Effects of Glucocorticoid-Induced Transcript 1 Gene Deficiency on Glucocorticoid Activation in Asthmatic Mice

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Abstract

Background: Glucocorticoid (GC) is the first-line therapy for asthma, but some asthmatics are insensitive to it. Glucocorticoid-induced transcript 1 gene (*GLCCI1*) is reported to be associated with GCs efficiency in asthmatics, while its exact mechanism remains unknown.
Methods: A total of 30 asthmatic patients received fluticasone propionate for 12 weeks. Forced expiratory volume in 1 s (FEV_1) and *GLCCI1* expression were detected. Asthma model was constructed in wild-type and *GLCCI1* knockout (*GLCCI1$^{-/-}$*) mice. Glucocorticoid receptor (GR) and mitogen-activated protein kinase phosphatase 1 (MKP-1) expression were detected by polymerase chain reaction and Western blotting (WB). The phosphorylation of p38 mitogen-activated protein kinase (MAPK) was also detected by WB.
Results: In asthmatic patients, the change of FEV_1 was well positively correlated with change of *GLCCI1* expression ($r = 0.430$, $P = 0.022$). In animal experiment, *GR* and *MKP-1* mRNA levels were significantly decreased in asthmatic mice than in control mice (wild-type: *GR*: 0.769 vs. 1.000, $P = 0.022$; *MKP-1*: 0.493 vs. 1.000, $P < 0.001$. *GLCCI1$^{-/-}$*: *GR*: 0.629 vs. 1.645, $P < 0.001$; *MKP-1:* 0.377 vs. 2.146, $P < 0.001$). Hydroprednisone treatment significantly increased *GR* and *MKP-1* mRNA expression levels than in asthmatic groups; however, *GLCCI1$^{-/-}$* asthmatic mice had less improvement (wild-type: *GR*: 1.517 vs. 0.769, $P = 0.023$; *MKP-1*: 1.036 vs. 0.493, $P = 0.003$. *GLCCI1$^{-/-}$*: *GR*: 0.846 vs. 0.629, $P = 0.116$; *MKP-1*: 0.475 vs. 0.377, $P = 0.388$). *GLCCI1$^{-/-}$* asthmatic mice had more obvious phosphorylation of p38 MAPK than wild-type asthmatic mice (9.060 vs. 3.484, $P < 0.001$). It was still higher even though after hydroprednisone treatment (6.440 vs. 2.630, $P < 0.001$).
Conclusions: *GLCCI1* deficiency in asthmatic mice inhibits the activation of GR and MKP-1 and leads to more obvious phosphorylation of p38 MAPK, leading to a decremental sensitivity to GCs.
Trial Registration: ChiCTR.org.cn, ChiCTR-RCC-13003634; http://www.chictr.org.cn/showproj.aspx?proj=5926.

Key words: Asthma; Glucocorticoid Receptor; Glucocorticoid-Induced Transcript 1; Glucocorticoids; Mitogen-Activated Protein Kinase Phosphatase-1

INTRODUCTION

Asthma is a chronic airway inflammatory disease that affects approximately 315 million people worldwide.[1] Inhaled corticosteroids (ICS) are the first-line anti-inflammatory therapy of asthma. However, not all asthmatic patients benefit from ICS treatment. In a study by Pignatti,[2] 22% of patients taking inhaled beclomethasone exhibited a decrease in forced expiratory volume in 1 s (FEV_1) after 12 weeks of therapy. Some asthmatics even experienced worsened symptoms after corticosteroid treatment.[3] Studies have revealed that several genes, such as *NR3C1*,[4] *STIP1*,[5] *CRHR1*,[6] and *FCER2*,[7] were associated with ICS efficiency of asthmatic patients.

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In 2011, Tantisira et al.[8] revealed that glucocorticoid-induced transcript 1 gene (*GLCCI1*) functional variant rs37973 was associated with marked attenuation of the response to treatment with ICS through changes in *GLCCI1* expression. Soon after that, Izuhara et al.[9] proposed that *GLCCI1* variant rs37973 was a risk factor for pulmonary function decline in Japanese patients with asthma receiving long-term ICS treatment. Xu et al.[10] also found that *GLCCI1* variant rs37973 was associated with poorer clinical therapeutic response to inhaled glucocorticoids (GCs) in a Chinese asthma population. Rijavec et al.[11] revealed that *GLCCI1* rs37973 was associated with short- and long-term treatment response. Brouwer et al.[12] revealed that *GLCCI1* variant rs37972 was also related with the dexamethasone responsiveness in bacterial meningitis as was found in asthma. Furthermore, researchers have found that *GLCCI1* polymorphism was involved in the GCs response in several diseases, such as graft-versus-host diseases,[13] rheumatoid arthritis,[14] and nephrotic syndrome.[15] The above reports suggest that *GLCCI1* plays an important and critical role in the GCs pathway, but the exact function of *GLCCI1* is still relatively unknown.

In our previous study, we found that asthmatic patients with rs37973 mutant genotype had diminished ICS response and less improvement in *GLCCI1* expression.[16] However, the association between *GLCCI1* expression and the response of ICS is only a statistical speculation. In the present study, we hypothesized that *GLCCI1* might affect the ICS efficiency in asthmatic patients by influencing the GC pathway. Thus, we investigated the effects of *GLCCI1* deficiency on the GC sensitivity in asthmatic mice model and detected the glucocorticoid receptor (GR), mitogen-activated protein kinase phosphatase 1 (MKP-1) expression, and p38 mitogen-activated protein kinase (MAPK) phosphorylation level in the lung tissue of these mice. Our findings demonstrated that *GLCCI1* deficiency in asthmatic mice inhibits the activation of GR and MKP-1 and leads to more obvious phosphorylation of p38 MAPK, leading to a decremental sensitivity to GCs.

METHODS

Ethical approval

This study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Chinese Ethnic Committee of Registering Clinical Trials and registered in the Chinese Clinical Trial Registry. Informed written consent was obtained from all patients before their enrollment in this study.

Participants and design

From September 2013 to December 2014, 36 adult asthmatic patients in mild-to-moderate stage were recruited from the Respiratory Clinic of Xiangya Hospital, Central South University in Changsha, Hunan, China. Asthma diagnosis and severity were established according to the Global Initiative for Asthma guidelines, based on the clinical symptoms and spirometry. Exclusion criteria included (1) intermittent

status asthma, severe asthma, or acute exacerbation of asthma; (2) occupational asthma or aspirin or nonsteroidal anti-inflammatory drug-related asthma; (3) life-threatening acute asthma exacerbation occurred within 1 year before the test; (4) recent asthma exacerbations requiring hospitalization or oral corticosteroids use; (5) history of significant lung diseases other than asthma; and (6) history of smoking in excess of 10 pack-years. After a 2-week run-in period, all the asthmatics were treated with fluticasone propionate (125 μg, twice a day, Glaxo Wellcome, S.A., UK) for 12 weeks. All patients received salbutamol (100 μg, Glaxo Wellcome, S.A., UK) as needed throughout the run-in and treatment period. No other asthma medications were permitted. Spirometry, blood sample, asthma control test (ACT), and asthma quality of life questionnaire (AQLQ) we collected at enrollment and at the 12th week. The blood sample was used for the detection of blood eosinophil count and *GLCCI1* mRNA expression in peripheral blood mononuclear cells (PBMCs).

