



Original article

Serum proteomics analysis based on label-free revealed the protective effect of Chinese herbal formula Gu-Ben-Fang-Xiao



Qiong-Qiong Xing^{a,b,c,d,1}, Li-Wei Liu^{a,b,c,d,1}, Xia Zhao^{a,b,c,d,*}, Yuan Lu^{a,b,c,d},
Ying-Mei Dong^{a,b,c,d}, Zhong-Qing Liang^{a,b,c,d}

^a Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing, 210029, China

^b Jiangsu Province Hospital of Chinese Medicine, Nanjing, 210029, China

^c Pediatric Institution of Nanjing University of Chinese Medicine, Nanjing, 210023, China

^d Jiangsu Key Laboratory of Pediatric Respiratory Disease, Nanjing, 210023, China

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ABSTRACT

Gubenfangxiao decoction (GBFXD) is a traditional Chinese medicine formula derived from Yupingfengsan, an ancient formula widely used to treat respiratory diseases. In recent years, GBFXD has been applied to efficaciously and safely treat asthma. However, the mechanism of GBFXD is still not fully elucidated. The aim of this study was to employ the label-free proteomic method to explore the protective mechanism of GBFXD in respiratory syncytial virus (RSV)-ovalbumin (OVA) induced chronic persistent asthmatic mice. After RSV-OVA challenge, mice were orally administered GBFXD at a dose of 36 g/kg accompanied with OVA nasal spray once every 3 days for 28 days. The label-free proteomics-based liquid chromatography-tandem mass spectrometry method was used to explore the differentially abundant proteins (DAPs) in the serum from model mice compared with that in control mice (M:C), and in GBFXD-treated mice compared with that in model mice (G:M). The mass spectrometry proteomics data have been deposited to the ProteomeXchange with identifier PXD013244. A total of 69 significant DAPs were identified including 39 in M:C, 46 in G:M, and 16 common differential proteins. Bioinformatics analysis revealed that the DAPs of M:C were mainly involved in inflammatory response and were related to lipid metabolism. However, the DAPs of G:M mostly participated in stress response, inflammatory response, and epithelial cell proliferation. Serum levels of Apoa-1, Apoc-1, Cfd, and Lrg1, EGFR and Lrg1 in the lungs were consistent with the results of proteomic analysis. Apoa-1 and Apoc-1 were closely related to cholesterol transport, lipid metabolism balance, and airway epithelial integrity; Cfd participated in immune response, affecting the occurrence and development of inflammation; EGFR and Lrg1 were involved in epithelial cell proliferation, influencing the process of airway remodeling.

In summary, these results indicated that GBFXD may affect inflammatory and immune response of asthma by regulating cholesterol transport and complement factor activation. Furthermore, it could repair damaged airway epithelium and avoid airway remodeling to prevent and treat asthma.

1. Introduction

Asthma is one of the most common chronic pulmonary diseases and affects approximately 334 million people worldwide [1]. It is therefore associated with substantial financial burden on industrialized and developing countries [2]. By the year 2025, it is estimated that more than 100 million individuals will have asthma [3]. Asthma has been described as a heterogeneous state manifested with variable respiratory symptoms and airflow limitation. Numerous underlying mechanisms are typically associated with airway inflammation and remodeling [1].

Currently, the standard asthma therapy includes corticosteroids and β_2 -adrenergic receptor agonists. Most patients' symptoms are controlled, and lung function can be ameliorated when these medicines are taken; however, other individuals still experience exacerbations or treatment-related side effect [4]. Asthma is associated with very high economic cost due to the limited availability of treatments that specifically target asthmatic symptoms in clinics [5]. Therefore, it is urgent and important to develop novel drugs to treat asthma. Fortunately, more and more new drugs, which are from natural products or natural product-like compounds, are being discovered with the rapid development of

* Corresponding author at: Affiliated Hospital of Nanjing University of Chinese Medicine, No. 155 Hanzhong Road, Nanjing, 210029, China.

E-mail address: zhaoxiahy@njucm.edu.cn (X. Zhao).

¹ These authors contributed equally to the study.

technology and the efforts of researchers [6–11].

Based on the fundamental theories of traditional Chinese Medicine (TCM), asthma is recurrent and lingering. The reasons for this condition are the superficial is not consolidating and the hidden phlegm is difficult to eradicate. Yupingfengsan (YPFS), recorded in the Dan Xi Xin Fa, is a traditional Chinese herbal formula of nourishing defense qi, which can superficially strengthen humans to resist pathogenic factors. Clinically, YPFS exerts immunomodulatory, anti-bacterial, and antiviral effects [12]. Therefore, it has been widely applied to treat respiratory diseases such as allergic rhinitis, asthma, repeated respiratory tract infections, and pneumonia, with less adverse reactions. Furthermore, reports indicated that asthma could be ameliorated by targeting multiple pathways [13,14]. Gubenfangxiao decoction (GBFXD) is an empirical formula prescribed by Professor Jiang Yuren, a prestigious TCM pediatrician in China. GBFXD has been used to treat pediatric asthma for more than 30 years in the Jiangsu Provincial Hospital of TCM. Meantime, a multicenter, randomized, parallel, controlled trial reported that GBFXD was efficacious in the treatment of chronic asthmatic children, and was especially effective in improving their lives and reducing the relapse rate [15]. Our previous study also has demonstrated that GBFXD could attenuate sustained airway inflammation by downregulating the asthma susceptibility gene ORMDL3 and suppressing ER stress response in murine asthma remission model [16,17]. However, the function of GBFXD on chronic persistent murine model of asthma remains elusive and further investigation is still required to elucidate the mechanisms by which chronic asthma is effectively treated upon GBFXD administration.

TCM, which involves the use of various herbal ingredients with multiple targets, is considered an effective alternative to western drugs for various multifactorial disorders [18]. However, it is extremely difficult to clearly identify the detailed mechanism of TCM formulas using conventional research methods. Mass spectrometry-based proteomics has emerged as a powerful and systematic approach for large-scale protein analysis [19]. Simultaneously, it is useful in revealing the potential targets or biomarkers of pharmaceutical products and analyzing major components of physiological pathways [20]. In recent years, this method has been rapidly employed to explore the mechanisms of TCM [21,22]. Therefore, a label-free quantitative mass spectrometry-based proteomic analysis was performed to elucidate the potential mechanism of chronic persistent asthma (CPA), and the protective effect demonstrated by GBFXD.

2. Materials and methods

2.1. Preparation of GBFXD and reagents

The formula consisted of the eleven herbs showed in Table 1. They were purchased from Jiangsu Province Hospital of Chinese Medicine, and were authenticated by Dr. Sheng-Jin Liu from the College of Pharmacy, Nanjing University of Chinese Medicine, China.

Briefly, the calcined Concha Ostreae was firstly decocted for 30 min,

while the other materials were soaked in eight aliquots of distilled water (v/w). All herbs were then mixed together and boiled twice in 8 and 6 aliquots of distilled water for 30 min, respectively. The supernatant acquired from the two decoctions was pooled together and concentrated to 3 g/mL at 65 °C by a rotavapor (Buchi, Switzerland). The extract was stored at 4 °C for further use and performed quality control according to the previous established method [17].

The ELISA kit for IgE was obtained from Multisciences (Hangzhou, China), and for Apoa-1, Apoc-1, Cfd, Lrg1, and 4-HNE from Nanjing Jin Yibai Biological Technology Co. Ltd. (Nanjing, China). Anti-mouse EGFR, Lrg1, and GAPDH antibody were purchased from Proteintech (Wuhan, China). Anti-mouse RAF1, P-MEK1/2 (Ser221), and MEK1/2 antibody were obtained from Affinity Biosciences (Cincinnati, OH, USA). Anti-mouse P-RAF1 (Ser296), P-ERK1/2 (Tyr222/205), ERK1/2 antibody were purchased from Immunoway (Plano, TX, USA). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG was obtained from Cell Signaling Technology Inc (USA).

2.2. Animals and experimental design

Four-week-old female SPF mice (weight, 16–18 g) were purchased from Suzhou JOINN Laboratories Co., Ltd., (Jiangsu, China). The animals were housed in the experimental animal center of the Nanjing University of Chinese Medicine, with a 12-h light/12-h dark cycle in a room with controlled temperature of 23 ± 2 °C. Mice were granted free access to sterilized water and food.

The animals were subjected to adaptive feeding for 7 days prior to initiation of the experiments. Mice were randomly divided into three groups (n = 12 each): C, control group; M, model group; and G, GBFXD (36 g/kg) treatment group. There were no significant differences in the weight of animals among the groups, prior to the start of the experiments.

The model was established as described previously, with some modifications [17]. In brief, mice in M and G groups were sensitized on days 1 and 8 with an intraperitoneal injection (i.p.) of 0.2 mL alum-precipitated antigen in normal saline (NS), including 100 µg of OVA (Ovalbumin, Grade II, Sigma-Aldrich) mixed with 1 mg aluminum potassium sulfate. Mice in M and G groups were then challenged once using an aerosolized 2.5% OVA solution for 30 min from day 15 to day 28, then for 30 min once every three days from days 32–85. In addition, mice in M and G groups were intranasally injected with 50 µL respiratory syncytial virus (RSV) ($1.0 \times 10^{4.167}$ TCID₅₀/mL) on days 29, 42 and 55. From days 56–86, mice in the GBFXD treatment group were intragastrically administered GBFXD at dose of 36 g/kg once a day, while mice in the control and model groups received the same volume of NS. The entire protocol was summarized in Fig. 1a. After the final intragastric administration, all animals were anesthetized with 3% sodium pentobarbital intraperitoneally. The serum (centrifugation of peripheral blood: 850 g, 4 °C, 10 min) and lung samples were collected and stored at −80 °C for further use. The above experimental procedures were conducted in compliance with the National Institute of

Table 1
Composition of GBFXD.

