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YTHDF1-mediated m6A modification of GBP4 promotes M1 macrophage polarization in acute lung injury

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Abstract

Background Acute lung injury (ALI) is a severe condition with multifaceted causes, including inflammation and oxidative stress. This research investigates the influence of m6A (N6-methyladenosine) modification on GBP4, a protein pivotal for macrophage polarization, a critical immune response in ALI.

Methods Utilizing a mouse model to induce ALI, the study analyzed GBP4 expression in alveolar macrophages. By overexpressing or knocking down GBP4, the study assessed its impact on M1 macrophage polarization. The role of YTHDF1 was also explored through knockdown experiments to determine its effect on GBP4 expression and macrophage polarization.

Results Increased GBP4 expression was noted in ALI model mice, promoting M1 macrophage polarization. YTHDF1 was found to enhance GBP4 expression by recognizing m6A sites on its mRNA, which was linked to reduced inflammation in MLE-12 cells upon YTHDF1 knockdown.

Conclusion The study emphasizes the crucial roles of GBP4 and YTHDF1 in ALI development and immune response regulation. It suggests m6A modification as a potential therapeutic target, contributing to the understanding of ALI's molecular mechanisms and guiding future treatment strategies.

Keywords m6A, ALI, Macrophage polarization, YTHDF1, GBP4

Introduction

In recent years, research on acute lung injury (ALI) has progressed rapidly, with a focus on its pathogenesis, prevention, and treatment. Studies have indicated that the pathogenesis of ALI involves multiple aspects, including inflammation, oxidative stress, and apoptosis, with cytokines and chemical mediators playing significant roles in the inflammatory process [1, 2]. In terms of prevention, the emphasis is on optimizing mechanical ventilation strategies, implementing lung-protective ventilation strategies, and applying measures such as fluid management [2, 3]. Regarding treatment, research has concentrated on the efficacy and safety of therapeutic measures such as glucocorticoids, bioresponse modifiers, oxygen therapy, and respiratory support [2, 4]. However, the pathogenesis of ALI remains not fully understood.

The regulatory mechanisms and biological functions of m6A modification within cells have garnered significant attention, and its role in ALI research is increasingly becoming a hot topic. As a common RNA chemical

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modification, m6A modification is involved in various biological processes, such as RNA stability, transcription, translation, and degradation, playing a crucial role in the regulation of gene expression [5]. In ALI research, m6A modification not only affects inflammatory responses and immune regulation but is also closely associated with pathological processes such as apoptosis [6] and oxidative stress [7, 8]. Recent findings have shown that m6A modification levels significantly change in ALI models [9], and both m6A modification enzymes and reader proteins play important roles in the development of ALI [9]. Additionally, some studies suggest that m6A modification may influence the occurrence and progression of ALI by regulating the expression of specific genes, affecting the function and immune response of alveolar epithelial cells [9]. Although research on m6A modification in ALI is still in its infancy, its potential role has already attracted widespread interest.

As a key inflammatory regulator, GBP4, a member of the Guanylate-binding protein (GBP) family and an interferon-induced gene, plays a significant role in immune regulation and antimicrobial defense [10]. Research indicates that GBP4 may play an important role in the occurrence and development of ALI [10–12]. Firstly, the expression of GBP4 in immune cells is regulated by various stimuli, and its levels are significantly upregulated under inflammatory conditions [11]. This suggests the potential role of GBP4 in inflammatory responses. Secondly, GBP4 may affect inflammation by regulating it, with some studies showing that overexpression of GBP4 promotes the activation of inflammasomes, thereby mediating pathogen clearance [10, 12]. Furthermore, GBP4 may also influence the aggregation of inflammatory cells and the formation of local inflammation by regulating the activity and migration of immune cells [11]. However, the specific mechanisms of GBP4 in ALI are not yet fully understood and require further research for clarification.

The aim of this study is to investigate the expression of GBP4 in macrophages in ALI, assess the impact of GBP4 on macrophage polarization, and elucidate the regulatory mechanism of m6A on GBP4. This research seeks to reveal the specific mechanisms of GBP4 in the development of ALI, providing a theoretical foundation and a new perspective for the formulation of future therapeutic strategies.

Materials and methods

Animal model and detection

Mice used in this study were housed with free access to food and water under protocols approved by the Zhengzhou University Institutional Animal Care and Use Committee. The animals were acclimated to laboratory conditions (23 °C, 12 h light/12 h dark, 50% humidity, with free access to food and water) for two weeks prior to experimentation. All animals were euthanized using barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection. After random assignment, mice were treated with LPS (concentration 100 μg/ml, dose 2 μl/g body weight) via intratracheal instillation, and 24 h later, their lungs were harvested for analysis.

HE staining

The mice were euthanized, and their lungs were removed and fixed in 4% paraformaldehyde overnight at 4 °C. Hematoxylin and eosin (H&E) staining was employed to analyze the morphology of lung tissues. Briefly, paraffin-embedded samples were deparaffinized and processed through a graded series of alcohol concentrations, followed by HE staining.

Preparation of alveolar macrophages

Dissociate lung tissue and incubate with anti-F4/80 biotinylated antibody for 2 h. Subsequently, incubate with streptavidin magnetic beads for 1 h, and then collect the cells using a magnetic rack. Fix the cells with 4% paraformaldehyde, and use an acidic elution buffer to remove the magnetic beads, followed by incubation with antibodies specific for M1 or M2 macrophage markers. Alternatively, skip cell fixation and directly add 5× SDS-PAGE Loading Buffer for Western Blot (WB) analysis.

Cell lines and cell culture

Mouse lung epithelial cell line MLE-12 and mouse mononuclear macrophage RAW264.7 were purchased from Servicebio (STCC20008P). MLE-12 was cultured in 90% DMEM/F-12 medium (HEPES, with L-glutamine) (Servicebio, G4612) supplemented with 10% FBS (Servicebio, G8002-100ML). RAW264.7 was cultured in 90% DMEM medium (Servicebio, G4511) with 10% FBS (Servicebio, G8002-100ML). All cells were cultured in a 37 °C, 5% CO2 incubator and passaged when reaching 90% confluence. Cells were seeded at 5×10^5 per well in a 6-well plate or 1×10^5 per well in a 12-well plate, and cultured until reaching 80% confluence for drug and transfection treatments. The concentration of LPS (Lipopolysaccharides, Macklin, L861706-5 mg) used for culturing cells was 100ng/ml, and cells were collected 24 h after LPS addition. The concentration of IL4 (Interleukin-4, Beyotime, P5849) used was 10ng/mL, with a treatment duration of 24 h. The sequences used for GBP4 knockdown were CTACGTGAGAAGAATGGGCTT, for YTHDF1 knockdown were GCTGAAGATTATCGCTTCCTA, and for METTL3 knockdown were CCTCAGTGGAT CTGTTGTGAT. Cells were transfected with plasmids using Lipo8000™ transfection reagent (Beyotime, C0533-1.5 ml) 2 h post-transfection, and the medium was

changed 24 h later, or drug treatment was applied. Stable knockdown cell lines were also constructed using these gene sequences.

