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Mucin 1 downregulation impairs the anti-necroptotic effects of glucocorticoids in human bronchial epithelial cells

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ABSTRACT

Aims: To investigate whether mucin 1 (MUC1) downregulation reduced the sensitivity of tumor necrosis factor-alpha (TNF-α)-induced bronchial epithelial cells to glucocorticoid-mediated necroptosis and explore the underlying mechanisms.

Main methods: The human lung bronchial epithelial cell line (16HBE) was transfected with small interfering RNA (siRNA) against MUC1 and then stimulated by TNF-α, where some cells were pretreated with dexamethasone. Flow cytometry was performed to analyze necroptosis in 16HBE cells, and western blot analysis was used to detect protein expression levels of MUC1, glucocorticoid receptor (GR)α, GRβ, NF-κB p65, phospho-p65 (p-p65), and histone deacetylase-2 (HDAC2). Additionally, nuclear translocation of MUC1 and GRα was assessed by immunofluorescence.

Key findings: We observed that MUC1 downregulation by siRNA significantly augmented TNF-α-induced necroptosis in 16HBE cells, and that dexamethasone showed impaired anti-necroptotic effects of MUC1 downregulation. Furthermore, we found that GRα nuclear translocation was inhibited in 16HBE cells with MUC1 downregulation, and that dexamethasone-mediated inhibition of p65 phosphorylation was lower in cells transfected with MUC1-siRNA compared to those transfected with negative control siRNA.

Significance: Impaired GRα nuclear translocation and inhibited p-p65 expression might contribute to glucocorticoid resistance caused by MUC1 deficiency in TNF-α-induced necroptosis in 16HBE cells, and should be considered as a potential target for the development of novel therapeutics for asthma.

1. Introduction

Asthma is an intricate inflammatory disease characterized by reversible airway obstruction, airway inflammation, mucus hypersecretion, and acute hyper-responsiveness [1]. Inhaled corticosteroids in combination with bronchodilators are the standard treatment for symptomatic control and improvement of lung function in patients with mild to moderate asthma. Approximately 5%–25% of patients with severe, steroid-resistant asthma have poor symptom control even with high-dose and/or systemic corticosteroids treatment, which comprise up to 50% of total asthma-related treatment costs [1–3]. Several studies showed that death of cell in asthmatic airways, including eosinophils and epithelial cells, play a regulatory role in severe asthma [4–8]. In addition, Cerps et al. showed that necroptosis occurs at a mouse model of viral stimulus-induced exacerbation of asthma, and interferon-β deficiency at asthma exacerbation promotes receptor-interacting protein kinase (RIPK3) and phosphorylation of mixed linage kinase domain-like protein (pMLKL) mediated necroptosis [9]. However, whether necroptosis of airway epithelial cells participates to the occurrence of steroid-resistant asthma remains unknown.

Glucocorticoids (GC) cross the cell membrane and bind with glucocorticoid receptors (GRs) to form glucocorticoid/GR complexes in the cytoplasm, which translocate to the nucleus and interact with glucocorticoid response elements (GREs), with subsequent increases in the expression levels of anti-inflammatory genes (transactivation) or proinflammatory genes (transrepression), respectively [3,10,11]. Molecular mechanisms of glucocorticoid resistance include increased GRβ expression or decreased GRα expression, defective glucocorticoid/GR nuclear translocation, and loss of histone deacetylase-2 (HDAC2) expression, among others [3,10–14]. In patients with uncontrolled severe asthma, tumor necrosis factor (TNF)-α expression is refractory to steroid treatment [15,16]. An increase in the level of TNF-α induces phosphorylation of mitogen-activated protein kinases (MAPKs), which in turn lead to GRα phosphorylation at Ser226, thereby inhibiting

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nuclear translocation of the glucocorticoid/GR complex and its subsequent binding to GREs [17,18]. Therefore, inhibitors for TNF-α and MAPKs have been tested as potential treatment options in patients with steroid-resistant asthma; however, studies found that the long-term administration of these biologics had significant adverse effects [17,19,20]. Effective molecules with good safety profiles are sorely needed for the treatment of steroid-resistant asthma.

Mucin 1 (MUC1), a membrane-bound mucin protein, has important anti-inflammatory roles in the airways in response to a variety of infectious insults [21–23]. MUC1 protein comprises two noncovalently associated polypeptide subunits, an N-terminal extracellular subunit and a C-terminal subunit containing a highly conserved cytoplasmic tail (CT) [24]. MUC1-CT modulates signal transduction events implicated in diverse cellular processes such as cell death and cell proliferation [25–27]. The interaction of MUC1-CT with signaling molecules is suggested to be regulated by specific protein-protein interactions. For example, MUC1-CT was demonstrated to interact with the IκB kinase complex and Fas-associated death domain to reduce cell apoptosis in response to TNF-α [28,29].

Recent studies indicated that MUC1 expression was downregulated in patients of chronic rhinosinusitis with nasal polyps. MUC1 inhibits phosphorylation of ERK1/2 and phosphorylation of GRα at Ser226; it was also shown to form a complex with GRα to promote the glucocorticoid/GR complex translocation to the nucleus [30]. Similarly, MUC1 expression was decreased in patients with severe asthma, whereas MUC1 deficiency impaired the anti-inflammatory effects of dexamethasone both in vitro and in vivo [31]. Moreover, recent evidence suggests that dexamethasone can repress TNF-α-induced necroptosis in intestinal epithelial cells and reduce the Ripk3 mRNA expression in $GR^{wt/wt}$ mice compared to $GR^{dim/dim}$ mice. These mice express a mutant version of GR, carrying a missense point mutation (A458T) which leads to a reduced GR dimerization [32].

Dexamethasone, a synthetic glucocorticoid, possesses potent anti-inflammatory and anti-necroptotic effects [30–32]. Recently, it has been suggested and supported by studies in asthma that dexamethasone could reduce airway inflammation both in vitro and in vivo [31,33,34]. Although evidence clearly shows that MUC1 serves an important role in the anti-inflammatory functions of dexamethasone, whether MUC1 alters the anti-necroptotic effects of dexamethasone to airway epithelial cells in asthma remains unknown. In this study, we investigated whether MUC1 downregulation reduced the sensitivity of TNF-α-induced epithelial cells to glucocorticoid-mediated necroptosis and explore the underlying mechanisms.

2. Materials and methods

2.1. Cell culture and stimulation

The human lung bronchial epithelial cell line (16HBE) was obtained from the Boster Biotech (Wuhan, China) and maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY USA) supplemented with 10% fetal bovine serum at 37 °C with 5% CO2. Cells were treated with 300 ng/ml of TNF-α (PeproTech, RH, USA) in the presence or absence of the dexamethasone (MedChemExpress, Shanghai, China), and the specific inductions were performed as indicated in the figure legends.

2.2. Assessment of necroptosis

TNF-α induced cellular death in 16HBE cells was quantified by flow cytometry using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (KeyGEN, Nanjing, China). The cells were washed in PBS and incubated in 500 μl binding solution containing 5 μl of annexin V-FITC and 5 μl of propidium iodide for 15 min at room temperature in the dark. Cell fluorescence was detected by Becton Dickinson LSR flow cytometer using the CellQuest software. Annexin V (+)/PI (+) cells in upper right quadrant were considered as necrotic.

2.3. Cell transfection

Human-specific MUC1 siRNA (sense 5′-GUU CAG UGC CCA GCU CUA Ctt-3′; antisense 5′-GUA GAG CUG GGC ACU GAA Ctt-3′) from Ruibo Biotechnology (Guangzhou, China) was used to silence MUC1 expression in 16HBE cells, and a non-targeting siRNA from the same company was used as control. Transfections were performed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, 4×10^5 cells were plated in 6-well plates for 24 h and transfected with 50 nM of each siRNA, or control siRNA, using 3.75 μl Lipofectamine 3000 in 250 μl Opti-MEM medium (Life Technologies, Carlsbad, CA, USA), then, the medium was changed 4–6 h after transfection. After 48 h, the effective gene silencing was confirmed by quantitative real-time polymerase chain reaction (PCR) and western blotting.