Component	Family	Part used	Batch number	Weight
<i>Astragalus membranaceus</i> (Fisch.) Bge. Var. <i>mongholicus</i> (Bge.) Hsiao; Zhi Huang Qi	Leguminosae	root	140903	15g
<i>Codonopsis pilosula</i> (Franch.) Nannf.; Dang Shen	Campanulaceae	root	140901	10g
<i>Poria cocos</i> (Schw.) Wolf; Fu Ling	Polygonaceae	sclerotia	140904	10g
<i>Atractylodes macrocephala</i> Koidz.; Bai Zhu	Composite	rhizome	140903	10g
<i>Citrus reticulata</i> Blanco; Chen Pi	Rutaceae	peel	140910	6g
<i>Saposhnikovia divaricata</i> (Turcz.) Schischk.; Fang Feng	Umbelliferae	root	140802	3g
<i>Cryptotympana pustulata</i> Fabricius; Chan Tui	Cicadidae	slough	140406	6g
<i>Magnolia denudata</i> Desr.; Xin Yi	Magnoliaceae	bud	140428	6g
<i>Schisandra chinensis</i> (Turcz.) Baill.; Wu Wei Zi	Magnoliaceae	fruit	140901	6g
<i>Ostrea gigas</i> Thunberg; Duan Mu Li	Ostreidae	shell	140801	15g
<i>Glycyrrhiza uralensis</i> Fisch.; Gan Cao	Leguminosae	rhizome and root	140516	3g

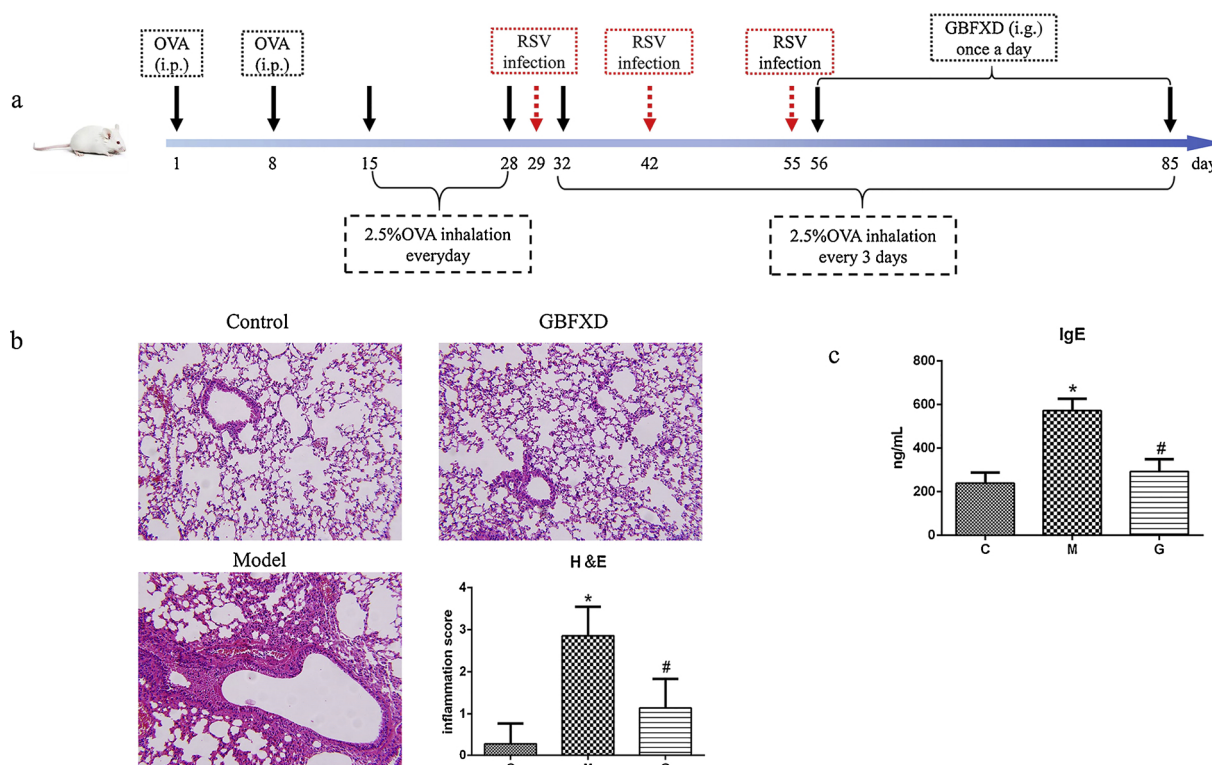


Fig. 1. The suppressive effect of GBFXD on airway inflammation and IgE level of serum in a chronic persistent murine asthma model. (a) Experimental protocol to establish the murine OVA-RSV asthma model and the treatment strategy. (b) Inflammatory changes within lungs were measured by H&E staining ($\times 200$). Total inflammation scores were calculated. (c) GBFXD regulated IgE level in serum as determined by ELISA. Values are presented as mean $\pm$ SD ($n = 4$ or 6 per group). # $p < 0.05$ compared to the control group, and * $p < 0.05$ compared with the model group. C, control group; M, model group; G, GBFXD group.

Health Guidelines for Laboratory Animals, and were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine [No.201810A026].

2.3. Lung histopathology

Following serum collection, lung tissue slices were fixed with 10% neutral-buffered formalin. To evaluate inflammatory cell infiltration, 3–4 μ m microsections from each group were stained with hematoxylin-eosin (H&E). Inflammation scores were determined as: grade 0, no inflammation; grade 1, occasional cuffing with inflammatory cells; and grades 2, 3, and 4 indicated that most bronchi or vessels were surrounded by a thin 1–2 cell layer, a moderate 3–5 cell layer, or a thick (> 5) cell layer of inflammatory cells, respectively.

2.4. Proteomics

2.4.1. Serum protein preparation

Firstly, 35 μ L of obtained serum samples from each mouse was processed using the ProteoExtract™albumin/IgG removal kit to eliminate albumin and IgG. This is because highly abundant proteins such as albumin and IgG in serum can cause ion suppression during LC–MS/MS analysis, and many low abundance proteins will not be detected. The filter-aided sample preparation (FASP) method was then employed to digest the eluted proteins [23]. Briefly, proteins were diluted with 8 M urea solution (with 10 mM DTT) and incubated for 1 h at 60 °C, then alkylated by iodoacetamide in the dark. Subsequently, protein samples were buffer-exchanged with 50 mM NH_4HCO_3 (pH 8.0–8.5) using 10 kDa molecular weight cut-off Amicon Spin Tube (Millipore, MA). For protein digestion, 4 μ g of sequencing-grade modified trypsin (Promega, USA) was respectively added to each sample, and the samples were incubated at 37 °C overnight (trypsin: protein ration = 1:50). Digested peptides were desalted using Ziptip C18 cartridges (Millipore, MA), and

a NanoDrop spectrophotometer (Thermo Scientific, USA) was used to estimate peptide concentrations by absorbance measurement at 280 nm.

2.4.2. LC–MS/MS analysis

Six biological replicates of the C, M, and G groups were analyzed by on-line nano LC system (DIONEX Thermo Scientific) combined with an LTQ–Orbitrap XL mass spectrometer (Thermo Scientific). The peptides (1.5 μ g) from each sample were first acidified with 0.1% formic acid (FA) and then loaded into the nano trap column (Acclaim PepMap100 C18, 75 μ m $\times$ 2 cm, 3 μ m, 100 Å, Thermo Scientific) at a flow rate of 4 μ L/min in loading buffer (2% acetonitrile, 0.1% FA in HPLC-grade water). Chromatographic separation was initiated in the analytical column (Acclaim PepMap® RSLC, C18, 75 μ m $\times$ 15 cm, 3 μ m, 100 Å, Thermo Scientific) using a linear gradient of 3–55% buffer B (80% acetonitrile and 0.1% FA) at a flow rate of 0.3 μ L/min over 112 min. Owing to the loading and washing steps, total time for an LC–MS/MS run was approximately 160 min.

Using an LTQ–Orbitrap XL mass spectrometer equipped with a nano-electrospray ion source, data-dependent MS/MS was implemented. A 2.2 kV electrospray voltage was utilized in the study, and from the resolution of 60,000 MS1 full scan (m/z 350–1800), the ten most outstanding ions were selected for MS/MS analysis, presuming they were at least doubly charged. The value of 35% for CID of the normalized collision energy was installed; lock mass calibration was activated, and the dynamic exclusion time was set to 30 s.