Flow cytometry

After RAW264.7 cells were polarized with LPS and IL4 induction (three repetitions per group), they were stained with CoraLite® Plus 488 Anti-Mouse CD86 (Proteintech, CL488-65068), CoraLite® Plus 647-conjugated iNOS (Proteintech, CL647-18985), CD206 Polyclonal antibody (Proteintech, 18704-1-AP) and CoraLite® Plus 488-Goat Anti-Rabbit Recombinant Secondary Antibody (H+L) (Proteintech, RGAR002), CD163 Polyclonal antibody (Proteintech, 16646-1-AP) and CoraLite® Plus 647-Goat Anti-Rabbit Recombinant Secondary Antibody (Proteintech, RGAR005). Reactive oxygen species (ROS) was detected using a reactive oxygen species detection kit (Beyotime, S0033S).

Western blot (WB)

Tissues or cells were lysed in RIPA buffer (50 mM Tris, 1% NP-40, 140 mM NaCl, and 0.1% sodium dodecyl sulfate, pH 7.6). Proteins were extracted and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Pall Corporation), which was then blocked with 5% BSA or skimmed milk in Tris-buffered saline containing 0.1% Tween 20 and probed with antibodies against GBP4 (17746-1-AP), GAPDH (60004-1-Ig), IL-1β (26048-1-AP), IL-6 (26404-1-AP), IL-10 (82793-16-RR), Arg1 (16001-1-AP), YTHDF1 (17479-1-AP), m6A (68055-1-Ig), METTL3 (15073-1-AP), respectively.

RNA extraction, cDNA synthesis, and PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was reverse-transcribed using SynScript® III RT SuperMix for qPCR (+gDNA Remover) (TSINGKE) followed by PCR. β-actin RNA was used as an endogenous control, and each reaction was run in triplicate. The primers used in PCR were 5'- GAAGGAAGCTGGAGC AGGAG −3' and 5'- CGACGCAATTCCTCAGCTTG −3' for GBP4; 5'- GATATCGCTGCGCTGGTCG −3' and 5'- TCCATGTCGTCCCAGTTGGT −3' for β-actin.

ELISA for TNF-α and IL-1β

Serum or supernatant levels of IL-1β and TNF-α were quantified by enzyme-linked immunosorbent assay (ELISA) system kits (Proteintech). Briefly, 100 μl of serum and standard samples were added in duplicate into the wells, covered, and incubated overnight at 4 °C. After removing unbound antigens, 100 μl of detection antibody was added. Excess polyclonal antibodies were removed, and 100 μl of HRP-conjugated IgG was added. Following a final wash, bound peroxidase activity was quantified using 100 μl of TMB. The reaction was stopped after 10 min, and OD values at 450 nm were measured using a plate reader. Levels in individual samples were calculated based on standard curves.

Detection of lactate dehydrogenase (LDH) and superoxide dismutase (SOD) activity

LDH activity in serum and cells was detected using the LDH Cytotoxicity Assay Kit (A020-2, Jiancheng Bioengineering Institute, Nanjing, China). Total SOD activity in serum and cells was measured using the Superoxide Dismutase (SOD) assay kit (WST-1 method) (A001-3-2, Jiancheng Bioengineering Institute, Nanjing, China).

Biotin-labeled RNA pulldown assay

Proteins were extracted from RAW264.7 cells using IP Lysis Buffer. The lysate was mixed with biotin-labeled RNA probes, allowing proteins to bind to the RNA probes. The mixture was incubated for 3 h at 4 °C, followed by the addition of streptavidin or streptomycin-coated magnetic beads. After incubation, non-specifically bound proteins and other cellular components were washed away. The magnetic beads, along with the pulled-down protein complexes, were collected and the proteins were separated by SDS-PAGE electrophoresis and subsequently analyzed by immunoblotting assay.

Statistical analysis

All summary results are presented as the mean ± the standard error of the mean (s.e.m.). Multiple groups were analyzed using one-way ANOVA, and differences between two groups were assessed using the Student's t-test; * indicates $P<0.05$, ** indicates $P<0.01$, and n.s. indicates not significant ($P>0.05$). Data analysis was performed using GraphPad Prism software, and each experiment was conducted at least three times unless otherwise specified. There were at least three replicates per group in all experiments.

Results

GBP4 expression is elevated in alveolar macrophages in ALI models

In this study, we aimed to investigate the expression changes of GBP4 in alveolar macrophages during acute lung injury (ALI) and its potential biological significance. To achieve this goal, we first used LPS to induce ALI in a mouse model and assessed the extent of lung injury through histological and biochemical analyses. LPS, as an exogenous pathogen-associated molecular pattern (PAMP), can simulate the inflammatory response caused by bacterial infection and is a commonly used model for studying the pathogenesis of ALI [13]. However, ALI is a complex clinical syndrome whose pathological process involves multiple factors. Although the LPS model can

mimic some characteristics of ALI, it is important to recognize its limitations in fully representing the entire spectrum of the disease [14, 15]. Subsequently, we isolated alveolar macrophages and analyzed GBP4 expression using flow cytometry and Western blot (WB) techniques. By preparing lung sections and performing hematoxylin-eosin (HE) staining, we observed significant tissue damage in the lungs of ALI model mice (Fig. 1A), characterized by the destruction of alveolar structures. To further quantify the level of inflammation, we measured the levels of reactive oxygen species (ROS), lactate dehydrogenase (LDH), and superoxide dismutase (SOD) in lung tissue. The results showed that compared to the control group, ALI model mice had significantly increased ROS and LDH levels (Fig. 1B and C), while SOD activity decreased (Fig. 1D), indicating that the inflammatory response was significantly activated during ALI. Additionally, we detected the levels of inflammatory factors IL1β and TNFα by ELISA, and the results also confirmed the enhancement of the inflammatory response (Fig. 1E and F). In the analysis of macrophages, we used flow cytometry to distinguish between M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. The results showed that the proportion of M1 macrophages significantly increased in ALI model mice, indicating a tendency for the lungs to produce more pro-inflammatory macrophages during ALI (Fig. 1G). This finding is consistent with the observed increase in inflammatory factor levels and further supports the activation of the inflammatory response during ALI. To directly assess GBP4 expression in macrophages, we performed Western blot analysis on isolated alveolar macrophages. The results showed that the expression level of GBP4 protein was significantly higher in macrophages from ALI model mice than in the control group (Fig. 1H). This finding suggests that GBP4 may play an important role in ALI, particularly in regulating the inflammatory response and macrophage polarization. In summary, our study results reveal an upregulation of GBP4 expression in alveolar macrophages during ALI and a possible association with the enhancement of the inflammatory response and the increase of M1 macrophages.

