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SETD7 promotes TNF-α-induced proliferation and migration of airway smooth muscle cells *in vitro* through enhancing NF-κB/CD38 signaling

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ABSTRACT

The inflammation-induced the excessive proliferation and migration of airway smooth muscle (ASM) cells in the airway wall contribute to airway remodeling in asthma pathogenesis. SET domain-containing lysine methyltransferase 7 (SETD7) has emerged as one of the key regulators of inflammation. Yet, the function of SETD7 in regulating inflammation-induced ASM cell proliferation and invasion remains unclear. In the present study, we aimed to investigate the function of SETD7 in regulating ASM cell proliferation and invasion induced by tumor necrosis factor (TNF)-α *in vitro*. Our results showed that SETD7 expression was upregulated in ASM cells stimulated with TNF-α. Silencing SETD7 significantly decreased TNF-α-induced ASM cell proliferation and migration, while SETD7 overexpression exhibited the opposite effect. Notably, silencing SETD7 decreased the activation of nuclear factor (NF)-κB and reduced the expression of CD38 induced by TNF-α. Blocking NF-κB activation significantly abrogated the promotional effect of SETD7 overexpression on CD38 expression. Moreover, overexpression of CD38 partially reversed the inhibitory effect of SETD7 silencing on TNF-α-induced ASM cell proliferation and migration. Overall, these results demonstrate that SETD7 regulates TNF-α-induced ASM cell proliferation and migration through modulation of NF-κB/CD38 signaling, suggesting a potential role of SETD7 in asthma airway remodeling.

1. Introduction

Asthma is a severe pulmonary disease characterized by airway inflammation, hyperresponsiveness, and remodeling that affects a large proportion of people globally [1,2]. Despite extensive research on asthma pathophysiology and pharmacology over the past decades, there is still a lack of efficacious anti-asthma medications. The dysregulation of airway smooth muscle (ASM) cells plays an important role in the pathogenesis of asthma [3]. ASM cells are a critical target of various mediators, such as growth factors and inflammatory mediators, which induce the excessive proliferation and migration of ASM cells in the airway wall, contributing to airway remodeling in asthma pathogenesis [4,5]. Therefore, a better understanding of the mechanism that underlies ASM cell hyperproliferation and migration in asthma may help identify novel targets for the development of anti-asthma medication.

SET domain-containing lysine methyltransferase 7 (SETD7) is a lysine methyltransferase that plays an important role in various physiological and pathological processes [6,7]. SETD7 was originally characterized as a H3K4-specific methyltransferase that methylates H3K4 [8]. Subsequent studies revealed that SETD7 also monomethylates non-histone substrates, such as p53 and Forkhead box O3 [9–11]. Accumulating evidence has shown that SETD7 contributes to the regulation of gene expression and the control of various cellular processes, including proliferation, apoptosis, and differentiation [12–16]. The dysregulation of SETD7 has been implicated in a diverse range of diseases, including cancer, pulmonary fibrosis, and viral infection [17–19], implicating SETD7 as a therapeutic target for disease treatment.

CD38 is a cell-surface protein that has pleiotropic functions that mediate numerous physiological and pathological processes [20,21]. The CD38 protein is a type II transmembrane glycoprotein expressed in a variety of mammalian cell types [22–24]. Notably, CD38 is expressed in ASM cells and plays an important role in the pathogenesis of asthma [25,26]. CD38 expression is induced by tumor necrosis factor (TNF)-α in ASM cells and regulates ASM airway hyperresponsiveness by

Abbreviations: SETD7, SET domain-containing lysine methyltransferase 7; ASM, airway smooth muscle; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; cADPR, cyclic ADP-ribose; IL, interleukin; qPCR, quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8

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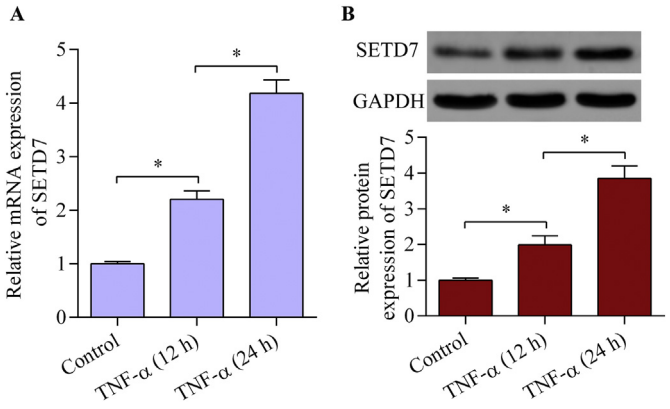

Fig. 1. SETD7 is induced by TNF-α in ASM cells. (A) Relative mRNA expression of SETD7 was examined by qPCR. (B) Protein expression of SETD7 was detected by Western blot. ASM cells were treated with 20 ng/ml TNF-α for 12 and 24 h before detection. N = 3, $*p < 0.05$. The differences among multiple experimental groups were detected using a one-way ANOVA followed by a *post hoc* Bonferroni test.

