Research Article

TNF-α Regulates the Glucocorticoid Receptor Alpha Expression in Human Nasal Epithelial Cells Via p65-NF-κb and p38-MAPK Signaling Pathways

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Background: Tumor necrosis factor (TNF)-α induces changes in the glucocorticoid receptor (GR) isoforms' expression in human nasal epithelial cells (HNECs) in chronic rhinosinusitis (CRS).

Objective: However, the underlying mechanism of TNF-α induced GR isoforms' expression in HNECs remains unclear. Here, we explored changes in inflammatory cytokines and glucocorticoid receptor alpha isoform (GRα) expression in HNECs.

Materials and Methods: To explore the expression of TNF-α in nasal polyps and nasal mucosa of CRS, fluorescence immunohistochemical analysis was employed. To investigate changes in inflammatory cytokines and GRα expression in HNECs, RT-PCR and western blotting were performed following the cells' incubation with TNF-α. Cells were pretreated with the nuclear factor-κB gene binding (NF-κB) inhibitor QNZ, the p38 inhibitor SB203580, and dexamethasone for one hour, then a TNF-α. Western blotting, RT-PCR, and immunofluorescence had been utilized for the cells' analysis and the ANOVA for the data analysis.

Results: The TNF-α fluorescence intensity was mainly distributed in nasal epithelial cells of nasal tissues. TNF-α prominently inhibited the expression of *GRα* mRNA from 6 to 24 h in HNECs. GRα protein was decreased from 12 to 24 h. Treatment with QNZ, SB203580, or dexamethasone inhibited the *TNF-α* and *interleukin (IL)-6* mRNA expression and increased the *GRα* levels.

Conclusion: TNF-α induced changes in the GR isoforms' expression in HNECs, and it was mediated through p65-NF-κB and p38-MAPK signal transduction pathways, which could be considered a promising neutrophilic CRS treatment.

Keywords: Chronic rhino sinusitis (CRS), Nuclear factor (NF)-κB, P38 mitogen-activated protein kinase (MAPK), Signaling pathways

1. Background

CRS is an inflammatory condition that affects the nose and the paranasal sinuses with a prevalence of 10% in the western population, which manifests as rhinorrhea, nasal obstruction, and olfactory dysfunction for at least three months (1). Endotypes of CRS were generally categorized into CRS with and without nasal polyps, (CRSwNP) and (CRSsNP), respectively. It's reported that CRSwNP in Asian individuals presents different immunopathological characteristics compared with patients in the western population. Most CRS with nasal polyps encountered in western countries is eosinophilic, while non-eosinophilic CRSwNP feature in a significant percentage of cases of CRSwNP reported in Asian countries (2).

Intranasal or oral glucocorticoid (GC) treatment is mainly applied to control nasal mucosal inflammatory gene expression in CRS patients and to inhibit the

activation of eosinophilic cells (3). Although GC therapy is valid for most CRS patients, many patients fail to respond to GC therapy, referred to as GC resistance (GCR). The glucocorticoid receptor (GR) is widely distributed in the nasal mucosa and nasal polyps (4), and the human GR receptor is described as two distinct isoforms, alpha isoform (GRα) and beta isoform (GRβ) (5). GRα regulates the GCs anti-inflammatory functions via the anti-inflammatory genes activation by the GR binding to glucocorticoid response elements (GREs) in the target genes' promoter region, the proinflammatory genes repression by the GR and proinflammatory transcription factors' interaction, like the p65-nuclear factor (NF)-κB gene binding, which also inhibits the epithelial-derived proinflammatory gene expression of numerous proinflammatory cytokines, like interleukin(IL)-1β, IL-6, and TNF-α, while GRβ functions as a predominantly GR-mediated transactivation negative inhibitor (6-11). Lower GRα expression in human nasal epithelial cells of nasal polyps in comparison to the healthy nasal mucosa has also been reported; expression of GRβ mRNA remains lower in all nasal samples (12). Wang et al. observed an imbalance in the GR subtype isoforms' expression in nasal polyps induced by IL-1β, which was regulated by p38-MAPK and c-Jun N-terminal kinase (JNK) signal transduction pathways (13).