A. Lung tissue was harvested at 48 h post-ALI induction with LPS in mice, followed by HE staining. Whole blood from NC and model group mice was collected for flow cytometry detection of ROS (B), biochemical lactate dehydrogenase (LDH) (C), and SOD (D), as well as ELISA detection of TNF-α (E) and IL-1β (F). G. After isolating macrophages from lung tissue, the ratio of M1 to M2 macrophages was assessed. H. Expression of GBP4 in macrophages from lung tissue was examined. There were at least three replicates per group in all experiments. Differences between two groups were assessed using the Student's t-test; * indicates $P<0.05$, ** indicates $P<0.01$.

Knockdown of GBP4 inhibits M1 macrophage polarization

To investigate the role of GBP4 in the polarization process of macrophages, especially in the context of acute lung injury (ALI), we used the mouse macrophage cell line RAW264.7 as a model to knock down the expression of GBP4 protein and further study its impact on LPS (lipopolysaccharide)-induced M1 polarization and IL4 (interleukin 4)-induced M2 polarization. First, we verified the efficiency of GBP4 knockdown using Western

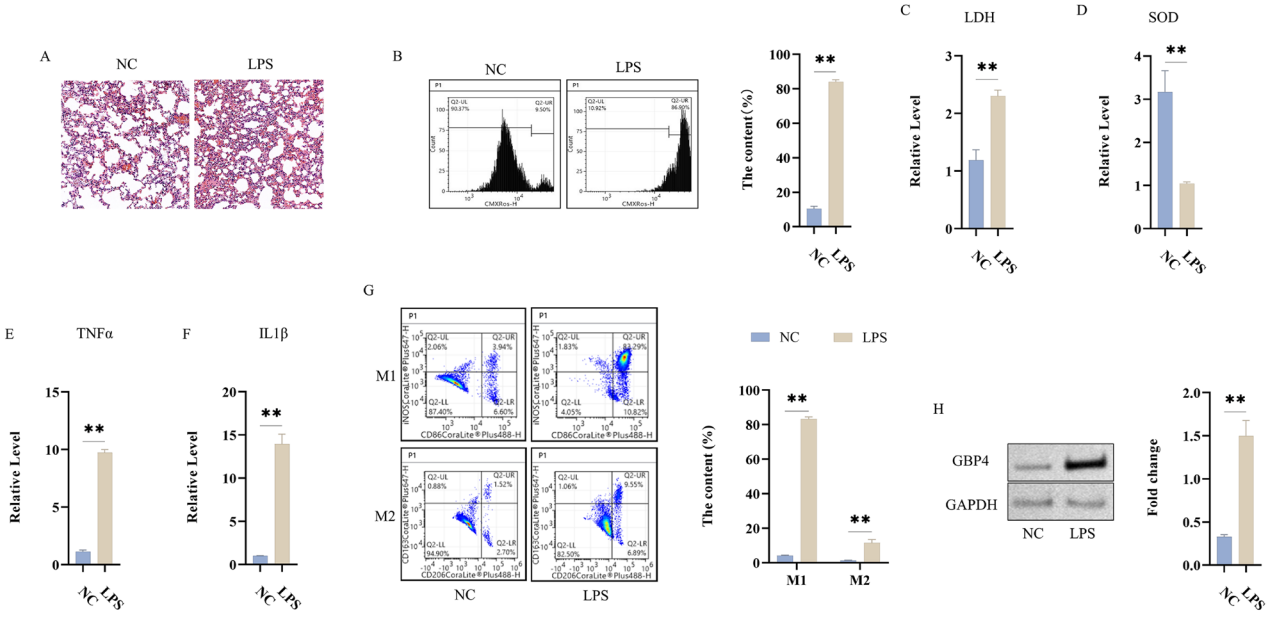

Fig. 1 Increased Expression of GBP4 in Macrophages during ALI

blot technology (Fig. 2A). After confirming a significant reduction in GBP4 expression levels, we treated RAW264.7 cells with LPS and IL4 to simulate M1 and M2 macrophage polarization, respectively. We observed that under LPS treatment, the expression level of GBP4 increased, suggesting that GBP4 may play a role in the M1 polarization process; however, IL4 treatment had no significant effect on the expression of GBP4, which may indicate a limited role of GBP4 in M2 polarization (Fig. 2B and C). After knocking down GBP4, we further analyzed the expression of M1 macrophage markers IL1β and IL6. The results showed that the expression levels of these pro-inflammatory factors significantly decreased in GBP4 knockdown cells treated with LPS, indicating that GBP4 promotes the polarization process of M1 macrophages (Fig. 2B). However, when we observed the expression of M2 macrophage markers IL10 and Arg1, we found that the expression levels of these anti-inflammatory factors did not significantly change in GBP4 knockdown RAW264.7 cells (Fig. 2B). This suggests that the role of GBP4 in M2 polarization may not be significant, or its effects are masked by other factors. Our study results reveal the dual role of GBP4 in the polarization process of macrophages, especially its key role in M1 polarization.

(A) Four shRNAs were used to knock down (KD) GBP4, followed by WB to assess the knockdown efficiency, with shRNA1 showing the most effective knockdown. (B) After knocking down or not knocking down GBP4, cells were treated with or without LPS or IL4, and the expression of GBP4 as well as M1 (IL1β, IL6) and M2 (IL10, Arg1) macrophage markers was detected. (C) Flow cytometry was used to assess the polarization of M1 and M2 macrophages. There were at least three replicates per group in all experiments.

YTHDF1 can regulate the expression of GBP4 through m6A modification

m6A modification is a widely prevalent RNA chemical modification on eukaryotic mRNA that has a significant impact on the regulation of gene expression. After analyzing the m6A sites on GBP4 mRNA, we found multiple potential m6A modification sites in its coding region (Fig. 3A). To verify the existence of these sites, we performed MeRIP-PCR experiments with m6A-specific antibodies. The results showed that compared to the IgG control group and the Input group, the MeRIP group had a significantly higher level of GBP4, confirming the presence of m6A modification in the coding region of GBP4 mRNA (Fig. 3B). Subsequently, we repeated this experiment in RAW264.7 cells treated with LPS to observe changes in m6A modification when GBP4 expression increased. The results indicated that the m6A level in the GBP4 coding region significantly increased after LPS treatment (Fig. 3C), suggesting that m6A modification may play a role in the regulation of GBP4 expression.