modulating cyclic ADP-ribose (cADPR)/Ca^{2+} signaling [27,28]. The expression of TNF-α is upregulated in asthma and the administration of TNF-α to experimental animals induces both airway hyperresponsiveness and airway inflammation [29,30]. *In vitro* treatment of TNF-α upregulates the proliferation and migration of ASM cells [31,32]. TNF-α-induced CD38 expression in ASM cells is regulated by numerous signaling pathways, such as nuclear factor (NF)-κB [33,34]. A lack of CD38 reduces airway hyperresponsiveness in a mouse model of interleukin (IL)-13-induced airway disease [35]. Therefore, CD38 has emerged as a promising target for treating asthma.

It has reported that SETD7 promotes TNF-α-induced inflammation through reinforcing NF-κB signaling [36]. Moreover, NF-κB is essential for induction of CD38 in TNF-α-treated ASM cells [33,34]. Considering that CD38 contributes to TNF-α-induced airway hyperresponsiveness in asthma [37,38], we hypothesized that SETD7 may be involved in regulating TNF-α-induced ASM cell proliferation and migration through NF-κB/CD38 signaling axis. In the present study, we aimed to explore the potential role of SETD7 in regulating the tumor necrosis factor (TNF)-α-induced proliferation and migration of ASM cells *in vitro*. We found that SETD7 expression was upregulated in ASM cells stimulated with TNF-α. Silencing SETD7 decreased TNF-α-induced proliferation and migration of ASM cells, whereas SETD7 overexpression showed the opposite effect. Importantly, silencing SETD7 decreased the activation of NF-κB and reduced the expression of CD38 induced by TNF-α. Blocking NF-κB activation significantly abrogated the promotional effect that SETD7 overexpression had on inducing CD38 expression. However, overexpression of CD38 partially reversed the inhibitory effect of SETD7 silencing on TNF-α-induced ASM cell proliferation and migration. Taken together, these results demonstrate that the inhibition of SETD7 restricts TNF-α-induced ASM cell proliferation and migration through the downregulation of CD38 expression *via* NF-κB, suggesting a potential relevance of SETD7 in asthma.

2. Materials and methods

2.1. Cell isolation, culture, and treatment

ASM cells were enzymatically dissociated from the tracheae of C57BL/6 mice as described previously [39]. In brief, the tracheae were excised, washed, and digested with 0.05% elastase and 0.2%

