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Original Research

Serum Pentaxin 3 (PTX3) Promotes NLRP3 Inflammasome and Pyroptosis in Patients with Up-Regulated Myasthenia Gravis

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Abstract

Background: Myasthenia gravis (MG) is an autoantibodies-mediated autoimmune disease with the complications of neuromuscular junction transmission. In this study, we aimed to investigate the molecular regulatory roles of pentaxin 3 (PTX3) in patients and in animal model with MG and to explore its underlying mechanism. **Methods**: Patients with MG were identified and enrolled at our designated hospital and animal model was utilized for the proposed study. Enzyme-linked immunosorbent assay (ELISA) kit were used to quantify the IL-1β, IL-6, INF-γ, IL-17, TNF-α, anti-TAChR IgG/IgG1/IgG2b/IgG2c levels. **Results**: Serum PTX3 expression level in patients with MG was up-regulated as compared to normal. Furthermore, we found increased expression level of mRNA and protein product of PTX3 in the mice with MG. PTX3 promoted inflammation, pyroptosis in patients as well as in the MG mouse model. In addition, PTX3 induced the STAT3/NLRP3 inflammasome and promoted gene synthesis of STAT3. We found that METTL3-mediated m6A modification decreases PTX3 stability. **Conclusions**: Our study suggests that the PTX3 is associated with the enhancement of inflammation and pyroptosis through regulating the STAT3/NLRP3 inflammasome signaling pathway at the early stage of the disease. The pro-inflammatory PTX3 facilitates the development of MG and it can be used as a potantial MG-associated diagnostic biomarker for MG.

Keywords: myasthenia gravis; NLRP3; inflammasome; PTX3; neuromuscular disease

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease caused by autoantibodies with neuromuscular junction transmission complications [1]. The global prevalence of MG is (150–250)/million, and the estimated annual incidence rate is (4–10)/million [2]. The incidence rate of MG in China is about 0.68/100,000, and interestingly, the female incidence rate is slightly higher [3]. The major reasons for death with MG included respiratory complications, infections in the lung, etc., with a 14.69% mortality rate in the hospital. It can occur at all ages, with double peaks at the age of 30 and 50 [4,5]. In China, myasthenia gravis (MG) is diagnosed in up to 50% of children and teenagers [6].

MG, as an autoimmune disease, can lead to deregulated levels of immune factors associated with anti-inflammation and pro-inflammation [7]. MG patients' symptoms have been shown to improve with some therapies that control the ratio of anti-inflammatory to pro-inflammatory factors [8]. Some of the previous studies indicated that the occurrence of MG is linked with the aberrant regulation of immune T cells and inflammatory factors in patients, in which the level of $CD4^+$ T cells is positively correlated with the development of MG, and OX40 + T is a synergistic factor expressed by activated T cells, which mainly acts on $CD4^+$ T cells for promoting the proliferation and differentiation of $CD4^+$ T cells with affecting body's immune responses [9,10].

The NLRP3 inflammasome is present primarily in immune and inflammatory cells following activation by inflammatory stimuli [11]; these cells include macrophages, monocytes, DCs, and splenic neutrophils [12]. It was found that NLRP3 inflammatory bodies activate caspase-1 and release the downstream cytokine IL-1β/IL-18 are important ways of regulating adaptive immunity [12,13]. The primary regulator of adaptive immune responses is IL-1β, which generates subsets of $CD4^+$ T and Th17 cells that secrete IL-17 to help the host resist extracellular bacteria, fungi, and autoimmunity [14]. The expression of NLRP3 inflammatory in MG is increased, and IL-1β could cause the corresponding pathological changes [15].

Pentaxin 3 (PTX3) is associated with the initiation and growth of cancer and has been regarded as a sign of cancer progression [16]. PTX3 regulates the TLR4/NF-κB signaling pathway to induce inflammation reaction. PTX3 is also an effector of the inflammatory response [17]. It can activate effectors under inflammatory conditions and is an important component of innate immunity [18,19]. It was reported that circulating PTX3 may reflect the extent of neuromuscular junction damage and might be involved in the pathogenesis of MG [19]. This study investigated the effects of PTX3 on patients with MG and to explore its underlying molecular mechanism utilizing MG mouse model.

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2. Materials and Methods

2.1 Patients

30 patients with MG were enrolled at designated hospital from May 2019 to July 2020. The blood samples from the patients were collected and immediately stored at 4 °C for further study. Informed consent was obtained from all the participants. The Medical Ethics committee of Beijing Friendship Hospital, Capital Medical University approved (Approve No. KR3234208) the study. All experiments were carried out following standard guidelines and regulations. The diagnosis of MG was made by an experienced neurologist based on clinical presentation, positive response to anticholinesterase medications, presence of autoantibodies, and characteristic electrophysiological findings. Patients with any history of inflammatory or autoimmune diseases such as autoimmune thyroid disease, inflammatory bowel disease, RA, and SLE were excluded from this study.

2.2 EAMG Mice Model

We built the EAMG model by using the C57BL/6 female mice [20]. Briefly, on day 1, mice were immunized with a synthetic immunogen containing complete Freund's adjuvant, and on days 30 and 50, mice were immunized with a synthetic immunogen containing incomplete Freund's adjuvant (SIMGA). After the first injection, we measured the EAMG scores every day; collected the serum; and sacrificed the model mice on the 21st day to conduct other essential experiments. All experiments were performed during the same period of the day (8:00 AM to 4:00 PM) to exclude diurnal variations in pharmacological effects. The animals were handled in compliance with the procedures approved by the Animal Resources Centre of Capital Medical University.