To further explore which specific m6A enzyme is involved in the regulation of GBP4 mRNA, we conducted an RNA pulldown-WB experiment. Western blot results showed that the YTHDF1 protein played a key role in the m6A modification of GBP4 mRNA (Fig. 3D). YTHDF1 is a protein involved in the methylation of adenosine residues (m6A), which regulates various physiological processes by controlling the stability, translation efficiency,

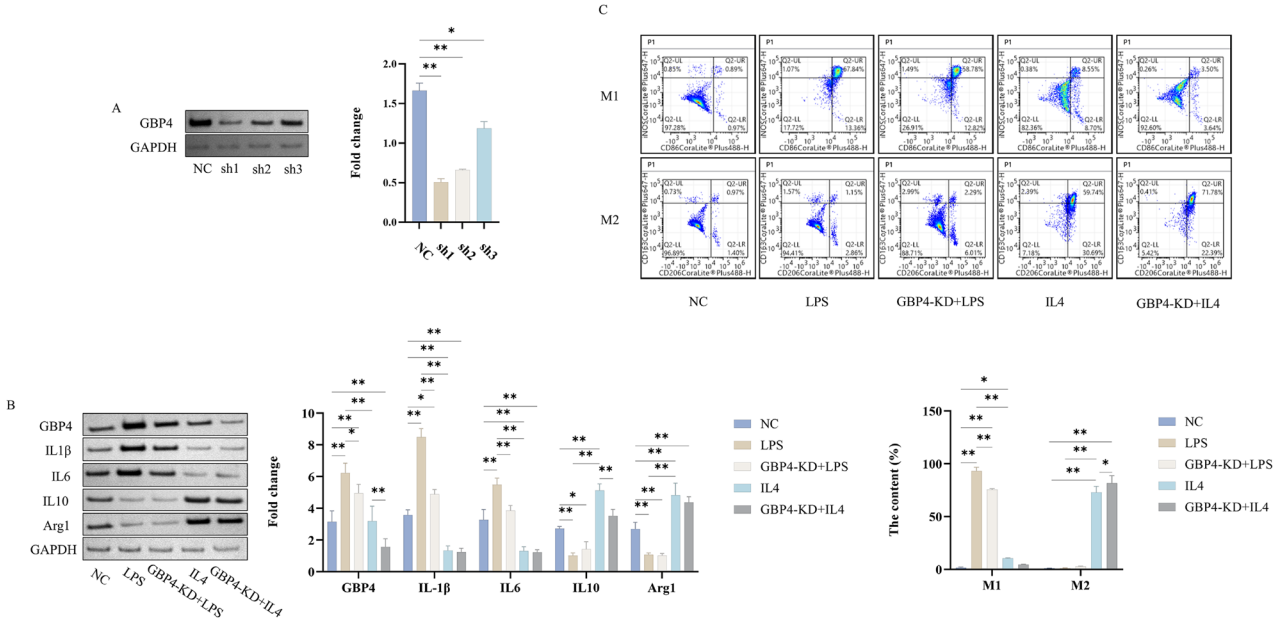

Fig. 2 Knockdown of GBP4 Inhibits M1 Macrophage Polarization

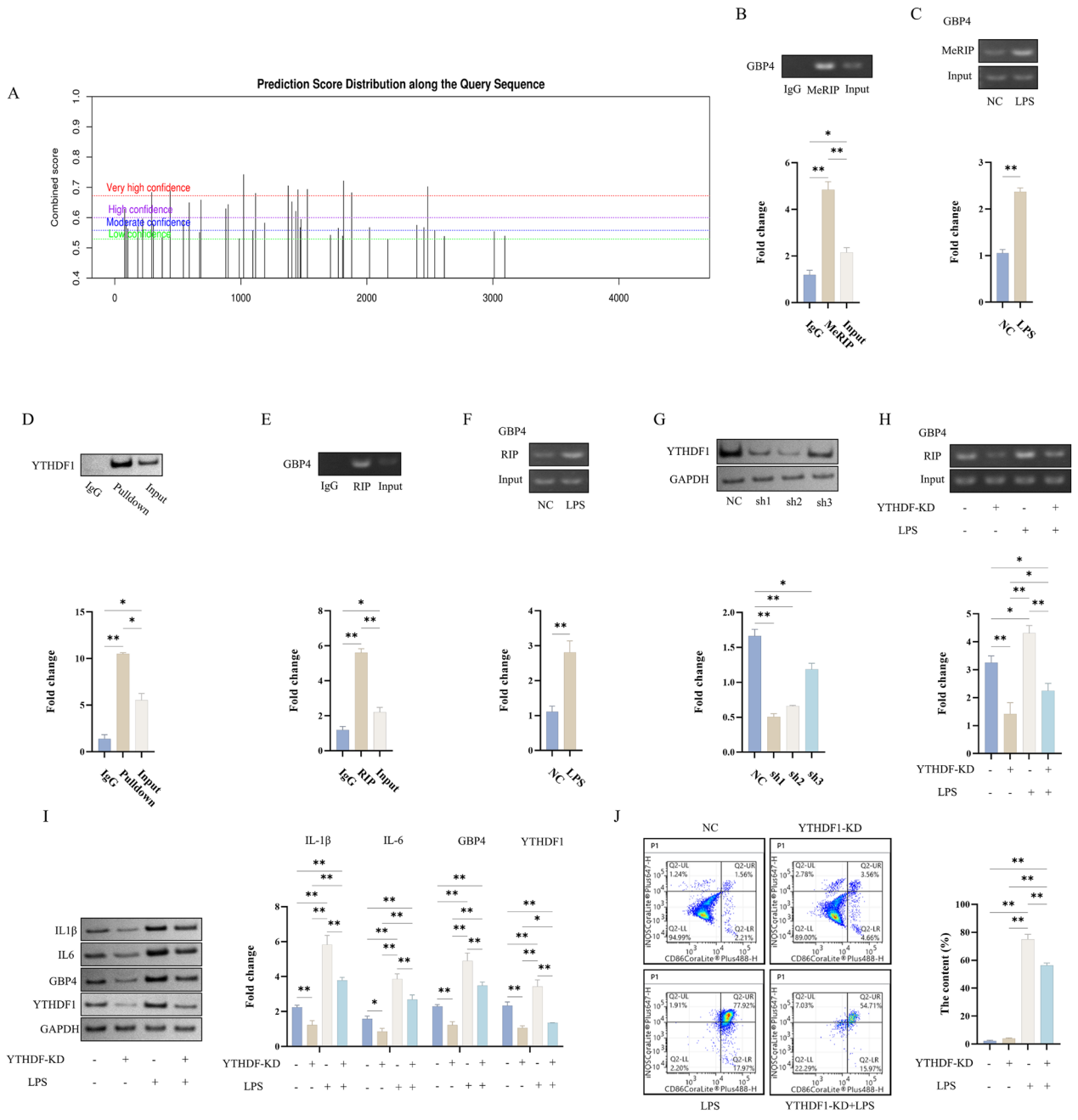

Fig. 3 YTHDF1 Regulates GBP4 Expression through m6A Modification

and RNA interactions of RNA [16]. This finding was further verified by YTHDF1 RIP-PCR experiments, which demonstrated that the YTHDF1 protein could recognize and bind to m6A sites in GBP4 mRNA (Fig. 3E). After confirming the interaction between YTHDF1 and GBP4 mRNA, we further studied the impact of LPS treatment on this process. RIP-PCR experiments revealed that after LPS treatment, the binding level of GBP4 mRNA to YTHDF1 significantly increased (Fig. 3F), indicating that the interaction between GBP4 mRNA and YTHDF1 is enhanced during the polarization process of M1 macrophages.