TNF-α acts as a mediator of proinflammatory cytokines and is widely involved in the pathogenesis of CRS. Increased TNF-α expression, mediated by p65-NF-κB and p38-MAPKs signaling, has been shown in CRS patients (1, 14). TNF-α can also impair GC anti-inflammatory actions and enhance GR expression in human airway smooth muscle (HASM) cells (15). This research aims analyzing the TNF-α role in regulating the GR isoforms expression in human nasal epithelial cells and CRS patients.

2.Objectives

In our study, we explored changes in inflammatory cytokines and GRα expression to reveal the underlying mechanisms of TNF-α induced GR isoforms' expression in HNECs.

3. Methods and Materials

3.1. Subjects and Specimens
Patients with CRS from the Shanghai Jiao Tong University, Medicine School, Affiliated Ren Ji Hospital, Shanghai, were enrolled in this study. CRS diagnoses depended on the patients' clinical history, clinical examination, nasal endoscopy, and the nasal sinuses' CT scans, following the European guidelines (16) for the CRS diagnosis and treatment (2020). Preoperative CT scans of the nose and paranasal sinuses were graded according to the Lund-Mackay classification. All patients had functional endoscopic sinus surgery (FESS) with paranasal sinuses clearance. No oral or nasal corticosteroids or antibiotics were used four weeks prior to the operation. The Shanghai Jiao Tong University, Medicine School, Affiliated Ren Ji Hospital's ethics committee approved this research. All participants had informed consent to the research content. This research followed the 1975 Declaration of Helsinki's ethical guidelines.

3.2. Cell Culture
Human nasal epithelial cells (HNECs, BNBIO, BNCC340481, China) were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, 11965-092, Australia) mixed with 10% fetal bovine serum (Gibco, 10099-141, Australia) and 100 U.mL^{-1} penicillin (Beyotime, C0222, China), in a cell incubator (Thermo Scientific™ HERACELL™ 150i CO_2 incubator) with 5% CO_2 at 37 °C.

3.3. Immunocytochemistry
For a whole day, HNECs were seeded on coverslips in 24-well cluster plates. HNECs were pre-treated with QNZ (Selleck, S4902, USA) or SB203580 (Selleck, S1076, USA) and then were treated with human recombinant TNF-α protein (R&D, 210-TA-020, USA). HNECs were fixed for ten minutes at room temperature with Immunol staining Fix solution. After intensive wash using 0.01 M PBS, the cell coverslips were pre-incubated in blocking buffer for Immunol staining (Beyotime, P0260, China) at room temperature for ten minutes. The cell coverslips were treated with polyclonal antibodies against p-P65 NF-κB, p-P38 MAPK in primary antibody dilution buffer for Immunol staining overnight at 4 °C. The cell coverslips were incubated with Alexa 488-conjugated donkey anti-rabbit antibodies in secondary antibody dilution buffer for Immunol staining for one hour at room temperature, following at least three times washing by PBS, then were put on glass slides with antifade mounting medium and seen by the inverted fluorescence microscope usage.

3.4. Real-Time PCR

HNECs were first incubated with ten ng.mL^{-1} TNF-α for three, six, twelve, and 24 hours. Cells were treated with ten ng.mL^{-1} TNF-α for six hours; after pre-treatment with QNZ, SB203580, and dexamethasone for one hour, cells were then treated with ten ng.mL^{-1} TNF-α for six hours. HNECs were harvested, and total RNA was isolated from HNECs by TRIzol reagent usage. Total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, from which, the complementary DNA (cDNA) was made by a 2× PCR Master Mix usage. Then, *TNF-α*, *IL-6*, *GRα*, and *β-actin* cDNA were amplified by the StepOnePlus Real-Time PCR System usage with the SYBR Green one-step qRT-PCR kit. Primers were as follows: *IL-6* forward, 5′ATGGCTGAAAAAGATGGATGCT-3′ and *IL-6*, reverse, 5′GCTCTGGCTTGTTCCTCAC TACTC-3′; *TNF-α*, forward, 5′-CCCAGGCAGTCAG ATCATCTTC-3′ and *TNF-α*, reverse, 5′-AGCTG CCCCTCAGCTTGA-3′; *GRα*, forward, 5′-AGCCA TTGTCAAGAGGGAAG-3′ and *GRα*, reverse, 5'-A GCAATAGTTAAGGAGATTTTCAACC-3'; *β-actin*, forward, 5′-GCCGATCCACACGGAGTACTT-3′ and *β-actin*, reverse, 5′-TTGCCGACAGGATGCAGAA-3′. These genes' average transcript levels were standardized to β-actin. To quantify the target genes' mRNA levels, the comparative threshold cycle (2-ΔΔCt) method had been utilized. Reactions were performed a minimum of three times in triplicate.