2.4.3. Protein identification and label-free quantitation (LFQ)

Raw data were analyzed by MaxQuant version 1.5.2.5 using the Andromeda algorithm [24,25], and were matched against the UniProtKB reference database for *Mus musculus* (86119 protein entries, version of September 20, 2018). In the Andromeda search, the search parameters were 6 and 20 ppm for the precursor and fragment ions

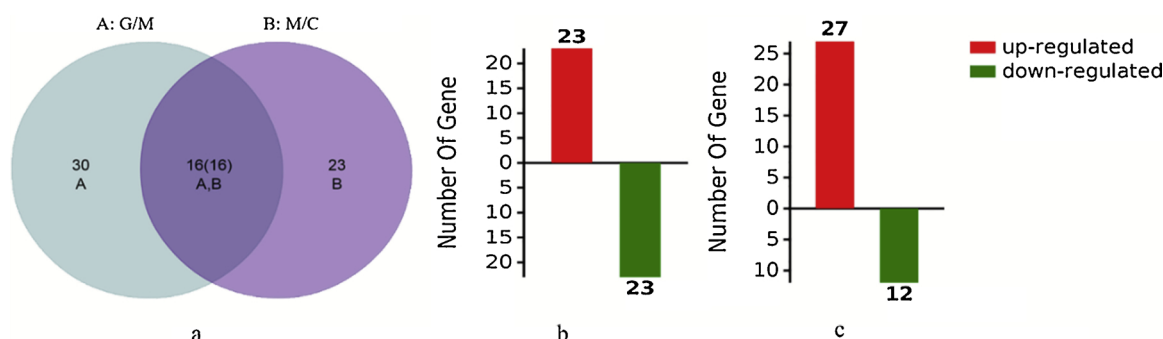


Fig. 2. The differentially abundant proteins (DAPs) in serum between M:C and G:M. (a) Venn-diagram showing the overlap of all DAPs; (b) DAPs involved in G:M; (c) DAPs involved in M:C. Red or Green represents the numbers of upregulated and downregulated proteins, respectively.

mass tolerances, respectively. The maximum number of missed cleavages was two. Carbamidomethylation of cysteine was set as a fixed modification, along with protein N-terminal oxidation of methionine as a variable modification. The value of 0.01 was set as the false discovery rate. The LFQ algorithm was applied to calculate protein abundances. After filtering of contaminants, reverse hits, and number of unique peptides (≥ 2) using Perseus version 1.5.3.2, the “proteinGroups.txt” was generated. Log2 transformation of the LFQ intensity values and the two-sample *t*-test were applied among the groups. Protein was considered to be a differentially abundant protein (DAP) based on the standard of the average of $|\log_2\text{Foldchange}| \geq 0.5849$ (1.5-fold cut-off), and a *p*-value < 0.05 .

2.4.4. Bioinformatics analysis of DAPs

The multi-omics data analysis tool, OmicsBean (<http://www.omicsbean.cn>), which includes Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and Protein-Protein Interaction (PPI) analysis, was applied to analyze the obtained DAPs.

2.4.5. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of immunoglobulin E (IgE), Apoa-1, Apoc-1, Cfd, Lrg1 in serum and 4-HNE in lungs were determined by ELISA, according to the manufacturer's instructions.

2.4.6. Western blotting

Approximately 100 mg of lung tissue from each mouse was used to extract protein using RIPA buffer containing PMSF and PhosStop (Boster, Wuhan, China). Protein concentration was determined by BCA assay, and total proteins were separated by 8–10% SDS-PAGE. The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (0.45 μm , Millipore, USA), and blocked with 5% BSA in TBS Tween 20 (TBST, 25 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween 20) for 2 h. After blocking, the membranes were washed three times with TBST, and incubated with primary antibodies including EGFR (1:3000 diluted), Lrg1, P-RAF1, RAF1, P-MEK1/2, MEK1/2, P-ERK1/2, ERK1/2 (1:1000 diluted), and GAPDH (1:5000 diluted) overnight at 4 °C; exposure to secondary HRP-conjugated antibody (1:10,000 diluted) for 1 h at room temperature to detect antibody binding was then performed. Protein bands were quantified using a ChemiDoc™ MP Imaging system (Bio-Rad Co., USA), and analyzed using the Image Lab software (Bio-Rad Co., USA). Finally, values were expressed as band intensity normalized to GAPDH or their respective total proteins.

2.4.7. Statistical analysis

The data were expressed as mean and standard deviation (SD). All tests were performed using Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's post hoc test to determine differences

among multiple comparisons. The significance level was set as $p < 0.05$.

3. Results

3.1. GBFXD attenuated airway inflammation and reduced IgE level of serum in a chronic persistent murine asthma model

To define the established murine asthma model and the effects of GBFXD on inflammatory changes, pathological examination of the lung was performed by H&E staining to evaluate the degree of inflammatory infiltration. Compared with the control mice, a remarkable infiltration of inflammatory cells around the airways and vessels was observed in model mice. Treatment with GBFXD dramatically attenuated the inflammatory cells present around the airways compared with the model group ($p < 0.05$, Fig. 1b).

Chronic inflammation associated with asthma is characterized by elevated IgE in serum [26]. Therefore, we measured the expression of IgE in each group of mice. As shown in Fig. 1c, the level of IgE in serum of the model group was upregulated while that of the GBFXD group was restored ($p < 0.05$). These findings indicated that GBFXD administration may attenuate airway inflammatory responses and our method successfully established a chronic persistent asthma model conforming to the required design specifications.

3.2. Identification of DAPs in serum of CPA and GBFXD-treated mice

To explore the potential mechanisms of GBFXD, the MaxQuant as previously mentioned, was used to acquire an overall summary of proteomics change in the model group compared with the control group, and in the GBFXD group compared with the model group. A total of 210 protein groups, 1390 peptides and 1235 unique peptides from LC-MS/MS were successfully identified among the three groups. The average LFQ intensities of each protein group over six biological replicates were compared as ratios of M:C and G:M to explore the important protein expression changes (1.5-fold cut-off, $p < 0.05$). A Venn diagram was created to show the details of 69 DAPs (Fig. 2a). Meanwhile, 39 proteins with differential accumulation were identified in M:C, 27 of which were upregulated and 12 were downregulated; and 46 proteins were identified in G:M, of which 23 were upregulated and 23 were downregulated (Fig. 2b and c). More details about the 69 DAPs were shown in Table S1, S2.

3.3. Functional annotation and categories of DAPs

To better understand the biological relevance of the changes in protein expression, and retrieve some information regarding the biological process (BP) or pathways, the identified DAPs were analyzed via bioinformatics approaches. By analyzing the 39 (M:C) DAPs, GO annotation showed significant enrichment of 368 BP terms, 27 cell

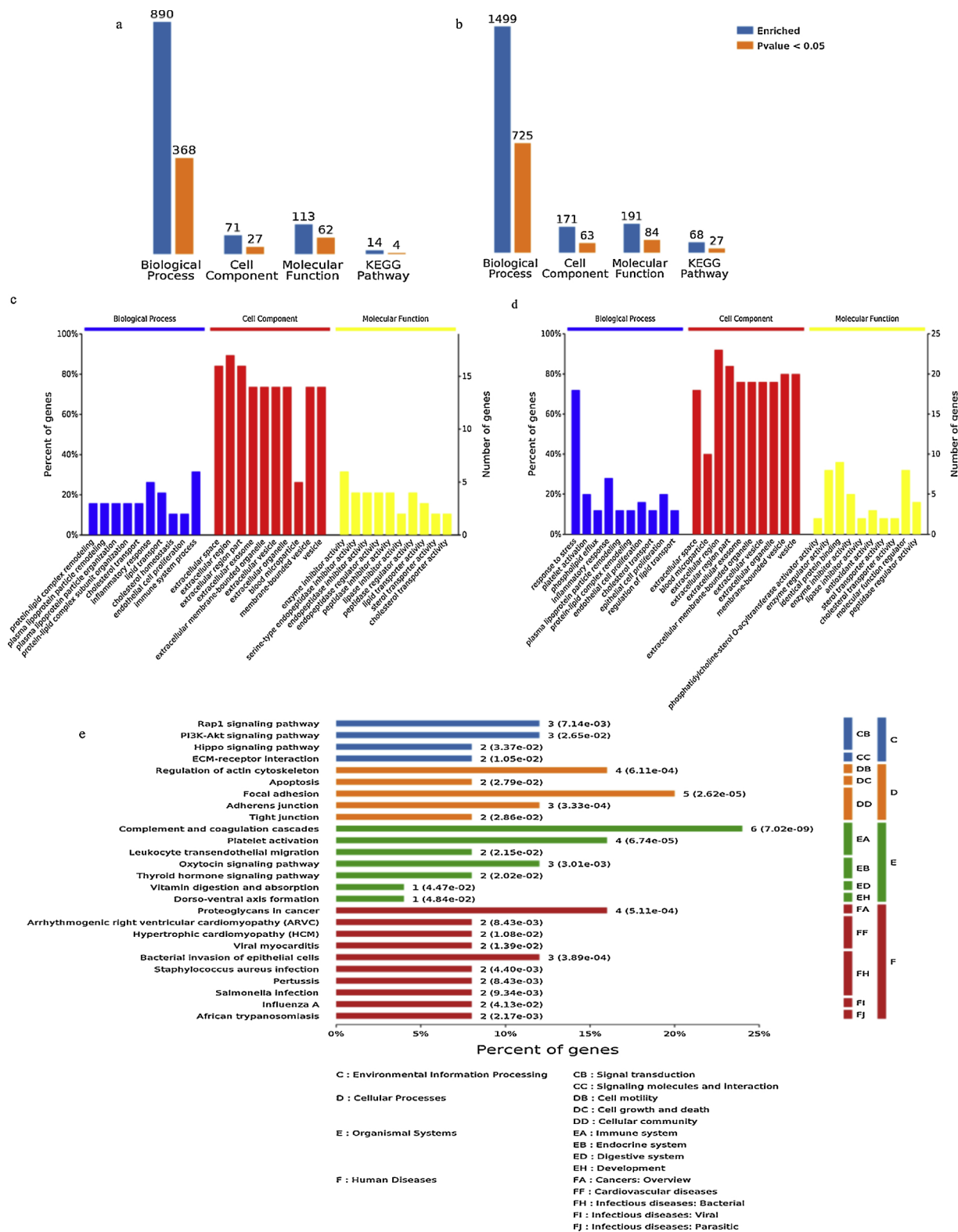


Fig. 3. Functional annotation and categories of DAPs. (a–b) Bioinformatics analysis of 39 (M:C), and 46 (G:M) DAPs using four analysis categories: BP, CC, MF, and KEGG pathway, respectively. Counts for each category represent the total associated terms in the database with the query protein list. Terms with a p -value < 0.05 are statistically significant. (c–d) The ten most significantly enriched terms of the GO hierarchy, information of percentage, and number of involved proteins in a term are shown in the left and right y-axes, respectively. (e) Enriched KEGG pathways of 46 (G:M) DAPs, the number of involved proteins in a specific pathway, and corresponding p -values are shown on the right side of the column.

component (CC) terms, and 62 molecular function (MF) terms. On the other hand, analysis of the 46 (G:M) DAPs showed enrichment of 725 BP terms, 63 CC terms, and 84 MF terms; the top ten significantly

enriched GO terms are shown in Fig. 3a and b. GO enrichment analysis was performed to clarify the changes in DAPs between the two groups. As shown in Fig. 3c, the M:C DAPs based

on BP classification were mainly involved in immune system process and the inflammatory response. In addition, the terms related to lipid metabolism such as lipid transport, cholesterol transport, and cholesterol homeostasis were evidently enriched. Many proteins possessed an extracellular region and extracellular region space for CC, and several major subcategories included enzyme inhibitor activity and peptidase regulator activity for MF. The GO annotation of G:M DAPs showed that the top three BP terms were response to stress, inflammatory response, and epithelial cell proliferation (Fig. 3d). Meantime, most of these proteins were involved in the extracellular region, extracellular region space (CC), and identical protein binding, molecular function regulator, and enzyme regulator activity (MF).