To assess the function of YTHDF1 in GBP4 expression and M1 macrophage polarization, we knocked down YTHDF1 in RAW264.7 cells (Fig. 3G) and treated them with LPS. The results showed that after knocking down YTHDF1, the binding of GBP4 mRNA to YTHDF1 significantly decreased (Fig. 3H), and the expression of GBP4 protein was also inhibited (Fig. 3I). Additionally, the expression levels of M1 macrophage markers decreased

(Fig. 3I). Flow cytometry analysis indicates that the polarization process of M1 macrophages is significantly inhibited after the knockdown of YTHDF1 (Fig. 3J).

In summary, our study reveals that YTHDF1 may regulate the expression of GBP4 by recognizing m6A modification sites on GBP4 mRNA and play a role in the polarization process of M1 macrophages.

(A) Prediction of m6A modification sites on GBP4 mRNA. (B) MeRIP assay was performed in RAW264.7 cells, followed by PCR to detect GBP4 levels. (C) MeRIP-PCR was used to detect GBP4 levels in control and LPS-treated RAW264.7 cells, with Input group GBP4 as an internal control. (D) RNA pulldown assay was conducted with a GBP4 RNA probe in RAW264.7 cells, followed by WB using a YTHDF1 antibody to detect YTHDF1 expression levels. (E) RIP assay using a YTHDF1 antibody was performed, followed by PCR to detect GBP4 levels. (F) RIP assay using a YTHDF1 antibody was conducted in LPS-treated or untreated RAW264.7 cells, to detect GBP4 expression levels, with Input group GBP4 as an internal control. (G) YTHDF1 was knocked down using shRNA, and WB confirmed the knockdown efficiency, with shRNA2 showing the most effective knockdown. After knocking down or not knocking down YTHDF1 in RAW264.7 cells, they were treated with or without LPS, followed by RIP using a YTHDF1 antibody and then PCR to detect GBP4 levels, with Input group GBP4 as an internal control (H); WB to detect M1 macrophage markers (IL1β, IL6) as well as YTHDF1 and GBP4 (I); and flow cytometry to assess M1 macrophage polarization (J). There were at least three replicates per group in all experiments.

The YTHDF1 protein can recognize m6A sites on GBP4 and promote the translation of GBP4 mRNA

To explore the regulatory mechanism of YTHDF1 on GBP4, we first determined the expression changes of GBP4 mRNA within 9 h of LPS treatment in RAW264.7 cells. We observed that within the first 6 h of LPS treatment, there were no significant changes in the mRNA and protein levels of GBP4 (Fig. 4A and B). This finding provided a basis for our subsequent experiments, indicating that the expression of GBP4 was not significantly affected in the early stages of LPS treatment. Subsequently, we investigated the changes in GBP4 mRNA and protein after knocking down YTHDF1 and treating with LPS for 6 h. We found that after knocking down YTHDF1, although the level of GBP4 mRNA remained unchanged, its protein level significantly decreased (Fig. 4C and D). This suggests that YTHDF1 can promote the translation of GBP4 mRNA.

To verify whether YTHDF1 regulates the expression of GBP4 through m6A modification, we knocked down the METTL3 gene (Fig. 4E), which is a known m6A methyltransferase. Using MeRIP technology, we detected the m6A modification level of GBP4 mRNA and found that after knocking down METTL3, the m6A level of GBP4 significantly decreased, meaning that the enrichment of GBP4 in the m6A antibody significantly reduced (Fig. 4F). Additionally, YTHDF1-RIP results also showed that after knocking down METTL3, the enrichment of GBP4 in YTHDF1 significantly decreased (Fig. 4G). In RAW264.7 cells with METTL3 knocked down, we further observed the changes in GBP4 expression after LPS treatment. The results showed that after knocking down METTL3, the increase in GBP4 was significantly inhibited (Fig. 4H).

To more precisely understand the impact of m6A modification on the translation of GBP4, we knocked down METTL3 and treated with LPS at 0, 3, 6, and 9 h to detect the expression of GBP4. We found that after knocking down METTL3, the translation of GBP4 was inhibited, especially at 9 h after LPS treatment, although the level of GBP4 mRNA increased, the protein level did not significantly change (Fig. 4I and J). Moreover, within 0–6 h after METTL3 knockdown, although the level of GBP4 mRNA did not significantly differ from before the knockdown, the protein level of GBP4 was significantly lower than that of the METTL3 group (Fig. 4I and J). The above results indicate that in the absence of m6A modification, the translation of GBP4 is inhibited.

To verify whether YTHDF1 regulates the translation of GBP4 mRNA through m6A, we overexpressed YTHDF1 and/or knocked down METTL3 in RAW264.7 cells, and treated RAW264.7 with LPS for 0, 3, 6, and 9 h to detect the levels of GBP4 mRNA and protein. The results showed that compared with the group with METTL3 knockdown alone, in the group where YTHDF1 was overexpressed while METTL3 was knocked down, the expression of GBP4 was not significantly affected within 0–9 h. That is, overexpression of YTHDF1 did not affect the expression of GBP4 when METTL3 was knocked down (Fig. 4K and L). The above results indicate that YTHDF1 affects the translation of GBP4 through m6A.

Finally, we validated that YTHDF1 regulates M1 macrophage polarization by controlling GBP4 through m6A. We knocked down METTL3 and/or overexpressed YTHDF1 in RAW264.7 cells, treated them with LPS for 24 h, and detected the markers of M1 polarization and the extent of M1 differentiation. The results showed that after knocking down METTL3, although LPS treatment could significantly increase the expression of GBP4, the degree of increase in GBP4 was significantly lower compared to the LPS treatment alone group, and even overexpression of YTHDF1 could not further increase the expression of GBP4 (Fig. 4M). The expression of IL1β and IL6 was also affected in the same way, that is, the knockdown of METTL3 significantly inhibited the expression