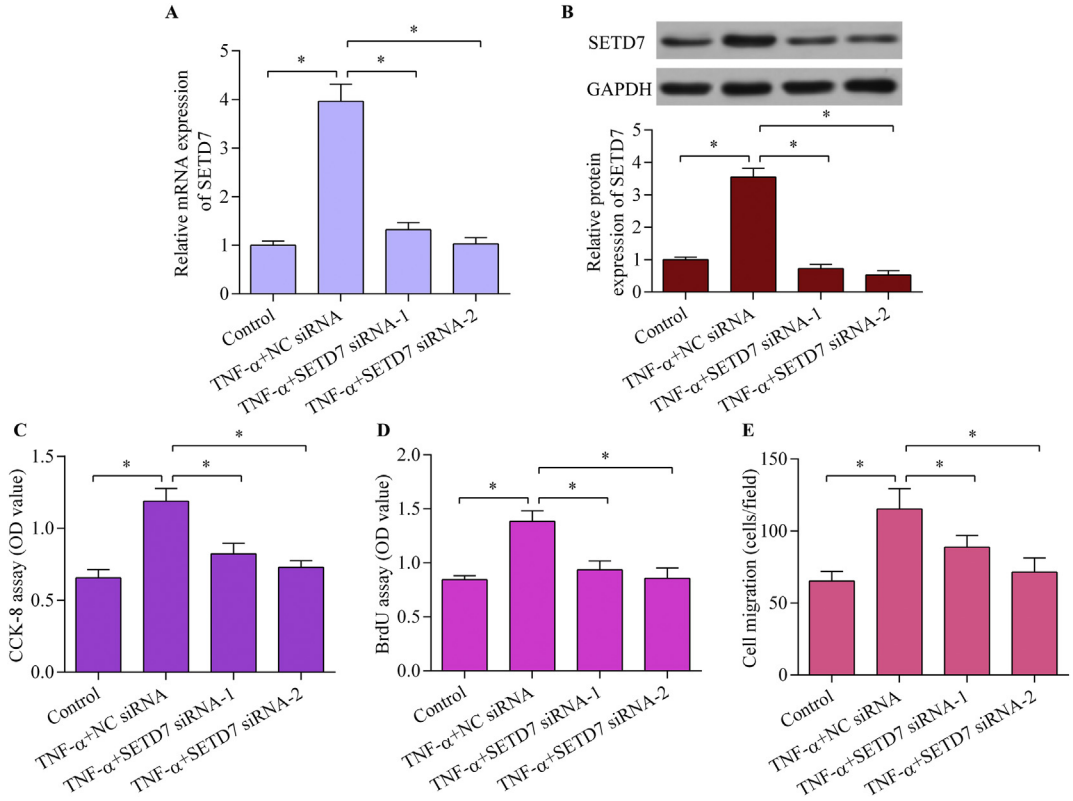

Fig. 2. SETD7 knockdown attenuates TNF-α-induced proliferation and migration. ASM cells were transfected with SETD7 siRNA or negative control (NC) siRNA for 24 h and then stimulated with 20 ng/ml TNF-α for 24 h. Relative mRNA (A) and protein (B) expression of SETD7 were examined by qPCR and Western blot, respectively. The effect of silencing SETD7 on cell proliferation was assessed by CCK-8 (C) and BrdU (D) assays. (E) The effect of SETD7 silencing on cell migration was evaluated by a Transwell assay. N = 3, $*p < 0.05$. The differences among multiple experimental groups were detected using a one-way ANOVA followed by a *post hoc* Bonferroni test.

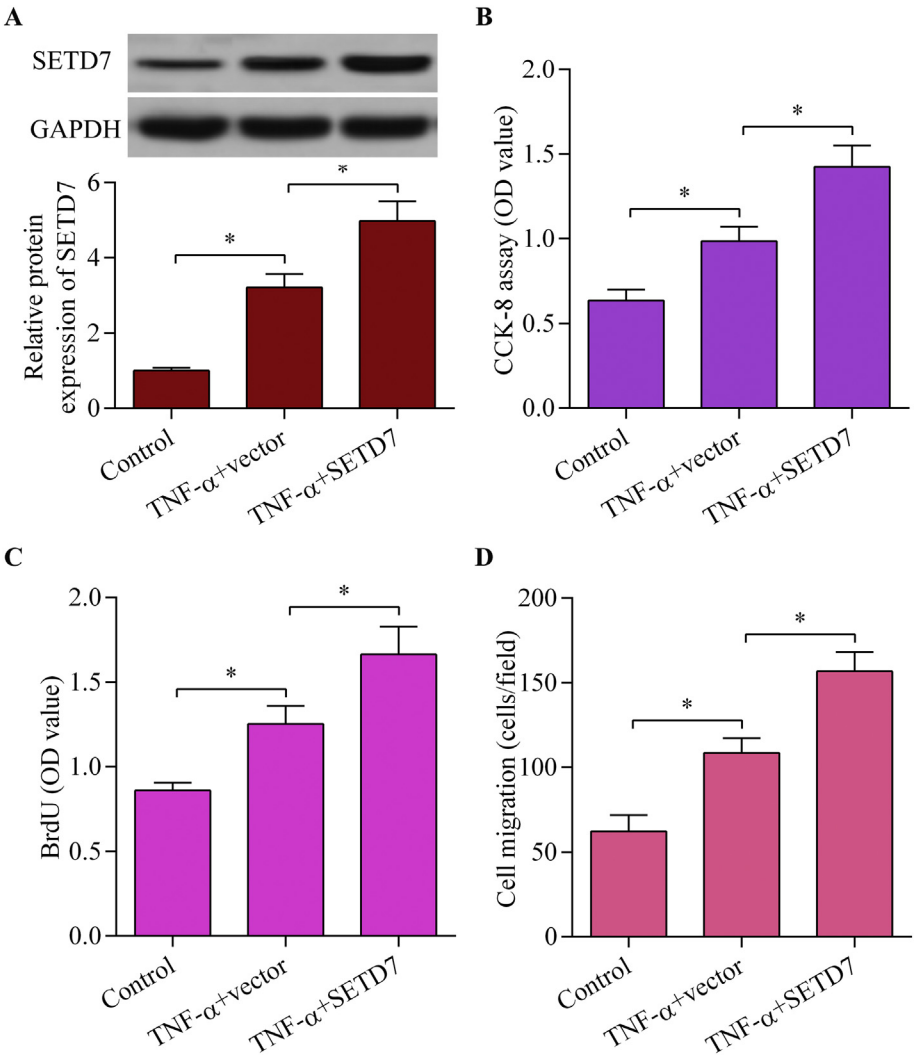

Fig. 3. Overexpression of SETD7 enhances TNF-α-induced proliferation and migration. ASM cells were transfected with pcDNA3.1/SETD7 vectors (SETD7) or pcDNA3.1/empty vectors (vector) for 24 h and then stimulated with 20 ng/ml TNF-α for 24 h. (A) The relative protein expression of SETD7 was examined by Western blot. The effect of SETD7 overexpression on cell proliferation was assessed by CCK-8 (B) and BrdU (C) assays. (D) The effect of SETD7 overexpression on cell migration was evaluated by a Transwell assay. N = 3, $*p < 0.05$. The differences among multiple experimental groups were detected using a one-way ANOVA followed by a *post hoc* Bonferroni test.

collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C. Then, the dissociated cells were collected by centrifugation and suspended into Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mix. Cells were maintained in a humidified incubator with 5% CO_2 at 37 °C. The culture medium was changed every 3 days and cells from passages 2–4 were utilized for the experiments. Cells were treated with 20 ng/ml TNF-α (R &D Systems, Minneapolis, MN, USA) to induce proliferation and migration according to a previous study with our modification [40].