Animals and design

Wild-type and *GLCCI1* knockout (*GLCCI1*-/-) C57BL/6N female mice of 6–8 weeks old weighing 18–20 g were bred under specific-pathogen-free conditions in the Experimental Animal Center of Central South University, Changsha, Hunan, China. The details of the construction of the *GLCCI1* knockout homozygous mice were displayed in the supplement file. All procedures performed on the animals were in compliance with the Chinese Council of Animal Care Guidelines, approved by the Central South University Animal Care Committee (No. 201603376). The wild-type and *GLCCI1*-/- mice were randomly divided into three groups (*n* = 8): control groups (ConWT and ConKO), asthma groups (AsWT and AsKO), and hydroprednisone-treated asthma groups (AsWT + GC and AsKO + GC). The mice were treated as follows: on day 0, day 7, and day 14, the asthmatic mice were sensitized with an intraperitoneal injection of 50 μg of chicken ovalbumin (OVA, Sigma, Grade V, USA) and 2 mg of aluminum hydroxide (Sigma, USA) in 0.2 ml of sterile saline. The control mice were treated with a sterile saline intraperitoneal injection for sham sensitization. The sensitized mice were exposed to 5% OVA (wt/vol) aerosol for 30 min every day from day 21 to day 27, while the control mice were exposed to sterile saline.[17] The hydroprednisone-treated asthmatic mice were handled with intraperitoneal injection of hydroprednisone (3 mg/kg, Lijun, China) every day 1 h before inhalation of 5% OVA aerosol.[18] Mice were sacrificed with 10% chloral hydrate (3.5 ml/kg) within 24 h after the last intervention. The right middle lungs were stored in 4% paraformaldehyde for hematoxylin and eosin (H and E) staining, and the rest were immediately stored in liquid nitrogen for polymerase chain reaction (PCR) and Western blotting (WB). Bronchoalveolar lavage fluid (BALF) was stored in cell preservation liquid for white blood cell counting.

Measurement of bronchial responsiveness

Pulmonary function was performed in asthmatic patients using the Jaeger Masterscope® spirometry system

(Jaeger, Wurzburg, Germany) according to the American Thoracic Society guideline.[19] All the asthmatic patients had their pulmonary function measured before and after 12 weeks' treatment of fluticasone propionate. The change of FEV_1 (ΔFEV_1) was calculated by FEV_1 measured after 12 weeks' treatment minus FEV_1 measured before treatment.

In vivo airway responsiveness of the mice to methacholine was measured within 24 h after the last OVA challenge using whole-body plethysmography (Buxco Electronics Inc., USA). The mice were challenged for 2 min with aerosolized solutions of methacholine (0, 2.5, 5.0, 10, and 20 mg/ml in normal saline) by an ultrasonic nebulizer, and airway resistance (R_L) was measured.[20,21] The results were expressed for each methacholine concentration as the percentage of baseline R_L values after saline exposure.

Bronchoalveolar lavage
After determination of bronchial responsiveness, the lungs were lavaged *in situ* with three successive 1 ml volumes of ice-cold phosphate-buffered solution (PBS) instilled by a syringe. The BALF was recovered manually by gentle aspiration with a disposable syringe after each infusion; the recovery rate of BALF was more than 80%. The lavage fluid was centrifuged at 1500 ×*g* for 10 min at 4°C; the sediment was resuspended in PBS and then stained with Wright–Giemsa. A total of 200 cells were counted for cell classification.[22,23]

Lung histopathology
The middle lobe of the right lung was fixed in 4% paraformaldehyde for 18–24 h, embedded in paraffin, and then routinely processed. Serial 3.5 μm tissue sections were stained with H and E. The morphological changes of the lung were observed under a light microscope (Leica Microsystems, Wetzlar, Germany).

Western blotting
Lung tissues of the mice were first ground by an electronic tissue grinder (Tiangen, China) and then incubated in RIPA lysis buffer (Servicebio, China) plus 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (Servicebio, China) and a protease inhibitor cocktail (Servicebio, China) for 30 min. The homogenates were centrifuged at 15,000 ×*g* for 15 min at 4°C. The concentration of proteins in the supernatant fractions was determined by BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein (50 μg) of each selected sample were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were transferred to polyvinylidene fluoride membranes after electrophoresis. Membranes were blocked with 5% nonfat dry milk or 5% bull serum albumin (for phosphorylated proteins) dissolved in Tris-buffered saline with Tween-20 buffer for 1 h and then incubated at 4°C overnight with primary antibodies against GLCCI1 (1:500; Hangzhou Goodhere Biotechnology, China), GR (1:1000; Cell Signaling Technology, USA), MKP-1 (1:1000; Merck-Millipore), p38 (1:1000; Cell Signaling Technology), and phosphorylated p38 (1:1000; Cell Signaling Technology) followed by incubation

with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The bands were detected by Image Lab system and quantified with densitometry. The results were expressed as the ratio of the mean band density of experimental groups to that of the control group after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

RNA preparation and real-time reverse transcription-polymerase chain reaction
We determined the relative mRNA levels of human *GLCCI1*, with human *GAPDH* mRNA as an internal control, using an ABI VIIA 7 real-time PCR system (ABI, USA). For the analysis, 4 ml of the collected blood was used for isolation of PBMCs, using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Total RNA in PBMC of the asthmatic patients and lung tissue of the mice was extracted by TRIzol reagent (Invitrogen, life technology). After testing the concentration and integrity of RNA by detecting optical density, RNA was reversely transcribed to cDNA according to the manufacturer's instructions. Primers for *GLCCI1*, *GR*, *MKP-1*, tumor necrosis factor-α (*TNF-α*), interferon-γ (*IFN-γ*), interleukin-6 (*IL-6*), and interleukin-13 (*IL-13*) used in real-time PCR were designed by primer premier 5.0 (Premier, Canada), compounded by Sangon Biotech (Shanghai, China), and are listed in Table 1. The PCR reactions were carried out in a 10 μl reaction volume containing 1 ng cDNA, 0.2 μmol/L forward primer, 0.2 μmol/L reverse primer, 5 μl 1× UltraSYBR Mixture, and 3.6 μl RNase-free double-distilled water. PCR conditions were as follows: step 1, 10 min at 95°C; step 2, 5 s at 95°C; and step 3, 34 s at 60°C; with step 2 and step 3 repeated for 40 cycles. The results were expressed as the ratio of the mean threshold cycle (Ct) values of experimental groups to that of the control group after normalization to *GAPDH*.

Statistical analysis
Statistical analysis of data was performed with SPSS 19.0 software (IBM, USA). All data were displayed as mean ± standard deviation (SD). Spearman's correlation was used to investigate the relationship between change in FEV_1 and change in *GLCCI1* mRNA expression. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* test was used to compare the differences among multiple groups for normally distributed variables. Otherwise, Kruskal–Wallis test followed by Mann–Whitney test was used to compare the differences among multiple groups for abnormally distributed variables. $P < 0.05$ was considered statistically significant.