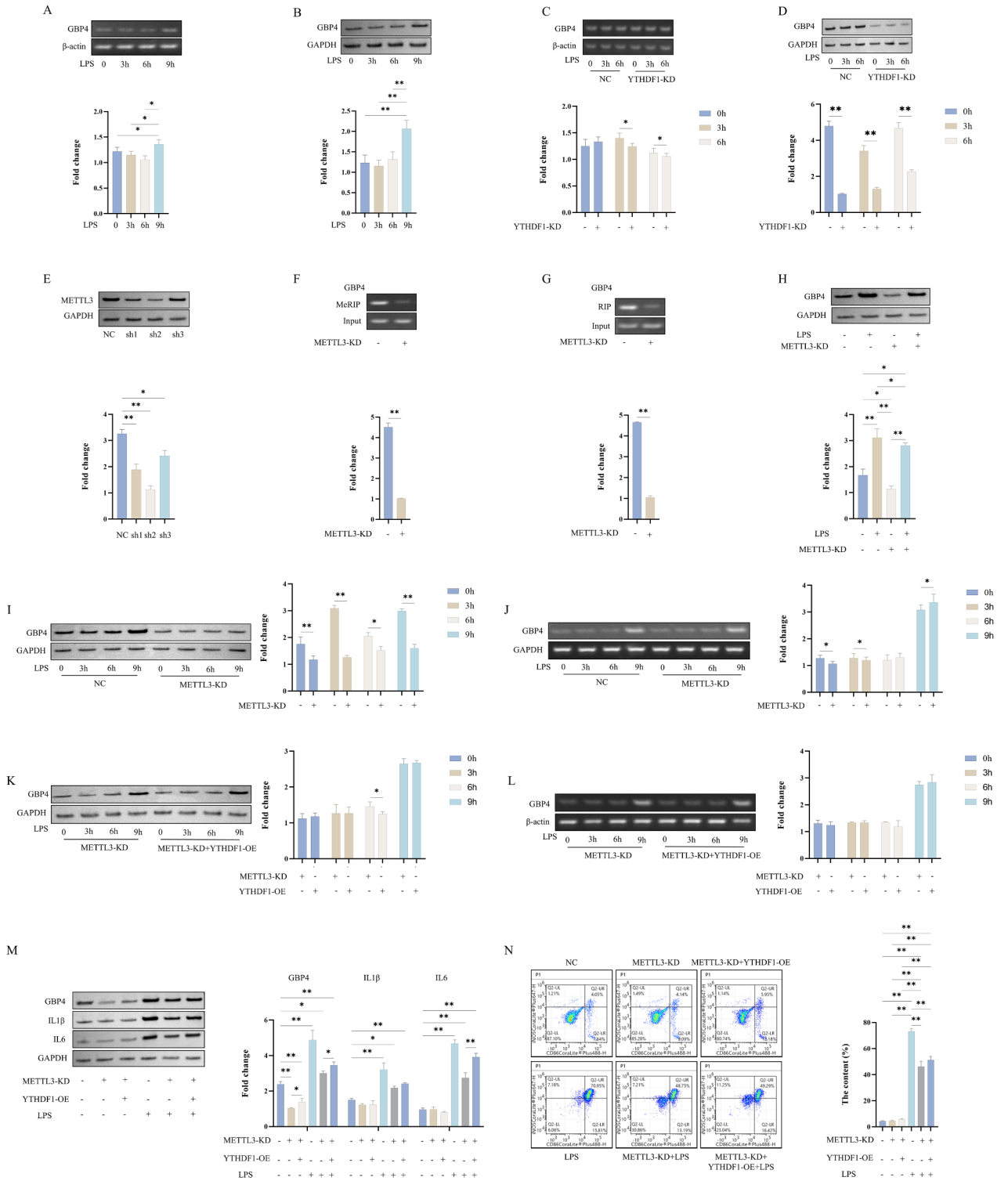

Fig. 4 YTHDF1 Protein Recognizes m6A Sites and Promotes mRNA Translation

of IL1β and IL6 after LPS treatment (Fig. 4M). We also used flow cytometry to detect the polarization of M1 macrophages, and the results showed that compared with the LPS treatment alone group, the knockdown of METTL3 followed by LPS treatment significantly inhibited the polarization of M1 macrophages, and overexpression of YTHDF1 did not enhance M1 macrophage polarization (Fig. 4N).

In summary, our study results reveal that YTHDF1 promotes the translation of GBP4 by recognizing m6A sites on GBP4 mRNA and further affects the polarization of M1 macrophages.

LPS-treated RAW264.7 cells for specified times were analyzed for GBP4 expression using PCR with β-actin as an internal control (A); GBP4 expression was also detected by WB (B). After knocking down YTHDF1, LPS-treated RAW264.7 cells for specified times were analyzed for GBP4 and YTHDF1 expression levels by PCR (C); GBP4 and YTHDF1 expression levels were also detected by WB (D). E. METTL3 was knocked down in RAW264.7 cells using shRNA, and WB confirmed the knockdown efficiency, with shRNA2 showing the most effective knockdown. F. MeRIP assay was performed in METTL3-knockdown or non-knockdown RAW264.7 cells, followed by PCR to detect GBP4 levels, with Input group GBP4 as a control. G. RIP assay using METTL3-knockdown or non-knockdown RAW264.7 cells and a YTHDF1 antibody was conducted, followed by PCR to detect GBP4 levels, with Input group GBP4 as a control. H. LPS-treated or non-treated METTL3-knockdown or non-knockdown RAW264.7 cells were analyzed for GBP4, YTHDF1, and METTL3 protein expression by WB. LPS-treated METTL3-knockdown or non-knockdown RAW264.7 cells for specified times were analyzed for GBP4 and METTL3 expression by WB (I) and PCR (J). After knocking down METTL3 and overexpressing YTHDF1, LPS-treated RAW264.7 cells were analyzed for GBP4, METTL3, YTHDF1, and M1 macrophage polarization marker (IL1β, IL6) expression levels by WB (M), and M1 macrophage polarization was assessed by flow cytometry (N). There were at least three replicates per group in all experiments.

Co-culture of RAW264.7 and MLE-12

First, we overexpressed GBP4 and knocked down YTHDF1 in RAW264.7 cells, and then detected the polarization of M1 macrophages under conditions with or without LPS treatment. Flow cytometry results showed that overexpression of GBP4 in the absence of LPS stimulation had no significant effect on M0 macrophages (Fig. 5A). However, under LPS stimulation, overexpression of GBP4 significantly promoted the polarization of M1 macrophages (Fig. 5A). In addition, when GBP4 was overexpressed and YTHDF1 was knocked down simultaneously, the polarization of M1 macrophages was significantly inhibited even under LPS treatment (Fig. 5A).

In the detection of GBP4 protein expression, we observed results consistent with previous findings, that is, overexpression of GBP4 with simultaneous knockdown of YTHDF1 can inhibit the protein expression of GBP4 (Fig. 5B). The enhancement of GBP4 expression by LPS treatment was significantly inhibited under conditions where YTHDF1 was knocked down and GBP4 was overexpressed (Fig. 5B). The detection results of YTHDF1 RIP-PCR further confirmed this, indicating that knockdown of YTHDF1 significantly inhibited the binding of GBP4 mRNA to YTHDF1, regardless of LPS treatment (Fig. 5C).