2.3 Quantitative PCR and Microarray Analysis

We used Light Cycler® 480 SYBR Mix (Roche, Mannheim, Germany) in the LightCycler® 480 real-time PCR system to quantify the genes expression in the PCR system. We utilized the Invitrogen SuperScript double-stranded cDNA synthesis kit for the reverse transcription. NimbleGen's one-color DNA labeling kit was used to synthesize double-stranded cDNA and then array hybridization was executed by using the NimbleGen hybridization system. In addition, the NimbleGen wash buffer kit was used for washing step. We employed *GenePix4000B* (*Axon*, Scottsdale, AZ, USA) and *GenePix* Pro 6.0 software (*Axon*, USA) microarray scanner (Molecular Devices) technology to scan the experimental outcomes. The sequences of primers for *PTX3* gene used is Forward 5′-CCTGCGATCCTGCTTTGTG-3′, Reverse 5′-GGTGGGATGAAGTCCATTGTC-3′.

2.4 Cell Culture and RNA Interference

Mouse myoblasts (C2C12) was cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal calf serum supplementation (FCS, Gibco, Carlsbad, CA, USA) in a humid environment of 5% CO_2 at 37 °C. We utilized Lipofectamine 2000 to transfect the PTX3 Plasmids with primer sequence of (5′-GTGCAGGGCTGGGCTGCCCG-3′) into C2C12 cells. After 48 h of transfection, LPS (200 ng) was added genetally to the C2C12 cells for 4 h and later replaced with the fresh medium. The cell line (C2C12 Mouse Myoblasts) used in this study was tested and authenticated using Short Tandem Repeat (STR) profiling, Which confirmed the identity by comparing the obtained profile with a reference databases of known cell lines. Furthermore, the cell line was tasted for Mycoplasma and other contamination using negative and positive controls with validated method. The cell line was free of mycoplasma and other species contamination.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA) Kit

We quantify the level of IL-1β (Abcam, Cambridge, UK, ab214025), IL-6 (Abcam, ab178013), INF-γ (Abcam, ab289902), IL-17 (Abcam, ab119535), TNF-α (Abcam, ab181421), anti-TAChR IgG/IgG1/IgG2b/IgG2c (Abcam, ab100548) by using an ELISA kit following the manufacturer's protocol.

2.6 Western Blot Analysis

Proteins were extracted from the cultured cells using RIPA and PMSF reagents (Beyotime, Beijing, China).We separated the protein lysates according to the molecular weight of respected protein on SDS/PAGE gels and then we transferred them to a Polyvinylidene Fluoride (PVDF, Millipore, Burlington, MA, USA) membrane system. The membranes containing protein were then blocked by using 5% non-fat-milk for 2 hours at room temperature and further, the membranes were placed on shaking incubator at 4 °C and incubated with anti-antibody of PTX3 (Abcam, ab90806), actin (Sigma, St. Louis, MO, USA, SAB4502543), Fl-GSDMD (Abcam, ab210070), N-GSDMD (Abcam, ab215203), NLRP3 (Abcam, ab263899), p-STAT3 (Abcam, ab30647), STAT3 (Abcam, ab68153). After incubation, the membranes were washed with 10% phosphate buffer saline (PBS) for ten minutes and repeated the washing steps three times. After that, membranes were incubated with a secondary antibody for 1 hour at room temperature and again repeated the washing step. The membranes were then visualized by machine.

2.7 RNA Microarrays

Microarray data were obtained from NCBI's Gene Expression Omnibus (GEO) database. All available studies were considered, but only investigations that included a DA/Ao comparison were selected for analysis. One-

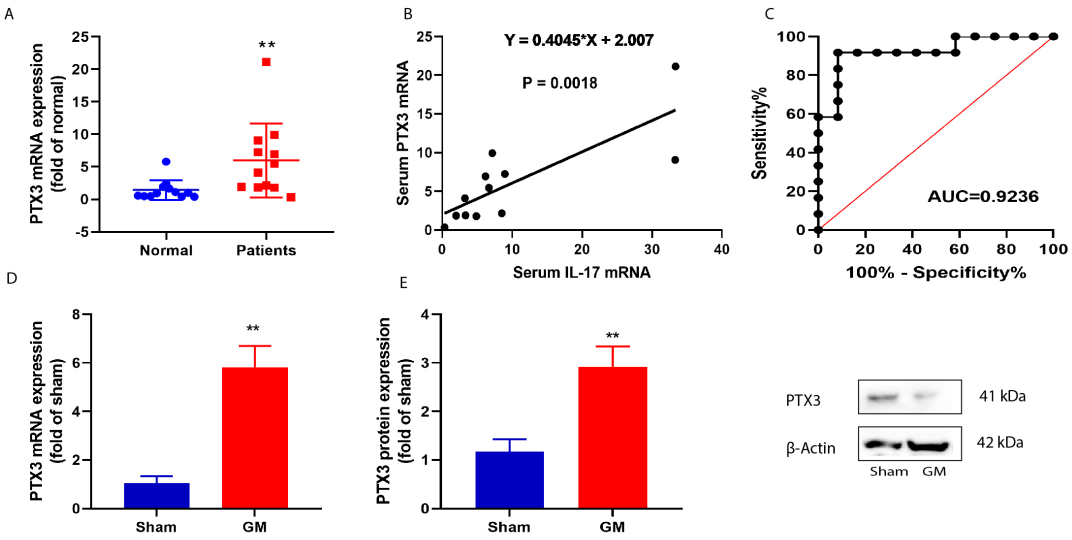

Fig. 1. Serum pentaxin 3 (PTX3) expression level in patients with Myasthenia gravis (MG) up-regulated. PTX3 mRNA expression (A), Serum mRNA expression of PTX3 was in negative correlation with serum IL-17 levels (B), AUC analysis results (C), Sensitivity analysis in patients with MG (D), PTX3 mRNA expression (E), while the western blot figure indicate protein expression in mice model. The experiments were performed in triplicate and data is presented an average of these experiments. Normal, normal volunteers' group; Patients, Patients with MG; Sham, sham control group; MG, mice with MG. $^{**}p < 0.01$ compared with normal volunteers group or sham control group.