3.5. Western Blotting

Cultured HNECs were seeded in a six-well plate, lysed, and then the protein was extracted by the RIPA lysis buffer combined with the protease and phosphatase inhibitor mix usage per well and quantified by the BCA kit usage. Proteins were separated using 10% polyacrylamide gel electrophoresis (PAGE). Every well was loaded with the protein sample and a protein marker on both sides, 5–10 μL, and 5 μL, respectively. After that, electrophoresis was done for 110 minutes at a 90 V constant voltage. The membrane was incubated with blocking buffer and incubated with GRα antibody (4 μg.mL^{-1}; Invitrogen, PA1516, USA), GRβ antibody (1:500; Invitrogen, PA3-514), p-P65 NF-κB (1:1000; Cell Signaling Technology, 3033T), p-P38 MAPK (1:1000; Cell Signaling Technology, 4511), and β-actin rabbit monoclonal antibody (1:3000; Beyotime, AF5003, China) overnight at 4 °C. The membranes were treated with the HRP-labeled secondary goat anti-rabbit IgG antibody. Immunoreactive proteins were measured using BeyoE-CL plus an Enhanced Chemiluminescence Western blot detection system.

3.6. Statistical Analysis

The SPSS 26.0 software and GraphPad Prism v5.0 had been utilized for the data analysis by conducting ANOVA.

4. Results

4.1 TNF-α is Involved in the CRS Pathogenesis

To determine the TNF-α expression in selected structures of nasal polyps and nasal mucosa of CRS patients, we employed fluorescence immunohistochemical analysis. TNF-α was observed in nasal polyps of CRSwNP and nasal mucosa of CRSsNP patients in comparison to the control group, which was mainly distributed in nasal epithelial cells. The expression of TNF-α was much higher in nasal polyps of CRSwNP than the nasal mucosa of CRSsNP patients (**Fig. 1**).
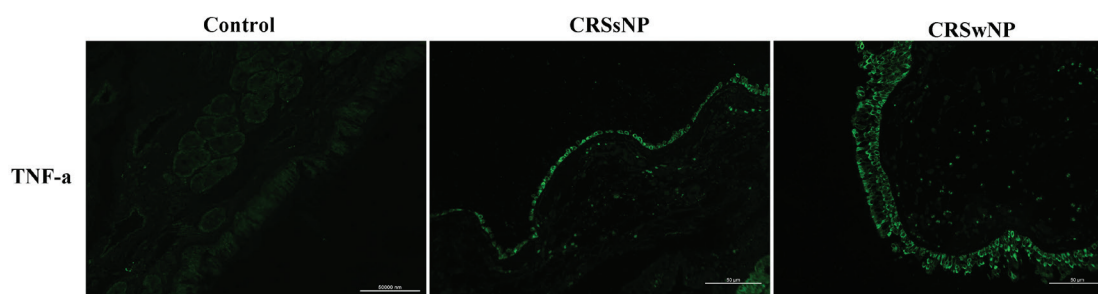
**Figure 1.** Immunostaining of TNF-α in the nasal mucosa and nasal polyps of CRS. Scale bars: 50 μm.

