of LPS-induced inflammatory cytokines (16, 17). These results shows that the phosphorylation of AKT, p38 and ERK1/2 was triggered at 30min after LPS treatment in Raw264.7 cells, but this activation was substantially suppressed by remimazolam (Fig. 3A-3D). And then we evaluated the role of NF-kB in the protection of inflammatory response by remimazolam. NF-kB activation is directly related to a sequential cascade, including inhibitor kappa B kinase (IKK)-dependent inhibitor kappa Bα(IkBa) phosphorylation, ubiquitination, and proteolytic degradation, accompanying subsequent translocation of cytosolic NF-kB to the nucleus. As show in Fig. 3E-3G, LPS significantly upregulated the phosphorylations of IKKa/b and IkBα but degraded IkBa expression in Raw264.7 cells. However, pretreatment with remimazolam reversed these effects, indicating that remimazolam was able to inhibit LPS-induced IKKa/b activation and IkBα degradation in Raw264.7 cells. These findings were further supported by immunofluorescent staining showing that remimazolam blocked the LPS-induced nuclear translocation of NF-kB p65 when compared with that of LPS stimulation alone at 10min (Fig. 3E). Taken together, these results elucidate that remimazolam inhibited LPS-induced NF-kB activation in Raw264.7 cells.

Furthermore, to determine the effect of remimazolam on advanced inflammatory response, we measured the phosphorylation of AKT, P38 and ERK1/2 after LPS treatment in macrophages. In Raw264.7 cells, ERK1/2, P38 and AKT phosphorylation were measured 1, 3 or 6 hours respectively after LPS treatment with or without remimazolam pretreatment. As shown in Fig. 3K-3M, LPS promoted the phosphorylation of ERK1/2, P38 and AKT at all time points and this effect could be inhibited by remimazolam. Then, we verified this result in BMDM (Fig. 3M-Q). Collectively, these results support the notion that the suppressive effect of remimazolam on LPS-induced macrophages activation is through preventing MAPK and PI3K signaling pathway.

4.Remimazolam significantly suppresses the macrophage surface expression of TLR4 after LPS treatment.

TLR4 activation is a tightly regulated process. In addition to directly regulating different signaling pathways, the amount of TLR4 / MD-2 present on the cell surface also controls the LPS response. Based on this reason, we measured the effect of remimazolam pretreatment in cell surface TLR4 expression after LPS treatment by using flow cytometry. At 12h after LPS treatment, TLR4 expression on the surface of Raw264.7 cells was increased ~ 6 fold as compared with the control group, and this increase could be partially inhibited by remimazolam(Fig. 4A and 4B). This alteration was observed in BMDM as well (Fig. 4C and 4D).

Next, we determined to verify if this change also happened in vivo. As shown in Fig. 4E and 4F, where in contract to LPS group, the amount of TLR4 on the surface of peritoneal macrophages from Re plus LPS group was markedly decreased than the former at 12h after LPS treatment. These findings indicate that remimazolam inhibits LPS-induced upregulation of macrophage surface TLR4 expression.

5.Remimazolam regulate the cell surface expression of TLR4 after LPS treatment by affecting functions and expressions of Rab5a.

Rab5a plays a crucial role in actin remodeling, TLR4-MyD88 interaction, and receptor internalization(18, 19). Our previous study demonstrated that Rab5a-mediated internalization of TLR4 results in increased cell surface expression of the receptors(20). Pretreatment of Raw264.7 cells and BMDM with remimazolam given 20min prior to LPS effectively inhibited the expression of Rab5a at protein level at 6, 12 and 24h after LPS treatment (Fig. 5A-5D). We further determined the effect whether remimazolam modulates Rab5a in the progress of blocking macrophage activation. As shown in Fig. 5E, remimazolam pretreatment attenuated the colocalization between early Rab5a and TLR4 at 1h after LPS treatment by confocal immunoluorescence microscopy. Collectively, these results indicate that remimazolam regulate functions and expressions of Rab5a, consequently enhanced late phase cell surface expression of TLR4 in response to LPS.

6. The anti-inflammatory effect of Remimazolam on macrophages was not exerted via the PBR

It is well known that the binding sites of benzodiazepine exist inside the central nerve system and also in peripheral tissues.(21) Peripheral benzodiazepine receptor (PBR) is located primarily on the outer membrane of mitochondria in steroid-producing cells(22, 23). Therefore, we studied macrophage with PBR knockout. Knockdown efficiency shown in Fig. 6A and 6B. However, we found that the inhibition of remimazolam on MAPK and PI3K signaling pathway proteins in response to LPS did not reverse after PBR knockout in Raw264.7 cells (Fig. 6C-F). These results indicated that remimazolam could not inhibit the inflammatory response by binding PBR in macrophage.