Results

Subject characteristics
Among the 36 asthmatic patients receiving fluticasone propionate treatment, 3 lost to follow-up, 2 withdrew consent during follow-up, and 1 was withdrawn by clinician for his poor compliance. As a result, 30 patients completed the 12-week

follow-up. Only individuals who completed the treatment were included in the analysis. The blood eosinophil count significantly decreased after fluticasone treatment for 12 weeks ($P = 0.024$). Significant improvement on FEV_1, $GLCCI1$ mRNA expression, ACT and AQLQ were also observed after fluticasone treatment ($P < 0.001$ for all). The characteristics of these 30 asthmatic patients are listed in Table 2.

Glucocorticoid-induced transcript 1 gene mRNA expression is associated with inhaled corticosteroid efficiency

We detected the $GLCCI1$ mRNA expression level in PBMCs of each patient before and after treatment (0 and 12th week). The change of $GLCCI1$ mRNA expression level (ΔGLCCI1 expression) was calculated by $GLCCI1$ mRNA expression at the 12th week minus $GLCCI1$ mRNA expression at the 0 week. ΔFEV1 was calculated by FEV1 at the 12th week minus FEV1 at the 0 week. Spearman's correlation analysis revealed that ΔFEV_1 was well positively correlated with $\Delta GLCCI1$ expression [Figure 1, $r = 0.430$, $P = 0.022$], suggesting that the change of $GLCCI1$ mRNA expression after GC treatment is associated with ICS efficiency in asthmatic patients. However, the baseline $GLCCI1$ mRNA expression was not correlated with ICS efficiency [Figure 1, $P > 0.05$]. Meanwhile, we also analyzed the relationship between $GLCCI1$ mRNA expression and the

blood eosinophil count, which found no correlation neither between baseline $GLCCI1$ mRNA expression and baseline blood eosinophils nor between $\Delta GLCCI1$ expression and change of blood eosinophils (data not shown).

GLCCI1 deficiency impaired the inhibitory effects of glucocorticoids on lung inflammation

To find out how $GLCCI1$ affects the ICS efficiency in asthmatics, we constructed asthma model in both $GLCCI1$ knockout (GLCCI1$^{-/-}$) and wild-type mice. The RL value in the asthmatic groups (AsWT and AsKO) was significantly elevated compared with the control groups (ConWT and ConKO) [$326.00 \pm 95.62\%$ vs. $68.83 \pm 35.32\%$, $t = -6.182$, $P = 0.001$ in wild-type group; $359.83\% \pm 76.03\%$ vs. $82.00\% \pm 35.39\%$, $t = -8.115$, $P < 0.001$ in GLCCI1-/- group; Figure 2a]. Administration of hydroprednisone (AsWT + GC and AsKO + GC) significantly decreased the RL value of the asthmatic mice (AsWT and AsKO) [$111.17\% \pm 42.91\%$ vs. $326.00\% \pm 95.62\%$, $t = 5.021$, $P = 0.002$ in wild-type group; $188.89\% \pm 49.85\%$ vs. $359.83\% \pm 76.03\%$, $t = 4.605$, $P = 0.001$ in GLCCI1-/- group; Figure 2a]. However, the RL was higher in $GLCCI1$-/- group than in the wild-type group. The proportion of eosinophils in BALF was elevated in asthmatic groups (AsWT and AsKO) than in control groups (ConWT and ConKO) [$0.57\% \pm 0.07\%$ vs. $0.38\% \pm 0.11\%$, $t = -2.934$, $P = 0.026$ in wild-type

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Table 1: Sequences of target genes for real-time PCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
GAPDH (mouse)	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC
GLCCI1 (mouse)	AGGCGAACCTCTTCTCTGGA	GTGAACATGAGGGTCCCGTG
GR (mouse)	GTGAGTTCTCCTCCGTCCAG	TACAGCTTCCACACGTCAGC
MKP1 (mouse)	AGGATATGCTTGACGCCTTG	GTCTGCCTTGTGGTTGTCCT
IFN-γ (mouse)	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
TNF-α (mouse)	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL13 (mouse)	CCTGGCTCTTGCTTGCCTT	GGTCTTGTGTGATGTTGCTCA
IL-6 (mouse)	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
GAPDH (human)	GGTGAAGGTCGGAGTCAACG	CAAAGTTGTCATGGATGACC
GLCCI1 (human)	ACTCGCAGCATTGACACTCA	ATGAGAGCTGCTCAACGGTC

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; *GLCCI1*: Glucocorticoid-induced transcript 1 gene; *GR*: Glucocorticoid receptor; *MKP-1*: Mitogen-activated protein kinase phosphatase 1; *IFN-γ*: Interferon-γ; *TNF-α*: Tumor necrosis factor-α; *IL-13*: Interleukin-13. *IL-6*: Interleukin-6; PCR: Polymerase chain reaction.

Table 2: Characteristics of the asthmatic patients receiving fluticasone treatment ($n = 30$)

Description	On admission	After treatment	t	P
Age (years), mean ± SD	42.10 ± 10.20		–	–
Gender (men/women), *n*	14/16		–	–
FEV₁ (L)	2.19 ± 0.60	2.39 ± 0.56	−4.327	<0.001
FEV₁% predicted	75.87 ± 11.48	83.26 ± 10.29	−4.376	<0.001
GLCCI1 mRNA expression	0.030 ± 0.015	0.068 ± 0.032	−8.101	<0.001
ACT	15.90 ± 3.57	23.67 ± 1.42	−11.267	<0.001
AQLQ	3.98 ± 0.48	4.68 ± 0.31	−6.863	<0.001
Blood eosinophil count (× 10⁹/L)	0.36 ± 0.22	0.28 ± 0.17	2.377	0.024

–: Not applicable; SD: Standard deviation; FEV₁: Forced expiratory volume in the 1st s; *GLCCI1*: Glucocorticoid-induced transcript 1 gene; ACT: Asthma control test; AQLQ: Asthma quality of life questionnaire; SD: Standard deviation.

group; 0.61% ± 0.08% vs. 0.37% ± 0.10%, $t = -3.688$, $P = 0.010$ in *GLCCI1*$^{-/-}$ group; Figure 2b]. Administration of hydroprednisone (AsWT + GC and AsKO + GC) significantly decreased the proportion of eosinophils in BALF of the asthmatic mice (AsWT and AsKO) [0.40% ± 0.12% vs. 0.57% ± 0.07%, $t = 2.603$, $P = 0.041$ in wild-type group; 0.42% ± 0.12% vs. 0.61% ± 0.08%, $t = 2.689$, $P = 0.036$ in *GLCCI1*$^{-/-}$ group; Figure 2b].