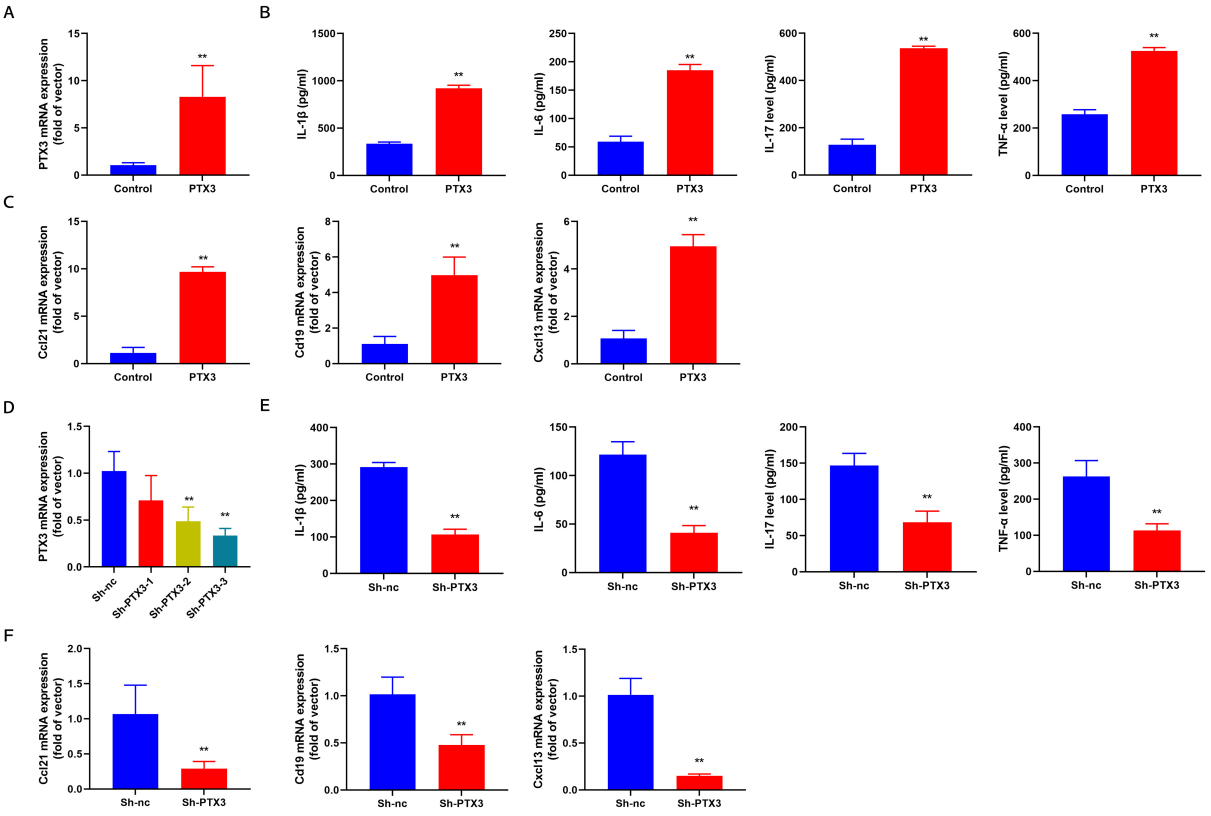

Fig. 2. PTX3 promoted inflammation level using *in vitro* model. PTX3 mRNA expression (A). IL-1β, IL-6, IL-17, TNF-α activity levels (B). Ccl12, Ccl19, and Ccl13 mRNA expressions (C) *in vitro* model by over-expression of PTX3. PTX3 mRNA expression (D). IL-1β, IL-6, IL-17, and TNF-α activity levels (E). Ccl12, Ccl19, and Ccl13 mRNA expressions (F) *in vitro* model by down-regulation of PTX3. All experiments were performed in triplicate. $^{**}p < 0.01$ compared with the control negative group or sh-nc group.

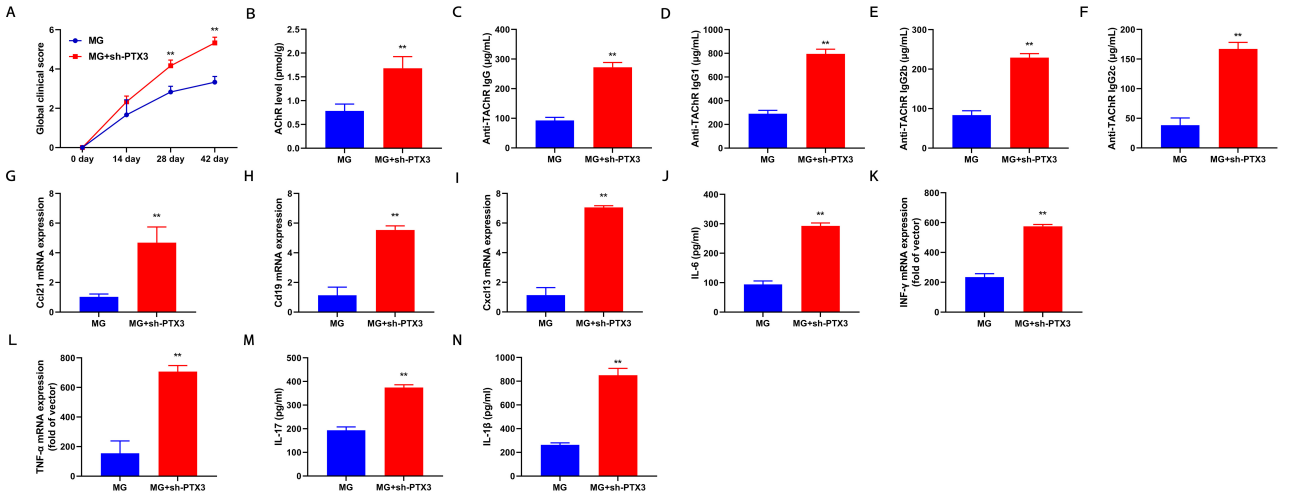

Fig. 3. The inhibition of PTX3 presented MG and reduced inflammation levels in the mice model. Global clinical score (A). AChR level (B). anti-TAChR IgG (C)/IgG1 (D)/IgG2b (E)/IgG2c (F) levels. Ccl12 (G), Ccl19 (H) and Ccl13 (I) mRNA expressions. IL-1β (J), IL-6 (K), INF-γ (L), IL-17 (M), TNF-α (N) activity levels. The experiments were performed in triplicate and data is presented an average of these experiments. $^{**}p < 0.01$ compared with the control negative group or sh-nc group.

way ANOVA test was used to analyze contrasts of interest, namely vessel type, to generate lists of DEGs between DA and Ao. Permissive DEG lists (fold change $\geq$1.2) were then separated by increased or decreased DA/Ao expression to generate UP and DOWN lists respectively.