2.2. Cell transfection

GenePharma (Shanghai, China) synthesized specific siRNAs targeting SETD7. The fragments of the open reading frame that encode SETD7 or CD38 were inserted into pcDNA3.1 vectors to generate pcDNA3.1/SETD7 or pcDNA3.1/CD38 expression vectors. Cell transfection of siRNAs and vectors were performed using Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as per the manufacturer's instructions.

2.3. RNA extraction and quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from cells using TRIzol Reagent (Thermo Fisher Scientific, Inc.) and was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocols. We amplified the cDNA templates using Power SYBR Green PCR Master Mix (Applied Biosystems) with appropriate primers with an Applied Biosystems 7500 Real-Time PCR System and the following thermal cycling parameters: 95 °C, 10 min and 40 cycles at 95 °C, 10 s at 60 °C, 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blot analysis

Cells were lysed using RIPA buffer (Beyotime Biotechnology, Shanghai, China) and the protein concentration was quantified using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology) as per the manufacturer's instructions. Protein lysates were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel for separation by electrophoresis. The resolved proteins were then transferred onto a polyvinylidene fluoride membrane and the membrane was immersed in 5% skim milk to block non-specific binding sites. Thereafter, the membrane was incubated with primary antibodies against SETD7, CD38, and GAPDH (Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing with Tris-buffered saline with Tween 20 (TBST), the membrane was probed with horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam) at room temperature for 1 h. After washing with TBST, the membrane was developed with Pierce ECL Western Blotting