Discussion

Sepsis represents a dysregulated host response to infection leading to organ dysfunction.(24) In response to pathogen, immune cells (such as macrophage) are activated to interact with the endothelium through corresponding recognition receptors producing cytokines, proteases, kinins, reactive oxygen species, and nitric oxide.(25) It is pathogen that triggers an initial exaggerated inflammatory-immune response, leading to activation or suppression of multiple endothelial, hormonal, bioenergetic, metabolic, immune, and other pathways.(26) These, in turn, produce the circulatory and metabolic perturbations resulting in organ dysfunction. Excessive cellular immune response leads to proinflammatory responses and multiple organ damage.(27–29) Therefore, immunotherapy is an important means for the treatment

of sepsis, such as anti-inflammatory and immunostimulation therapy.(30) Judicious and early antimicrobial administration and early goal-directed therapies have significantly and positively impacted sepsis-related mortality.

Remimazolam is a new sedative drug which combines the properties of two other anesthetic drugs – Midazolam and remifentanil. It acts on GABA receptors like midazolam and has organ-independent metabolism like remifentanil.(31, 32) Since it was approved for use by state drug administration on December 2020 in China, Remimazolam is widely applied in general anesthesia and Intensive care unit (ICU) sedation in adults including septic patients. So far there is no study reporting the role of remimazolam on the pathophysiological process of sepsis, except for its sedative effect. Here, we demonstrated that compared with LPS group, remimazolam effectively decreased LPS induced the release of serum pro-inflammatory cytokines and multiple organ damage such as lung and liver. As a consequence, remimazolam treatment dramatically increased the survival rate of endotoxemia mice indicating a potential therapeutic benefit for septic patients.

Lipopolysaccharide (LPS) is a macromolecular glycolipid in the outer leaflet of the outer membrane of most of the Gram-negative bacteria. It is specifically recognized by transmembrane protein TLR4 which is a member of the toll-like receptor family, leading to inflammatory cytokine production and innate immune system activation(33). When activated by LPS, TLR4 recruited the adaptor protein myeloid differentiation factor 88 (MyD88), leading to NF-kB and MAPK pathways activation following induction of proinflammatory cytokines(33–35). In addition, the signal transduction pathway mediated by phosphoinositide 3-kinase (PI3K) and its substrate, protein kinase B (AKT), have been attracted extensive attention in cellular inflammatory response(33, 35). PI3K/AKT also involved in LPS-induced cell inflammatory response. Here, we found remimazolan could inhibit the activation of both MAPK and PI3K signal pathway at 15min after LPS treatment. This may partially explain why pro-inflammatory cytokines, such as TNF-a, IL-6 and IL-1b decreased, and organ damage alleviated in remimazolan pretreated group.

Cell surface expression of TLR4 on innate immune cells critically regulates host responses and progression of inflammation(36). In macrophage, cell surface TLR4 expression is mainly determined by the balance between receptor trafficking from the Golgi apparatus to the cell membrane, and internalization of the cell surface receptor into endosomal compartments(37). Our previous study revealed that Rab family protein Rab5a is a key molecule mediating the circulation of TLR4 from the cell surface and back in response to LPS. Internalization of TLR4 mediated by Rab5a augment late phase cell surface expression of TLR4 in response to LPS. Therefore, we observed the effect of remimazolam on Rab5a expression and activity. LPS increased the expression of Rab5a both in BMDM and Raw264.7 cells and this effect could be inhibited by remimazolam. As a consequence, cell surface expression of TLR4 decreased dramatically in remimazolam plus LPS group. The amount of TLR4 on the cell surface can to some extent determine the intensity of the inflammatory response induced in cells stimulated by LPS. This may be one of the reasons why remimazolam can suppress the activation of P38 and ERK1/2 and the expression of inflammatory cytokines in response to LPS. In addition to the central nervous system, benzodiazepines action peripheral tissues performed with PBR or mitochondria translocator

proteins(38). It has been shown that PBR distributes ubiquitously in most types of tissues and was highly expressed in activated macrophages(39, 40). Therefore, after we knockout the PBR of Raw264.7 cells, we found that the anti-inflammatory effect of remimazolam was not reversed. With a negative outcome, we could conclude that binding to PBR was not responsible for the anti-inflammatory effect of remimazolam on LPS-activated macrophages.

Conclusion

In summary, our study first elucidated that remimazolam can inhibit LPS-induced inflammatory responses of macrophages and obviously improve the survival rate of endotoxemia mice. According to our results, remimazolam suppresses inflammatory response of sepsis at least from two aspects. First, it inhibits the activation of MAPK and PI3K signal pathway at 15min after LPS treatment. Later remimazolam also disturbs Rab5a related TLR4 expression at cell surface in response to LPS. These results suggest that remimazolam might be beneficial to septic patients who are suffering from uncontrolled inflammatory responses.