2.8 Immunofluorescence Analysis

We fixed the cells by using 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 15 minutes, and then the cells slides were blocked using the 5% BSA for 30 minutes at 37 °C. We then treated the cells with primary antibodies at 4 °C overnight. Then, incubated the cells by using the Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG DyLight 488-conjugated secondary antibodies for 2 hours at room temperature. Furthermore, we stained the nuclei of the cells using the DAPI and we utilized a fluorescent illumination microscope (Olympus IX71, Tokyo, Japan) for observing the cells.

2.9 Methylation Modification Sites of PTX3

DNA was amplified using a methylation-specific primer set, PTX3-MF: 5′-CGTTTGCGGTTAGGAGTATTC-3′ and PTX3-MR: 5′-CAAAACGTCGTCCGTAACTTA-3′, or a non-methylation-specific primer set, PTX3-UF: 5′-TGTGT TTGTGGTTAGGAGTATTTG-3′ and PTX3-UR: 5′-CAA AACATCATCCATAACTTA-3′ [18], in a total volume of 20 µL, using 0.5 units of hot-start Taq-polymerase (Takara, Japan) per reaction. The size of the non-methylated amplicon was 105 bp, and the methylated amplicon was 103 bp.

2.10 Statistical Analysis

We employed GraphPad 8.0 Software for analyzing the data and presented the data as mean $\pm$ SD. The statistical threshold was set as the $p < 0.05$. We analyzed the differentiation of two groups by utilizing the Student's *t*-test or performing the Tukey post hoc test after applying two-way ANOVA.

3. Results

3.1 Serum Level of PTX3 was Increased in Patients with MG

To investigate the expression level of the *PTX3* gene in MG, we used the PCR assay to identify the PTX3 expression level in patients with MG and mice. Our results revealed that the level of the PTX3 mRNA expression is significantly elevated (Fig. 1A). Serum mRNA expression of PTX3 showed a significant positive correlation with the serum IL-17 levels in patients with MG and the AUC = 0.9236 (Fig. 1B,C). In mice models of MG, we found an elevated level of PTX3 mRNA and protein quantity (Fig. 1D,E).

3.2 PTX3 is Associated with the Enhancement of Inflammation Levels in Vitro

Next, we analyzed the correlation between PTX3 expression level and inflammation level in MG models. PTX3 plasmid increased PTX3 mRNA expression and enhanced the inflammatory factors, including IL-1β, IL-6, IL-17, and TNF-α activity levels in the *in-vitro* model (Fig. 2A,B). PTX3 up-regulation increased Ccl12, Ccl19, and Ccl13 mRNA expressions in the *in-vitro* model (Fig. 2C). Furthermore, Sh-PTX3 plasmid reduced PTX3 mRNA expression and decreased inflammatory regulators in the *in-vitro* model