To further study the impact of these changes on the inflammatory response of alveolar epithelial cells, we conducted co-culture experiments with RAW264.7 and MLE-12. Before co-culturing, MLE-12 cells were treated with LPS for 6 h. Then, we replaced the culture medium and placed RAW264.7 cells in the transwell chamber above MLE-12 cells for co-culture. After 12h of co-culture, we examined the inflammatory phenotype of MLE-12 cells. The detection of ROS showed that overexpression of GBP4 in macrophages, regardless of whether YTHDF1 was knocked down, did not affect the ROS level in MLE-12 cells in the absence of LPS treatment (Fig. 5D). However, after LPS treatment, overexpression of GBP4 significantly increased the ROS level in MLE-12 cells, and overexpression of GBP4 and knockdown of YTHDF1 significantly inhibited the increased ROS (Fig. 5D). This result indicates that overexpression of GBP4 may enhance the oxidative stress of alveolar epithelial cells by promoting the polarization of M1 macrophages.

In subsequent assay, we found that the expression results of LDH, IL1β, and TNFα were consistent with the results of ROS, that is, overexpression of GBP4 promoted the production of these inflammatory factors after LPS treatment, and knockdown of YTHDF1 inhibited this process (Fig. 5E-G). In contrast, the detection result of SOD showed that the expression level of SOD increased when GBP4 was overexpressed and YTHDF1 was knocked down (Fig. 5H).

In summary, our study results indicate that overexpression of GBP4 and knockdown of YTHDF1 in RAW264.7 cells significantly inhibit the polarization of M1 macrophages and the inflammatory response of alveolar epithelial cells.

After overexpressing GBP4 in macrophages and knocking down or not knocking down YTHDF1, cells were treated with LPS and analyzed for M1 macrophage polarization by flow cytometry (A), GBP4 expression by WB (B), and YTHDF1 RIP-PCR to detect GBP4 levels (C). After co-culturing MLE-12 cells with the aforementioned macrophages, ROS (D), lactate dehydrogenase (E), IL-1β (F), TNF-α (G), and SOD (H) levels were detected. There were at least three replicates per group in all experiments. Multiple groups were analyzed using one-way ANOVA, * indicates $P<0.05$, ** indicates $P<0.01$, and n.s. indicates not signiicant ($P>0.05$).

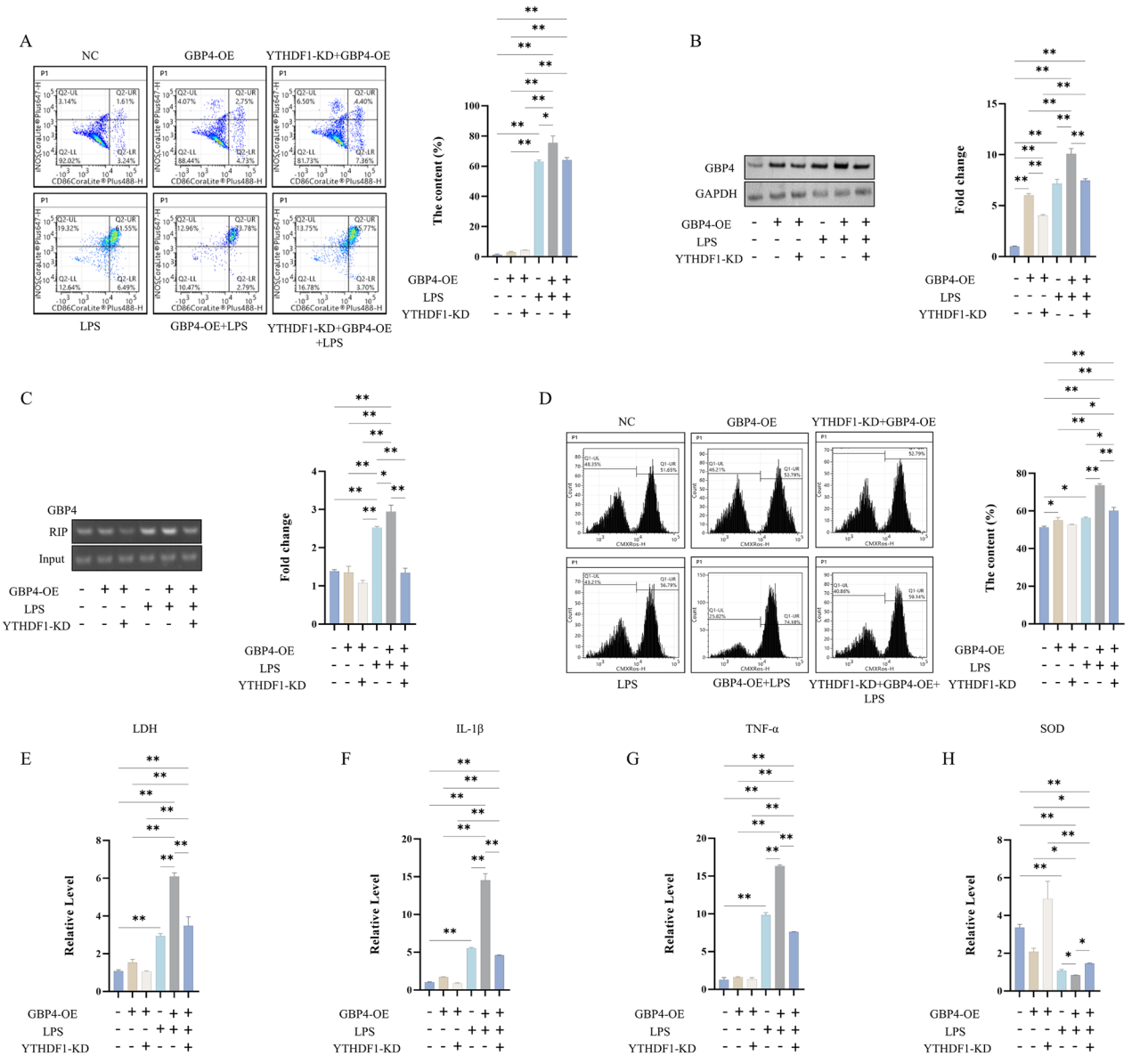

Fig. 5 Co-culture of RAW264.7 and MLE-12

Discussion

Acute lung injury (ALI) is a severe pathological condition characterized by pulmonary inflammation and damage. Macrophages, particularly alveolar macrophages, play a pivotal role in the development and progression of ALI. In the context of ALI, macrophages exacerbate inflammatory responses by releasing inflammatory cytokines such as IL1β and TNFα. Research has shown that HMGB1 can participate in LPS-induced ALI by activating the AIM2 inflammasome and inducing M1 macrophage polarization through TLR2, TLR4, and RAGE/NF-κB signaling pathways [17].