4.2. TNF-α Induced Inflammatory Cytokine mRNA Expression and Regulated GRα Release in HNECs via p65-NF-κB and p38 MAPK Signaling

To investigate the changes in inflammatory cytokine and GRα mRNA expression, HNECs were incubated with ten ng.mL^{-1} TNF-α for 3, 6, 12, and 24 hours. Treatment with TNF-α induced TNF-α and IL-6 mRNA significantly increased in all-time courses in comparison to the control group and peaked at 3 hours (**Fig. 2A, B**). Interestingly, TNF-α significantly inhibited the expression of GRα mRNA from six to 24 hours in comparison to the control group (**Fig. 2C**). To explore the p65-NF-κB and p38 MAPK signaling role in TNF-α–stimulated HNECs, the p65 and p38 phosphorylation was examined via western blotting. HNECs were treated with TNF-α (10ng.mL^{-1}) for 7.5, 15, 30, and 60 minutes. Phosphorylation of p38 and p65 began at 15 min and reached peak levels simultaneously, and then it degraded at one h (**Fig. 2D**). After the treatment with TNF-α (10ng.mL^{-1}) for 3, 6, 12, and 24 h, GR and GR-α proteins were significantly increased from 3 to 12 h following a decrease lower

than the control group at 24 h (**Fig. 2E**). Then, we pretreated cells with the p65-NF-κB inhibitor QNZ (500 nM), the p38 MAPK inhibitor SB203580 (5 μM), and dexamethasone (100 nM) for one hour, then treated with TNF-α (10ng.mL^{-1}) for six hours. Pre-incubation with QNZ, SB203580, and dexamethasone decreased the TNF-α and IL-6 mRNA expression significantly (**Fig. 3A, B**). Interestingly, treatment with QNZ and SB203580 also remarkably increased the GRα mRNA expression. Dexamethasone slightly increased the GRα mRNA expression in comparison to the control group, but there is no statistical significance (**Fig. 3C**). Consistent with the qPCR results, the p65 and p38 phosphorylation were inhibited, while protein levels of GR and GRα were both increased following pretreatment with QNZ and SB203580. Dexamethasone also significantly increased the GR and GRα protein levels and decreased the p65 and p38 phosphorylation in HNECs (**Fig. 3D, E**). To further prove the p65-NF-κB and p38 MAPK signaling role in TNF-α–stimulated HNECs in protein levels, fluorescence immunohistochemical analysis was performed. The