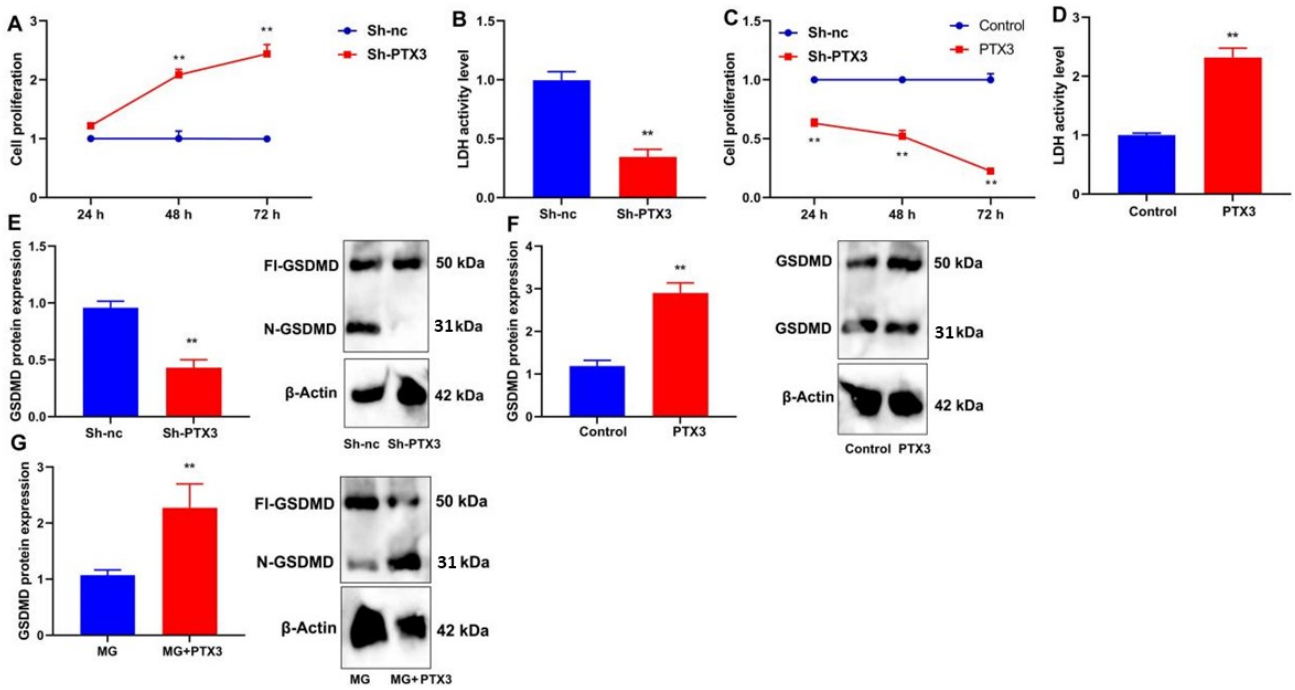

Fig. 4. PTX3 promoted pyroptosis in mice model or *in vitro* model. Cell growth and LDH activity levels (A and B) *in vitro* model by over-expression of PTX3. Cell growth and LDH activity levels (C and D) *in vitro* model by down-regulation of PTX3. GSDMD protein expression *in vitro* model by over-expression of PTX3 (E). Full-length GSDMD (Fl-GSDMD) and N-terminal domain of GSDMD (N-GSDMD) protein expression *in vitro* model by down-regulation of PTX3 (F). GSDMD protein expression in mice with MG by sh- PTX3 (G). The experiments were performed in triplicate and data is presented an average of these experiments. $^{**}p < 0.01$ compared with the control negative group or sh-nc group or MG group.

(Fig. 2D,E). Our results also concluded that the down-regulation of PTX3 is associated with the reduction level of Ccl12, Ccl19, and Ccl13 mRNA expressions in the *in-vitro* model (Fig. 2F).

3.3 PTX3 Expanded MG and Promoted Inflammation Levels in the Mice Model

We next further examined the function of PTX3 in MG using PTX3 recombinant protein. PTX3 recombinant protein increased global clinical score and AChR level and expanded anti-TAChR IgG/IgG1/IgG2b/IgG2c levels, induced Ccl12, Ccl19, and Ccl13 mRNA expressions, while increased the levels of inflammatory regulators, including IL-1β, IL-6, INF-γ, IL-17, and TNF-α in the model of MG (Fig. 3).

3.4 PTX3 Promoted Pyroptosis in Mice Model of MG

Furthermore, we analyzed the regulatory roles of PTX3 on pyroptosis in the *in-vitro* model of MG. PTX3 up-regulation decreased cell proliferation and increased the activity of LDH in the *in-vitro* model (Fig. 4A,B). Down-regulation of PTX3 enhanced the cellular proliferative characteristics and decreased the activities of LDH in the *in-vitro* model (Fig. 4C,D). Additionally, we discovered in the *in-vitro* model that downregulating PTX3 decreased the protein level of GSDMD while upregulating PTX3 increased the expression of GSDMD (Fig. 4E,F). Furthermore, in the mice model, PTX3 recombinant protein promoted GSDMD protein expression (Fig. 4G).

3.5 PTX3 Induced STAT3/NLRP3 Inflammasome and Promoted the Expression of STAT3

To understand the molecular regulatory role of PTX3 in the MG model, we used microarray technology to perform gene expression profiling (Fig. 5A,B). Our results indicated that PTX3 might regulate STAT3 and NLRP3 levels (Fig. 5B). The recombinant protein product of PTX3 stimulated the expression of p-STAT3/NLRP3 protein in the mice model (Fig. 5C). In the mice model of MG, PTX3 up-regulation induced PTX3 and p-STAT3/NLRP3 protein expressions (Fig. 6A) while, PTX3 down-regulation suppressed PTX3 and p-STAT3/NLRP3 protein expressions *in vitro* model (Fig. 6B). Confocal showed that PTX3 up-regulation increased PTX3 and p-STAT3 expression levels *in vitro* model (Fig. 6C). We searched that the gene promoter of PTX3 and STAT3, showing the molecular interaction between PTX3 and STAT3 (Fig. 7A,B). Based on the luciferase reporter assay, the PTX3 WT substantially correlated with the expression of STAT3 (Fig. 7C). Then, the STAT3 inhibitor (2 μM of STAT3-IN-10) downregulated the protein level of p-STAT3 and reduced the protein expression level of GSDMD/NLRP3 in the *in-vitro* model

