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Xuanfei Baidu Decoction suppresses complement overactivation and ameliorates IgG immune complex-induced acute lung injury by inhibiting JAK2/STAT3/SOCS3 and NF-κB signaling pathway

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ABSTRACT

Background: The significant clinical efficacy of Xuanfei Baidu Decoction (XFBD) is proven in the treatment of patients with coronavirus disease 2019 (COVID-19) in China. However, the mechanisms of XFBD against acute lung injury (ALI) are still poorly understood.

Methods: In vivo, the mouse model of ALI was induced by IgG immune complexes (IgG-IC), and then XFBD (4g/kg, 8g/kg) were administered by gavage respectively. 24 h after inducing ALI, the lungs were collected for histological and molecular analysis.

In vitro, alveolar macrophages inflammation models induced by IgG-IC were performed and treated with different dosage of XFBD-containing serum to investigate the protective role and molecular mechanisms of XFBD.

Results: The results revealed that XFBD mitigated lung injury and significantly downregulated the production of pro-inflammatory mediators in lung tissues and macrophages upon IgG-IC stimulation. Notably, XFBD attenuated C3a and C5a generation, inhibited the expression of C3aR and C5aR and suppressed the activation of JAK2/STAT3/SOCS3 and NF-κB signaling pathway in lung tissues and macrophages induced by IgG-IC. Moreover, in vitro experiments, we verified that Colivelin TFA (CAF, STAT3 activator) and C5a treatment markedly elevated the IgG-IC-triggered inflammatory responses in macrophages and XFBD weakened the effects of CAF or C5a.

Conclusion: XFBD suppressed complement overactivation and ameliorated IgG immune complex-induced acute lung injury by inhibiting JAK2/STAT3/SOCS3 and NF-κB signaling pathway. These data contribute to understanding the mechanisms of XFBD in COVID-19 treatment.

Introduction

The coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has become a serious global disease (Loo et al., 2021). Currently, COVID-19 management is limited to symptomatic and palliative treatment. While an increasing number of studies have developed more effective therapies, including various antiviral, anti-inflammatory drugs and the...
neutralizing antibodies, they are still have limitations or lack clinical effectiveness (Drozdzal et al., 2021). During the struggle against COVID-19 in China, it was found that for patients with mild and moderate disease, early intervention with traditional Chinese medicine (TCM) can effectively prevent disease progression. According to “Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia” in China, “three medicines and three prescriptions” were recommended for COVID-19 treatment, of which “three tripartite” includes Qingfei Paidu Decotion, Huashui Baidu Decotion, and Xuanfu Baidu Decotion (XFBD) (Huang et al., 2020). A pilot randomized clinical trial indicated that XFBD combined with conventional medicine significantly improved the symptoms of short of breath, chest tightness, increased white blood cell or lymphocyte count and reduced the level of C-reactive protein (CRP) in plasma (Xiong et al., 2020). Network pharmacology analysis demonstrated that XFBD play a role in balancing immunity and eliminating the inflammation (Wang et al., 2020b). Furthermore, studies showed that XFBD prevented the cyclophosphamide-induced immunosuppression (Yan et al., 2021) and inhibited the production of cytokine in macrophages (Yang et al., 2021). However, the molecular mechanisms of XFBD are still poorly understood.

Complement system is an important component of nonspecific innate immunity and participates in the regulation of defense function. However, it also induces immune pathological damage mediated by the excessive activation of complements. The complement system is activated by three pathways: classical pathway, alternative pathway and mannose binding lectin associated serine proteinase pathway. These three pathways converge in the activation of complement C3, and C3 is further transformed into the main components of complement activation, C3a, C5a and C5b. Among them, C3a and C5a, known as sensitizing toxins, can enhance adhesion molecules expression of monocytes/macrophage, stimulate neutrophil degranulation, and provoke pro-inflammatory cytokines. Previous studies have confirmed that complement system was significantly activated in ALI (Cleary et al., 2020), ischemia-reperfusion injury (Howard et al., 2022), xenotransplantation (Grafals and Thurman, 2019) and severe abdominal infection (Mizuno et al., 2012). Accumulating evidence suggested that complement dysregulation is a key driver of hyper-inflammation, immune thrombus, and microvascular endothelial injury in COVID-19 (Bosmann, 2022; Boussier et al., 2022; Cugno et al., 2022). Plasma levels of C3a and C5a were elevated respectively (Jodele and Kohl, 2021) and the concentration of plasma C3a was correlated positively with plasma CRP in patients with COVID-19 (Zinellu and Mangoni, 2021). In addition, another study identified strong staining for the terminal complement complex in alveolar epithelial cells, inflammatory cells, and even in exudates in alveolar spaces with necrotic cell debris at autopsy (Qin et al., 2021). Furthermore, the transcribe analyse of the bronchoalveolar lavages and the lung epithelial cells of COVID-19 patients showed that the complement system is one of the most active intracellular pathways (Yan et al., 2020). According to the current research results, complement activation is one of the core events of COVID-19.