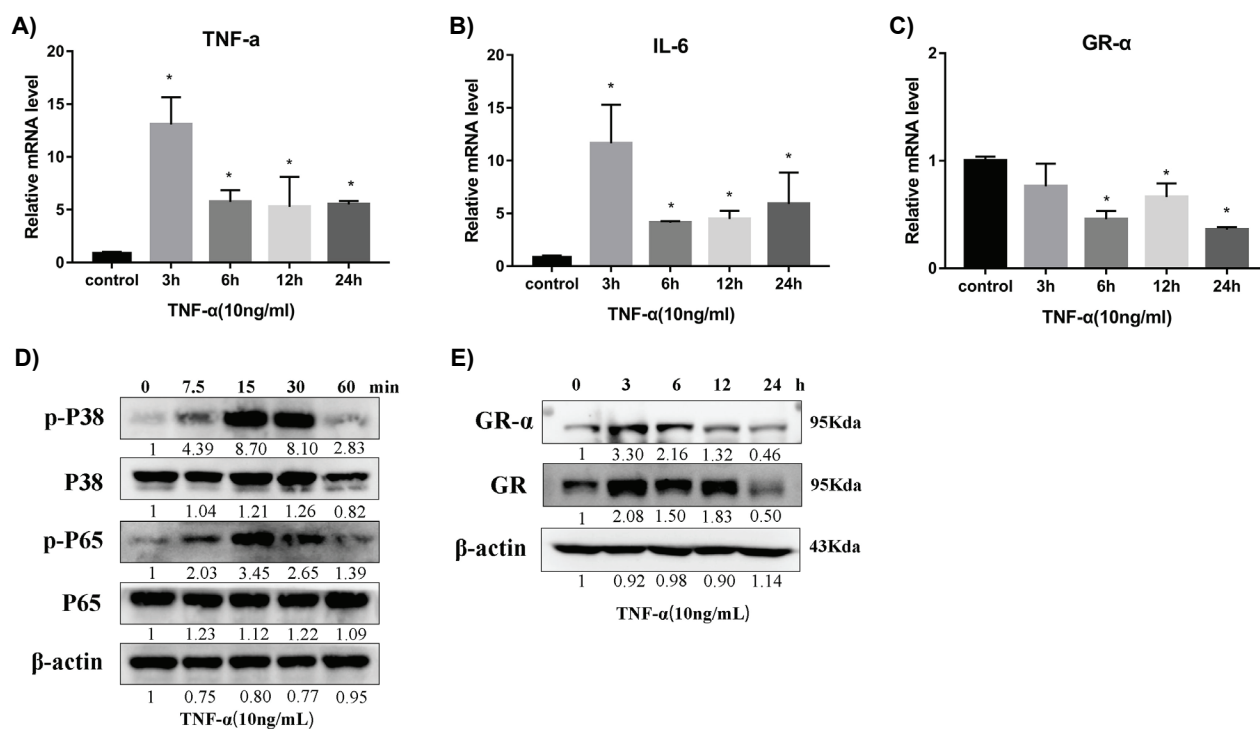
Figure 2. TNF-α induced inflammatory cytokines and regulated mRNA and protein levels of GR isoforms in HNECs. (A-C) TNF-α (10 ng.mL^{-1}) increased TNF-α/IL-6 and decreased GR-α mRNA expression in HNECs. **(D-E)** TNF-α (10 ng.mL^{-1}) increased phosphorylation of P38/P65 and decreased expression of GR-α/GR protein levels in HNECs. Values are expressed as the mean± SEM of separate experiments.*p< 0.05 vs. control.
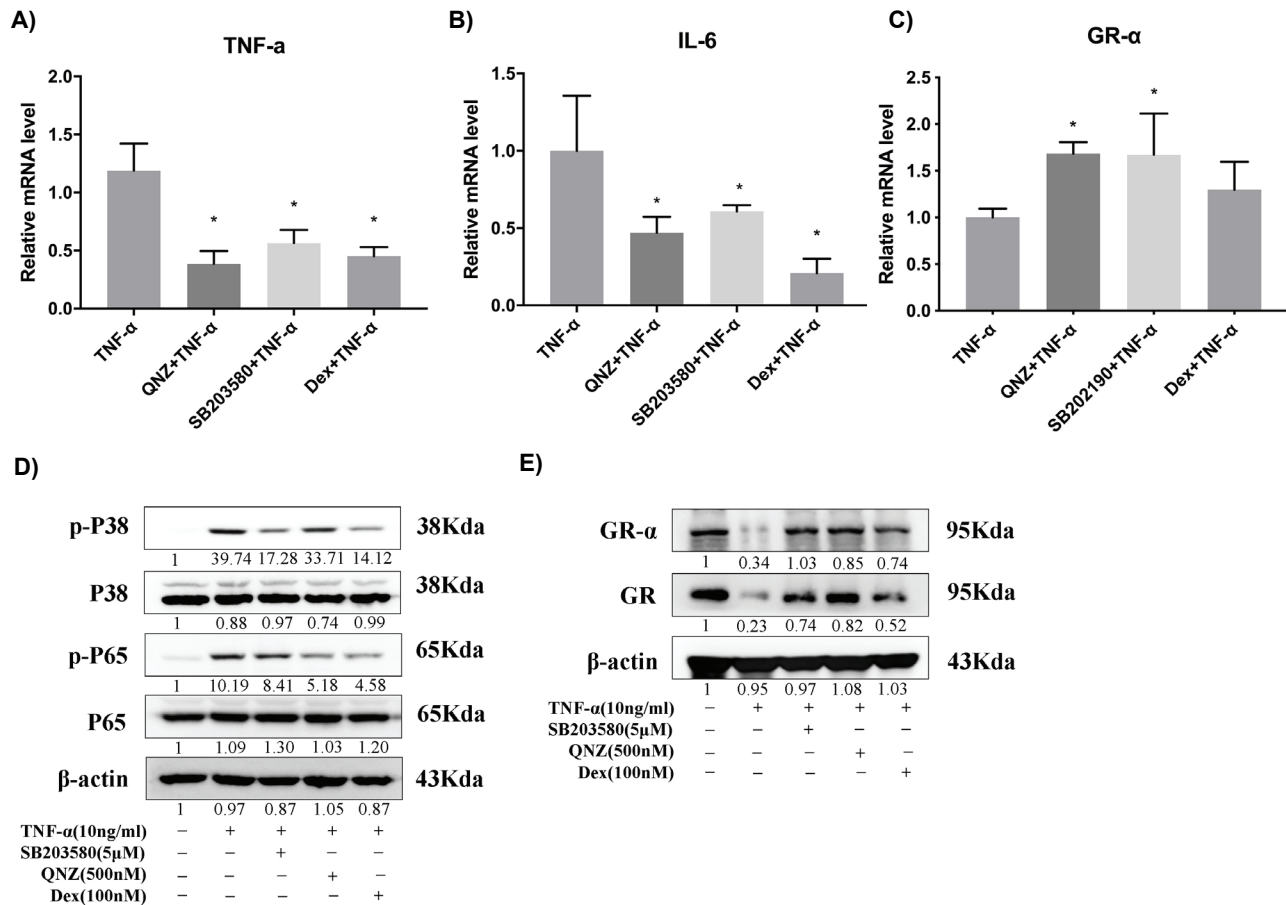
Figure 3. The p65-NF-κB and p38 MAPK pathways mediated. TNF-α induced inflammatory cytokine release and regulated *GRα* expression in HNECs. **(A-C)** TNF-α-induced expression of TNF-α and IL-6 mRNA were inhibited and GR-α mRNAwas incereased in HNECs with treatment of P38 MAPK inhibitor SB203580, P65 NF-κB inhibitor QNZ and Dexamethasone. **(D-E)** The phosphorylation of P38 and P65 were inhibited and GR-α protein levels was incereased in HNECs after incubation with SB203580, QNZ and Dexamethasone. Values are expressed as the mean ± SEM of separate experiments. *$p < 0.05$ vs. control group.