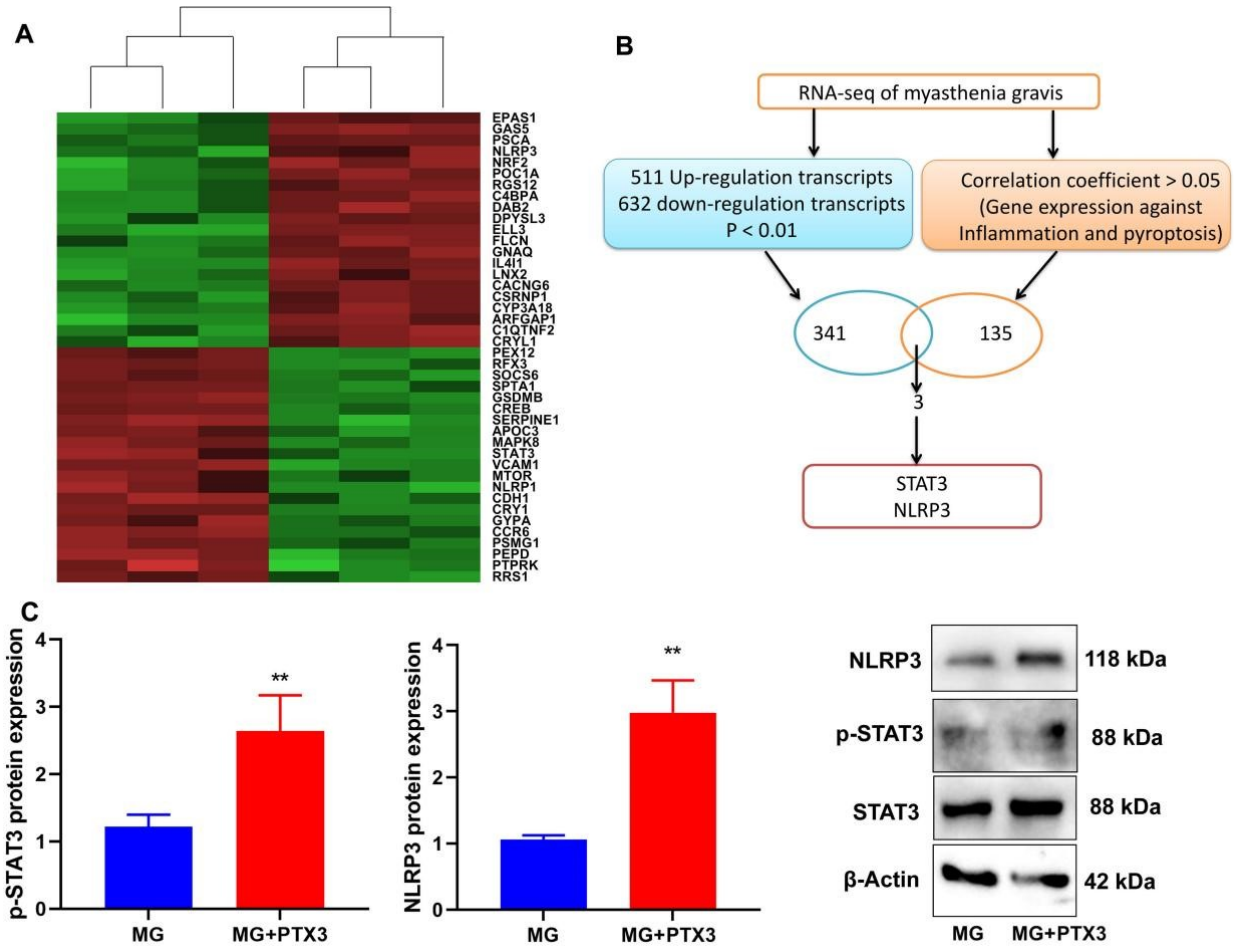

Fig. 5. PTX3 induced STAT3/NLRP3 inflammasome. Heat map (A). Result workflow and analysis (B). Presentation of p-STAT3/NLRP3 protein expression in mice with MG (C). $^{**}p < 0.01$ compared with the MG group.

(Fig. 7D). Moreover, the STAT3 agonist (5 µM of Oleoylcarnitine) induced the protein level of p-STAT3 and stimulated the protein level of GSDMD/NLRP3 in the *in-vitro* model (Fig. 7E).

3.6 METTL3-Mediated m6A Modification PTX3 Stability

We further examined the mechanism of PTX3 to check the progression of MG. We found that serum mRNA level of PTX3 was positively correlated with the level of METTL3 in MG patients (Fig. 8A). However, PTX3 level (serum mRNA) is not correlated with METTL14 levels in MG patients (Fig. 8B). In the immunoprecipitated fraction, an elevated level of PTX3 was found by using the m6A antibody (Fig. 8C). METTL3 reduced the m6A methylation level of PTX3 (Fig. 8D). Si-METTL3 decreased PTX3 expression levels (Fig. 8E), indicating the stable stimulation of m6A methylation of PTX3. The suspicious methylation modification sites of PTX3 are present at the stop codon region (Fig. 8F). METTL3 reduced PTX3 mRNA levels expression levels (Fig. 8G). In conclusion, these results suggest that METTL3-mediated m6A modification decreases PTX3 stability.

4. Discussion

MG, an autoimmune disorder, is crucially regulated by immune mediators, including antibodies of acetylcholine receptor, complement, and cytokines. In neuromuscular junction, the postsynaptic membrane acetylcholine receptors are substantially associated with the development of MG [21]. It is often manifested as pathological fatigue of partial or systemic skeletal muscle [22]. The development of MG is related to the change in the expression level of inflammatory factors, which participate in the pathogenesis of MG through immune regulation [23,24]. Here in, we quantified the elevated level of serum PTX3 in MG patients. A study concluded that dysregulated PTX3 contributes to ischemia and reperfusion injury [25]. Thus, it indicates that the PCBP2 regulates the disease progression of MG.

IL-1β can promote the differentiation and proliferation of thymocytes, promote the release of IL-2 inflammatory factors, improve the activation level of T cells, and promote the occurrence of autoimmune response [26]. Therefore, the quantity of inflammation-associated regulators and T cells variations are of great significance in evaluating