In addition, we even found some organelle-specific proteins in CC terms and the details of GO/CC terms and enrichment analysis were displayed in supplementary data. For example, Ces1c was mainly classified into CC terms (the organelle aspects) about endoplasmic reticulum (ER). Moreover, our previous study also demonstrated that GBFXD could regulate the ER stress [17]. There were close relationships between lipid metabolism and lipid transfer pathways in ER. It had also been reported that Ces1c was involved in lipid metabolism [27]. Furthermore, BP terms enriched in both sets of comparisons (M:C, G:M) including inflammatory response, endothelial cell proliferation, protein-lipid complex remodeling, plasma lipoprotein particle remodeling, and cholesterol transport.

For further analyzing the biological pathways that responded to the protective effect of GBFXD, all DAPs in G:M were explored using the KEGG pathway annotation. The results revealed that most proteins were significantly enriched in complement and coagulation cascades (Fig. 3e). Many signaling pathways were also enriched, including the PI3K-AKT signaling pathway and Hippo signaling pathway. Importantly, these pathways have been found to be involved in asthma [28,29].

After searching the obtained DAPs in the STRING database, a PPI network was constructed (Fig. 4e). As shown in Fig. 4a–d, the important DAPs were involved in BPs mainly enriched in G:M and M:C. More importantly, some of these DAPs such as Apoa-1, Apoc-1, and EGFR did not only participate in one BP.

3.4. Validation of DAP levels in serum by ELISA

ELISA was performed to verify the accuracy of proteomics. The results showed that the levels of Apoc-1 and Lrg1 in serum were significantly increased, Apoa-1 and Cfd were significantly decreased when compared the model group with the control group. After 28 days of GBFXD treatment, the serum proteins mentioned above were restored in the GBFXD group compared with the model group (Fig. 5a–d).

3.5. Validation of DAPs in lungs by western blotting

As presented in Fig. 5e–g, the expression levels of EGFR and Lrg1 in lungs were significantly elevated in the model group, and down-regulated in the GBFXD group; these results were consistent with the label-free proteomics and ELISA results. We also detected the RAF/MEK/ERK pathway in lungs using Western blotting assay, one of the most important downstream signal pathway of EGFR, and found that the expression of P-RAF1, P-MEK1/2, P-ERK1/2 significantly up-regulated in the model group. Meantime, the phosphorylation of RAF1, MEK1/2, ERK1/2 were remarkably decreased after GBFXD treatment as shown in Fig. S2. These results further confirmed the credibility of our proteomic analysis.

4. Discussion

The latest Global Initiative for Asthma (GINA) recommended that asthma management goals should not only include risk reduction of asthma exacerbations, but also improvement of asthma control. Inhaled

corticosteroids (ICSs) are considered the gold-standard treatment for long-term management of mild persistent asthma in children [26]. However, some research has suggested that ICSs may potentially exhibit long-term systemic side effects, such as reduced growth velocity, bone density, and fractures [30]. Bone health is especially important during childhood; therefore, concerns regarding the potential adverse effects of ICSs may possibly lead to poor medication adherence, subsequently resulting in more frequent and severe asthma exacerbations. GBFXD has been an effective treatment for asthma, and is not associated with any adverse events [15]. In the present study, we used a label-free proteomic based method to investigate the mechanism of CPA on a murine model, and developed a better understanding of GBFXD.

The underlying mechanisms of asthma are complex and represent a multitude of host-environment interactions that occur over different spatial (i.e., genes, cells, tissue, and organ) and time scales. Although multiple studies have been conducted on asthma, the pathogenesis of asthma remains unclear. Chronic airway inflammation is a major feature of asthma. Usually, inflammatory response is considered as a protective mechanism in restoring tissue homeostasis by immune system response, but persistent and excessive inflammation may lead to diseases [31] such as asthma, which is characterized by abnormal airway epithelial cells, and the activation of various inflammatory cells. In brief, the occurrence and development of asthma is closely related to immune imbalance, which could lead to chronic airway inflammation. Efforts to target inflammation in the management of asthma have garnered great interest. Importantly, studies have implicated lipid homeostasis in pulmonary inflammation [31,32]. Statins, which are HMG-CoA inhibitors, are used to decrease cholesterol levels and have also been described to exert many pleiotropic effects that contain anti-inflammatory, anti-oxidative, immunomodulatory properties. Even several research reported that statin use could have been tried as therapies for asthma [33,34].

In our study, the main bioactive compounds of GBFXD which included liquiritin, prim-o-glucosylcimifugin, lobetyolin, magnolin, and schisandrol were standardised using HPLC (Fig. S1). As is widely known, IgE is the major inflammation characteristic of asthma not only for the acute phase, but also for the chronic phase [35]. The results of lung histopathology and IgE revealed that GBFXD could inhibit chronic airway inflammation to alleviate asthma. In our proteomics experiment, a total of 69 DAPs was identified. GO clearly identified that the DAPs of M:C participated in various BPs, especially in inflammatory response, lipid transport, and immune system. The results indicated that the occurrence and development of asthma is closely related to lipid and immunity balance; meantime, they also demonstrated that our animal model could mimic chronic persistent asthma. Several proteins exhibited the tendency to recover after GBFXD treatment, which was also involved in the inflammatory response. These proteins were significantly enriched in cholesterol transport and immune response, and they were also observed to function collectively in networks, as shown in PPI.

Apoa-1 plays an important role in cholesterol transport. The anti-inflammatory function of HDL is the reduction of the cholesterol content of immune cells by decreasing receptor localization and signaling within lipid rafts [36]. It has also been increasingly recognized that both HDL and Apoa-1 play key roles in modulating normal lung health and disease [37]. More studies have suggested that Apoa-1 and HDL may modulate both airflow obstruction and airway inflammation in asthma, and murine studies have demonstrated that Apoa-1 or its mimetic (5A, D-4 F) peptides possessed protective function in asthma. For example, administering D-4 F to OVA-sensitized mice reduced pulmonary inflammation and airway resistance by decreasing oxidative stress [38]. Similarly, administration of 5A could also attenuate the development of allergen-induced airway inflammation, AHR, and airway remodeling by reducing goblet cell hyperplasia and collagen gene expression [39]. Clinical studies have also supported the important role of Apoa-1 in asthma. A study reported that the Apoa-1 level