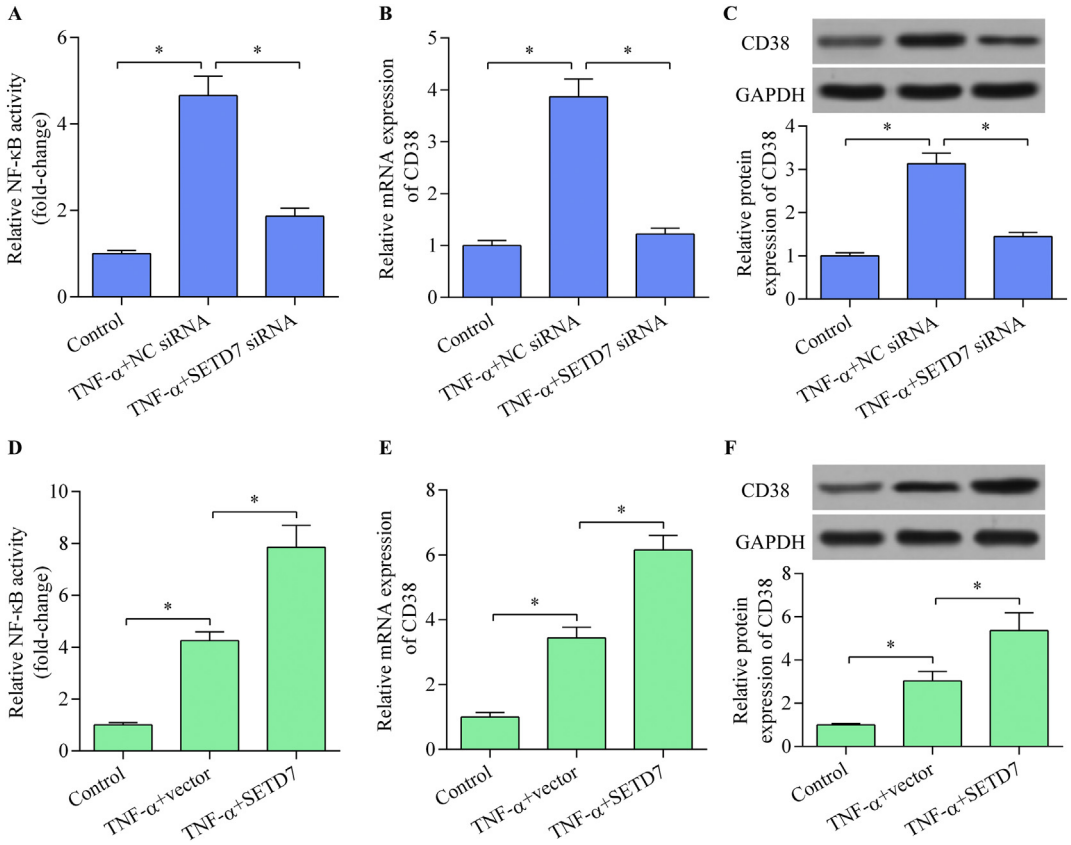

Fig. 4. SETD7 regulates TNF-α-induced NF-κB/CD38 signaling in ASM cells. (A) We determined the effect of silencing SETD7 on NF-κB activation using a luciferase reporter assay. We used qPCR and Western blot to examine the effect of silencing SETD7 on SETD7 mRNA (B) and protein (C) expression, respectively. (D) The effect of SETD7 overexpression on NF-κB activation was assessed by a luciferase reporter assay. The effect of SETD7 overexpression on SETD7 mRNA (E) and protein (F) expression was detected by qPCR and Western blot, respectively. N = 3, $*p < 0.05$. The differences among multiple experimental groups were detected using a one-way ANOVA followed by a *post hoc* Bonferroni test.

Substrate (Thermo Fisher Scientific, Inc.). The relative intensity of protein bands was quantified using Image-Pro Plus 6.0 software.

2.5. Cell proliferation assay

We used Cell Counting Kit-8 (CCK-8) and BrdU assays to determine cell proliferation. For the CCK-8 assay, cells were plated into 96-well tissue culture plates and then we performed cell transfection. After the indicated treatment and time, 10 μl of CCK-8 solution (Beyotime Biotechnology) was added to each well and incubated for 2 h at 37 °C. Then, the optical density (OD) value at a wavelength of 450 nm was measured using a microplate reader (Bio-Rad, Sunnyvale, CA, USA). For the BrdU assay, cells were seeded into 96-well plates and detected using a BrdU Cell Proliferation ELISA Kit (colorimetric) according to the manufacturer's protocols.

2.6. Transwell assay

Cell migration was detected by a Transwell assay using transwell chambers in 24-well plates. Briefly, after transfection cells were suspended in 200 μl of serum-free medium and placed in the upper chamber. Meanwhile, the lower chamber was filled with 500 μl of medium containing 20% FBS. After 24 h of incubation at 37 °C, the residual cells that remained on the upper surface of the filters were wiped off using cotton swabs and the migrated cells on the lower filter surface were fixed with 4% formaldehyde and stained with 0.1% crystal violet for visualization. The stained cells were counted under an optical microscope.

2.7. Luciferase reporter assay

The transcriptional activity of NF-κB was detected by a luciferase reporter assay using a pNF-κB-luc reporter plasmid (Beyotime Biotechnology). Cells were plated in 24-well plates and transfected with a pNF-κB-luc reporter plasmid and SETD7 siRNA or SETD7 expression vector for 24 h. Then, cells were stimulated with TNF-α for 24 h before being harvested for detection. Luciferase activities were determined using a Firefly Luciferase Reporter Gene Assay Kit (Beyotime Biotechnology) following the manufacturer's protocols.

2.8. Statistical analysis

The quantitative data were expressed as means ± standard deviations (SD). Data were analyzed using SPSS Statistics Version 19.0 (SPSS Inc., Chicago, IL, USA). The differences among multiple experimental groups were detected using a one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. SETD7 is increased in ASM cells stimulated with TNF-α

To investigate the potential role of SETD7 in airway remodeling, we examined the expression change of SETD7 in TNF-α-treated ASM cells. The results of the qPCR analysis showed that treatment with TNF-α induced a significant increase in SETD7 mRNA expression (Fig. 1A). The protein expression of SETD7 is also consistently upregulated by