2.4. RNA isolation and quantitative real-time PCR

Total RNA from 16HBE cells was extracted using TRIzol reagent (Takara Bio, Otsu, Japan). RNA was reversed transcribed into cDNA with a cDNA reverse transcription-PCR kit (Takara Bio) according to the manufacturer's instructions. Subsequently, cDNA was used as a template for quantification of gene expression using a SYBR Green real-time PCR kit (Takara Bio) and an ABI PRISM Fast 7500 system (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed at 95 °C for 5 min, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (ΔΔCt = (target gene Ct of experimental group – reference gene Ct of experimental group) – (target gene Ct of control group – reference gene Ct of control group)). The sequences of the primers used were as follows: *β-actin* forward 5′-AGA AAA TCT GGC ACC ACA CCT-3′, reverse 5′-GAT AGC ACA GCC TGG ATA GCA-3′; *MUC1* forward 5′-TCA GCT TCT ACT CTG GTG CAC AA-3′, reverse 5′-ATT GAG AAT GGA GTG CTC TTG CT-3′, *GRα* forward 5′-GAA GGA AAC TCC AGC CAG AAC-3′, reverse 5′-CTG ATT GGT GAT GAT TTC AGC TA-3′, *GRβ* forward 5′-GAA GGA AAC TCC AGC CAG AAC-3′, reverse 5′-TGA CTT ATT ATT GAC AAC GAA GTG C-3′.

2.5. Western blot analysis

Total or nuclear protein lysates were prepared from the cultured 16HBE cells. The protein concentration was measured using the BCA protein assay kit (Aspen Biological, Wuhan, China). Equal amounts of protein were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The band with the desired protein was cut and blocked in 5% non-fat dried milk in 1× TBS for 1 h at room temperature. The membranes were then incubated in the appropriate primary antibodies (anti-MUC1-CT (Thermo Fisher Scientific, MA5-11202); anti-GRα (abcam, ab3580); anti-GRβ (abcam, ab3581); anti-nuclear factor (NF)-κB p65 (Proteintech, 10745-1-AP); anti-phospho-NF-κB p65 (Cell Signaling Technology, 3033), and anti-HDAC2 (Proteintech, 12922-3-AP)) overnight at 4 °C. Subsequently, the membranes were washed with 1× TBST 3 times and incubated with an appropriate HRP-labeled secondary antibody for 90 min at room temperature on a shaker. Finally, the membranes were again washed with 1× TBST 3 times and assayed using a ChemiDoc XRS gel imaging system (Bio-Rad, Hercules, CA, USA). The protein expression level was quantified by Image J (NIH Image, Bethesda, MD).

2.6. Immunofluorescence staining

After the treatment with TNF-α of corresponding groups, the culture medium was removed and the adherent cells were washed once in PBS.

Cells were then fixed in 4% paraformaldehyde at 4 °C for 15 min, followed by rinsing with PBS 3 times. Cells were incubated with 0.5% Triton X-100 at room temperature for 15 min to rupture their cell membranes, and they were rinsed again with PBS 3 times. Subsequently, cells were blocked with 2% bovine serum albumin and 5% fetal calf serum for 1 h at room temperature and incubated with hamster anti-MUC1-CT and rabbit anti-GRα primary antibodies at 4 °C overnight. After rinsing with PBS 3 times, cells were incubated with Alexa Fluor 488-labeled goat anti-hamster and Alexa Fluor 594-labeled goat anti-rabbit antibodies at room temperature for 60 min. Eventually cells were rinsed with PBS 3 times and counterstained with DAPI (4′,6-diamidino-2-phenylindole; Aspen Biological, Wuhan, China) in a dark place at room temperature for 10 min. Images were visualized at × 400 by laser scanning confocal microscopy using a Leica Sp5 microscope (Leica Microsystems, Buffalo Grove, IL USA).

2.7. Statistical analysis

Data were expressed as the means ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Two-tailed Student *t*-test was used for comparison between two groups, and one-way analysis of variance with Tukey post hoc test was used for comparison of more than two groups. A P value < 0.05 was considered to indicate statistically significant differences.

3. Results

3.1. MUC1 downregulation augments TNF-α-induced necroptosis in 16HBE cells

Our preliminary studies revealed that TNF-α induced necroptosis in 16HBE cells. As shown in Fig. 1A, the number of necroptotic cells was significantly increased in the TNF-α-treated cells compared with the control group. Additionally, we also determined that the MUC1 expression was increased in a time-dependent manner after TNF-α stimulation (Fig. 1B). To further investigate whether MUC1 impacted TNF-α-induced necroptosis, the cells were transfected with MUC1-siRNA. As shown in Fig. 1C, cells transfected with MUC1-siRNA exhibited a significant reduction in the expression levels of MUC1 as confirmed by both quantitative real-time PCR and western blot analysis. In addition, our findings demonstrated that the percentage of cell death was significantly higher in the MUC1-siRNA cells than the negative control-siRNA cells after treatment with TNF-α (Fig. 1D). These results were consistent with our preliminary findings that MUC1 downregulation contributed to TNF-α-induced necroptosis of 16HBE cells.

3.2. MUC1 downregulation attenuates dexamethasone-mediated inhibition of TNF-α-induced necroptosis in 16HBE cells

To determine whether Glucocorticoids could inhibit TNF-α-induced necroptosis in 16HBE cells with MUC1 downregulation, the 16HBE cells were treated with dexamethasone. As displayed in Fig. 2A, we found that dexamethasone efficiently decreased TNF-α-induced necroptosis, which indicated that dexamethasone exerted anti-necroptotic functions in 16HBE cells expressing MUC1. However, dexamethasone did not reduce cells necroptosis in MUC1-siRNA + TNF-α group (Fig. 2B). Together, these findings suggested that cells with MUC1 downregulation were more resistant to dexamethasone's inhibitory effect on TNF-α-induced necroptosis.

3.3. Glucocorticoid resistance is not associated with GRα and GRβ protein expression

Recent studies showed that the molecular mechanisms of steroid resistance included increased GRβ or decreased GRα expression. Therefore, we first determined whether MUC1 knockdown altered the expression of GRα and GRβ by real-time PCR and western blotting. As demonstrated in Fig. 3A, the mRNA expression levels of GRα were significantly higher in the MUC1-siRNA + TNF-α group compared with the NC + TNF-α group. However, the protein expression levels of GRα did not show an upregulation in the MUC1-siRNA + TNF-α group compared with the NC + TNF-α group (Fig. 3B). Moreover, there was not statistically significant for an increase in GRβ expression levels at both the mRNA and protein levels in the MUC1-siRNA + TNF-α group compared with the NC + TNF-α group (Fig. 3C, D). Altogether, these results suggested that glucocorticoid resistance might be not associated with GRα and GRβ expression.

3.4. MUC1 downregulation reduces glucocorticoid sensitivity via a reduction in the nuclear translocation of GRα

Upon glucocorticoid binding to GR, the glucocorticoid/GR complex translocates to the nucleus to activate transcription factors that modulate the expression of glucocorticoid-responsive genes. Therefore, we next determined whether MUC1 knockdown could impair the GRα nuclear translocation and whether it was associated with glucocorticoid insensitivity following TNF-α treatment. We found that dexamethasone enhanced the GRα fluorescence intensity in the nuclei of 16HBE cells. However, the effect of dexamethasone on GRα nuclear translocation was lower in 16HBE cells with MUC1 downregulation. Conversely, TNF-α reduced the efficacy of dexamethasone on GRα translocation to the nucleus, and this effect was stronger in 16HBE cells with MUC1 downregulation (Fig. 4A).

To further confirm that MUC1 suppression inhibited GRα nuclear translocation, we determined the expression levels of GRα in both the cytoplasm and nucleus of 16HBE cells. Western blot analysis showed that dexamethasone increased GRα nuclear protein expression in the negative control group compared with the MUC1-siRNA group. Treatment with TNF-α on the NC + Dex group led to a decrease in GRα nuclear protein, similar results were also observed in the MUC1-siRNA + Dex group (Fig. 4B).

We also investigated the subcellular localization of MUC1 and GRα by confocal microscopy. As shown in Fig. 4C, under basal conditions, MUC1 and GRα were localized in the cytoplasm and nucleus of 16HBE cells. After dexamethasone exposure, the nuclear colocalization of MUC1 with GRα was increased. Together, these results suggested that MUC1 downregulation could reduce glucocorticoid sensitivity by decreasing nuclear translocation of GRα (Fig. 6).