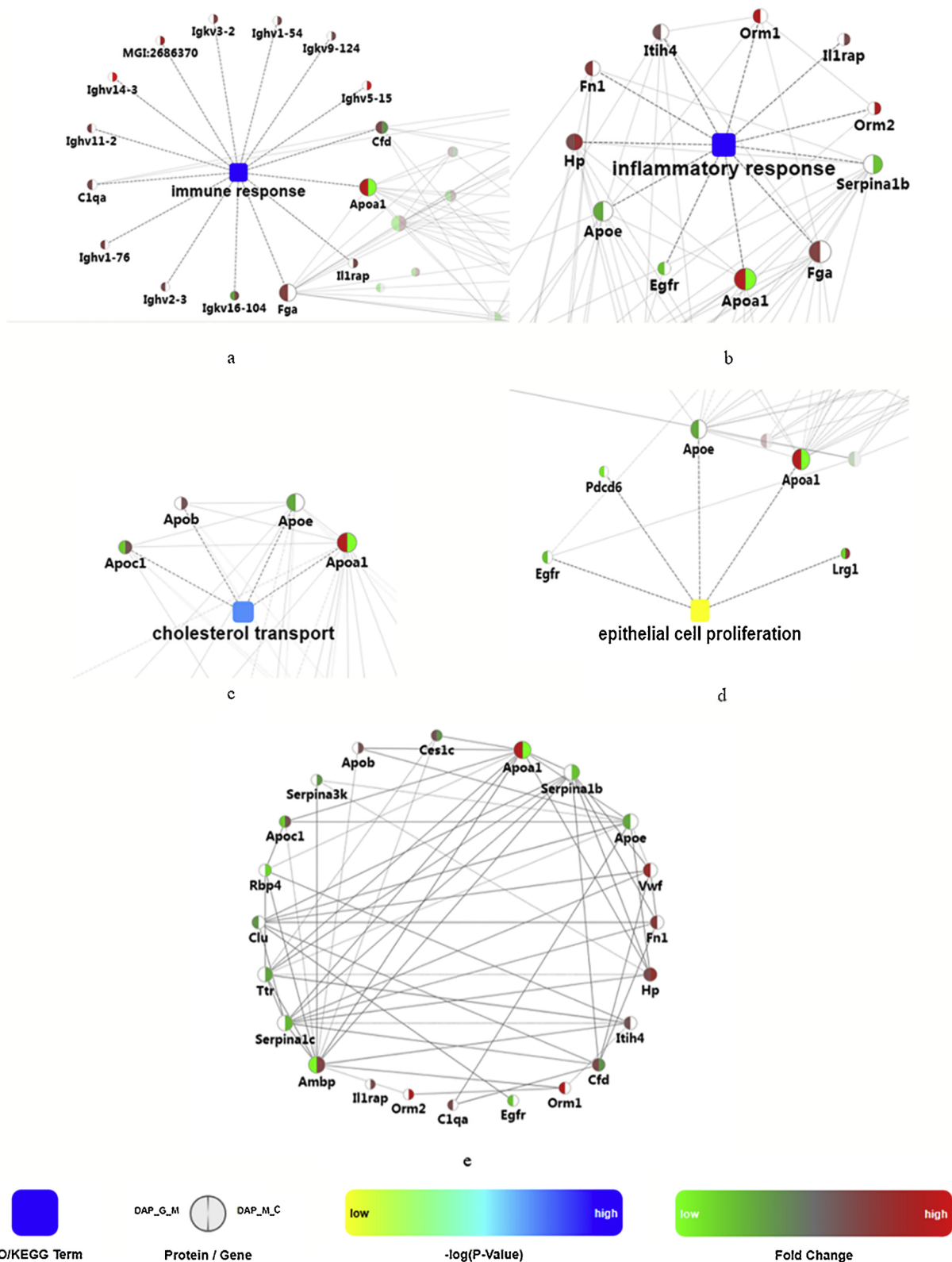


Fig. 4. Biological process enrichment analysis of differentially abundant proteins (DAPs) and network of DAPs. (a) Apo-1 and Cfd were related to immune response; (b) Apo-1 and EGFR were related to inflammatory response; (c) Apo-1 and Apoc-1 were related to cholesterol transport; and (d) Apo-1, EGFR, and Lrg1 were related to epithelial cell proliferation. (e) DAPs were visualized by OmicsBean based on fold change of protein and BP enrichment. Proteins are indicated by circle nodes with gradient colors (red, upregulation; green, downregulation). The left half represents DAPs in G:M while the right half represents DAPs in M:C. Rectangles indicate BPs, which are colored with a yellow to blue gradient (smaller to larger p -value, respectively).

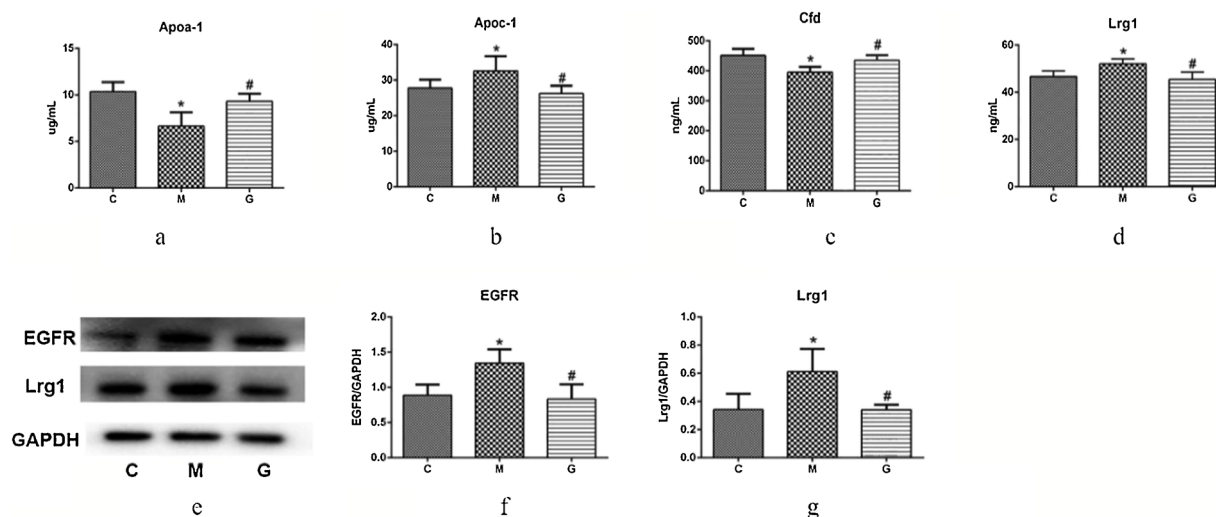


Fig. 5. Validation of the differentially abundant proteins identified in proteomics. (a–d) Levels of ApoA-1, Apoc-1, Cfd and Lrg1 in serum were measured by ELISA; (e–g) Western blot analyses demonstrated that the levels of EGFR and Lrg1 in the lung were downregulated after GBFXD treatment.

in bronchoalveolar lavage fluid (BALF) collected from mild-to-moderate asthmatics, were significantly lower than in healthy controls. ApoA-1 was also proposed to promote recovery of disrupted tight junction proteins in cultured primary bronchial epithelium obtained from asthmatics [40].

Apoc-1, a 6.6 kDa plasma protein with 57 amino acid residues, has different function compared with ApoA-1. Studies have shown that Apoc-1 could increase plasma triglycerides by inhibiting the enzyme lipoprotein lipase (LPL) [41], and exert a proinflammatory effect as it can stimulate TNF- α production by macrophages following inflammatory stimulus [42]. Administration of an endogenous inhibitor of TNF- α mediated signaling could inhibit bronchial smooth muscle hyper-responsiveness in animal models of asthma [43]. ApoA-1 and Apoc-1 are associated with the maintenance of lipid balance through the regulation of cholesterol transport. In our study, ApoA-1 was found to significantly increase, while Apoc-1 decreased after GBFXD treatment. We even detected the expression of 4-HNE and MDA, which were the products of lipid peroxidation [44], and found that GBFXD treatment markedly decreased the levels of them compared with the model group (Fig S3). These results suggested that GBFXD may alleviate inflammatory response and maintain epithelial integrity by regulating lipid balance. Further study need to be conducted to confirm the conclusion.

Complement activation is traditionally viewed to occur in blood, owing to abundant hepatic production and the secretion of most of its components [45]. However, a recent study clarified that resident and invading immune cells could also synthesize complement proteins [46]. There is some evidence that complement can serve a protective role in allergic disease. Using a bioinformatics approach, significant interactions between T cell activation and the complement system in patients with allergic rhinitis were identified, including reductions in the expression of most complement species containing complement factor D (Cfd) [47]. It has also been reported that changes in pulmonary Cfd expression coincided temporally with changes in pulmonary IL-17A expression and a significant inverse correlation between pulmonary expression of Cfd and IL-17A, AHR [48]. In our study, the decreased Cfd in the model group showed evident elevation following GBFXD treatment. Therefore, we hypothesized that GBFXD may play a role in the complement pathway, the most significant pathway in our KEGG pathway annotation, to regulate immune response to reduce airway hyper-responsiveness and inflammation.

Neovascularization around the airways is a key feature of airway remodeling. Lrg1 has been reported to be a pro-angiogenic factor through its modulation of the TGF- β pathway [49]. Importantly, Honda

H et al found that not only Lrg1 levels of BALF and lung in asthma model mice were significantly higher than in control mice, but also Lrg1 concentrations of sputum were significantly higher in patients with asthma than normal subjects [50]. Dysregulation of the tissue repair process could also lead to airway remodeling and EGFR plays a predominant role in airway remodeling and tissue repair in asthma [51]. Increased EGFR level was related to the severity of airway thickening in patients with chronic asthma, which was induced by asthma-related inflammation as well as cytokines [52]. Recently, one study reported that Lrg1 could remarkably promoted the proliferation, migration of PDAC cells by selectively interaction with EGFR and subsequent activation of the p38/MAPK signaling pathway [53]. In the current study, we found that Lrg1 and EGFR were downregulated after GBFXD treatment. Furthermore, GBFXD could regulate the activity of RAF/MEK/ERK pathway and obviously decreased the phosphorylation of RAF1, MEK1/2, ERK1/2. As is known that EGFR and its downstream signaling pathways, such as RAF/MEK/ERK and PI3K/AKT/mTOR pathway, play key roles in cell over proliferation [54–58]. Therefore, our results indicated that GBFXD could alleviate airway remodeling, which might be ascribed to influencing the interaction between Lrg1 and EGFR, even inhibiting RAF/MEK/ERK signaling pathway.

Previous studies have showed that complement system is involved in the regulation of inflammation by HDL and lack of HDL can lead to excessive activation of complement [59,60]. Moreover, cholesterol crystals, whose formation were mainly caused by lipid abnormalities, have been reported to affect inflammasome activation by a complement dependent mechanism [61]. Recent studies also demonstrated that HDL could activate PI3K/AKT and MEK/ERK was involved in hypoxia-Induced expression of ApoA-1 [60,62]. We found there were close relationship among those BPs (Fig. 4a–d) based on those research, even though the correlation among ApoA-1, Apoc-1, CFD, EGFR and Lrg1 (Fig. 4e) according to the existing literature have been merely demonstrated. The study only discovered some important targets of GBFXD. Therefore, the detailed mechanism among them will be the important focus of our next study.

5. Conclusion

In summary, the present study provided some evidence of the protective mechanisms of GBFXD in CPA. The data revealed that GBFXD could play roles in the regulation of cholesterol transport, and complement factor activation to improve inflammatory and immune responses in asthma. GBFXD may also possess the ability to repair damaged airway epithelium, and avoid airway remodeling to alleviate CPA.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2019.109390>.