p65-NF-κB and p38 MAPK fluorescence intensity was significantly observed in TNF-α-treated HNECs, which could be inhibited by QNZ and SB203580 (**Fig. 4**).

5. Discussion

CRS is a chronic inflammatory disease that mainly occurs in the nasal mucosa and the paranasal sinuses, divided into with or without nasal polyps depending on the polyp's presence (17). Eosinophilic infiltration is prominent in the Western population with CRSwNP, whereas the eosinophilic endotype constitutes less than 50% percent of CRSwNP patients in East Asia (18, 19). This research aimed determining why the GCs fail to reduce inflammation in non-eosinophilic infiltration CRS. Proinflammatory cytokines like IL-1β/IL-6,

IL-8/MPO, IL-17A, and TNF-α were upregulated in neutrophilic inflammation of CRS. Cytokines like TNF-α and IL-6 could decrease the expression of GRs, inhibiting the GRs translocation from the cytoplasm to the nucleus and subsequent binding to DNA (20). GRs have been reported as two distinct isoforms, GRα and GR-β, which distribute in the nasal mucosa and nasal polyps (4, 21). GCs bind to GRs in the nasal mucosa and nasal polyps, affecting the inflammatory mRNA expression and inhibiting eosinophils' activation and T-cell-associated inflammatory responses (22). TNF-α was widely found in inflammatory cells of CRSwNP tissues, which reduced the expression of GR of monocytes (23). Our study found that TNF-α was mainly distributed in nasal epithelial cells of NPs in
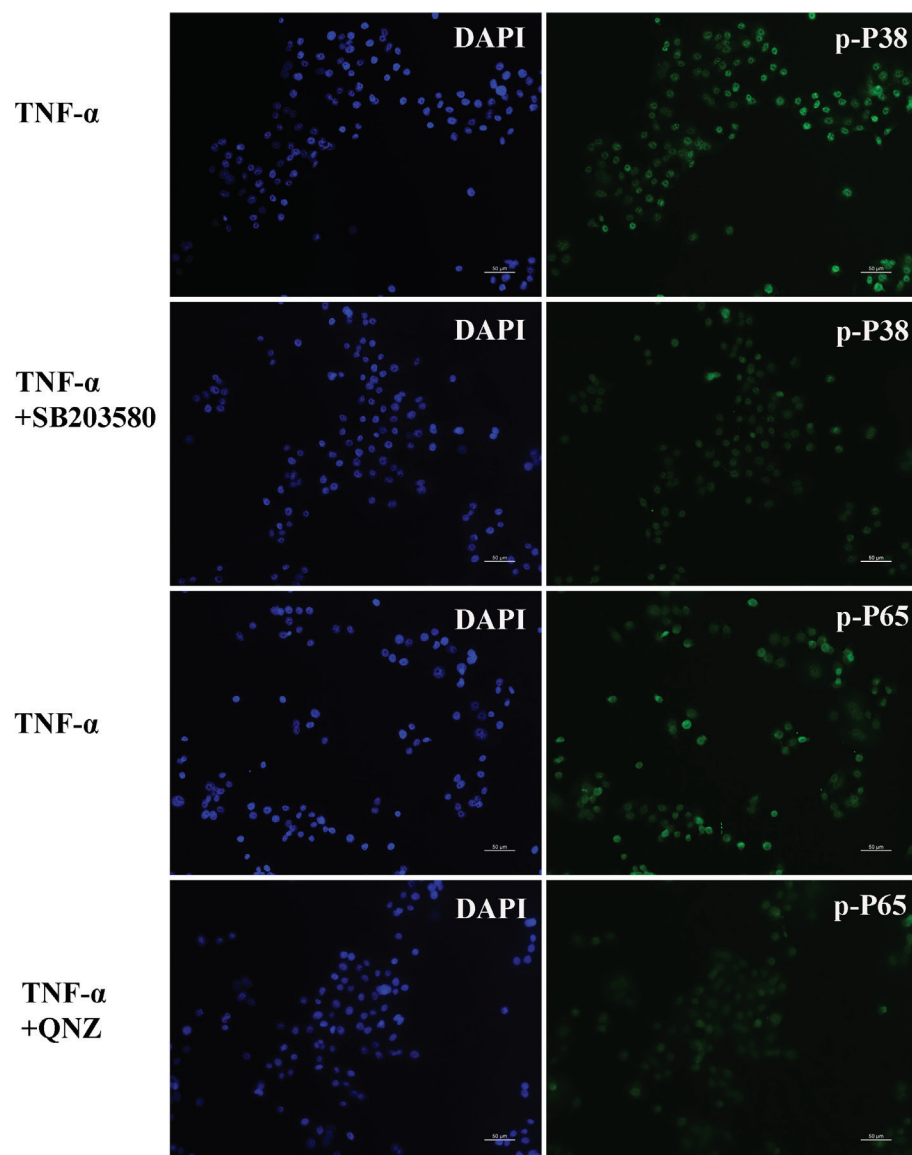
Figure 4. Immunostaining of p-p38 MAPK and p-p65 NF-κB in HNECs. Fluorescence intensity of p65-NF-κB and p38 MAPK was observed in TNF-α-treated HNECs, which could be inhibited with QNZ and SB203580. Scale bars: 50 μm.

CRS patients (**Fig. 1**). Recently, p65-NF-Kb and p38-MAPK pathways were widely involved in CRS tissue and human nasal epithelial cell lines (2, 24).