3.5. MUC1 downregulation reduces glucocorticoid sensitivity via inhibiting p-p65 expression

Since the NF-κB pathway and attenuated HDAC2 signaling were shown to be strongly involved in steroid resistance, we also investigated the effect of MUC1 downregulation on the activation of these two pathways. We found that MUC1 downregulation did not lead to any changes in the expression of NF-κB p65 or HDAC2 in cells treated with TNF-α or in those cells in the presence or absence of dexamethasone (Fig. 5A, B).

Therefore, we focused on whether MUC1 downregulation might affect the phosphorylation of NF-κB p65 by measuring p-p65 levels. We found that p-p65 levels were increased by TNF-α as an early and transient event, with the most effect observed at 5 min, which was lost after 60 min (Fig. 5C). Therefore, we chose the 5-min treatment with TNF-α for further evaluation. As shown in Fig. 5D, the p-p65 protein expression induced by TNF-α was increased further in the negative control-siRNA cells compared with the MUC1-siRNA cells. However, the inhibitory effect of dexamethasone on p-p65 was lower in the MUC1-siRNA cells compared with the negative control-siRNA cells. These results suggested that p-p65 but not HDAC2 was involved as the

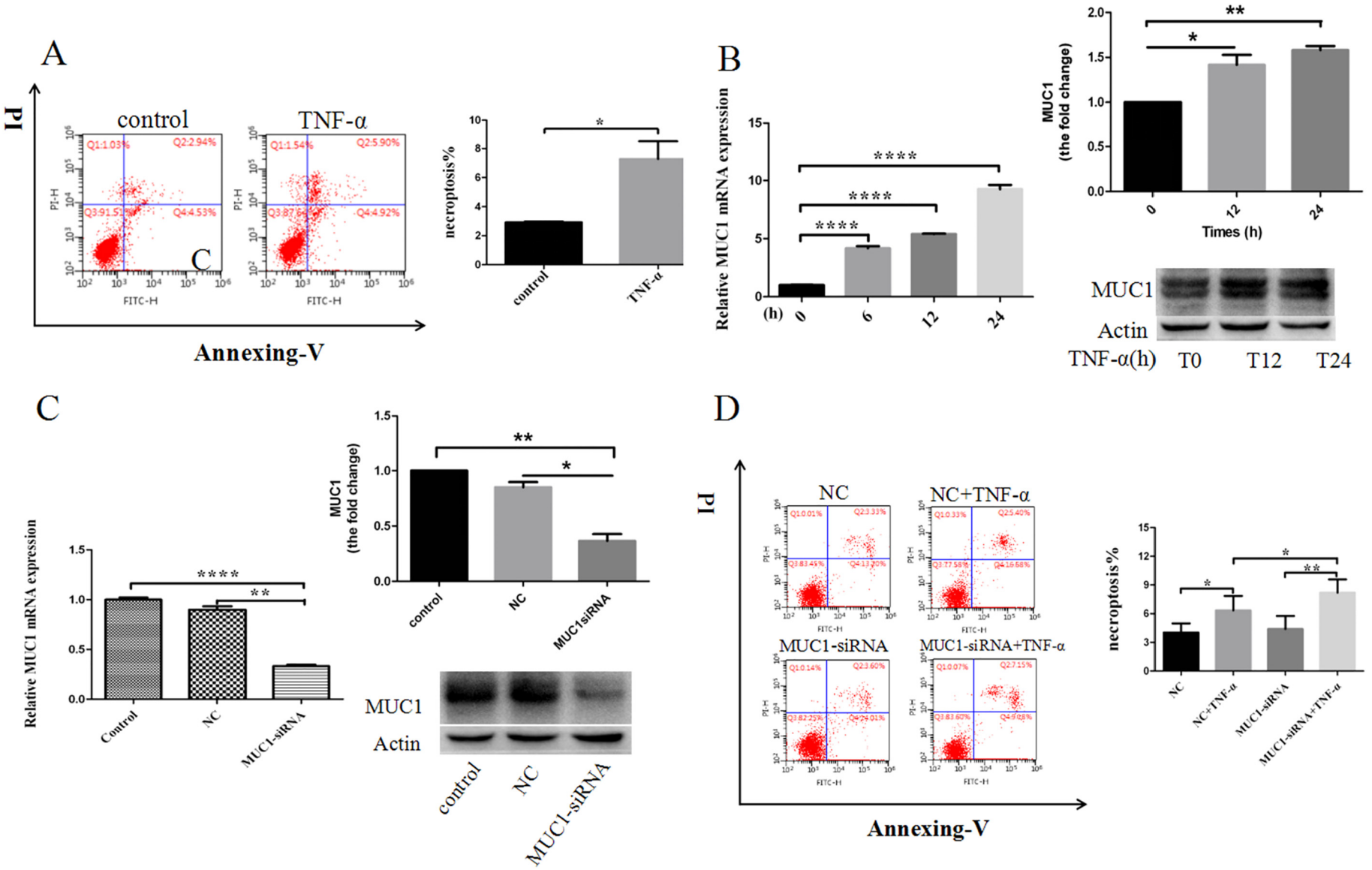

Fig. 1. MUC1 downregulation augments TNF-α-induced necroptosis in 16HBE cells.
(A) 16HBE cells were treated with TNF-α (300 ng/mL) for 24 h, flow cytometry was conducted to detect the cell necroptosis. (B) MUC1 expression was measured by real-time PCR and western blot analysis in 16HBE cells treated with TNF-α (300 ng/mL) for the indicated times. (C) mRNA and protein expression levels of MUC1 in negative control-siRNA and MUC1-siRNA transfected cells were determined by real-time PCR and western blot. (D) At 24 h after transfection with MUC1-siRNA or negative control-siRNA, 16HBE cells were treated with TNF-α (300 ng/mL) for 24 h, flow cytometry was used to assess the cell necroptosis. Data are expressed as means ± SD ($n = 3$) and were analyzed by Student *t*-test or one-way analysis of variance, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$. NC group (transfected with negative control-siRNA); MUC1-siRNA group (transfected with MUC1-siRNA).