The role of GBP4 in neutrophil clearance of Salmonella Typhimurium via inflammasome-dependent prostaglandin production underscores its importance in immune responses to bacterial infections [10]. Our study reveals that GBP4 overexpression enhances M1 macrophage polarization under LPS stimulation, increasing IL1β, TNFα, and oxidative stress in MLE-12 cells. This suggests GBP4 modulates pulmonary macrophage inflammation, affects macrophage polarization, and is involved in immune signaling pathways. This suggests that GBP4 may function in ALI by modulating the inflammatory response of pulmonary macrophages, affecting macrophage polarization, and participating in the regulation of related signaling pathways. The role of GBP4 in promoting M1 macrophage polarization and its potential involvement in the inflammasome-dependent

clearance of bacteria underscore its significance in the pathogenesis of ALI and the body's defense mechanisms against infection. In summary, GBP4 appears to be a multifaceted protein that could play a crucial role in both the inflammatory response and the immune defense against pathogens in the context of ALI.

Macrophage polarization into M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes plays a crucial role in the development and progression of ALI. A study found that HMGB1 promotes M1 macrophage polarization and exacerbates ALI inflammation by activating the AIM2 inflammasome and through TLR2, TLR4, and RAGE/NF-κB signaling pathways [17]. Our research indicates that overexpression of GBP4 significantly promotes M1 macrophage polarization, and knocking down GBP4 can inhibit macrophage polarization towards the M1 phenotype. Although direct research on GBP4 and macrophage polarization is scarce, exploring the general mechanisms of macrophage polarization can infer the potential role of GBP4. Studies suggest that macrophage polarization is influenced not only by environmental factors such as cytokines and microbial products but also by intracellular signaling pathways and transcription factors [18]. For example, bioelectricity regulation can modulate the polarization state of macrophages by affecting their membrane potential, further influencing macrophage phenotype and function [19]. Additionally, the metabolic and transcriptional networks during macrophage polarization have been proven to be closely related to their functions [20]. These studies provide a scientific basis for understanding the potential role of GBP4 in regulating macrophage polarization, implying that GBP4 may participate in the regulation of the polarization process by affecting the intracellular signaling and transcriptional regulatory networks of macrophages, thereby influencing immune responses and inflammatory reactions.

m6A modification is a critical RNA modification that dynamically regulates gene expression by adding or removing m6A marks on mRNA, involving "writers," "erasers," and "readers" that influence RNA processing, stability, translation, and splicing [21]. This modification is essential for splicing efficiency, RNA maturation, and cellular functions, with roles in both coding and non-coding RNAs, impacting tumor development, drug response, and highlighting the therapeutic potential of targeting m6A regulators [22]. Our research indicates that YTHDF1, an m6A reader protein, directly affects GBP4 expression by recognizing and promoting the translation of m6A-modified GBP4 mRNA. This regulation significantly impacts cellular functions in acute lung injury (ALI), shaping the lung's inflammatory environment, dictating immune cell polarization, and aiding in resolving inflammation and restoring lung homeostasis [23–25]. YTHDF1 modulation of GBP4 expression influences M1 macrophage polarization and pro-inflammatory functions, potentially affecting the release of cytokines such as TNFα and IL6, playing a significant role in ALI development. These findings underscore the importance of YTHDF1 in immune regulation and highlight its role in modulating GBP4 expression and macrophage functions in ALI pathogenesis.

LPS is an exogenous PAMP, primarily simulating the inflammatory response induced by bacterial infections [13]. However, ALI is a complex clinical syndrome whose pathological processes involve various factors, including endogenous and exogenous elements. Although the LPS model can mimic certain characteristics of ALI, it cannot fully replicate the complexity of human ALI. Despite the significant value of the LPS model in studying the pathogenesis and therapeutic strategies of ALI, its clinical relevance to human ALI may be limited [14, 15]. The LPS-induced ALI model mainly reflects ALI caused by bacterial infections, while the etiology of ALI in clinical settings is more diverse, including non-infectious factors.

Our research reveals that GBP4 plays a crucial role in regulating the inflammatory response of alveolar epithelial cells, potentially exacerbating ALI by promoting the amplification of inflammatory signals and M1 macrophage polarization. YTHDF1, as an m6A modification reader protein, regulates the translation efficiency of GBP4 mRNA by recognizing and binding to m6A sites on GBP4 mRNA, thereby affecting the expression of GBP4 protein. This regulatory mechanism may have a significant impact on the inflammatory response of alveolar epithelial cells and the progression of ALI. The interaction between YTHDF1 and GBP4 mRNA suggests a sophisticated level of post-transcriptional regulation that could influence the severity and resolution of the inflammatory cascade in ALI. By modulating GBP4 expression, YTHDF1 may directly influence the polarization of macrophages towards a pro-inflammatory phenotype, which in turn releases a plethora of cytokines and chemokines that can damage the alveolar-capillary membrane, a hallmark of ALI. This insight into the role of the m6A-YTHDF1-GBP4 axis not only sheds light on the intricate network of molecular interactions underlying ALI pathogenesis but also highlights potential therapeutic targets for mitigating lung injury and improving patient outcomes. Studies also show that m6A modification and YTHDF1 play important roles in regulating the expression of inflammation-related genes, providing a new perspective for understanding the molecular mechanisms of alveolar epithelial cell inflammatory responses [26].

Conclusion

In summary, GBP4 and YTHDF1 play key roles in the inflammatory response of alveolar epithelial cells and acute lung injury (ALI). The upregulation of GBP4

expression is closely associated with exacerbated inflammatory responses, particularly by promoting M1 macrophage polarization and the release of pro-inflammatory factors, worsening pulmonary damage. Concurrently, m6A modification, especially mediated by YTHDF1, plays a significant regulatory role in the translation of GBP4 mRNA, affecting the expression and function of GBP4 protein. YTHDF1 not only regulates the translation of GBP4 but may also comprehensively influence the inflammatory response of alveolar epithelial cells by regulating the expression of other inflammation-related genes. These findings reveal the important roles of GBP4 and YTHDF1 in regulating the inflammatory response of alveolar epithelial cells and macrophage polarization, providing important clues for a deeper understanding of the molecular mechanisms of ALI and exploring new therapeutic strategies. These results emphasize the complexity of inflammation regulation in ALI and the potential value of modulating m6A modification in disease management.

Supplementary Information

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Supplementary Material 1

Author contributions

Conceptualization, Fengan Cao, Shilei Wang, Qiuyue Tan, Junna Hou, Yunlu Li, Wentao Ma and Shilong Zhao; Data curation, Junna Hou; Funding acquisition, Fengan Cao, Shilei Wang, Qiuyue Tan and Jing Gao; Investigation, Fengan Cao, Shilei Wang and Qiuyue Tan; Methodology, Fengan Cao, Shilei Wang, Qiuyue Tan and Shilong Zhao; Project administration, Jing Gao; Resources, Yunlu Li; Supervision, Jing Gao; Validation, Fengan Cao, Shilei Wang, Qiuyue Tan and Wentao Ma; Writing – original draft, Fengan Cao, Shilei Wang, Qiuyue Tan and Jing Gao; Writing – review & editing, Jing Gao.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval

Mice used in this study were housed with free access to food and water under protocols approved by the Zhengzhou University Institutional Animal Care and Use Committee (2020-KY-462).

Competing interests

The authors declare no competing interests.

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