The *IFN-γ*, *TNF-α*, *IL-6*, *IL-13*, and *IL-4* mRNA expression levels in lung tissue are shown in Table 3 and Figure 2c. The *IFN-γ*, *TNF-α*, *IL-6*, *IL-13*, and *IL-4* mRNA expression levels were significantly elevated in asthmatic groups (AsWT and AsKO) than in control groups (ConWT and ConKO) ($P < 0.05$). Administration of hydroprednisone significantly decreased *IFN-γ*, *TNF-α*, *IL-6*, *IL-13*, and *IL-4* mRNA expression levels in wild-type asthmatic group ($P < 0.05$), but not in *GLCCI1*$^{-/-}$ asthmatic group (except for *IFN-γ* and *TNF-α*, $P < 0.05$). The *IFN-γ*, *TNF-α*, *IL-6*, *IL-13*, and *IL-4* mRNA expression levels in GC-treated *GLCCI1*$^{-/-}$ asthmatic mice (AsKO + GC) were significantly decreased than those in GC-treated wild-type asthmatic mice (AsWT + GC) ($P < 0.05$). As shown in Figure 3, the asthmatic groups displayed more severe infiltration of inflammatory cells in lung tissue; the infiltration of inflammatory cells was more serious in *GLCCI1*$^{-/-}$ asthmatic mice than in wild-type asthmatic mice. Subsequent administration of hydroprednisone decreased the severity of inflammation in the lung tissue.

Glucocorticoid-induced transcript 1 gene deficiency impaired the effects of glucocorticoids on glucocorticoid receptor and mitogen-activated protein kinase phosphatase-1

We first detected *GLCCI1* mRNA and protein expression in the lung tissue of each group of mice. In wild-type mice, the *GLCCI1* mRNA and protein expression levels significantly decreased in asthmatic group (AsWT) than those in control group (ConWT) (mRNA: 0.547 ± 0.180 vs. 1.000 ± 0.000, $t = 6.170$, $P = 0.002$; protein: 0.350 ± 0.044 vs. 1.000 ± 0.000, $t = 32.500$, $P < 0.001$). After administration of hydroprednisone (AsWT + GC), the *GLCCI1* mRNA and protein expression levels significantly increased than those in AsWT group (mRNA: 1.419 ± 0.447 vs. 0.547 ± 0.180, $t = -4.429$, $P = 0.001$; protein: 1.222 ± 0.062 vs. 0.350 ± 0.045, $t = -25.450$, $P < 0.001$). While in *GLCCI1*$^{-/-}$ mice, *GLCCI1* was almost not expressed, neither at the mRNA nor at protein level, indicating that the *GLCCI1* gene was successfully knocked out (mRNA: 0.0028 ± 0.0017 in ConKO, 0.0028 ± 0.0017 in AsKO, 0.0032 ± 0.0031 in AsKO + GC; protein: 0.372 ± 0.061 in ConKO, 0.200 ± 0.054 in AsKO, 0.308 ± 0.043 in AsKO + GC). The data are shown in Figure 4.

To evaluate whether *GLCCI1* influences the expression of *GR* and *MKP-1* or not, we detected the mRNA and protein levels of *GR* and *MKP-1* in the lung tissue of each group of mice. In wild-type mice, the *GR* and *MKP-1* mRNA and protein expression levels significantly decreased in asthmatic group (AsWT) than those in control group (ConWT) (*GR* mRNA: 0.769 ± 0.172 vs. 1.000 ± 0.000, $t = 3.292$, $P = 0.022$;
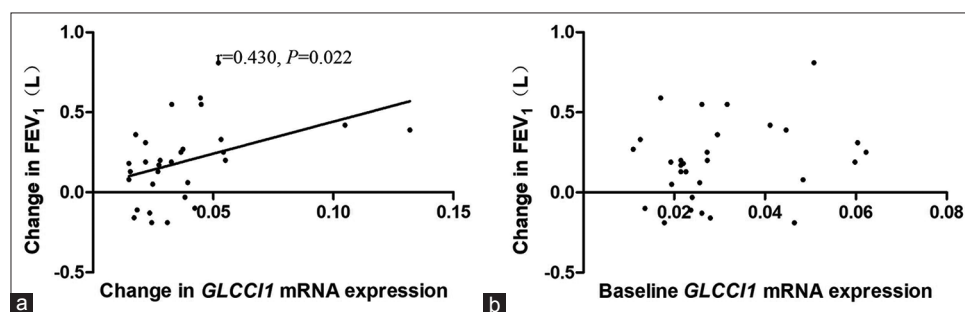
Figure 1: The correlation between the change in FEV$_1$ and *GLCCI1* mRNA expression in asthmatic patients ($n = 30$). (a) Spearman's correlation analysis revealed that change in FEV$_1$ was well positively correlated with change in *GLCCI1* expression after fluticasone treatment for 12 weeks ($r = 0.430$, $P = 0.022$). (b) The baseline *GLCCI1* mRNA expression was not correlated with ICS efficiency ($P > 0.05$). FEV$_1$: Forced expiratory volume in 1 s; *GLCCI1*: Glucocorticoid-induced transcript 1 gene.

Table 3: *IFN-γ*, *TNF-α*, *IL-6*, *IL-13*, and *IL-4* mRNA expression in lung tissue

Groups	TNF-α	IFN-γ	IL-13	IL-6	IL-4
ConWT	1.000	1.000	1.000	1.000	1.000
AsWT	1.518 ± 0.374*	2.245 ± 0.627*	15.711 ± 0.664*	4.024 ± 2.027*	2.086 ± 0.795*
AsWT + GC	1.120 ± 0.105†	1.228 ± 0.072†	4.184 ± 2.732†	1.529 ± 0.455†	1.173 ± 0.304†
ConKO	1.017 ± 0.291	1.293 ± 0.302	1.066 ± 0.090	0.779 ± 0.685	1.165 ± 0.241
AsKO	2.275 ± 0.343†,‡	2.904 ± 0.373†,‡	15.677 ± 5.557‡	4.176 ± 2.744‡	3.000 ± 1.190‡
AsKO + GC	1.533 ± 0.243§,‖	2.199 ± 0.457§,‖	9.781 ± 3.246‖	4.096 ± 1.628‖	2.109 ± 0.475‖