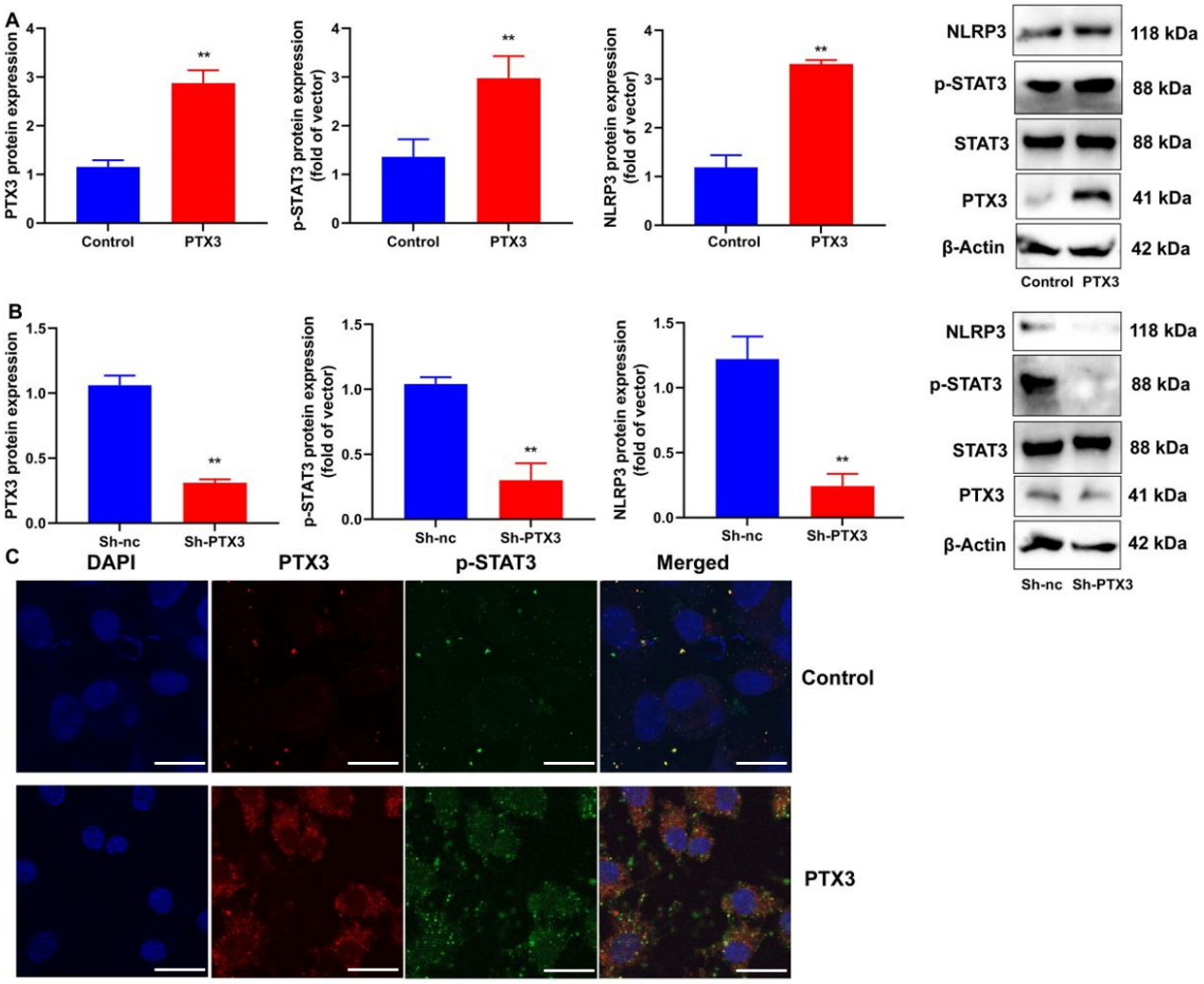

Fig. 6. PTX3 promoted gene synthesis of STAT3. PTX3/p-STAT3/NLRP3 protein expression in the *in-vitro* model by over-expression of PTX3 (A). PTX3/p-STAT3/NLRP3 protein expression *in vitro* model by down-regulation of PTX3 (B). PTX3/p-STAT3 expression by confocal microscope with the scale bar of 10 μM (C). The data is presented an average of triplicated experiments. $^{**}p < 0.01$ compared with the control negative group or sh-nc group.

the therapeutic effect and severity of MG patients [27]. Caspase-1 activated by NLRP3 can cleave IL-1β and IL-18 precursors, which produce corresponding productive cytokines, and can also stimulate inflammatory necrosis of cells and tissue cell death [28,29]. In this study, PTX3 promoted inflammation levels and pyroptosis by utilizing *in-vitro* model, and the inhibition of PTX3 presented MG phenotypes and reduced inflammation levels and pyroptosis in the mice model. Li *et al*. [30] identified that the LPS-induced inflammation was modulated by PTX3 in human nasal epithelial cells. Furthermore, Gu *et al*. [31] indicated that PTX3 promoted LPS-stimulated pyroptosis and inflammation in the stem cells of a human through activation of NLRP3 inflammasome. Specifically, PCBP2 induced NLRP3 inflammasome to promote pyroptosis and inflammation in the course of MG.

The development of MG and the expression level of inflammatory factors are activated, and participate in the response of cell apoptosis, while the STAT3 regulatory signal is associated with the inflammatory and cell death process mediated by MG [32–34]. The development of myasthenia gravis has been shown to regulate inflammatory response through the STAT3 signaling pathway. STAT3 can activate the IL-1β signal pathway to promote the development of MG [35]. We demonstrated that PTX3 promoted pyroptosis in mice model or vitro model. Gao *et al*. [36] suggested that PTX3 promoted airway inflammation in experimental asthma by STAT3. Thus, PTX3 played a repair factor in the occurrence and development of MG by STAT3. As a humoral mediator of innate immunity, PTX3 opsonizes pulmonary pathogens promoting the clearance by phagocytosis and triggers the mucosal immune response to fungal or bacterial infections and respiratory viruses. Furthermore, PTX3 also has relevant roles in non-infectious pulmonary diseases. Altogether, PTX3 exerts multiple roles in respiratory diseases. However, its involvement in the development