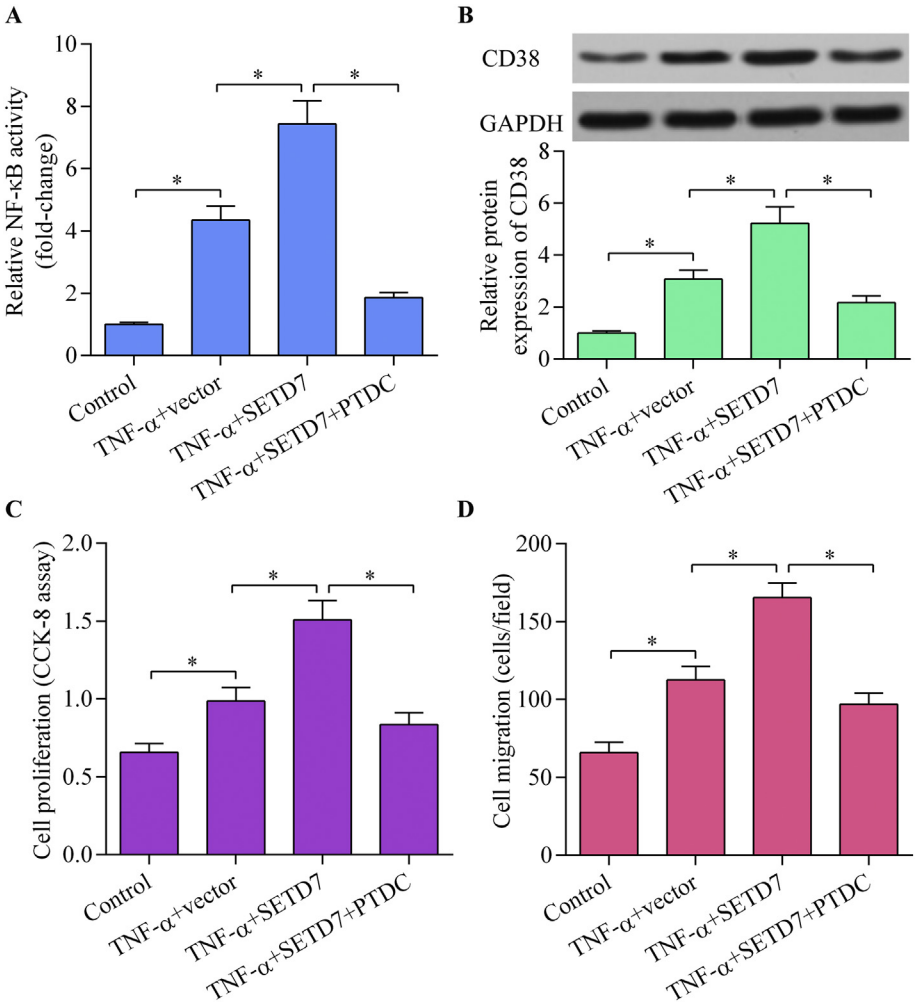

Fig. 5. SETD7 modulates CD38 expression by NF-κB. ASM cells were transfected with SETD7 expression vectors for 24 h in the presence of PTDC (50 μM) and were then stimulated with 20 ng/ml TNF-α for 24 h. (A) NF-κB activity was determined by a luciferase reporter assay. (B) Protein expression of CD38 was examined by a Western blot. (C) Cell proliferation was assessed by a CCK-8 assay. (D) Cell migration was detected by a Transwell assay. N = 3, $*p < 0.05$. The differences among multiple experimental groups were detected using a one-way ANOVA followed by a *post hoc* Bonferroni test.

TNF-α treatment in ASM cells (Fig. 1B). These data indicate that SETD7 is induced by TNF-α in ASM cells.

3.2. Silencing SETD7 restricts TNF-α-induced proliferation and migration of ASM cells

To investigate the biological function of SETD7 in airway remodeling, we detected the regulatory effect of silencing SETD7 on TNF-α-induced ASM cell proliferation and migration. Our results showed that transfection of SETD7 siRNA significantly downregulated the expression of SETD7 in ASM cells (Fig. 2A and B). The treatment of TNF-α induced a significant increase in cell proliferation and migration (Fig. 2C–E). Notably, TNF-α-induced ASM cell proliferation and migration were markedly attenuated by SETD7 depletion in ASM cells (Fig. 2C–E). Overall, these results suggest that silencing SETD7 restricts TNF-α-induced ASM cell proliferation and migration.

3.3. SETD7 overexpression exacerbates TNF-α-induced proliferation and migration of ASM cells

To validate the regulatory effect of SETD7 on TNF-α-induced proliferation and migration of ASM cells, we performed gain-of-function experiments on SETD7. The overexpression of SETD7 was achieved by transfecting SETD7 expression vectors into ASM cells (Fig. 3A). Notably, we found that SETD7 overexpression significantly upregulated the proliferation and migration of ASM cells treated with TNF-α (Fig. 3B–D). These results indicate that SETD7 overexpression exacerbates TNF-α-induced ASM cell proliferation and migration.

3.4. SETD7 modulates NF-κB activation and CD38 expression induced by TNF-α

TNF-α-induced NF-κB/CD38 signaling activation contributes to airway remodeling during asthma [33]. NF-κB has been reported as a downstream target of SETD7 [41], so we examined whether SETD7 is involved in regulating TNF-α-induced NF-κB/CD38 signaling activation in ASM cells. Our results showed that silencing SETD7 significantly decreased TNF-α-induced activation of NF-κB in ASM cells (Fig. 4A). Interestingly, silencing SETD7 also significantly attenuated TNF-α-induced CD38 expression (Fig. 4B and C). In contrast, SETD7 overexpression had the opposite effect on TNF-α-induced NF-κB/CD38 signaling (Fig. 4D–F). Collectively, these results indicate that SETD7 is involved in regulating NF-κB activation and CD38 expression induced by TNF-α in ASM cells.