Wang (13) found that IL-1β reduced the ratio of GRα/GRβ in nasal polyp cells by activating the p38-MAPK pathway to reduce the effectiveness of GC treatment. IL-1β also mediated the expression of the GC receptor in nasal epithelial cells via p65-NF-κB. GRα is the physiological and pharmacological receptor for GCs and the primary isoform via which the majority of GCs act. GR-β fails to transactivate GC-responsive genes in the nuclei for short of the GC ligand-binding domain and functions as an overt negative regulator of GR transcriptional activity (25). The studies have also indicated that GRα mRNA expression is significantly reduced by stimulation with TNF-α. However, protein levels of GRα and GR were significantly increased following stimulation with TNF-α for 3 and 6 h, then decreased obviously at 24 h (**Fig. 2**). However, Webster found that HeLa cells' TNF-α-treatment significantly

increased the GRα accumulation compared with GRβ, which is contrary to our results (26). Many studies showed that GRβ exerts a strong inhibitory effect on the GR-mediated transactivation (25, 27).

The present study found that pre-incubation with SB203580 and QNZ significantly increases GRα mRNA expression and protein levels. These results revealed, the TNF-α regulates the GR isoforms expression in HNECs via p65-NF-κb and p38-MAPK signal transduction pathways, implying that the p65-NF-κb and p38-MAPK signaling inhibition might be effective in the CRS treatment. As reported, IL-1β decreases the ratio of GRα/GRβ from mRNA and protein levels via p38 MAPK and JNK pathways, which induces GC resistance (13). As reported, TNF-α leads to an interferon regulatory factor's (IRF)-1 better expression, that could abolish GR-interacting protein (GRIP)-1 from GR complex, hence reducing the GR-dependent genes' transcription and inducing the IRF-1–dependent pro-inflammatory genes like CD38 expression (28). In human bronchial epithelial 16HBE cells, IL-17A induced GC insensitivity is mainly regulated by the phosphoinositide-3-kinase (PI3K) pathway and subsequent reduction of histone deacetylases 2 (HDAC2) activity (29). Our research is consistent with previous results regarding the signaling pathways involved in GR mRNA expression and GC sensitivity in rat brain tissue (30) and CRS (13). Decreased GRβ expression observed in the GTM cells made the cells more sensitive to GCs, while the increased expression made them more resistant (31). The asthmatic GC resistance is thought to be caused by increased GRβ levels functioning as an overt negative receptor (32). Increased GRβ expression in PBMCs by interleukin (IL)-17/ interleukin (IL)-23 cytokines is linked to steroid insensitivity (33). Evidence also indicates that increased GRα and GRβ can significantly repress the AP-1 and NF-κB activities even in the hormone absence, suggesting GRβ does not act as a negative factor (34). However, GRβ has been reported to be related to enhanced cell migration, growth, as well as decreased sensitivity to GC-induced apoptosis in recent studies (35). Our study reveals the mechanism of TNF-α induced GCR via p65-NF-κb and p38-MAPK signal transduction pathways in HNECs, which has its limitation due to the lack of mature animal models of chronic rhinosinusitis. For the complex pathogenic factors of chronic rhinosinusitis, the problem of establishing appropriate cell models or animal models need to be solved.

6. Conclusions

Our results show that TNF-α is involved in CRS patients' nasal mucosa and nasal polyps. TNF-α significantly increased TNF-α and IL-6 mRNA expression and decreased mRNA and protein levels of GRα via NF-κB and p38 MAPK signaling. Treatment with QNZ, SB203580, or dexamethasone inhibited the TNF-α and IL-6 mRNA expression and increased GRα mRNA and protein levels, which may be useful in treating the neutrophilic CRS.

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Ethics Declaration Additional Information
The text was submitted by the author(s) in English. We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

Compliance with Ethical Standards
No interest conflicts were declared. The Shanghai Jiao Tong University, Medicine School, Affiliated Ren Ji Hospital's ethics committee approved this research. All participants had informed consent to the research content.

Author Contributions
This research was conceived by JP. L. who helped in writing the manuscript too.
The experiments and the data analysis were done by YQ. J., B. L., and XM. B.
Some experiments were done by P. Z.

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The manuscript was written by YQ. J., B. L., and XM. B. The manuscript's final version was approved by all authors.

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