Declarations

Ethics approval and consent to participate

Animals were obtained from the SeBiona Bio-Tech (Guangdong, China). All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Guangdong Medical University. Guide for the Care and Use of Laboratory Animals and Animal Welfare Act are followed to guide 3M's animal research program.

Consent for publication

The authors agree to publication.

Availability of data and materials

The datasets used and analysed during the study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Not applicable.

Details of authors' contributions

X.L., S.L., Y.Z., J.S., X.Z., S.L., S.Z., J.T. planned and did experiments including cell isolation and treatment, confocal microcopy, Western blotting, and flow cytometry; X.L., S.L. and Y.Z. did animal experiments; X.L., S.L., Y.Z., J.S., X.Z., S.L. and J.T. planned the project and conceived the experiments; X.L., S.L., S.Z., and J.T. conceived the data and wrote the manuscript.

References

1. Drewry AM, Ablordeppey EA, Murray ET, et al. Antipyretic Therapy in Critically Ill Septic Patients: A Systematic Review and Meta-Analysis. CRIT CARE MED. 2017;45(5):806–13.

2. Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. Crit Rev Eukaryot Gene Expr. 2010;20(2):87–103.

3. Schulte W, Bernhagen J, Bucala R. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets–an updated view. Mediators Inflamm. 2013;2013:165974.

4. Wesolowski AM, Zaccagnino MP, Malapero RJ, et al. Remimazolam: Pharmacologic Considerations and Clinical Role in Anesthesiology. PHARMACOTHERAPY. 2016;36(9):1021–7.

5. Keam SJ. Remimazolam. First Approval DRUGS. 2020;80(6):625–33.

6. Zhou J, Curd L, Lohmer LL, et al. Population Pharmacokinetics of Remimazolam in Procedural Sedation With Nonhomogeneously Mixed Arterial and Venous Concentrations. Clin Transl Sci; 2020.

7. Wang F, Zhou Q, Shen M, et al. Efficacy and safety of remimazolam in procedural sedation and analgesia: A protocol for systematic review and meta analysis. Med (Baltim). 2020;99(27):e20765.

8. Kilpatrick GJ, McIntyre MS, Cox RF, et al. CNS 7056: a novel ultra-short-acting Benzodiazepine. ANESTHESIOLOGY. 2007;107(1):60–6.

9. Kanto JH. Midazolam: the first water-soluble benzodiazepine. Pharmacology, pharmacokinetics and efficacy in insomnia and anesthesia. PHARMACOTHERAPY. 1985;5(3):138–55.

10. Chawla N, Boateng A, Deshpande R. Procedural sedation in the ICU and emergency department. Curr Opin Anaesthesiol. 2017;30(4):507–12.

11. Li Z, Scott MJ, Fan EK, et al. Tissue damage negatively regulates LPS-induced macrophage necroptosis. CELL DEATH DIFFER. 2016;23(9):1428–47.

12. Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. IMMUNITY. 2014;41(1):21–35.

13. Huen SC, Cantley LG. Macrophages in Renal Injury and Repair. ANNU REV PHYSIOL. 2017;79:449–69.

14. Foster SL, Medzhitov R. Gene-specific control of the TLR-induced inflammatory response. CLIN IMMUNOL. 2009;130(1):7–15.

15. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 2003;348(2):138–50.

16. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. SCIENCE. 2003;300(5625):1524–5.

17. Ojaniemi M, Glumoff V, Harju K, et al. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. EUR J IMMUNOL. 2003;33(3):597–605.

18. Chen YJ, Hsieh MY, Chang MY, et al. Eps8 protein facilitates phagocytosis by increasing TLR4-MyD88 protein interaction in lipopolysaccharide-stimulated macrophages. J BIOL CHEM. 2012;287(22):18806–19.

19. Langemeyer L, Fröhlich F, Ungermann C. Rab GTPase Function in Endosome and Lysosome Biogenesis. TRENDS CELL BIOL. 2018;28(11):957–70.

20. Tang J, Zhou B, Scott MJ, et al. EGFR signaling augments TLR4 cell surface expression and function in macrophages via regulation of Rab5a activation. PROTEIN CELL. 2020;11(2):144–9.

21. Kaynar G, Yurdakan G, Comert F, et al. Effects of peripheral benzodiazepine receptor ligand Ro5-4864 in four animal models of acute lung injury. J SURG RES. 2013;182(2):277–84.

22. Papadopoulos V, Baraldi M, Guilarte TR, et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. TRENDS PHARMACOL SCI. 2006;27(8):402–9.

23. Guilarte TR. TSPO in diverse CNS pathologies and psychiatric disease: A critical review and a way forward. Pharmacol Ther. 2019;194:44–58.

24. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315(8):801–10.