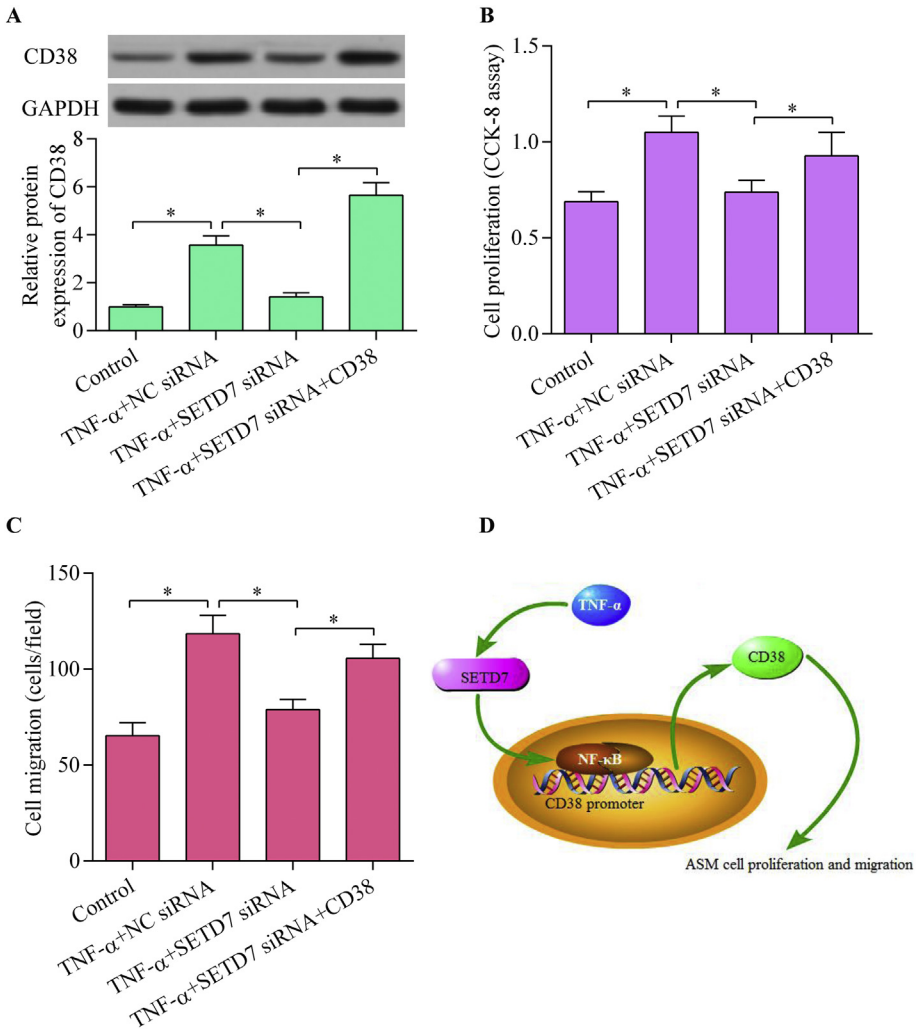

Fig. 6. Restoration of CD38 expression reverses the inhibitory effect of silencing SETD7 on TNF-α-induced proliferation and migration. ASM cells were cotransfected with SETD7 siRNA and pcDNA3.1/CD38 expression vectors for 24 h and were then stimulated with 20 ng/ml TNF-α for 24 h. (A) Protein expression of CD38 was detected by a Western blot. (B) Cell proliferation was examined by a CCK-8 assay. (C) Cell migration was assessed by a Transwell assay. N = 3, $*p < 0.05$. The differences among multiple experimental groups were detected using a one-way ANOVA followed by a *post hoc* Bonferroni test. (D) A schematic model of the SETD7-regulated NF-κB/CD38 axis in modulating TNF-α-induced ASM cell proliferation and migration.

3.5. Inhibition of NF-κB blocks the promotional effect of SETD7 overexpression on TNF-α-induced CD38 expression

To investigate whether SETD7 regulates CD38 expression by NF-κB, we detected the effect of NF-κB inhibition on SETD7-induced CD38 expression in TNF-α-treated ASM cells. We found that the NF-κB inhibitor PTDC significantly attenuated TNF-α-induced NF-κB activation in ASM cells (Fig. 5A). The inhibition of NF-κB significantly reversed the promotional effect of SETD7 overexpression on TNF-α-induced CD38 expression (Fig. 5B). Moreover, the promotional effect of SETD7 overexpression on TNF-α-induced proliferation and migration was also markedly reversed by NF-κB inhibition (Fig. 5C and D). Overall, these results suggest that SETD7 modulates CD38 expression, proliferation, and migration by targeting NF-κB.

3.6. Restoration of CD38 expression reverses the inhibitory effect of SETD7 silencing on TNF-α-induced proliferation and migration

To investigate whether SETD7 regulates TNF-α-induced proliferation and migration by CD38, we performed a rescue assay of CD38 in SETD7 siRNA-transfected ASM cells. Our results showed that transfecting CD38 expression vector significantly restored the expression of CD38 in SETD7 siRNA-transfected ASM cells (Fig. 6A). As expected, the inhibitory effect of silencing SETD7 on TNF-α-induced proliferation and migration was partially reversed by CD38 overexpression (Fig. 6B and C). These results suggest that SETD7 regulates TNF-α-induced ASM cell proliferation and migration by CD38.