*$P < 0.05$ versus ConWT group, †$P < 0.05$ versus AsWT group, ‡$P < 0.05$ versus ConKO group, §$P < 0.05$ versus AsKO group, ‖$P < 0.05$ versus AsWT + GC group. ConWT: Wild-type control group; AsWT: Wild-type asthmatic group; AsWT + GC: Hydroprednisone-treated wild-type asthmatic group; ConKO: *GLCCI1* knockout control group; AsKO: *GLCCI1* knockout asthmatic group; AsKO + GC: Hydroprednisone-treated *GLCCI1* knockout asthmatic group; $n = 6$ in each group. TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; IL-13: Interleukin-13; IL-6: Interleukin-6; IL-4: Interleukin-4; *GLCCI1*: Glucocorticoid-induced transcript 1 gene.
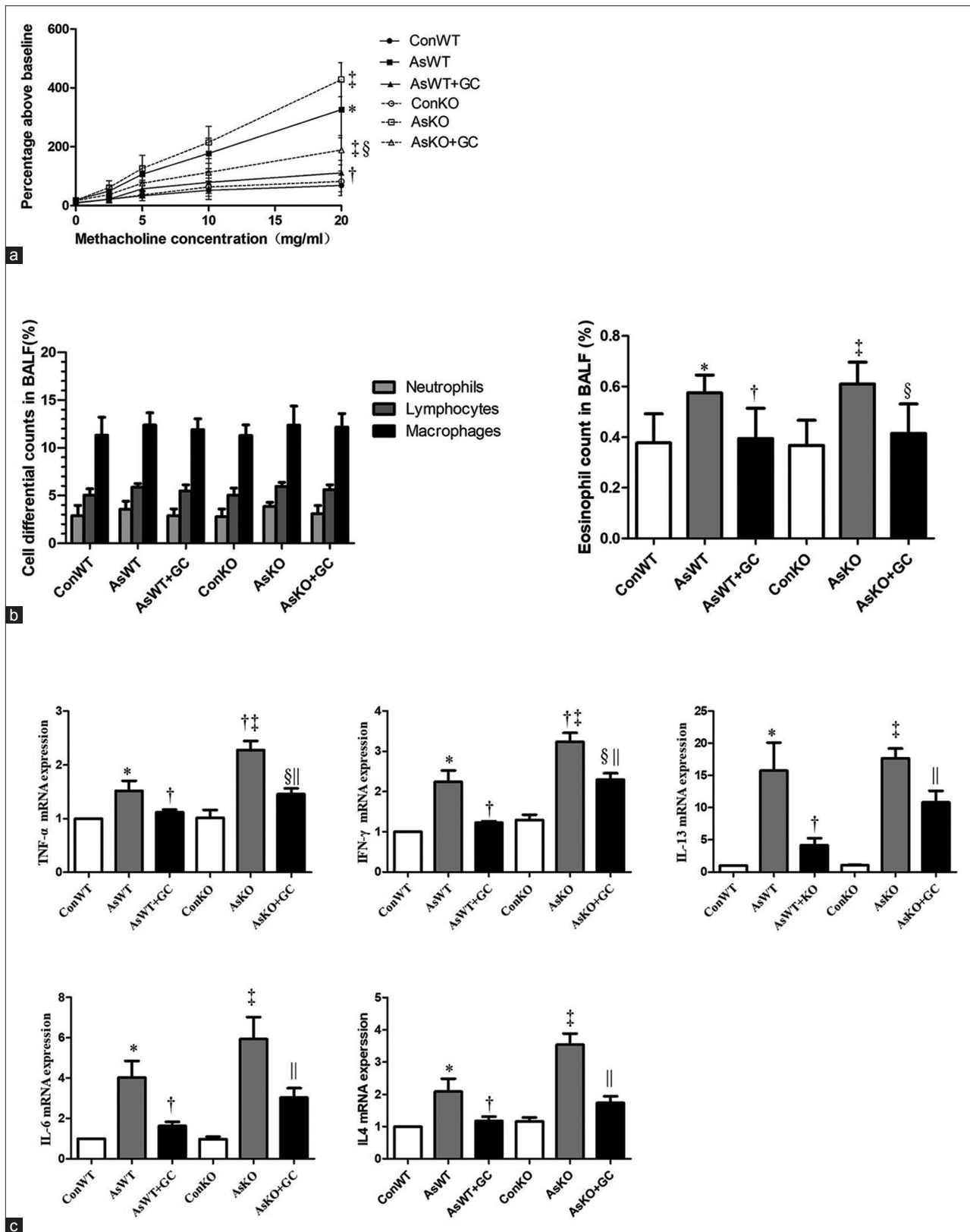
Figure 2: Effect of *GLCCI1* deficiency on lung inflammation in asthmatic mice under glucocorticoid treatment. (a) Airway resistance in each group (*n* = 6). (b) Cell differential counts in bronchoalveolar lavage fluid (%) (*n* = 4). (c) The mRNA expression of inflammatory genes in lung tissue (*n* = 6). $*P < 0.05$ versus ConWT group, $†P < 0.05$ versus AsWT group, $‡P < 0.05$ versus ConKO group, $§P < 0.05$ versus AsKO group, $\|P < 0.05$ versus AsWT + GC group. ConWT: Wild-type control group; AsWT: Wild-type asthmatic group; AsWT + GC: Hydroprednisone-treated wild-type asthmatic group; ConKO: *GLCCI1* knockout control group; AsKO: *GLCCI1* knockout asthmatic group; AsKO + GC: Hydroprednisone-treated *GLCCI1* knockout asthmatic group; BALF: Bronchoalveolar lavage fluid; TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; IL-13: Interleukin-13; IL-6: Interleukin-6; IL-4: Interleukin-4; GLCCI1: Glucocorticoid-induced transcript 1 gene.
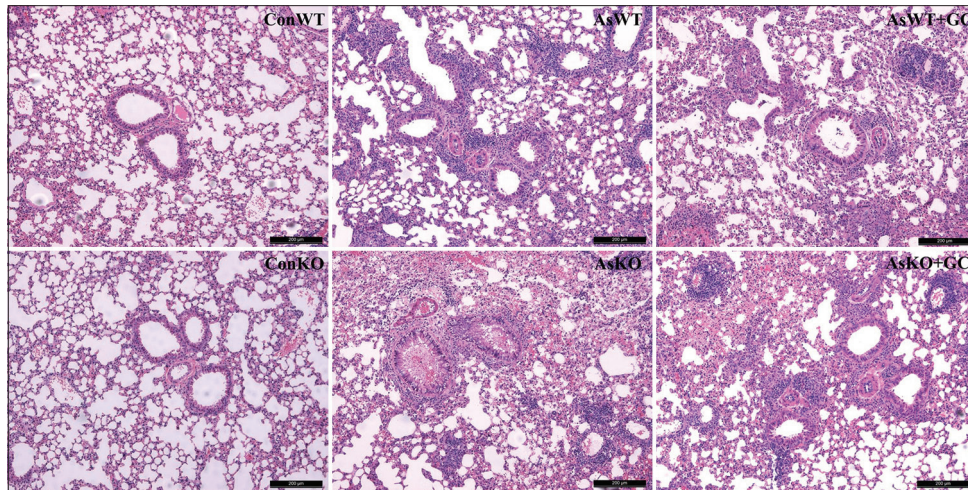
Figure 3: The histopathological structure of lung tissue (H and E, ×100. Scale bar = 200 μm). ConWT: Wild-type control group; AsWT: Wild-type asthmatic group; AsWT + GC: Hydroprednisone-treated wild-type asthmatic group; ConKO: *GLCCI1* knockout control group; AsKO: *GLCCI1* knockout asthmatic group; AsKO + GC: Hydroprednisone-treated *GLCCI1* knockout asthmatic group; *GLCCI1*: Glucocorticoid-induced transcript 1 gene.
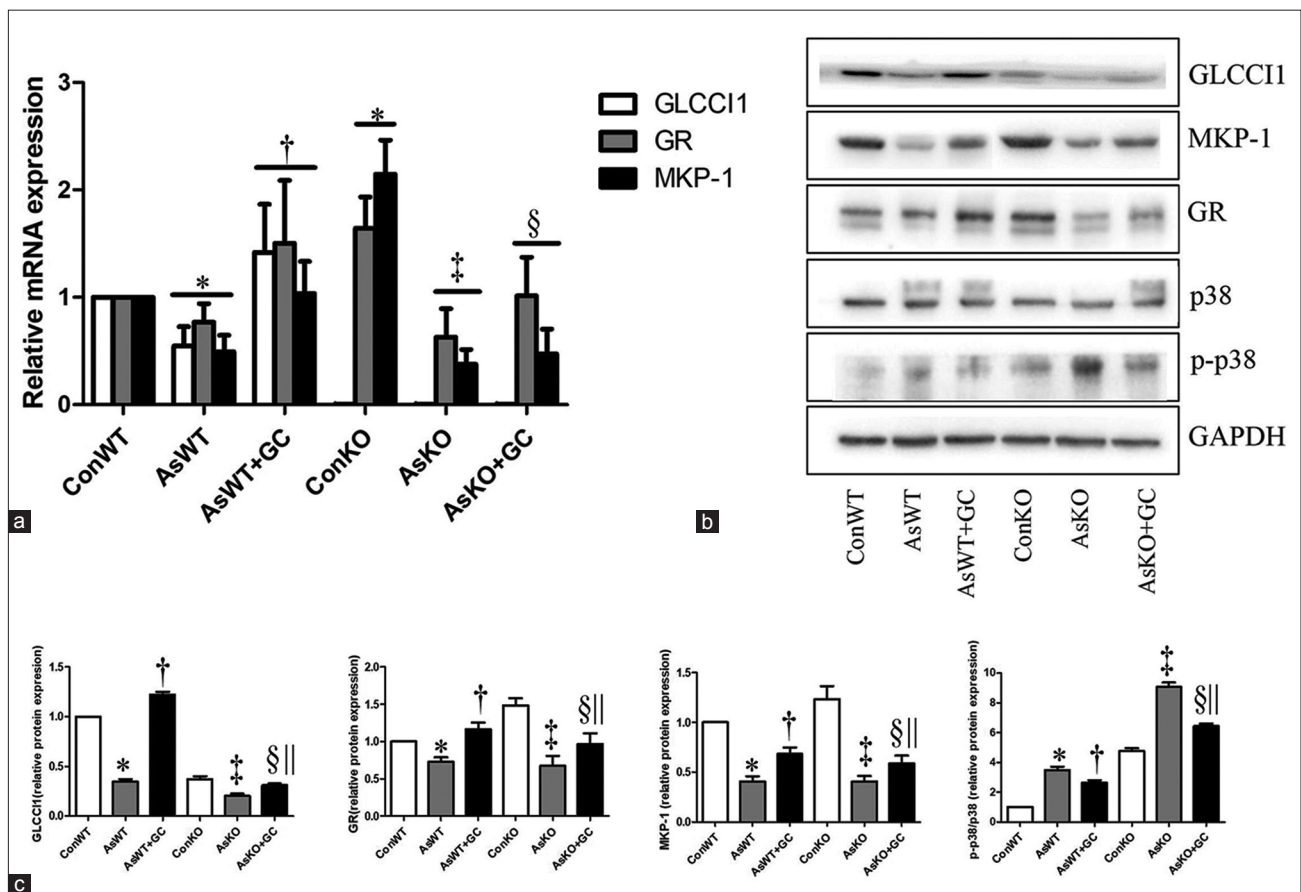