25. Mahapatra S, Heffner AC. Septic Shock. 2021.

26. Arina P, Singer M. Pathophysiology of sepsis. Curr Opin Anaesthesiol. 2021;34(2):77–84.

27. Ramachandran G. Gram-positive and gram-negative bacterial toxins in sepsis: a brief review. VIRULENCE. 2014;5(1):213–8.

28. Cecconi M, Evans L, Levy M, et al. Sepsis and septic shock. LANCET. 2018;392(10141):75–87.

29. Brevetti G, Giugliano G, Brevetti L, et al. Inflammation in peripheral artery disease. CIRCULATION. 2010;122(18):1862–75.

30. Rubio I, Osuchowski MF, Shankar-Hari M, et al. Current gaps in sepsis immunology: new opportunities for translational research. LANCET INFECT DIS. 2019;19(12):e422–36.

31. Rogers WK, McDowell TS. Remimazolam, a short-acting GABA(A) receptor agonist for intravenous sedation and/or anesthesia in day-case surgical and non-surgical procedures. IDrugs. 2010;13(12):929–37.

32. Goudra BG, Singh PM. Remimazolam. The future of its sedative potential. Saudi J Anaesth. 2014;8(3):388–91.

33. Lakhani SA, Bogue CW. Toll-like receptor signaling in sepsis. CURR OPIN PEDIATR. 2003;15(3):278–82.

34. Bode JG, Ehlting C, Häussinger D. The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. CELL SIGNAL. 2012;24(6):1185–94.

35. Lim KH, Staudt LM. Toll-like receptor signaling. Cold Spring Harb Perspect Biol. 2013;5(1):a11247.

36. Parnas O, Jovanovic M, Eisenhaure TM, et al. A Genome-wide CRISPR Screen in Primary Immune Cells to Dissect Regulatory Networks. CELL. 2015;162(3):675–86.

37. Saitoh S. Chaperones and transport proteins regulate TLR4 trafficking and activation. IMMUNOBIOLOGY. 2009;214(7):594–600.

38. Li F, Liu J, Liu N, et al. Translocator Protein 18 kDa (TSPO): An Old Protein with New Functions? BIOCHEMISTRY-US. 2016;55(20):2821–31.

39. Vowinckel E, Reutens D, Becher B, et al. PK11195 binding to the peripheral benzodiazepine receptor as a marker of microglia activation in multiple sclerosis and experimental autoimmune encephalomyelitis. J NEUROSCI RES. 1997;50(2):345–53.

40. Kumar A, Muzik O, Chugani D, et al. PET-derived biodistribution and dosimetry of the benzodiazepine receptor-binding radioligand (11)C-(R)-PK11195 in children and adults. J NUCL MED. 2010;51(1):139–44.
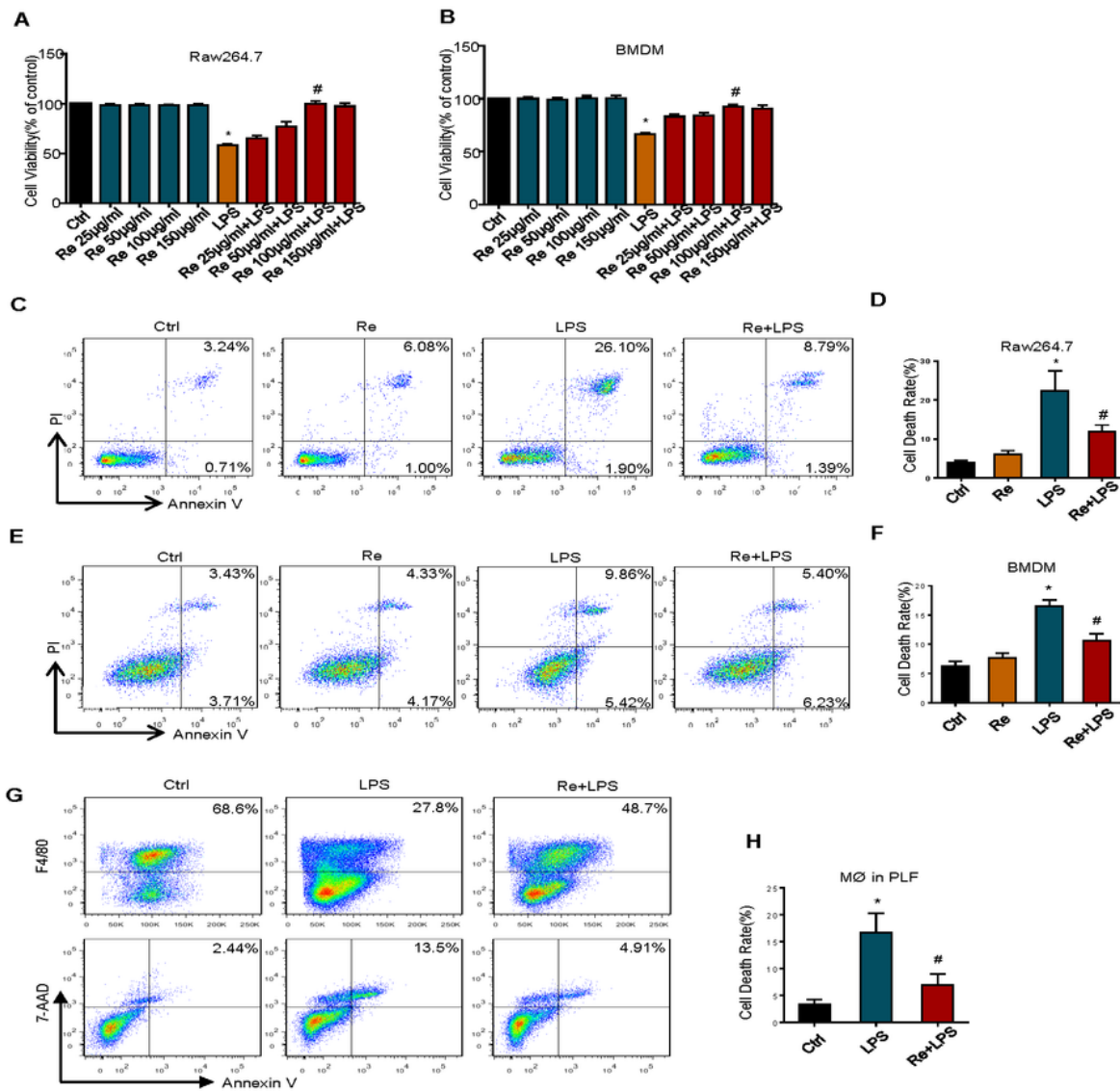
41. Declarations.