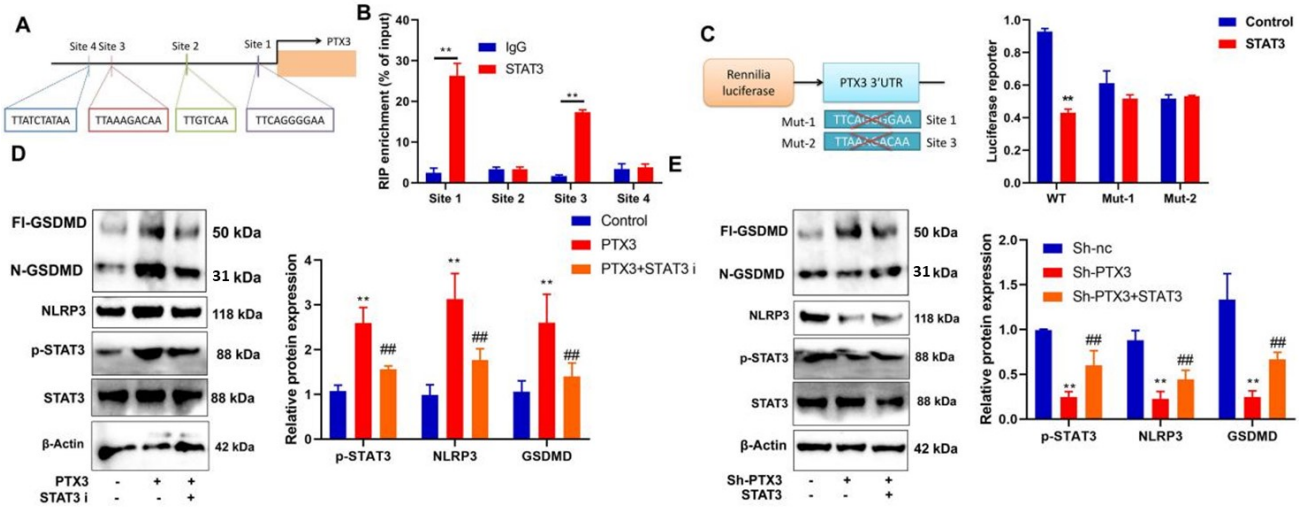

Fig. 7. Gene promoter of PTX3 and STAT3. The gene promoter of PTX3 and STAT3 (A), molecular interaction within PTX3 and PTX3 (B), luciferase reporter (C), p-STAT3/GSDMD/NLRP3 protein expression *in vitro* model by PTX3+STAT3 inhibitor (D), p-STAT3/GSDMD/NLRP3 protein expression *in vitro* model by sh-PTX3+STAT3 agonist (E). All experiments were performed in triplicates and the data is presented an average of these experiments. $^{**}p < 0.01$ compared with the control negative group or sh-nc group.

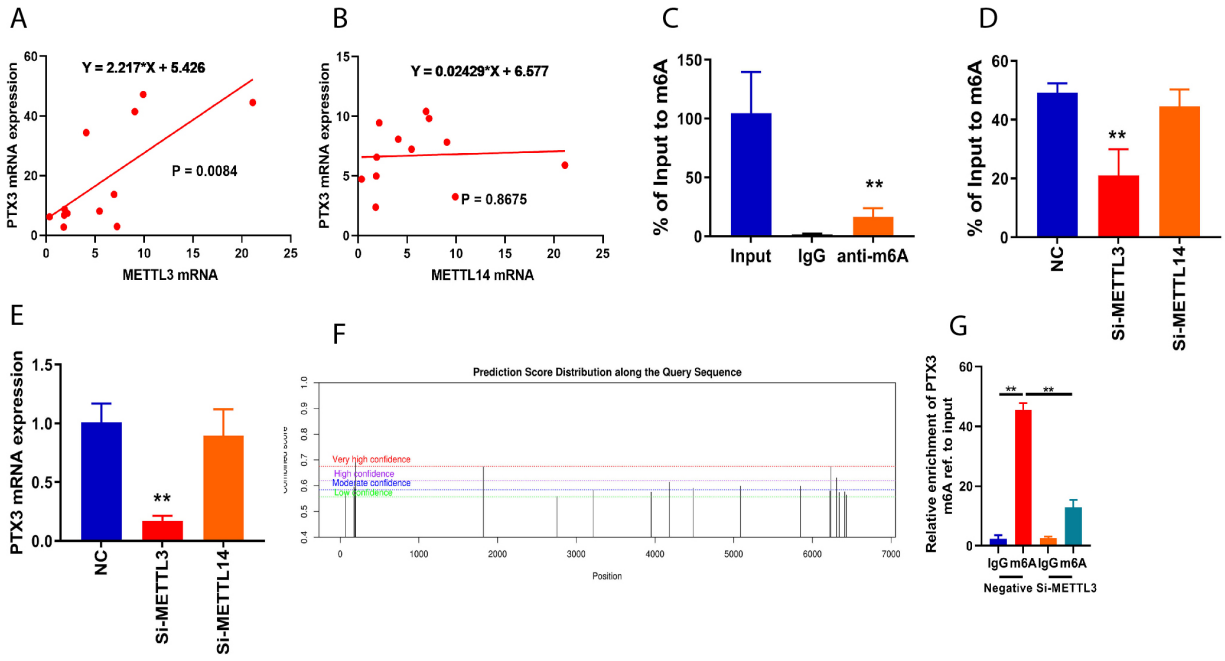

Fig. 8. METTL3-mediated m6A modification PTX3 stability. Serum mRNA of PTX3 was in negative correlation with METTL3 or METTL14 levels in patients with MG (A and B), fraction immunoprecipitated by m6A antibody (C), m6A methylation level of PTX3 (D), presentation of PTX3 expression levels (E), multiple suspicious methylation modification sites (F), PTX3 mRNA levels expression levels (G). The experiments were performed in triplicate and the data is an average of these expeirments. $^{**}p < 0.01$ compared with the lgG or NC group.

of IPF remains poorly explored. Many questions remain open, starting from the therapeutic effect of PTX3 to the mechanisms involved in the protective role of the protein and the relationships of PTX3 with the homeostasis of the airway epithelium and with the collagen fibers of the ECM. Little is known about the effect of IPF therapy with Pirfenidone/Nintedanib on PTX3. Further clinical studies will be necessary to answer these and questions and explore the enderlying mechanisms.

In the end, the first time, we demonstrated that the expression level of PTX3 is elevated in patients with MG. PTX3 promoted pyroptosis and inflammation in the pro-

gression of MG through STAT3/NLRP3 signaling pathway. Moreover, PTX3 represents a potential therapeutic target/spot for the treatment of MG. Finally, this research discovered the mechanisms at molecular levels for suppressing pyroptosis and inflammation in patients with MG.

5. Conclusions

Our study suggests that the PTX3 is associated with the enhancement of inflammation and pyroptosis through regulating the STAT3/NLRP3 inflammasome signaling pathway at the early stage of the disease. The pro-inflammatory PTX3 facilitates the development of MG and it can be used as a potantial MG-associated diagnostic biomarker for MG.

Availability of Data and Materials

The data used to support this study is available from the corresponding author upon request.

Author Contributions

Conceptualization, YZ; Data curation, YPL; Formal analysis, YL, SW and YPL; Investigation, YL and SW; Methodology, YL and YZ; Resources, YZ; Software, YL and SW; Validation, YZ; Writing – original draft, YL, SW, YPL and YZ; Writing – review & editing, YZ. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by The Medical Ethics committee of Beijing Friendship Hospital, Capital Medical University (Approval No. KR3234208). Informed consent was obtained from all the participants.

Acknowledgment

Not applicable.

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This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2811306.

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