IgG immune complexes (IC)-induced mice acute lung injury (ALI) model is suitable for revealing the mechanism of immunological injury. Previous studies have shown that C5a was highly activated in the model (Ward et al., 2016) and IC induced pulmonary hyper-inflammation was C5a-dependent (Tang et al., 2014). Thus, the IgG-IC-induced ALI model has histopathological damage similar to that of infected SARS-CoV-2. In addition, the transcriptions such as the nuclear factor-kappa B (NF-xB) and signal transducer and activator of transcription 3 (STAT3) have been shown to be activated in both IC-induced ALI (Lentsch et al., 1998; Tang et al., 2011) and COVID-19 (Leng et al., 2020; Matsuyama et al., 2020). Therefore, we selected IC-induced ALI and alveolar macrophages to explore the effectiveness and mechanisms of XFBD against COVID-19.

Materials and methods

Materials and regents

Bovine serum albumin (BSA) and Rabbit anti-BSA IgG were purchased from sigma-Aldrich Chemical Co (StLouis, MO, USA). Anti-C3a (JF10-30) and anti-C3αR (NBP2-1569) were purchased from Novus Biologicals (Littleton, CO, United States). Anti-C5a (ab194637), anti-C5αR (ab59390) and IL-1p (ab254360) were obtained from Abcam (Cambridge, MA, United States). Anti-p-JAK2 (7771s), anti-JAK2 (3230s), anti-p-STAT3 (9145s), anti-STAT3 (12640s), anti-SOCS3 (9021s), anti-p-IκκBβ (2694s), anti-IκκBβ (8943s), anti-p-NF-xB p65 (3033s), anti-NF-xB p65 (8242s) were obtained from Cell Signaling Technologies (Beverly, MA, USA). Anti-IL-18 (A1115) was purchased from Abclonal Technology (Wuhan, China). The complement C5a(HY-P7695) and STAT3 activator Colivelin TFA(CAF, HY-P1061A) were obtained from MCE (Shanghai, China).

IgG IC-induced acute lung injury

Pathogen-free male C57BL/6 mice (8–12 weeks old) were obtained from Beijing HFK Biotechnology Co., Ltd. (Beijing, China), and housed in a pathogen-free conditions. The research was conducted in accordance with the Guidelines for Animal Ethics Committee of Tianjin Nankai Hospital. The XFBD was provided by Tianjin Modern TCM Innovation Center (TRT, 200302) in the form of a freeze-dried powder. The preparation and raw materials of XFBD extract was performed according to the previously published protocols (Yan et al., 2021). All mice were randomly divided into six groups (n=6 per group): control group (Con), anti-BSA IgG group (anti-BSA), anti-BSA IgG + XFBD high dose group (anti-BSA + XFBD-H), anti-BSA IgG + BSA group (IgG IC, anti-BSA IgG + BSA + XFBD low dose group (IgG IC + XFBD-L), anti-BSA IgG + BSA + XFBD high dose group (IgG IC + XFBD-H). The IgG IC-induced acute lung injury model were performed according to the previously published protocols (Gao et al., 2006a). Briefly, mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (12.5 mg/kg body weight), and then injected intratracheally with 120 μg of rabbit anti-BSA IgG in 40 μl of PBS, which was followed by 2 mg of BSA via tail vein injection immediately. The control mice received no treatment; anti-BSA IgG group received anti-BSA IgG intratracheally in the absence of an intravenous infusion of BSA. 6 h later, the XFBD-L group received XFBD solution (4 g/kg) by gavage, and the XFBD-H group was administered with XFBD solution (8g/kg) by gavage. The dosage of XFBD in mice was converted from the clinical dosage, that is, all the granules’ weight 29.988g/70 kg (human body weight) × 9.1 (conversion coefficient for humans and mice) = 4 g/kg per day. 24 h after inducing ALI, the mice were anesthetized, the serum and lung tissues were obtained for experiments.