Figures

Figure 1

Remimazolam improved LPS-induced cell death in vitro and vivo. Raw264.7 cells(A) and BMDM(B) were exposed to LPS (1μg/mL, 24h) with or without different concentration of remimazolam. The CCK8 assay assessed cell viability. C, D Raw264.7 cells were treated with LPS (1 μg/ml) for 24 h in the presence or absence of pretreatment of remimazolam (Re) for 20 min followed by flow cytometry analysis of cell death. E, F BMDM were treated with LPS (1 μg/ml) for 24 h in the presence or absence of pretreatment of

remimazolam (Re) for 20 min followed by flow cytometry analysis of cell death. G, H Wild type mice were treated as described in materials and methods. Peritoneal lavage fluids were collected at 12 h after LPS treatment and peritoneal macrophages were identified with F4/80. Cell death was analysis by flow cytometry. All flow cytometric plots are the representative from at least 5 experiments. Each bar represents the mean ± S.D, * p < 0.05, compared with control group; # p <0.05, compared with LPS group.
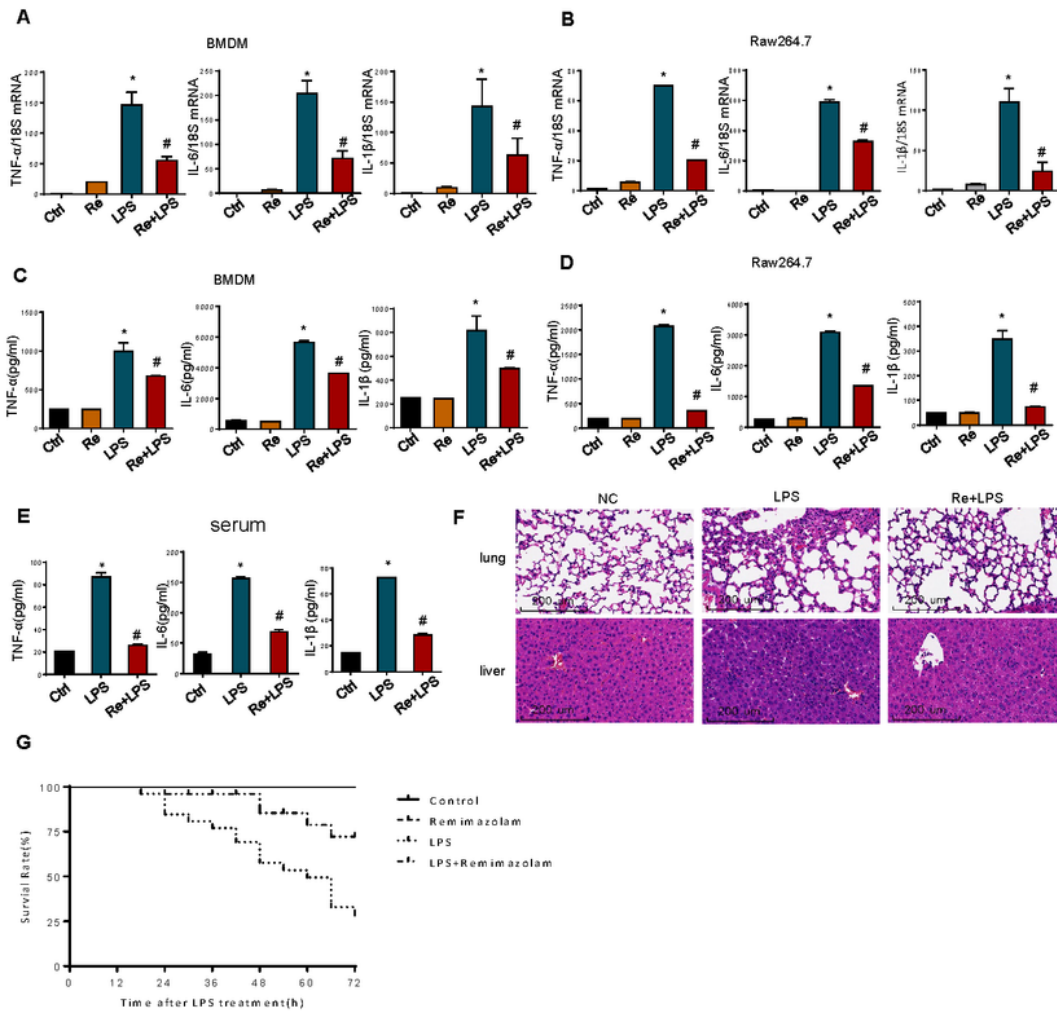