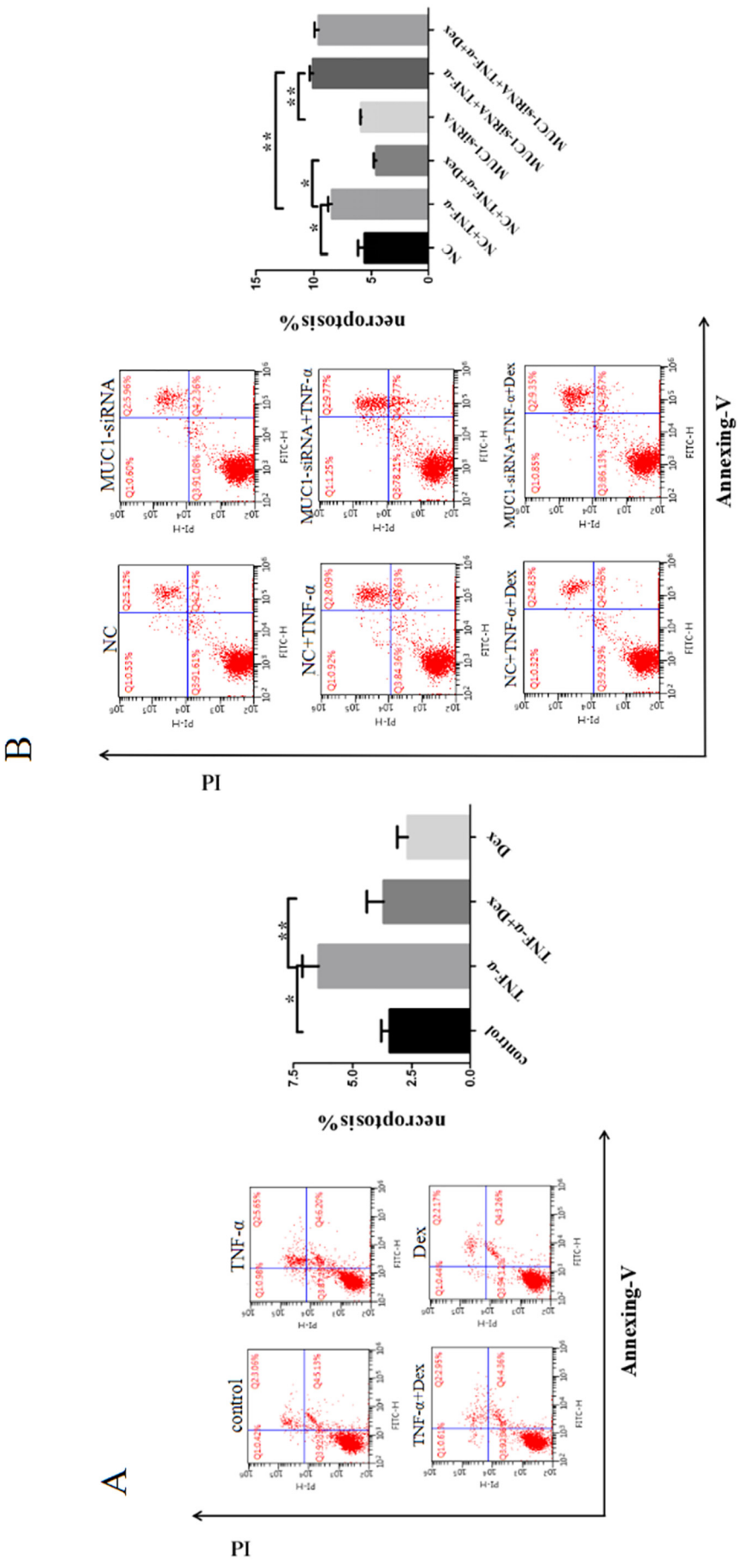

Fig. 2. MUC1 downregulation attenuates dexamethasone-mediated inhibition of TNF-α-induced necroptosis in 16HBE cells.
(A) 16HBE cells were pre-treated with 1 μM dexamethasone for 2 h, then stimulated with TNF-α (300 ng/mL) for 24 h. Cell death was analyzed by flow cytometry. (B) 16HBE cells were transiently transfected with MUC1-siRNA or negative control-siRNA followed by 1 μM dexamethasone pretreatment for 2 h and stimulation with TNF-α for 24 h, cell death was measured by flow cytometry. Data are expressed as means ± SD ($n = 3$) and were analyzed by one-way analysis of variance, $^{*}P < 0.05$; $^{**}P < 0.01$. Dex (dexamethasone); NC group (transfected with negative control-siRNA); MUC1-siRNA group (transfected with MUC1-siRNA).

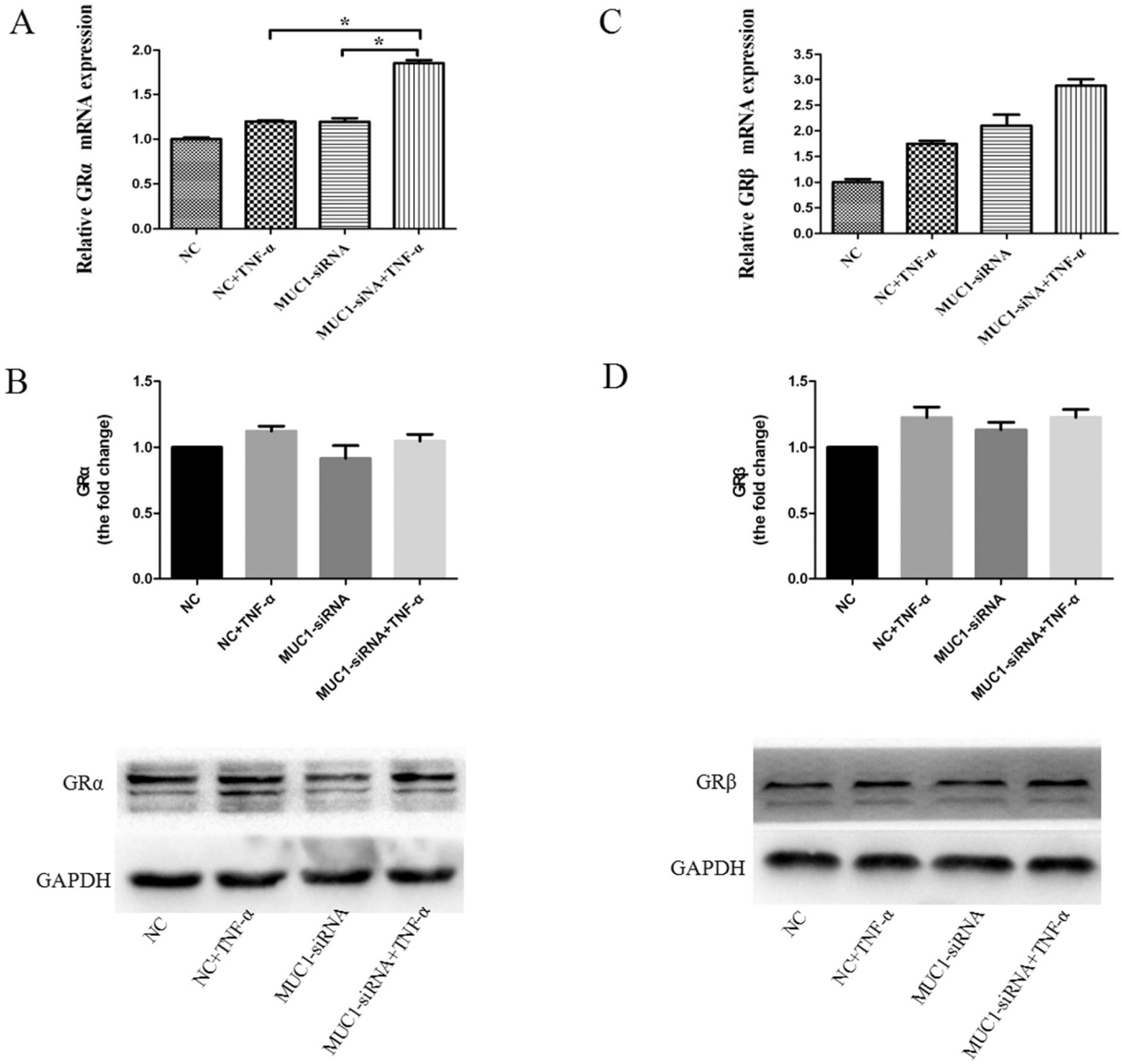

Fig. 3. Glucocorticoid resistance is not associated with GRα and GRβ protein expression.
16HBE cells were transfected with MUC1-siRNA or negative control-siRNA, then treated with TNF-α (300 ng/mL) for 24 h. (A, B) GRα expression was detected by real-time PCR and western blot. (C, D) GRβ expression was detected by real-time PCR and western blot. Data are expressed as means ± SD ($n = 3$) and were analyzed by one-way analysis of variance, $^*P < 0.05$. NC group (transfected with negative control-siRNA); MUC1-siRNA group (transfected with MUC1-siRNA).

downstream effector of TNF-α-induced glucocorticoid resistance in 16HBE cells with MUC1 downregulation (Fig. 6).

4. Discussion

In the present study, we provided the new evidence supporting a role for MUC1 in response to dexamethasone during TNF-α-induced necroptosis. MUC1 downregulation augmented TNF-α-induced necroptosis in 16HBE cells, whereas which showed a lack of response to dexamethasone. Furthermore, the elucidation of the mechanism underlying the glucocorticoid resistance revealed that MUC1 downregulation impaired GRα nuclear translocation and inhibited p-p65 expression.

Severe asthma is characterized by poor symptom control and persistent airway inflammation despite treatment with systemic corticosteroids [1–3]. Bronchial epithelial cells (BECs) are the first line of defense against pathogens and inhaled allergens that may cause inflammation [35]. Recent study demonstrated that cell death in BECs induced by Dermatophagoides farinae was associated with airway inflammation in asthma [36]. Furthermore, several studies showed that TNF-α was increased in the airways of patients with severe asthma, which contributes to airway inflammation [15,16]. However, whether necroptosis occurs in response to TNF-α in BECs is still not known. In our previous study, we found that TNF-α induced necroptosis in 16HBE cells, which was promoted by MUC1 downregulation [37]. The findings of the current study are consistent with these previous results and support for MUC1 as a novel protective molecule in asthma.