Figure 4: Effect of *GLCCI1* deficiency on *GR*, *MKP-1* expression and the phosphorylation of p38 MAPK. (a) The *GLCCI1*, *GR*, and *MKP-1* mRNA expression in lung tissue. Data were shown as mean ± SD ($n = 6$). (b) The Western blotting analyses of GLCCI1, GR, MKP-1, and p38 MAPK. (c) Relative band density values in b. Data are shown as mean ± SD ($n = 5$). *$P < 0.05$ versus ConWT group, †$P < 0.05$ versus AsWT group, ‡$P < 0.05$ versus ConKO group, §$P < 0.05$ versus AsKO group, ‖$P < 0.05$ versus AsWT + GC group. ConWT: Wild-type control group; AsWT: Wild-type asthmatic group; AsWT + GC: Hydroprednisone-treated wild-type asthmatic group; ConKO: *GLCCI1* knockout control group; AsKO: *GLCCI1* knockout asthmatic group; AsKO + GC: Hydroprednisone-treated *GLCCI1* knockout asthmatic group; GLCCI1: Glucocorticoid-induced transcript 1 gene; GR: Glucocorticoid receptor; MKP-1: Mitogen-activated protein kinase phosphatase-1; MAPK: Mitogen-activated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SD: Standard deviation.

GR protein: 0.730 ± 0.061 vs. 1.000 ± 0.000, $t = 9.859$, $P = 0.001$. *MKP-1* mRNA: 0.493 ± 0.152 vs. 1.000 ± 0.000, $t = 8.152$, $P < 0.001$; *MKP-1* protein: 0.406 ± 0.053 vs. 1.000 ± 0.000, $t = 24.968$, $P < 0.001$). After administration of

hydroprednisone (AsWT + GC), the *GR* and *MKP-1* mRNA and protein expression levels significantly increased than those in AsWT group (*GR* mRNA: 1.517 ± 0.573 vs. 0.769 ± 0.172, $t = -3.065$, $P = 0.023$; *GR* protein: 1.164 ± 0.090 vs. 0.730 ± 0.061, $t = -8.922$, $P < 0.001$. *MKP-1* mRNA: 1.036 ± 0.297 vs. 0.493 ± 0.152, $t = -3.981$, $P = 0.003$; *MKP-1* protein: 0.684 ± 0.065 vs. 0.406 ± 0.053, $t = -7.398$, $P < 0.001$). In *GLCCI1*^{-/-} mice, the *GR* and *MKP-1* mRNA and protein expression levels significantly decreased in asthmatic group (AsKO) than those in control group (ConKO) (*GR* mRNA: 0.629 ± 0.265 vs. 1.645 ± 0.289, $t = 6.336$, $P < 0.001$; *GR* protein: 0.678 ± 0.130 vs. 1.488 ± 0.095, $t = 11.267$, $P < 0.001$; *MKP-1* mRNA: 0.377 ± 0.138 vs. 2.146 ± 0.319, $t = 12.458$, $P < 0.001$; *MKP-1* protein: 0.410 ± 0.052 vs. 1.234 ± 0.130, $t = 13.134$, $P < 0.001$), the same as wild-type mice. However, administration of hydroprednisone (AsKO + GC) resulted in less increase of *GR* and *MKP-1* mRNA and protein expression levels than those in wild-type asthmatic mice (*GR* mRNA: 0.846 ± 0.137 vs. 0.629 ± 0.265, $t = -1.776$, $P = 0.116$; *GR* protein: 0.968 ± 0.142 vs. 0.678 ± 0.130, $t = -3.369$, $P = 0.010$. *MKP-1* mRNA: 0.475 ± 0.228 vs. 0.377 ± 0.138, $t = -0.903$, $P = 0.388$; *MKP-1* protein: 0.568 ± 0.055 vs. 0.410 ± 0.052, $t = -4.671$, $P = 0.002$). Furthermore, the GR and *MKP-1* mRNA and protein expression levels in hydroprednisone-treated *GLCCI1*^{-/-} asthmatic group were significantly less than that in hydroprednisone-treated wild-type asthmatic group (*GR* mRNA: 0.846 ± 0.137 vs. 1.517 ± 0.573, $t = 2.793$, $P = 0.034$; *GR* protein: 0.968 ± 0.142 vs. 1.164 ± 0.090, $t = 2.608$, $P = 0.031$. *MKP-1* mRNA: 0.475 ± 0.228 vs. 1.036 ± 0.297, $t = 3.665$, $P = 0.004$; *MKP-1* protein: 0.568 ± 0.055 vs. 0.684 ± 0.065, $t = 3.057$, $P = 0.016$). The data are shown in Figure 4.