Figure 2

Remimazolam inhibits LPS-induced inflammatory response in vitro and vivo. Please see manuscript for complete figure caption.
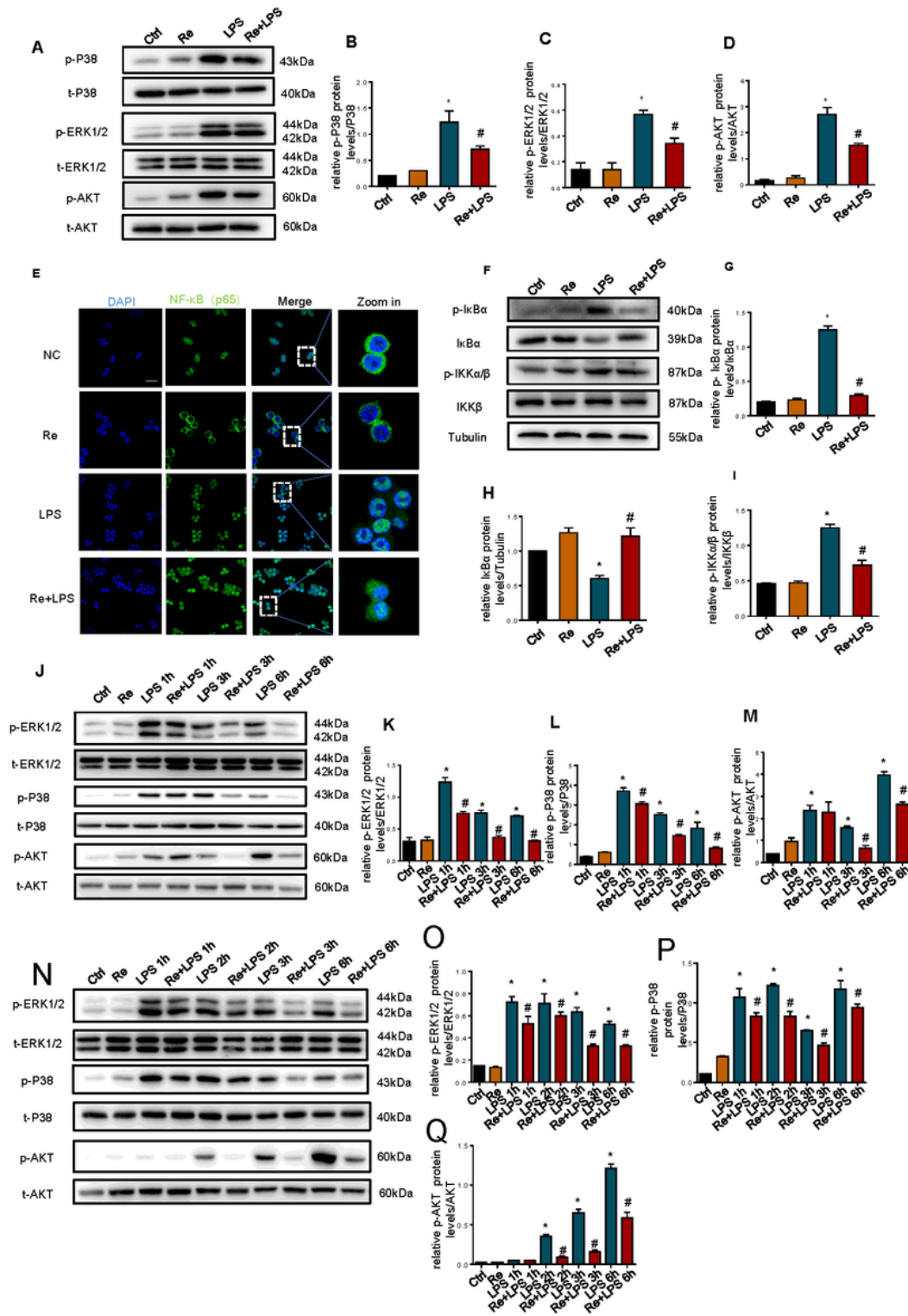