Micro-computed tomography (micro-CT)

All micro-CT images were acquired with a micro-CT scanner (Micro-CT QuantumFX μCT Software, PerkinElmer). 24 h after inducing ALI, animals were accepted CT scanning while breathing freely under anesthesia. The mice are positioned in the CT scanner. The x-ray source parameters were 90 kV and 160 mA. A total of 360 views were obtained over a full 360 rotation. The total acquisition time of each mouse is 5 min. The maximum width of the image field is 68 mm and the voxel size is 35 × 35 × 35 μm.

Histological examinations

At 24 h after IgG IC deposition, lung tissues were fixed in 4% paraformaldehyde, then dehydrated, embedded in paraffin and cut into 5 μm thick serial sections for hematoxylin and eosin (H&E) staining and visualized under a light microscope.

Materials and regents

Micro-computed tomography (micro-CT)
Immunocytochemistry Staining (IHC)

For IHC analysis, the prepared lung tissues sections were deparaffinized to hydrate, subjected to antigen retrieval with EDTA antigen retrieval solution, followed by cell penetration with 0.5% triton x-100 solution. Then the sections detected with Bioss Kit (SP0022, Beijing, China) according to the manufacturer’s instructions. After blocking with 10% goat serum, the slides were immunostained using the primary antibodies C3a (dilution 1:100), C3aR (dilution 1:500), C5a (dilution 1:200), and C5aR (dilution 1:500) overnight at 4 °C. Then, the horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody was incubated 1h at room temperature, and the DAB substrate kit (ZLI-9018, ZSGB-BIO, Beijing, China) was used to display the positive cells. Finally, the nucleus was redyed with hematoxylin. The sections were photographed using a microscope (Leica DMI4000B, Germany).

ELISA

The complement C3a (NBP2-70037, Novus, United States) and C5a (ELM-CC5a, RayBiotech, United States) in serum were detected according to the manufacturer’s protocols. The levels of IL-6, IL-1β and TNFs and MCP-1 in lung tissues were estimated by using the ELISA kits (RK00008, RK00006, RK00027, RK00381; ABclonal, Wuhan, China) according to the manufacturer’s protocols.

Preparation of XFBD-containing serum

We prepared control serum (CONs) and XFBD-containing serum (XFBDs) in advance. Twenty male Wistar rats weighing 180-200g were divided into two groups (n=10 each group), namely CONs and XFBDs. The rats in the XFBD group were intragastrically administered at a dose of 4 g/kg twice daily for three consecutive days. The CONs group was given an equal dose of water. Three days later, after all rats were anesthetized, the blood was taken from the abdominal aorta, and the serum was obtained by centrifugation at 3000 rpm for 10 min. Subsequently, after water bath treatment at 56 °C for 30 min, the serum was filtered with a 0.22 μm filter membrane and stored in aliquots at −80 °C. Then, CONs (10%, 20%) and XFBDs (10%, 20%) were added on macrophages respectively.

Cell culture and in vitro IgG immune complex treatment

MH-S cells (Mouse alveolar macrophages) were obtained from ProCell (CL-0597, Wuhan, Hubei, China). Cells were cultured in RPMI-1640 medium supplemented with 0.05 mM β-mercaptoethanol, 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37 °C under 5% CO2. For IgG immune complex formation in vitro, 100 μg BSA in 100 μl PBS buffer was incubated with 25 μl 2.5 mg/ml anti-BSA IgG at 37 °C for 30 min.

RNA extraction and real time PCR

Total RNAs were isolated by using Trizol (Takara, China). Then 1 μg total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (RR037A, Takara, Beijing, China). The mRNA expression levels were semi-quantified by using GoTaq® QPCR Master MixKit (A6001, Promega, Wisconsin, United States) on the Applied Biosystems 7500 Fast Real-Time PCR System. The sequences of the primers are listed in Table 1, and they were synthesized by Sangon Biotech (Shanghai, China). The relative mRNA expression levels of the target genes were quantified using the 2−ΔΔCT method and showed as the relative fold change normalized to GAPDH.