Glucocorticoid-induced transcript 1 gene deficiency impaired the inhibitory effects of glucocorticoids on p38 mitogen-activated protein kinase pathway

To clarify whether *GLCCI1* involved in the p38 MAPK pathway, we evaluated the phosphorylation ratio of the p38. As shown in Figure 4, WB revealed that phospho-p38 (p-p38) was prominent in asthmatic group than in control group, both in wild-type and *GLCCI1*^{-/-} asthmatic mice (wild-type: 3.484 ± 0.235 vs. 1.000 ± 0.000, $t = -23.613$, $P < 0.001$; *GLCCI1*^{-/-}: 9.060 ± 0.313 vs. 4.766 ± 0.177, $t = -26.663$, $P < 0.001$). Furthermore, the level of p-p38 in *GLCCI1*^{-/-} asthmatic mice was significantly much higher than that in wild-type asthmatic mice (9.060 ± 0.313 vs. 3.484 ± 0.235, $t = -31.816$, $P < 0.001$). Administration of hydroprednisone in asthmatic mice significantly decreased the p-p38 both in wild-type and *GLCCI1*^{-/-} mice (wild-type: 2.630 ± 0.181 vs. 3.484 ± 0.235, $t = 6.434$, $P < 0.001$; *GLCCI1*^{-/-}: 6.440 ± 0.160 vs. 9.060 ± 0.313, $t = 16.637$, $P < 0.001$), but the level of p-p38 in hydroprednisone-treated *GLCCI1*^{-/-} asthmatic group was still significantly higher than that in hydroprednisone-treated wild-type asthmatic group (6.440 ± 0.160 vs. 2.630 ± 0.181, $t = -35.223$, $P < 0.001$).

DISCUSSION

GCs are the most important anti-inflammatory drugs used in asthma as they inhibit the expression of inflammatory mediators by macrophages and other cells. However, a proportion of asthmatic patients do not benefit from their GC treatment and bear from uncontrolled and worsened condition of asthma. Until now, the mechanism of GCs insensitivity in asthma remains poorly understood.

We have previously found that the *GLCCI1* mRNA expression was correlated with asthma susceptibility, but not correlated with asthma severity.[16] In this study, we observed that the change of *GLCCI1* mRNA expression was significantly well positively correlated with ΔFEV_1 in asthmatic patients treated with ICS in a Chinese Han adult population, which was consistent with the previous study by Tantisira *et al*.[8] Many researchers have studied the effect of *GLCCI1* polymorphism on ICS efficiency,[8,24] lung function,[9] and asthma control of asthmatics.[25] *GLCCI1* was identified as a novel pharmacogenetics determinant of the response of asthmatic patients to ICS. Tantisira *et al*.[8] proposed that *GLCCI1* might be an early marker of GC-induced apoptosis as it was a key mechanism through which GCs resolve lymphocytic and eosinophilic inflammation in asthma. However, the exact mechanism of how *GLCCI1* affects the ICS response of asthmatics still remains unknown. It is well known that the GC pathway plays a critical role in the GCs efficiency; thus, we hypothesized that *GLCCI1* might affect the ICS efficiency of asthmatic patients by influencing the GC pathway.

In 2011, Nishibori *et al*.[26] discovered that *GLCCI1* promotes the normal development and maintenance of podocyte structure and function using *GLCCI1* gene knockdown zebrafish. As zebrafish is not available for pulmonary research, we constructed *GLCCI1* knockout homozygous mice to explore how *GLCCI1* affects the ICS response of asthmatics. We determined the role of *GLCCI1* on the effects of GCs in asthma by constructing an asthma model in both wild-type and *GLCCI1*^{-/-} mice. As expected, both the wild-type and *GLCCI1*^{-/-} mice of the allergic asthma model exhibited airway hyperresponsiveness (AHR), eosinophilic airway inflammation, elevated lung immunological cytokines, and infiltration of inflammatory cells. In wild-type asthmatic mice, administration of GC significantly decreased AHR and alleviated airway inflammation. On the other hand, the function of GC in *GLCCI1*^{-/-} asthmatic mice was weakened, with less decrease in AHR and less alleviation of airway inflammation, which indicated that *GLCCI1* deficiency might lead to GCs inefficiency in asthmatic condition.

The effects of GCs are mediated by GR. GR is a member of the nuclear hormone receptor superfamily of transcription factors.[27] In the absence of GC, GR stays in the cytoplasm in a complex with a number of proteins such as heat shock protein 90 (HSP 90) at an inactivated status. After crossing the cell membrane by passive diffusion, the GC binds to its receptor GR and activates GR by dissociation with HSP 90. The GC-GR complex then migrates to the nucleus, binds to the glucocorticoid response element, and activates the transcription of target genes,[28] including *MKP-1*,[29] *GILZ*,[30] and *SGK1*.[31] Via binding to the receptor, GCs are able to

inhibit p38 MAPK by inducing MKP-1, which in turn results in de-phosphorylation of p38.[32] MKP-1 is a phosphatase that dephosphorylates and inactivates MAPKs, including p38 MAPK,[33] and inhibits production of pro-inflammatory cytokines, which is critical for the anti-inflammatory functions of GCs.[29] Clark *et al*.[29] proposed that MKP-1 played a role in the inhibition of p38 MAPK and the consequent destabilization of pro-inflammatory mRNAs by GCs. Keränen *et al*.[34] found that dexamethasone increased MKP-1 expression and resulted in the suppression of p38 MAPK signaling leading to the inhibition of cytokine production in human bronchial epithelial cells. Thus, they revealed that MKP-1 was an important factor in the therapeutic effects of GCs in the treatment of inflammatory lung diseases. MKP-1 may be involved in GC response since GCs inhibit p38 MAPK in macrophages from *MKP1*^{+/+} mice but not in those from *MKP-1*^{-/-} littermates.[35] As a result, MKP-1 has been identified as a marker of responsiveness to GC treatment.[36]

In order to explore how *GLCCI1* deficiency leads to GCs inefficiency in asthmatic condition, we detected both the GR and MKP-1 expression in the lung tissue of each group. Interestingly, we found that GCs significantly increase the expression of GR and MKP-1 in wild-type asthmatic mice. Although the GR and MKP-1 expression also increase after GC treatment in *GLCCI1*^{-/-} asthmatic mice, the *GR* and *MKP-1* mRNA and protein expression levels in GC-treated *GLCCI1*^{-/-} asthmatic group were significantly less than that in GC-treated wild-type asthmatic group. Bhattacharyya *et al*.[37] discovered that dexamethasone could induce MKP-1 expression and inhibit p38 MAPK pathway in mice with normal macrophages, but not in mice with conditional deletion of GR in macrophages, suggesting that GR and its activation are essential for GCs to induce MKP-1 expression and inhibit p38 MAPK activity. We then concluded that *GLCCI1* deficiency might suppress the activation of GR and inhibit the formation of GC-GR complex, resulting in less induction of MKP-1 expression under GCs stimulation. However, the mechanism of how *GLCCI1* deficiency suppresses the activation of GR needs further research.