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Supplementary data

Serum proteomics analysis based on label-free revealed the protective effect of Chinese herbal formula Gu-Ben-Fang-Xiao

Qiong-Qiong Xing^{a,b,c,d,1}, Li-Wei Liu^{a,b,c,d,1}, Xia Zhao^{a,b,c,d}, Yuan Lu^{a,b,c,d}, Ying-Mei Dong^{a,b,c,d}, Zhong-Qing Liang^{a,b,c,d}

^aAffiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, China

^bJiangsu Province Hospital of Chinese Medicine, Nanjing 210029, China

^cPediatric Institution of Nanjing University of Chinese Medicine, Nanjing 210023, China

^dJiangsu Key Laboratory of Pediatric Respiratory Disease, Nanjing 210023, China

Corresponding author: Xia Zhao, Affiliated Hospital of Nanjing University of Chinese Medicine, No. 155 Hanzhong Road, Nanjing 210029, China. Tel: +86 25 86618946; Fax: +86 25 86618942. Email: zhaoxiahy@njucm.edu.cn

¹These authors contributed equally to the study.

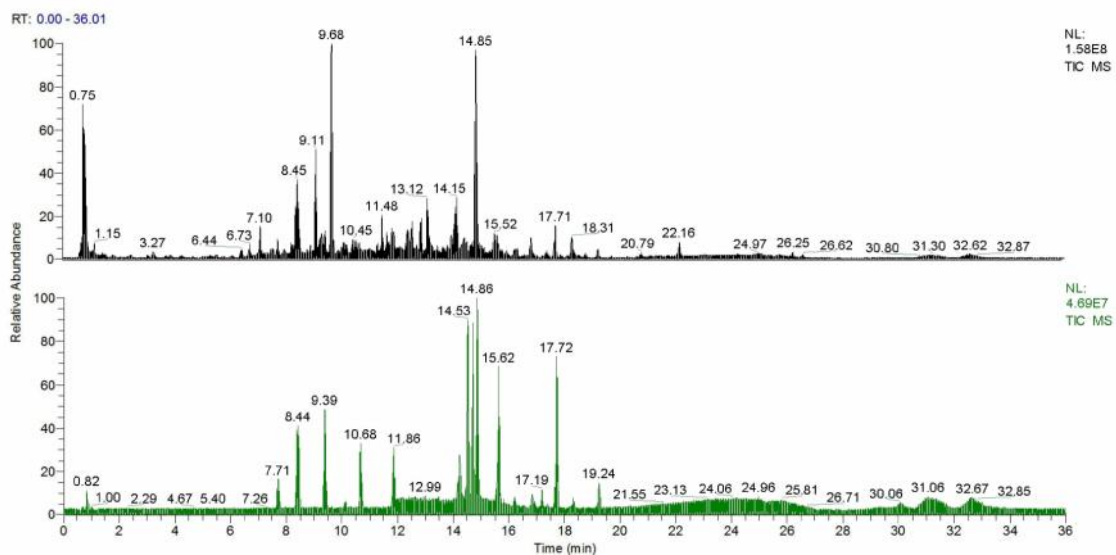
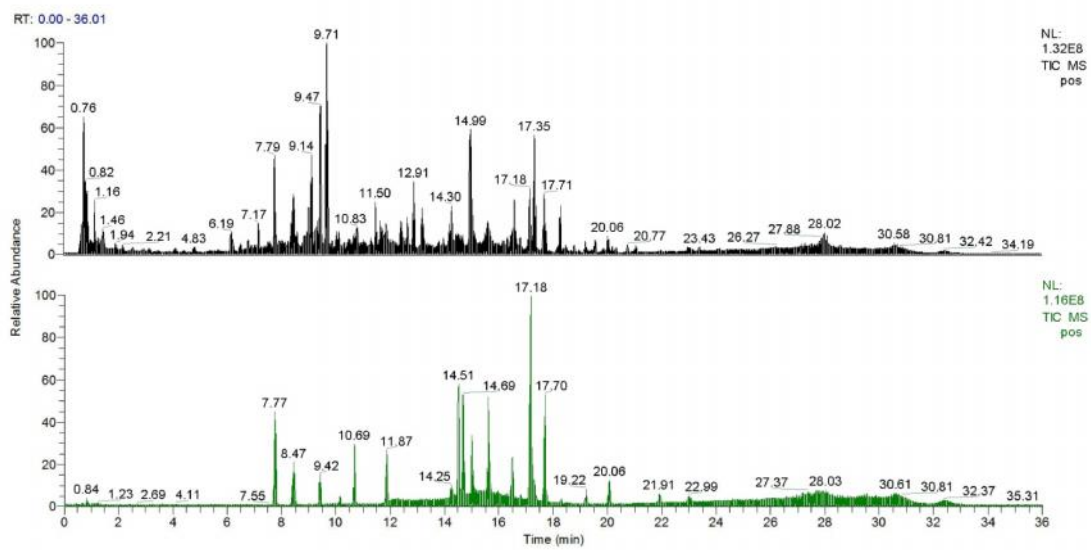
Supplementary materials and methods

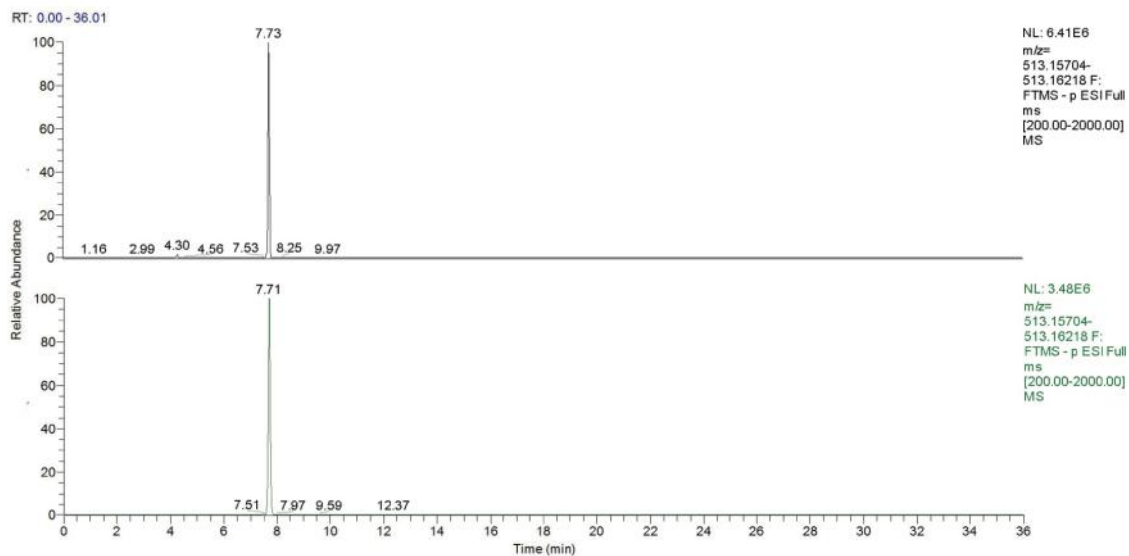
Lipid Peroxidation MDA assay

Determination of malondialdehyde (MDA) in lung samples was performed according to the instructions of the Lipid Peroxidation MDA Assay Kit (Beyotime biotechnology, Beijing, China). Briefly, the lungs were homogenized and then the supernatant was used for the further MDA detection. 100 uL supernatant was added with 200 μL MDA detection working fluid. After mixing, samples were incubated at 100 °C for 15 min, then cooled to room temperature. The mixture was centrifuged at 1000 g for 10 min at room temperature. 200 μl supernatant was added to a 96-well plate, and absorbance was measured at 532 nm using a microplate reader. The concentration of MDA in samples was calculated by the standard curve.

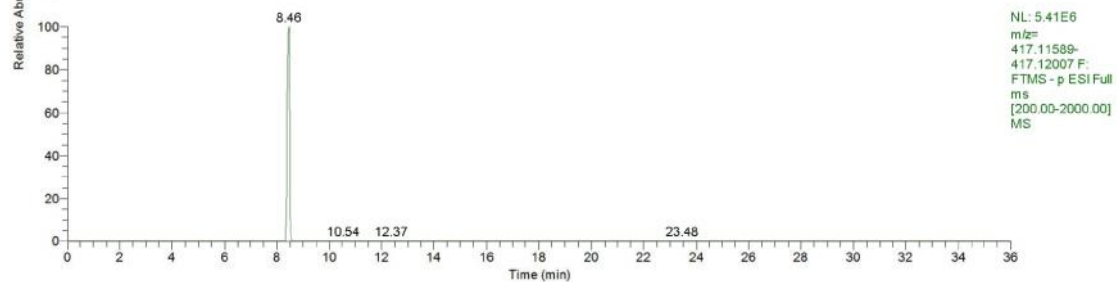
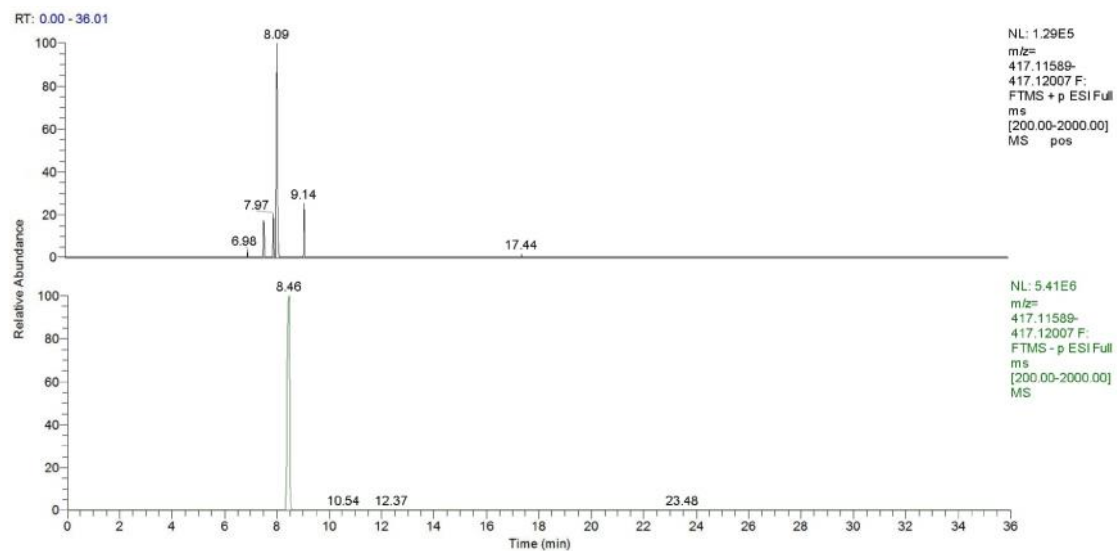
Supplementary Figures