Figure 3

Suppressive effects of remimazolam on LPS induced activation of the MAPK and PI3K signaling pathway in macrophage. Please see manuscript for complete figure caption.
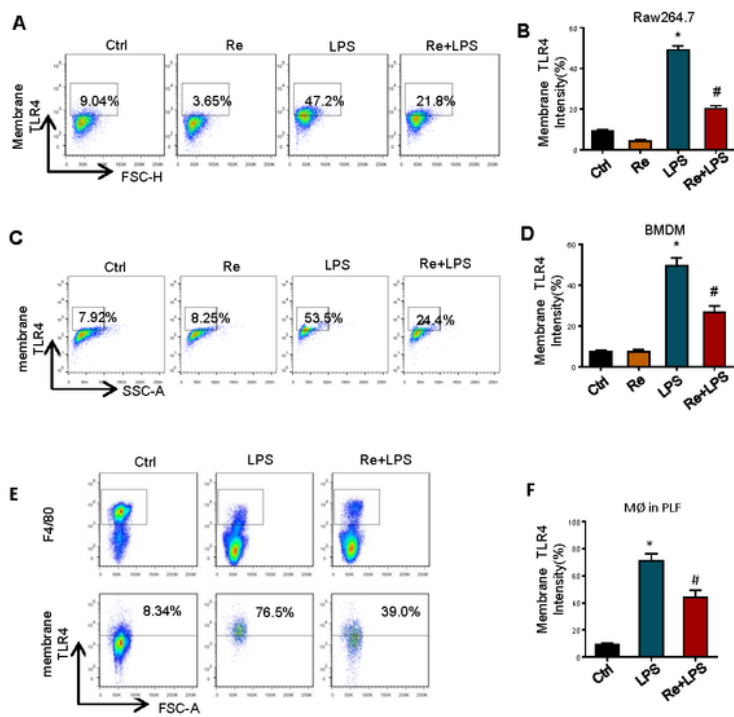
Figure 4

Remimazolam improved LPS-induced cell death in vitro and vivo. Raw264.7 cells(A) and BMDM(B) were exposed to LPS (1μg/mL, 24h) with or without different concentration of remimazolam. The CCK8 assay assessed cell viability. C, D Raw264.7 cells were treated with LPS (1 μg/ml) for 24 h in the presence or absence of pretreatment of remimazolam (Re) for 20 min followed by flow cytometry analysis of cell death. E, F BMDM were treated with LPS (1 μg/ml) for 24 h in the presence or absence of pretreatment of