Dexamethasone exerts strong anti-inflammatory and anti-necroptotic activity in response to certain cytokines [10–14,30–32]. Recent evidences indicate that BECs transfected with MUC1-siRNA were resistant to inhibition of dexamethasone-mediated interleukin-8 and granulocyte-macrophage colony-stimulating factor expression after stimulation of the cells with lipopolysaccharide or peptidoglycan, suggesting that MUC1 deficiency might lead to an attenuation of the anti-inflammatory functions of glucocorticoids [30,31]. In the current study, the number of cells undergoing necroptosis induced by TNF-α was reduced in dexamethasone-treated 16HBE cells. Interestingly, we also observed that dexamethasone failed to reduce TNF-α-induced

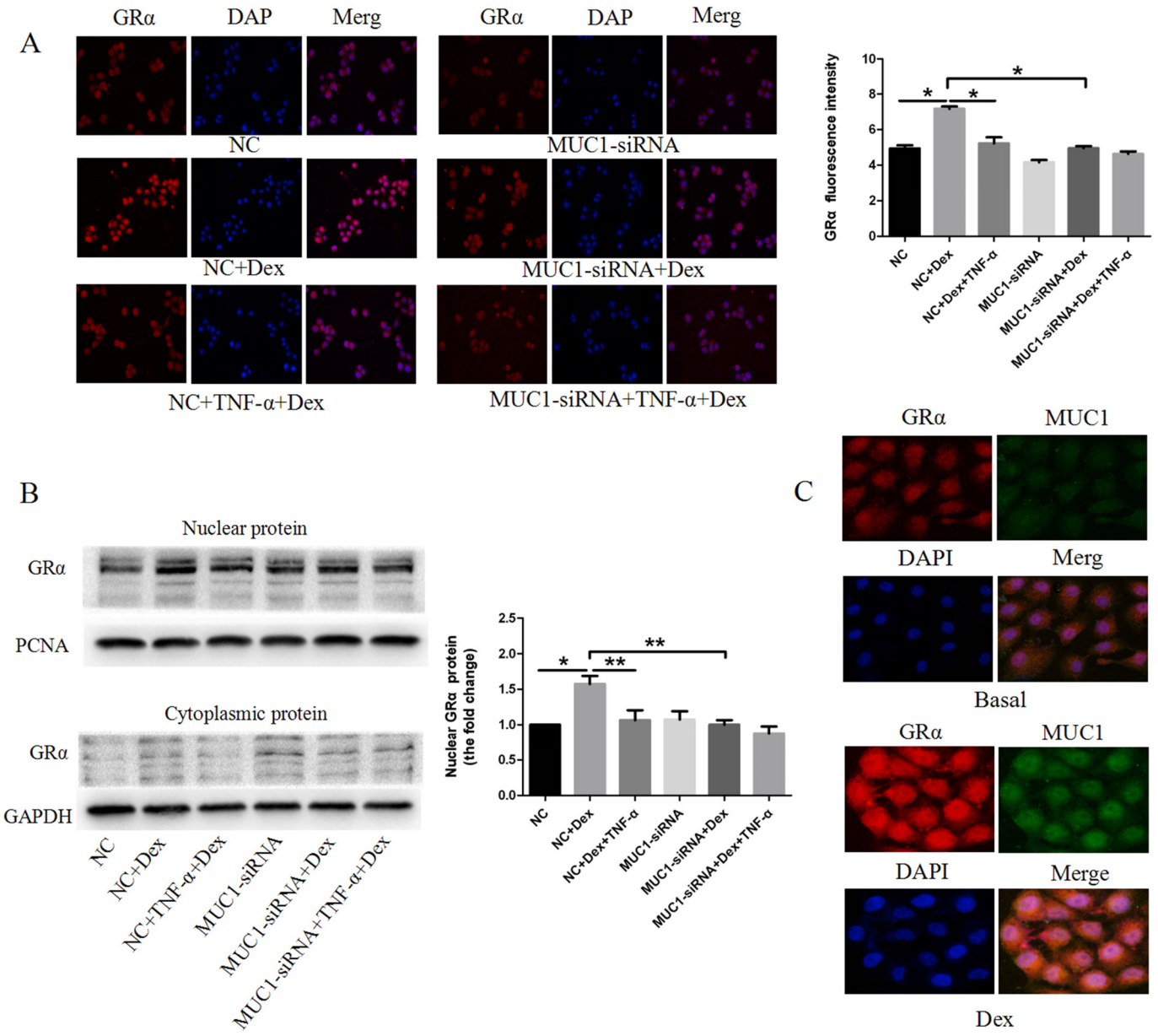

Fig. 4. MUC1 downregulation reduces glucocorticoid sensitivity via a reduction in the nuclear translocation of GRα.
(A) 16HBE cells were transiently transfected with MUC1-siRNA or negative control-siRNA, followed by stimulation with 1 μM dexamethasone for 12 h in the presence or absence of TNF-α (300 ng/mL), immunofluorescence was assessed with an anti-GRα antibody. (B) 16HBE cells were transiently transfected with MUC1-siRNA or negative control-siRNA, followed by stimulation with 1 μM dexamethasone for 12 h in the presence or absence of TNF-α (300 ng/mL), cytosolic and nuclear proteins of GRα were assayed by immunoblot analysis. (C) 16HBE cells were incubated in the presence or absence (basal) of 1 μM dexamethasone for 12 h, immunofluorescence was used to label anti-MUC1 and anti-GRα antibody. Data are expressed as means ± SD (n = 3) and were analyzed by one-way analysis of variance, $^{*}P < 0.05$; $^{**}P < 0.01$. Scale bar = 50 μm. Dex (dexamethasone); NC group (transfected with negative control-siRNA); MUC1-siRNA group (transfected with MUC1-siRNA).

necroptosis in 16HBE cells with MUC1 downregulation. These results suggest that MUC1 downregulation contributes to insensitivity to the anti-necroptotic effect of glucocorticoids. Certainly, it remains to be determined the in vivo efficacy of MUC1 for necroptosis in asthma. Our next step is to investigate the effect of MUC1 on necroptosis in animal model of asthma.

Recent studies reported that an imbalance between GRα and GRβ expression was associated with glucocorticoid insensitivity [38–40]. Glucocorticoids exert their anti-inflammatory functions mainly through GRα. GRβ, an alternative splicing isoform, functions as a dominant-negative inhibitor of GRα [10–14,38–40]. In addition, GRβ expression was shown to be induced by proinflammatory cytokines such as TNF-α and interferon γ [41,42]. Silencing MUC1 expression did not lead to a significant increase in the protein levels of GRα and GRβ, suggesting that the molecular mechanism underlying resistance to glucocorticoids does not involve upregulation of GRβ.

GRα nuclear translocation is a key step in the glucocorticoid-mediated gene regulation. In the absence of its ligand, GR resides predominantly in the cytoplasm in a complex with the heat-shock protein (HSP) 90, the immunophilins FK506 binding protein 51 (FKBP5) and other co-chaperone proteins to maintain its activity. Upon ligand binding, the GR complex translocates to the nucleus, where it mediates transcription of numerous target genes [43]. Recent evidence has demonstrated that MUC1 may interact with HSP 90 by being translocated to the nucleus as a MUC1-HSP 90 transcription complex in response to fibroblast growth factor (FGF) receptor 1 [44].