Figure S1

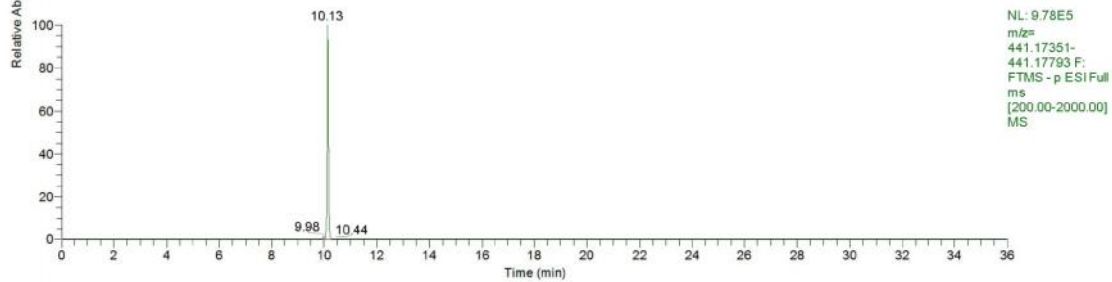
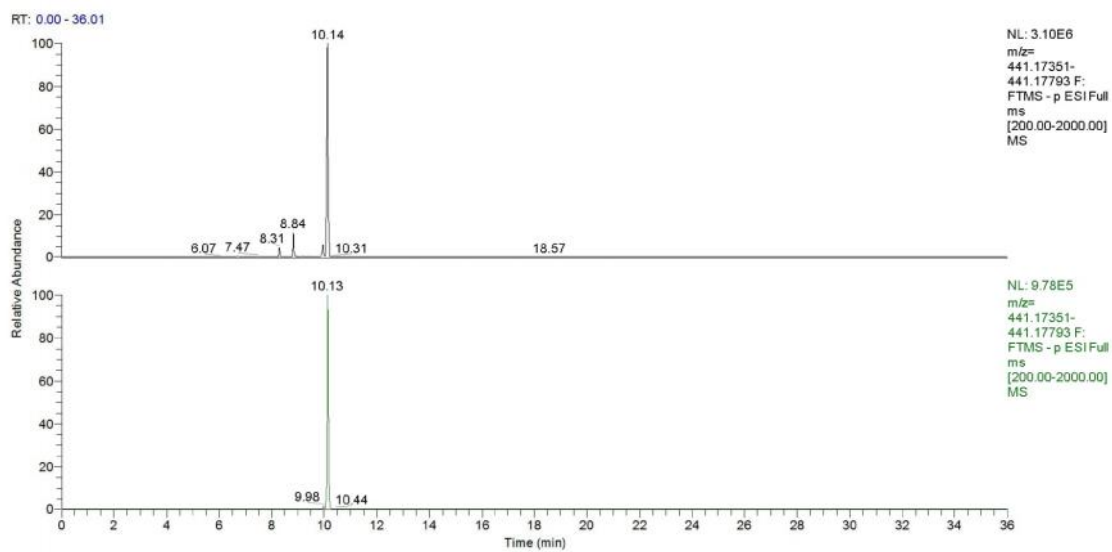




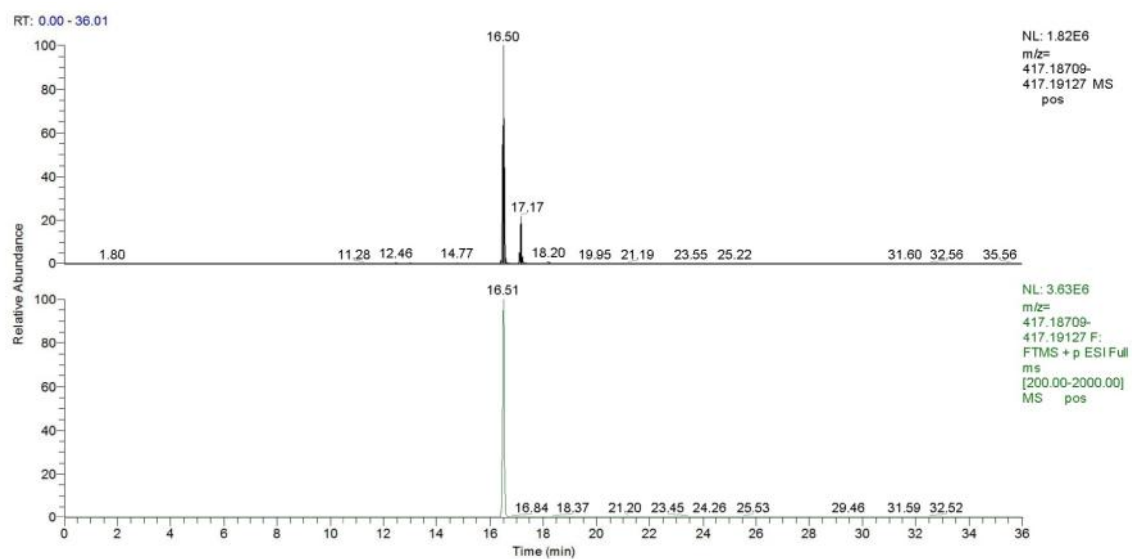
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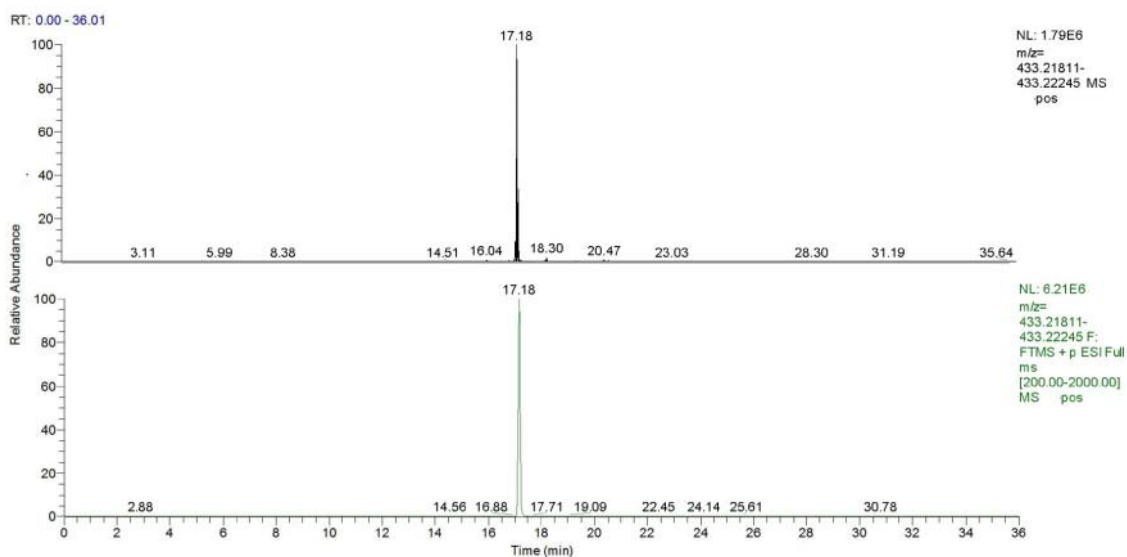
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Figure S2