4. Discussion

In this study, we demonstrated that SETD7 is involved in regulating ASM cell proliferation and migration. The C57BL/6 mice have been widely used to establish asthma model [42–44]. We therefore isolated the ASM cells from the tracheae of C57BL/6 mice as described previously [39]. We found that the inhibition of SETD7 restricted TNF-α-induced ASM cell proliferation and migration. The underlying mechanism was associated with its regulatory effect on the NF-κB/CD38 signaling axis (Fig. 6D). Our study suggests a potential relevance of SETD7 in asthma.

SETD7 has been reported as an important regulator that controls cell proliferation and migration. A high expression of SETD7 promotes the proliferation of various tumor cells [13,17,45]. SETD7 is involved in promoting the proliferation and migration of vascular endothelial cells [46]. In addition, SETD7 has been reported to promote the migration of renal fibroblasts during the progression of chronic kidney disease [47]. To date, little is known about the role of SETD7 in regulating ASM cell proliferation and migration. Consistent with these findings, our results revealed that SETD7 induced by TNF-α promoted the proliferation and migration of ASM cells, reinforcing the significance of SETD7 in regulating cell proliferation and migration. Considering that TNF-α-induced proliferation and migration of ASM cells contribute to asthma progression, SETD7 may serve as a potential target for treating asthma.

SETD7 has been reported as an important regulator of NF-κB signaling. The inhibition of SETD7 results in significant reduction in NF-κB p65 expression in high glucose-treated macrophages [48]. SETD7

overexpression inhibits the nuclear translocation of NF-κB p65 in hepatocytes during hepatitis C virus replication [19]. Notably, SETD7 has been reported to contribute to TNF-α-induced methylation of NF-κB p65 subunit p65, which is required for the expression of a subset of NF-κB target genes in response to TNF-α stimulation [41]. Moreover, knockdown of SETD7 decreases the expression of NF-κB target genes induced by TNF-α in monocytes [36]. Therefore, these findings suggest that SETD7 functions as a positive regulator of TNF-α-induced activation of NF-κB signaling. In line with these findings, our study demonstrated that the inhibition of SETD7 significantly downregulated TNF-α-induced activation of NF-κB in ASM cells, reinforcing the significance of SETD7 in regulating NF-κB activation. However, the opposite effect of SETD7 on NF-κB activation has also been reported. Yang et al. reported that SETD7-mediated lysine methylation of NF-κB p65 promotes the degradation of p65 and decreases the TNF-α-induced expression of NF-κB target genes [49]. In multiple myeloma cells, SETD7 inhibits the activation of NF-κB signaling to induce cell apoptosis and the generation of reactive oxygen species [50]. These discrepancies indicate that the precise role of SETD7 in regulating NF-κB activation may be related to the cell type and the context.

CD38 has been reported as an important regulator of asthma. CD38 is induced by inflammatory cytokines in ASM cells and contributes to the regulation of cyclic cADPR/Ca^{2+} signaling, which plays an important role in airway remodeling and hyperresponsiveness in asthma [27,28]. The increased expression of CD38 promotes the proinflammatory cytokine release in ASM cells [51]. Notably, knockout of CD38 effectively reduces the asthmatic phenotype in mouse models [35,37,52]. Interestingly, TNF-α-induced CD38 expression in ASM cells is regulated by NF-κB. The promoter region of CD38 possesses a NF-κB-binding site and a lack of this binding site significantly blocks the regulatory effect of TNF-α on CD38 [53]. Given that NF-κB is regulated by SETD7, we investigated whether SETD7 contributes to regulating CD38 expression in ASM cells. As expected, we found that SETD7 regulates the expression of CD38 in NF-α-treated ASM cells. We also found that the inhibition of NF-κB significantly reversed the promotional effect of SETD7 on the induction of CD38 expression. Moreover, our results showed that the restoration of CD38 expression partially reversed the SETD7 inhibition-mediated effect on TNF-α-induced ASM cell proliferation and migration. Overall, our study suggests that the SETD7 contributes to regulation of TNF-α-induced ASM cell proliferation and migration through upregulation of CD38 expression *via* enhancing NF-κB signaling.

In conclusion, our findings demonstrate that the inhibition of SETD7 restricts TNF-α-induced ASM cell proliferation and migration by blocking the NF-κB/CD38 signaling axis. These results highlight a potential relevance of the SETD7/NF-κB/CD38 axis in the pathogenesis of asthma. Therefore, understanding such a potential mechanism might be critical for validating SETD7 as a therapeutic target for asthma. However, the precise role and molecular mechanism of SETD7 in asthma needs further investigation using animal models *in vivo*.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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