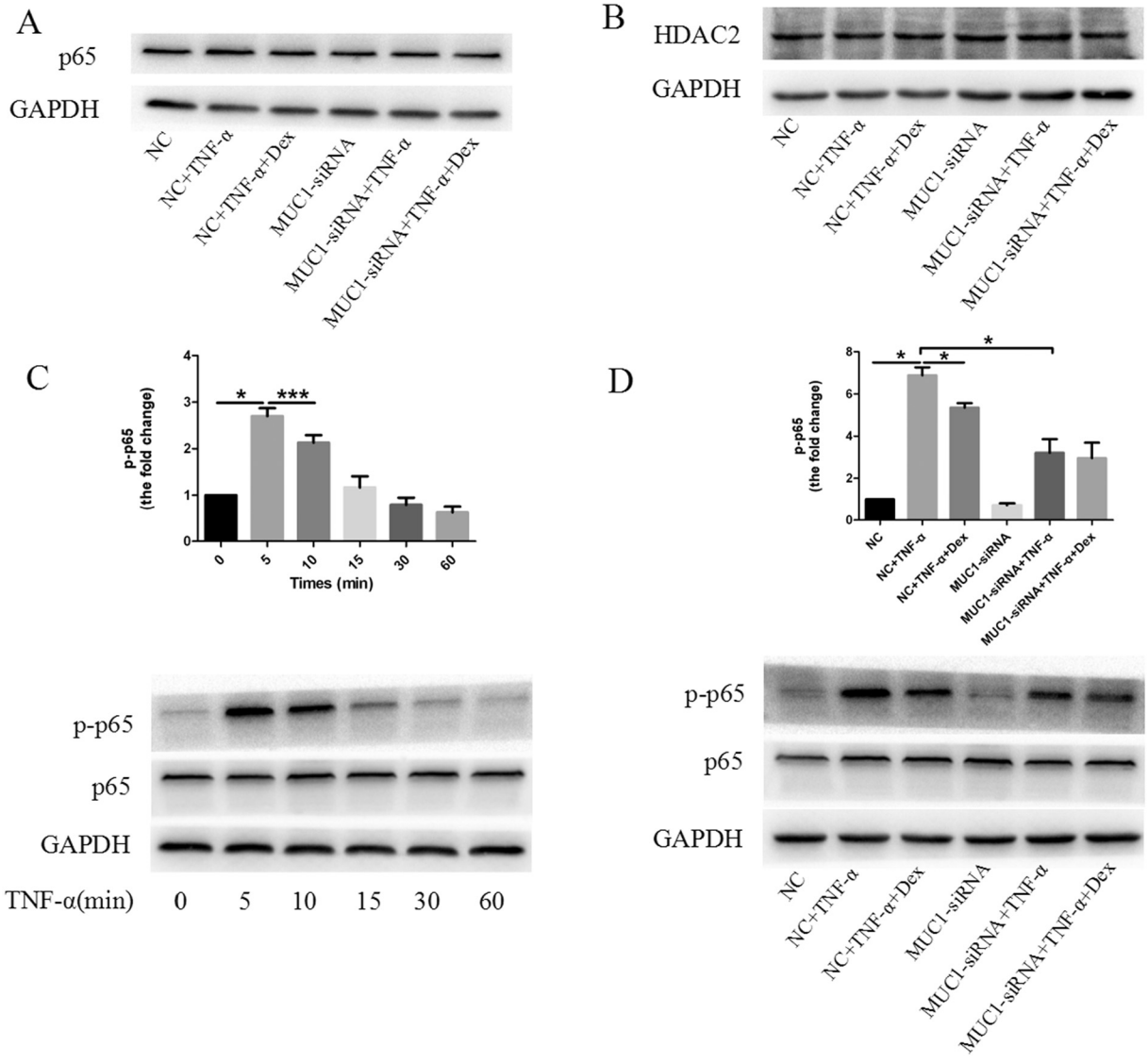

Fig. 5. MUC1 downregulation reduces glucocorticoid sensitivity via inhibiting p-p65 expression.
(A, B) MUC1-siRNA cells and negative control-siRNA cells were treated with 1 μM dexamethasone for 2 h, followed by stimulation with TNF-α (300 ng/mL) for 24 h, then p65 and HDAC2 expression was detected by western blot. (C) 16HBE cells were stimulated with TNF-α (300 ng/mL) for the indicated times and p-p65 was analyzed by western blot. (D) MUC1-siRNA cells and negative control-siRNA cells were treated with 1 μM dexamethasone for 2 h, followed by TNF-α (300 ng/mL) stimulation. After TNF-α stimulation for 5 min, p-p65 expression levels were determined by immunoblotting. Data are expressed as means ± SD ($n = 3$) and were analyzed by one-way analysis of variance, $^{*}P < 0.05$; $^{***}P < 0.001$. Dex (dexamethasone); NC group (transfected with negative control-siRNA); MUC1-siRNA group (transfected with MUC1-siRNA).

Additionally, Javier et al. reported that MUC1 suppression reduced the efficacy of dexamethasone in translocating GRa to the nucleus after stimulation with lipopolysaccharide, peptidoglycan, or flagellin [30,31]. We thus hypothesized that MUC1 might affect GRα nuclear translocation in TNF-α-induced necroptosis. Our immunofluorescence-based analysis revealed that TNF-α decreased the efficacy of dexamethasone in translocating GRα to the nucleus; this effect of TNF-α was stronger in MUC1-siRNA cells. To further support of this finding, we determined the expression levels of GRα in both the cytoplasm and the nucleus of 16HBE cells. Consistently, we found a similar trend in the nuclear protein levels of GRα. Additionally, co-immunofluorescence analysis showed that GRα and MUC1 could translocate from the cytoplasm to the nuclear in the presence of dexamethasone. In addition, we have tried to detect a protein-protein interaction between MUC1 and GRα in vitro, but the results were negative (data not show), which suggested that other mechanisms may be further explored. Nonetheless, these results overall indicate that MUC1 participates in TNF-α-induced resistance to glucocorticoids by modulating GRα nuclear translocation.

A common signaling pathway in glucocorticoids resistance is the NF-κB p65 signaling cascade. GC activate the GR, and then induce transrepression of inflammatory transcription factors, such as NF-kB [45–47]. In this regard, recent evidence suggested that particulate matter-induced necroptosis in human bronchial epithelial cells were increased through upregulation of p-p65 expression [48]. In addition, receptor interacting protein kinase 3 deficiency impaired TNF-α-induced necroptosis in smooth muscle cell by reducing p-p65 expression [49]. Interestingly, we found that the expression of p-p65 induced by TNF-α was significantly increased in 16HBE cells. However, some studies showed that MUC1 could bind IKKβ and IKKγ to activate p65 phosphorylation and that silencing MUC1 was accompanied with a decrease in p-p65 and nuclear NF-κB levels [50–52]. In this work, the expression of p-p65 was significantly reduced in 16HBE cells with MUC1 downregulation, and the dexamethasone-mediated inhibition of p65 phosphorylation was lower in the MUC1-siRNA cells than in the

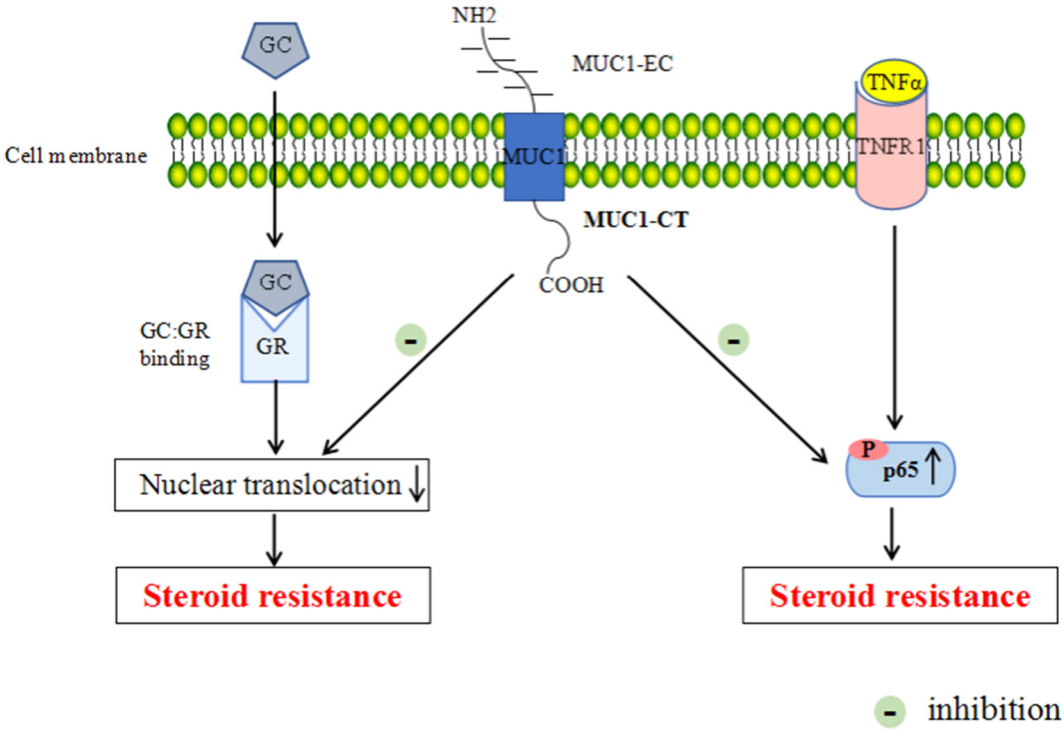

Fig. 6. MUC1 downregulation contributes to steroid-resistant. Two possible mechanisms may explain the effect of MUC1on corticosteroid efficacy. First (left side), MUC1 participates in TNF-α-induced resistance to glucocorticoids by inhibiting GRα nuclear translocation. Second (right side), MUC1 downregulation shows a low response to the dexamethasone-mediated inhibition of p-p65 expression.

negative control-siRNA cells. These data confirmed that MUC1 was able to activate the NF-κB pathway and that MUC1 downregulation showed a low response to the dexamethasone-mediated inhibition of p-p65 expression.