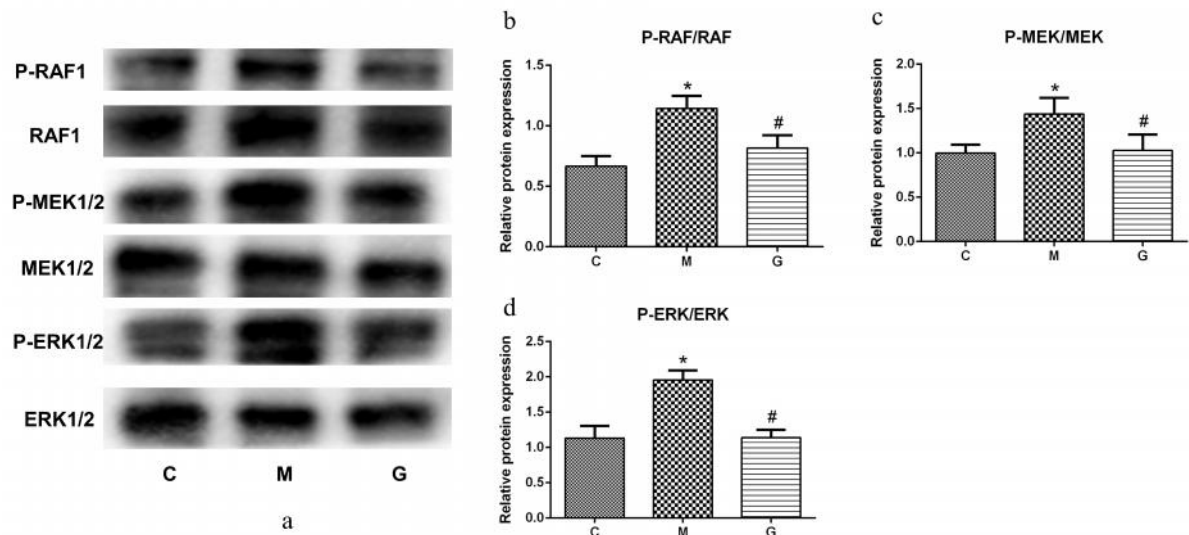
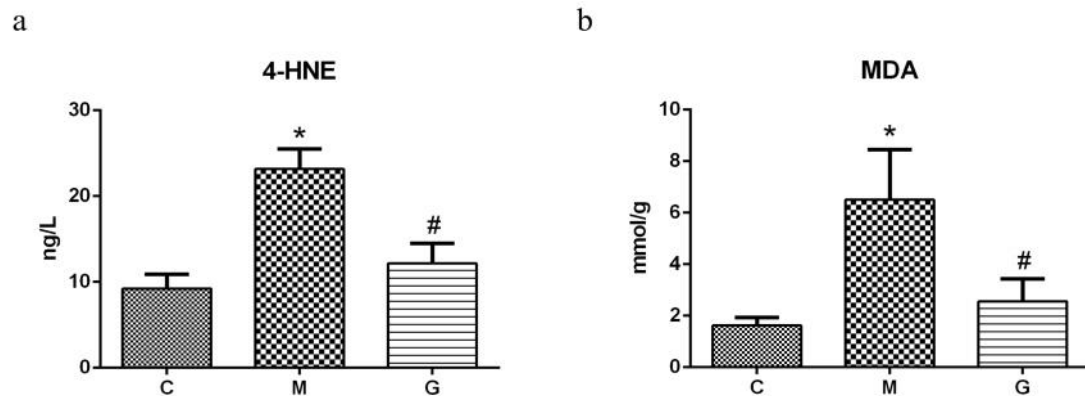


Figure S3



Supplementary Figure Legends

Figure S1. HPLC chromatograms of GBFXD with the representative active ingredients. (a) The total chromatograms in positive ion mode; (b) The total chromatograms in negative ion mode; (c) Prim-O-glucosylcimifugin; (d) Liquiritin; (e) Lobetyolin; (f) Magnolin; (g) Schisandrol A. (black, GBFXD; green, standards)

Figure S2. The effect of GBFXD on RAF/MEK/ERK signaling pathway. Western blotting was used to detect the relative protein expressions of P-RAF/RAF, P-MEK/MEK, and P-ERK/ERK.

Figure S3. The effect of GBFXD on lipid peroxidation in CPA model. (a) The levels of 4-HNE in lung tissue measured by ELISA; (b) The MDA levels in lung tissue detected using a Lipid

Supplementary Tables

Table S1. Significant DAPs of serum in the model group compared to those in the control group

Gene name	Ratio	Protein names	Uniprot ID
Serpina3k	-0.73	Serine protease inhibitor A3K	P07759
Igkv10-96	2.07	Igk protein	A1A540
Igkc	1.43	Kappa light chain	A2P1G9
Igkv9-124	0.59	Immunoglobulin kappa chain variable 9-124	A0A0G2JDR3
MGI:2686370	1.92	Immunoglobulin kappa variable 4-55	A0A0B4J1I9
Ighv14-3	3.41	Immunoglobulin heavy variable V14-3	A0A075B5R9
Apob	0.59	Apolipoprotein B-100	E9Q414
Ttr	-1.03	Transthyretin	P07309
Gm14743	-0.69	Predicted gene 14743	A2BHD2
C1ra	-0.68	-	Q3UU42
Igkv5-39	2.08	Anti-HIV-1 reverse transcriptase	Q65ZI1
Ighv1-69	1.25	Ig heavy chain V region 3	A0A0G2JFS5
A1bg	-2.83	Alpha-1B-glycoprotein	Q19LI2
MGI:4439812	3.03	Immunoglobulin heavy variable 5-15	A0A075B5Q9
Rbp4	-1.81	Retinol-binding protein 4	Q00724
Igkv17-127	1.92	Immunoglobulin kappa variable 17-127	A0A075B5J9

Il1rap	0.77	Interleukin-1 receptor accessory protein	Q61730
Igkv3-2	1.03	Immunoglobulin kappa variable 3-2	A0A075B5P0
Orm2	2.50	Alpha-1-acid glycoprotein 2	P07361
Igkv6-14	1.72	Anti-DNA light chain	Q8VIJ0
Serpina1b	-1.54	Alpha-1-antitrypsin 1-2	P22599
Serpina1c	-1.36	Alpha-1-antitrypsin 1-3	Q00896
Ighv1-54	0.65	Immunoglobulin heavy variable V1-54	A0A075B5W5
Cfd	-0.64	Complement factor D	P03953
Igkv16-104	0.89	Immunoglobulin kappa variable 16-104	A0A0B4J1I1
Hbbt1	-0.77	Beta-globin	A8DUK4
Hp	1.73	Haptoglobin	Q61646
Ighv1-18	1.90	Immunoglobulin heavy variable V1-18	A0A0A6YXN4
Ambp	1.05	Protein AMBP	Q07456
Lrg1	1.30	Leucine-rich alpha-2-glycoprotein 1	Q91XL1
Gpx3	0.72	Glutathione peroxidase 3	P46412
IgG1 TS1 VH	0.68	-	-
Apoc1	0.60	Apolipoprotein C-I	P34928
Apcs	0.60	Pentaxin	Q4JFI8
Ces1c	-0.66	Carboxylesterase 1C	P23953
Igkv8-27	1.81	Immunoglobulin kappa chain variable 8-27	A0A075B5N4

Igk	3.21	Kappa light chain variable region	Q5F217
Apoa1	-2.56	Apolipoprotein A-I	Q00623
Igh	2.41	-	-

Table S2. Significant DAPs of serum in the GBFXD group compared to those in the model group

Gene name	Ratio	Protein name	Uniprot ID
Actg1	1.36	Actin	P63260
Lcat	-1.46	-	Q9CW47
Serpina3g	-0.61	Serpina3g protein	Q4V9W0
B2m	2.10	Beta-2-microglobulin	Q9D239
Apoc2	1.75	Apolipoprotein C-II	Q3UJG0
Ahsg	0.82	-	Q3UEK5
Mup10	0.93	Major urinary protein 10	A2BIN1
Apoe	-1.09	Apolipoprotein E	P08226
Actb	1.77	Actin	P60710
Ighv2-3	0.71	Immunoglobulin heavy variable 2-3	A0A075B696
C1qa	0.80	Complement C1q subcomponent subunit A	P98086
C4bp	-1.87	C4b-binding protein	P08607
Iglv1	0.65	Immunoglobulin lambda chain variable region	Q8VDE2
Itih4	0.60	Inter alpha-trypsin inhibitor, heavy chain 4	A6X935
Tf	-0.67	-	-

Pi16	-0.94	Peptidase inhibitor 16	Q9ET66
Fga	1.14	Fibrinogen alpha chain	E9PV24
Qsox1	-0.79	Sulfhydryl oxidase 1	Q8BND5
Ighv11-2	1.05	Immunoglobulin heavy variable V11-2	A0A075B5R8
Pdcd6	-2.08	Programmed cell death protein 6	P12815
Clu	-0.77	Clusterin	Q06890
Vwf	1.87	von Willebrand factor	Q8CIZ8
Orm1	2.57	Alpha-1-acid glycoprotein 1	Q60590
Fn1	1.35	Fibronectin	P11276
Igfals	-0.65	Als splice variant 1	Q791Q5
Plg	-0.61	Angiostatin	Q6TCI0
Ighv1-76	1.03	Immunoglobulin heavy variable 1-76	A0A0G2JFE9
Egfr	-1.53	Epidermal growth factor receptor	Q01279
Hbb-b2	-1.29	Beta globin	Q9R1N1
Lifr	-1.36	-	Q3UKU5
Cfd	0.92	Complement factor D	P03953
Igkv16-104	-0.94	Immunoglobulin kappa variable 16-104	A0A0B4J1I1
Hbbt1	-0.86	Beta-globin	A8DUK4
Hp	0.85	Haptoglobin	Q61646
Ighv1-18	-0.73	Immunoglobulin heavy variable V1-18	A0A0A6YXN4

Ambp	-2.41	Protein AMBP	Q07456
Lrg1	-1.69	Leucine-rich alpha-2-glycoprotein 1	Q91XL1
Gpx3	-1.07	Glutathione peroxidase 3	P46412
IgG1 TS1 VH	0.89	-	-
Apoc1	-1.62	Apolipoprotein C-I	P34928
Apcs	-0.79	Pentaxin	Q4JFI8
Ces1c	0.64	Carboxylesterase 1C	P23953
Igkv8-27	1.19	Immunoglobulin kappa chain variable 8-27	A0A075B5N4
Igk	-1.69	Kappa light chain variable region	Q5F2I7
Apoa1	2.67	Apolipoprotein A-I	Q00623
Igh	2.75	-	-

Table S3. Qualitative parameters

Standard compounds	Molecular formula	Retention time	m/z
Prim-O-glucosylcimifugin	C ₂₀ H ₂₈ O ₁₁	7.73	513.15961
Liquiritin	C ₂₁ H ₂₂ O ₉	8.46	417.11798
Lobetyolin	C ₂₀ H ₂₈ O ₈	10.13	441.17572
Magnolin	C ₂₃ H ₂₈ O ₇	16.51	417.18918
Schisandrol A	C ₂₄ H ₃₂ O ₇	17.18	433.22028