To deeply determine whether p38 MAPK is influenced by *GLCCI1* deficiency, we observed the phosphorylation of p38 MAPK and the expression of TNF-α and IL-6 of lung tissue in each group as p38 MAPK pathway mediates the expression of them.[38] We found that the phosphorylation of p38 MAPK was more obvious in *GLCCI1*^{-/-} asthmatic mice than that in wild-type asthmatic mice. Treatment of GCs decreased the phosphorylation of p38 MAPK, but the phosphorylation level of p38 MAPK in GC-treated *GLCCI1*^{-/-} asthmatic group was still significantly higher than that in GC-treated wild-type asthmatic group. At the same time, GC intervention significantly decreased the expression of TNF-α and IL-6 in wild-type asthmatic mice, but showed less decrease in *GLCCI1*^{-/-} asthmatic mice than that in wild-type asthmatic mice. Those results indicated that *GLCCI1* deficiency resulted in more obvious phosphorylation of p38 MAPK and led to more serious inflammation in the asthmatic model, and GCs
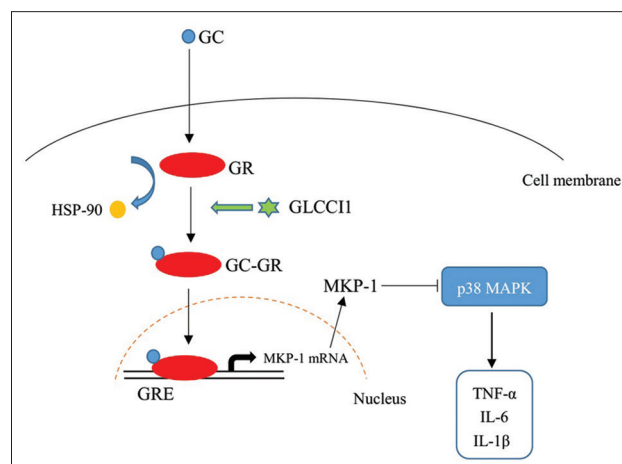

Figure 5: Schematic summary of the possible mechanism for GLCCI1 in glucocorticoids pathway. *GLCCI1* might modulate the formation of GC-GR complex or the activation of GR. *GLCCI1* deficiency leads to less activation of GR, leading to less improvement of MKP-1 pathway, more obvious phosphorylation of p38 MAPK and less inhibition for the pro-inflammatory genes under glucocorticoids, which induce poor response to glucocorticoids in asthma. GC: Glucocorticoid; GR: Glucocorticoid receptor; GRE: Glucocorticoid response element; HSP 90: Heat shock protein 90; MKP-1: Mitogen-activated protein kinase phosphatase-1; MAPK: Mitogen-activated protein kinase; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IL-1β: Interleukin-1β.

had a mild effect on them. There were various indications that *MKP-1* deficiency is associated with an increase in the production of many cytokines including TNF-α, IL-6, and IL-1β.[39] It was reasonable to conclude that MKP-1 decreased the production of many cytokines by inhibiting the phosphorylation of p38 MAPK.

In summary, we found that *GLCCI1* is closely related to GC efficiency in the clinical trial with 30 asthmatic patients receiving fluticasone treatment. Furthermore, we speculated that *GLCCI1* deficiency might inhibit the activation of GR, leading to less improvement of MKP-1 pathway and less inhibition for the pro-inflammatory genes under GCs, which induce poor response to GCs in asthma [Figure 5]. The mechanism of how *GLCCI1* deficiency inhibits the activation of GR still needs further research.

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Conflicts of interest

There are no conflicts of interest.

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GLCCI1基因敲除参与支气管哮喘小鼠糖皮质激素通路活化下调

摘要

背景：糖皮质激素是支气管哮喘治疗的一线药物，但部分哮喘患者对激素不敏感。研究发现，糖皮质激素诱导的转录基因1（GLCCI1）与哮喘患者糖皮质激素疗效有关，但其具体机制尚不明确。

方法：30例哮喘患者接受为期12周的丙酸氟替卡松吸入治疗，检测FEV_1及GLCCI1表达水平。利用野生型及GLCCI1基因敲除小鼠构建哮喘模型。造模成功后，通过PCR及Western blotting检测肺组织中GR、MKP-1表达水平；Western blotting检测p38 MAPK信号通路的磷酸化程度.

结果：哮喘患者经丙酸氟替卡松吸入治疗后FEV1改变量与*GLCCI1* mRNA表达水平改变量呈正相关（r＝0.430,*P*＝0.022）。在动物实验中，哮喘小鼠肺组织*GR*及*MKP-1 mRNA*表达水平低于对照组(野生型组: *GR*：0.769 vs.1.000，*P*＝0.022;*MKP-1*：0.493 vs.1.000，*P* < 0.001；*GLCCI1*⁻ᐟ⁻组: GR：0.629 vs.1.645，*P* < 0.001；*MKP-1*：0.377 vs.2.146，*P* <0.001)。与哮喘组小鼠相比，经氢化泼尼松治疗后的哮喘小鼠*GR*及*MKP-1* mRNA表达水平显著升高，但*GLCCI1*⁻ᐟ⁻组升高程度低于野生型组(野生型组: *GR*: 1.517 vs.0.769,*P*＝0.023;*MKP-1*: 1.036 vs.0.493,*P*＝0.003.*GLCCI1*⁻ᐟ⁻组: *GR*: 0.846 vs.0.629,*P*＝0.116;*MKP-1*: 0.475 vs.0.377,*P*＝0.388)。与野生型哮喘小鼠相比，*GLCCI1*基因敲除哮喘小鼠p38 MAPK磷酸化水平升高 (9.060 vs.3.484,*P* <0.001)。经氢化泼尼松治疗后*GLCCI1*基因敲除哮喘小鼠p38 MAPK磷酸化水平仍高于野生型哮喘小鼠(6.440 vs.2.630,*P* <0.001)。

结论：GLCCI1基因消除哮喘小鼠GR及MKP-1表达受抑制，导致p38 MAPK磷酸化，从而导致糖皮质激素敏感性下降。