Another molecular mechanism of glucocorticoid insensitivity is attributed to decreased HDAC2 expression [3,10–14,53]. GR is a substrate of HDAC2, and following ligand binding, HDAC2 mediates GR deacetylation which enables GR binding to the NF-κB complex [54]. In the present study, we find that HDAC2 level did not change following the TNF-α treatment in the MUC1-siRNA cells. Collectively, these findings suggest that NF-κB and not HDAC2 might be the downstream molecule involved in TNF-α-induced glucocorticoid insensitivity caused by MUC1 deficiency.

5. Conclusion

In summary, we have demonstrated that MUC1 downregulation augmented TNF-α-induced necroptosis in 16HBE cells, and which could not be inhibited by dexamethasone. Furthermore, we found that impaired GRα nuclear translocation and inhibited p-p65 expression involved to the glucocorticoid resistance caused by MUC1 deficiency. Our findings suggest a possible explanation for why steroid-resistant asthma is not well sensitive to glucocorticoid, thus contributing to the amount of knowledge of the loss of corticosteroid efficacy processes in steroid-resistant asthma caused by MUC1 deficiency which may be of potential target for the development of new therapeutics for asthma treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] E.D. Bateman, S.S. Hurd, P.J. Barnes, J. Bousquet, J.M. Drazen, J.M. FitzGerald, ... H.J. Zar, Global strategy for asthma management and prevention: GINA executive summary, Eur. Respir. J. 51 (2) (2018).
[2] R.Y. Kim, B. Rae, R. Neal, C. Donovan, J. Pinkerton, L. Balachandran, ... P.M. Hansbro, Elucidating novel disease mechanisms in severe asthma, Clin. Transl. Immunol. 5 (7) (2016) e91.
[3] P.M. Hansbro, R.Y. Kim, M.R. Starkey, C. Donovan, K. Dua, J.R. Mayall, ... J.C. Horvat, Mechanisms and treatments for severe, steroid-resistant allergic airway disease and asthma, Immunol. Rev. 278 (1) (2017) 41–62.
[4] C. Persson, L. Uller, Theirs but to die and do: primary lysis of eosinophils and free eosinophil granules in asthma, Am. J. Respir. Crit. Care Med. 189 (6) (2014) 628–633.
[5] B. Naylor, The shedding of the mucosa of the bronchial tree in asthma, Thorax 17 (1962) 69–72.
[6] L. Uller, C.G. Persson, J.S. Erjefalt, Resolution of airway disease: removal of inflammatory cells through apoptosis, egression or both? Trends Pharmacol. Sci. 27 (9) (2006) 461–466.
[7] G. Kano, M. Almanan, B.S. Bochner, N. Zimmermann, Mechanism of Siglec-8-mediated cell death in IL-5-activated eosinophils: role for reactive oxygen species-enhanced MEK/ERK activation, J. Allergy Clin. Immunol. 132 (2) (2013) 437–445.
[8] C. Persson, Primary lysis of eosinophils in severe desquamative asthma, Clin. Exp. Allergy 44 (2) (2014) 173–183.
[9] S.C. Cerps, M. Menzel, I.M. Persson, L. Bjermer, H. Akbarshahi, L. Uller, Interferon-β deficiency at asthma exacerbation promotes MLKL mediated necroptosis, Sci. Rep. 8 (1) (2018) 42–48.
[10] J.M. Rodriguez, M. Monsalves-Alvarez, S. Henriquez, M.N. Llanos, R. Troncoso, Glucocorticoid resistance in chronic diseases, Steroids 115 (2016) 182–192.
[11] P.J. Barnes, Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease, J. Allergy Clin. Immunol. 131 (3) (2013) 636–645.
[12] E. Goleva, L.B. Li, P.T. Eves, M.J. Strand, R.J. Martin, D.Y. Leung, β Increased Glucocorticoid Receptor, Alters steroid response in glucocorticoid-insensitive asthma, Am. J. Respir. Crit. Care Med. 173 (6) (2006) 607–616.
[13] J. Xie, X. Long, L. Gao, S. Chen, K. Zhao, W. Li, E. Liu, Respiratory syncytial virus nonstructural protein 1 blocks glucocorticoid receptor nuclear translocation by targeting IPO13 and may account for glucocorticoid insensitivity, J. Infect. Dis. 217 (1) (2017) 35–46.
[14] K. Ito, S. Yamamura, S. Essilfie-Quaye, B. Cosio, M. Ito, P.J. Barnes, I.M. Adcock, Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor

enables NF-κB suppression, J. Exp. Med. 203 (1) (2006) 7–13.
[15] P.H. Howarth, K.S. Babu, H.S. Arshad, L. Lau, M. Buckley, W. McConnell, ... S.T. Holgate, Tumour necrosis factor (TNFα) as a novel therapeutic target in symptomatic corticosteroid dependent asthma, Thorax 60 (12) (2005) 1012–1018.
[16] P.M. Hansbro, G.E. Kaiko, P.S. Foster, Cytokine/anti-cytokine therapy-novel treatments for asthma? Br. J. Pharmacol. 163 (1) (2011) 81–95.
[17] M. Khorasanizadeh, M. Eskian, E.W. Gelfand, N. Rezaei, Mitogen-activated protein kinases as therapeutic targets for asthma, Pharmacol. Ther. 174 (2017) 112–126.
[18] E. Ayroldi, L. Cannarile, G. Migliorati, G. Nocentini, D.V. Delfino, C. Riccardi, Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways, FASEB J. 26 (12) (2012) 4805–4820.
[19] S.A. Antoniu, Cytokine antagonists for the treatment of asthma: progress to date, BioDrugs 23 (4) (2009) 241–251.
[20] S.E. Wenzel, P.J. Barnes, E.R. Bleecker, J. Bousquet, W. Busse, S.E. Dahlen, ... P. Chanez, A randomized, double-blind, placebo-controlled study of tumor necrosis factor-alpha blockade in severe persistent asthma, Am. J. Respir. Crit. Care Med. 179 (7) (2009) 549–558.
[21] K. Kato, E.P. Lillehoj, W. Lu, K.C. Kim, MUC1: the first respiratory Mucin with an anti-inflammatory function, J. Clin. Med. Res. 6 (12) (2017).
[22] K. Kato, E.P. Lillehoj, Y.S. Park, T. Umehara, N.E. Hoffman, M. Madesh, K.C. Kim, Membrane-tethered MUC1 mucin is phosphorylated by epidermal growth factor receptor in airway epithelial cells and associates with TLR5 to inhibit recruitment of MyD88, J. Immunol. 188 (4) (2012) 2014–2022.
[23] K. Kato, A.D. Hanss, M.A. Zemskova, N.E. Morgan, M. Kim, K.S. Knox, ... K.C. Kim, Pseudomonas aeruginosa increases MUC1 expression in macrophages through the TLR4-p38 pathway, Biochem. Biophys. Res. Commun. 492 (2) (2017) 231–235.
[24] V. Apostolopoulos, L. Stojanovska, S.E. Gargosky, MUC1 (CD227): a multi-tasked molecule, Cell. Mol. Life Sci. 72 (23) (2015) 4475–4500.
[25] K. Kato, E.P. Lillehoj, K.C. Kim, MUC1 regulates epithelial inflammation and apoptosis by PolyI:C through inhibition of Toll/IL-1 receptor-domain-containing adapter-inducing IFN-b (TRIF) recruitment to toll-like receptor 3, Am. J. Respir. Cell Mol. Biol. 51 (3) (2014) 446–454.
[26] P. Zhao, M. Meng, B. Xu, A. Dong, G. Ni, L. Lu, Decreased expression of MUC1 induces apoptosis and inhibits migration in pancreatic cancer PANC-1 cells via regulation of Slug pathway, Cancer Biomark. 20 (4) (2017) 469–476.
[27] T. Xu, D. Li, H. Wang, T. Zheng, G. Wang, Y. Xin, MUC1 downregulation inhibits non-small cell lung cancer progression in human cell lines, Exp. Ther. Med. 14 (5) (2017) 4443–4447.
[28] R. Ahmad, D. Raina, V. Trivedi, J. Ren, H. Rajabi, S. Kharbanda, D. Kufe, MUC1 oncoprotein activates the IκB kinase β complex and constitutive NF-κB signaling, Nat. Cell Biol. 9 (12) (2007) 1419–1427.
[29] N. Agata, R. Ahmad, T. Kawano, D. Raina, S. Kharbanda, D. Kufe, MUC1 oncoprotein blocks death receptor-mediated apoptosis by inhibiting recruitment of caspase-8, Cancer Res. 68 (15) (2008) 6136–6144.
[30] J. Milara, T. Peiró, M. Armengot, S. Frias, A. Morell, A. Serrano, J. Cortijo, Mucin 1 downregulation associates with corticosteroid resistance in chronic rhinosinusitis with nasal polyps, J. Allergy Clin. Immunol. 135 (2) (2015) 470–476.
[31] J. Milara, A. Morell, A. de Diego, E. Artigues, E. Morcillo, J. Cortijo, MUC1 deficiency mediates corticosteroid insensitivity in asthma, Allergy 74 (1) (2019) 111–121.
[32] M. Ballegeer, K. Van Looveren, S. Timmermans, M. Eggermont, S. Vandevyver, F. Thery, ... C. Libert, Glucocorticoid receptor dimers control intestinal STAT1 and TNF-induced inflammation in mice, J. Clin. Invest. 128 (8) (2018) 3265–3279.
[33] A.L. da Silva, R.F. Magalhães, V.C. Branco, J.D. Silva, F.F. Cruz, P.S. Marques, ... P.R. Rocco, The tyrosine kinase inhibitor dasatinib reduces lung inflammation and remodelling in experimental allergic asthma, Br. J. Pharmacol. 173 (7) (2016) 1236–1247.
[34] M. Gurusamy, S. Nasseri, H. Lee, B. Jung, D. Lee, G. Khang, ... D. Wu, Kinin B1 receptor antagonist BI113823 reduces allergen-induced airway inflammation and mucus secretion in mice, Pharmacol. Res. 104 (2016) 132–139.
[35] B.N. Lambrecht, H. Hammad, The airway epithelium in asthma, Nat. Med. 18 (5) (2012) 684–692.
[36] Y.M. Tsai, K.H. Chiang, J.Y. Hung, W.A. Chang, H.P. Lin, J.M. Shieh, I.W. Chong, Y.L. Hsu, Der f1 induces pyroptosis in human bronchial epithelia via the NLRP3 inflammasome, Int. J. Mol. Med. 41 (2) (2018) 757–764.
[37] H. Zhang, J. Ji, Q. Liu, S. Xu, MUC1 downregulation promotes TNF-α-induced necroptosis in human bronchial epithelial cells via regulation of the RIPK1/RIPK3 pathway, J. Cell. Physiol. (2019) 1–9.
[38] J.C. Webster, R.H. Oakley, C.M. Jewell, J.A. Cidlowski, Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative b isoform: a mechanism for the generation of glucocorticoid resistance, Proc. Natl. Acad. Sci. 98 (12) (2001) 6865–6870.
[39] E. Goleva, L.B. Li, P.T. Eves, M.J. Strand, R.J. Martin, D.Y. Leung, Increased glucocorticoid receptor β alters steroid response in glucocorticoid-insensitive asthma, Am. J. Respir. Crit. Care Med. 173 (6) (2006) 607–616.
[40] A. Vazquez-Tello, R. Halwani, Q. Hamid, S. Al-Muhsen, Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells, J. Clin. Immunol. 33 (2) (2013) 466–478.
[41] S.H. Kim, D.H. Kim, P. Lavender, J.H. Seo, Y.S. Kim, J.S. Park, ... Y.K. Jee, Repression of TNF-alpha-induced IL-8 expression by the glucocorticoid receptor-beta involves inhibition of histone H4 acetylation, Exp. Mol. Med. 41 (5) (2009) 297–306.
[42] O. Tliba, J.A. Cidlowski, Y. Amrani, CD38 expression is insensitive to steroid action in cells treated with tumor necrosis factor-alpha and interferon-gamma by a mechanism involving the up-regulation of the glucocorticoid receptor beta isoform, Mol. Pharmacol. 69 (2) (2006) 588–596.
[43] M. Bekhbat, S.A. Rowson, G.N. Neigh, Checks and balances: the glucocorticoid receptor and NFκB in good times and bad, Front. Neuroendocrinol. 46 (2017) 15–31.
[44] J. Ren, D. Raina, W. Chen, G. Li, L. Huang, D. Kufe, MUC1 oncoprotein functions in activation of fibroblast growth factor receptor signaling, Mol. Cancer Res. 4 (11) (2006) 873–883.
[45] F. Xu, M. Luo, L. He, Y. Cao, W. Li, S. Ying, Z. Chen, H. Shen, Necroptosis contributes to urban particulate matter-induced airway epithelial injury, Cell. Physiol. Biochem. 46 (2) (2018) 699–712.
[46] S. Qin, C. Yang, W. Huang, S. Du, H. Mai, J. Xiao, T. Lü, Sulforaphane attenuates microglia-mediated neuronal necroptosis through down-regulation of MAPK/NF-κB signaling pathways in LPS-activated BV-2 microglia, Pharmacol. Res. 133 (2018) 218–235.
[47] X. Zhao, T. Laver, S.W. Hong, G.B. Twitty Jr., A. Devos, M. Devos, E.N. Benveniste, S.E. Nozell, An NF-κB p65-cIAP2 link is necessary for mediating resistance to TNF-a induced cell death in gliomas, J. Neuro-Oncol. 102 (3) (2011) 367–381.
[48] F. Xu, M. Luo, L. He, Y. Cao, W. Li, S. Ying, Z. Chen, H. Shen, Necroptosis contributes to urban particulate matter-induced airway epithelial injury, Cell. Physiol. Biochem. 46 (2) (2018) 699–712.
[49] Q. Wang, Z. Liu, J. Ren, S. Morgan, C. Assa, B. Liu, Receptor-interacting protein kinase 3 contributes to abdominal aortic aneurysms via smooth muscle cell necrosis and inflammation, Circ. Res. 116 (4) (2015) 600–611.
[50] R. Ahmad, D. Raina, V. Trivedi, J. Ren, H. Rajabi, S. Kharbanda, D. Kufe, MUC1 oncoprotein activates the IκB kinase β complex and constitutive NF-κB signalling, Nat. Cell Biol. 9 (12) (2007) 1419–1427.
[51] T. Kawano, R. Ahmad, H. Nogi, N. Agata, K. Anderson, D. Kufe, MUC1 oncoprotein promotes growth and survival of human multiple myeloma cells, Int. J. Oncol. 33 (1) (2008) 153–159.
[52] R. Ahmad, D. Raina, M.D. Joshi, T. Kawano, J. Ren, S. Kharbanda, D. Kufe, MUC1-C oncoprotein functions as a direct activator of the nuclear factor-kappaB p65 transcription factor, Cancer Res. 69 (17) (2009) 7013–7021.
[53] Y. Chen, A.M. Watson, C.D. Williamson, M. Rahimi, C. Liang, A.M. Colberg-Poley, M.C. Rose, Glucocorticoid receptor and histone deacetylase–2 mediate dexamethasone-induced repression of MUC5AC gene expression, Am. J. Respir. Cell Mol. Biol. 47 (5) (2012) 637–644.
[54] K. Ito, S. Yamamura, S. Essilfie-Quaye, B. Cosio, M. Ito, P.J. Barnes, I.M. Adcock, Histone deacetylase 2–mediated deacetylation of the glucocorticoid receptor enables NF-κB suppression, J. Exp. Med. 203 (1) (2006) 7–13.