remimazolam (Re) for 20 min followed by flow cytometry analysis of cell death. G, H Wild type mice were treated as described in materials and methods. Peritoneal lavage fluids were collected at 12 h after LPS treatment and peritoneal macrophages were identified with F4/80. Cell death was analysis by flow cytometry. All flow cytometric plots are the representative from at least 5 experiments. Each bar represents the mean ± S.D, * $p < 0.05$, compared with control group; # $p < 0.05$, compared with LPS group.
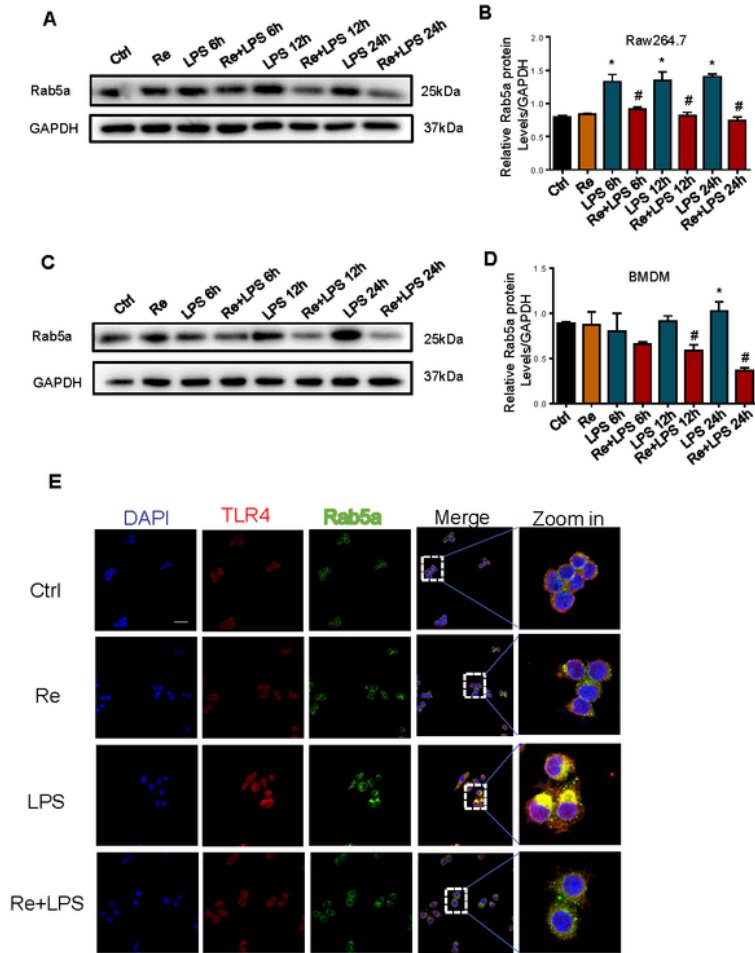

Figure 5

Remimazolam regulate the cell surface expression of TLR4 after LPS treatment by affecting functions and expressions of RAB5a. A (Raw264.7 cells), C (BMDM): Western blot analysis of Rab5a expression. B, D Protein presence of Rab5a was normalized to GAPDH. E Immune-staining of TLR4 with Rab5a (up panel; Scale bar, 100μm.). Each bar represents the mean ± S.D (n = 3), * p < 0.05, compared with control group; # p <0.05, compared with LPS group.
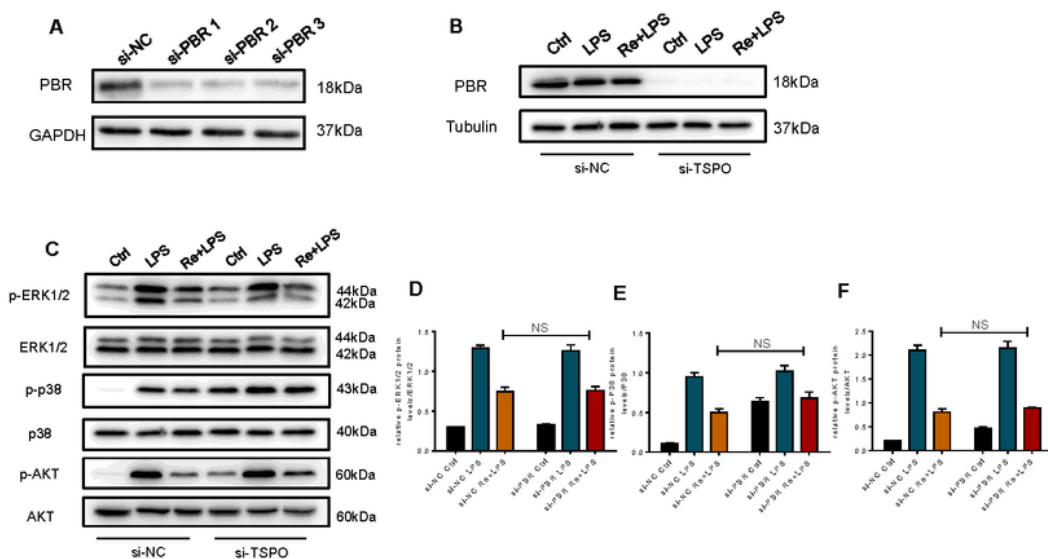

Figure 6

The anti-inflammatory effect of Remimazolam on macrophages was not exerted via the PBR. A, B Efficiency of si-PBR in Raw265.7 cells. Raw264.7 cells were treated with LPS for 3 h. In some groups, Raw264.7 cells were treated with remimazolam (Re) for 20 min or pre-transfected with si-NC, si-TSPO for 48 h before LPS treatment. C Western blot analysis of p-ERK1/2, ERK1/2, p-P38, P38, p-AKT and AKT protein expression. D, E, F Protein presence of p-ERK1/2, p-P38 and p-AKT was normalized to ERK1/2, P38 and AKT respectively. Each bar represents the mean ± S.D, $* p < 0.05$, compared with control group; $\# p < 0.05$, compared with LPS group; NS no significance.

Supplementary Files

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- Graphicalabstract.pdf