Western blot analysis

Lung tissues and MH-S cells were lysed in cold RIPA buffer (Solarbio, Beijing, China) supplemented with PMSF (11359061001, Roche, Basel, Switzerland) and phosphatase inhibitors (MCE, Shanghai, China). 40 μg (lung tissues) or 15 μg (MH-S cells) of proteins were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane followed by blocking with 5% skim milk solution for 2 h at room temperature. The specific primary antibodies were incubated at 4 °C overnight, followed by HRP-conjugated secondary antibody for 2 h at room temperature. The immunoreactivity can be visualized using enhanced chemiluminescent reagents (Millipore Corporation, Billerica, MA, USA) and quantified using Quantity One, which is a digital gel image analysis system (Bio-Rad, California, USA).

Immunofluorescence staining

The cells were fixed with ethanol. After blocking with 10% goat serum for 30 min, the cells were incubated with the primary antibodies overnight at 4 °C, followed by the corresponding fluorescent secondary antibody for 1h at room temperature in the dark. The following antibodies were used: anti-C3aR (dilution 1: 500) or anti-C5aR (dilution 1: 500). The nuclei were visualized with DAPI. Images were obtained using a fluorescence microscope (Leica, Germany).

Statistical analysis

Results were shown as mean ± SD. The statistical analysis between multiple groups was performed by one-way ANOVA or two-way ANOVA by Prism version 8.0. Differences between groups at p < 0.05 was considered statistically significant.

Results

XFBD mitigated IgG IC-induced ALI

The ingredients of XFBD were illustrated in the previously study by using the UHPLC-PDA method (Wang et al., 2021; Zhao et al., 2021). The in silico pharmacological analysis speculated that immunity and inflammation are the major biological pathways regulated by XFBD targets (Huang et al., 2021; Zhao et al., 2021). In this study, we sought to explore whether XFBD alleviated the lung injury induced by IgG-IC. As shown in Fig. 1A, the pathological examination revealed that the mice of the control group exhibit normal pulmonary architecture without any signs of inflammatory responses. The slight inflammatory responses in the lung could be observed in the mice which receiving airway

| Primers | Forward sequence 5’-3’ | Reverse sequence 5’-3’ |
|---------|-------------------------|---------------------|
| IL-6    | AGCCAGAGCTTCTGAGGAT   | AGGAGACATTGAAATTGGG |
| IL-1β   | CACTCACCCTGCTGCTACTCA | CTGATGCTGAGGTTCGAGA |
| IL-18   | TTCTACTGAGGACATCATCA  | TGAAGTTGGTGACAAAACTACG |
| MCP-1   | GGCTCATCTGGCTAACAT   | ATGGCGGTAGTGTCTG   |
| C3aR    | AGGAGAGTGTTTCTCGTCC  | AGGCGGCTCGGTCGATGA |
| GAPDH   | AGGCAGAGCTTCTGAGGAT | AGGAGACATTGAAATTGGG |

Table 1
Primers used for RT-qPCR analysis.
administration of anti-BSA IgG alone. In the mice receiving IgG-IC, obvious pulmonary inflammatory characteristics are discovered including infiltration of inflammatory cells, hemorrhage, alveolar cavity expansion and thickened alveolar wall. In contrast, the tissue inflammatory features caused by IgG-IC were significantly reduced by XFBD treatment at both low and high doses.

In addition, Micro-CT images showed an increase in lung density of the mice 24 h after airway deposition of IgG-IC, whereas XFBD at low dosage (XFBD-L group) and XFBD at high dosage (XFBD-H group) significantly attenuated the lung density induced by IgG-IC (Fig. 1B).

**XFBD decreased the generation of C3a and C5a, and attenuated the expressions of C3aR and C5aR in IgG IC-injured lungs**

Complement, especially C3a and C5a, is well known for promoting the activation immune cells including activation monocytes/macrophages, T cells, B cells, and neutrophils, which further induced pro-inflammatory responses (Java et al., 2020). Complement activation is one of the core events of both COVID-19 patients and IgG IC-induced ALI. In this study, we detected the inhibitory efficacy of XFBD on C3a/C3aR, C5a/C5aR in lung tissues and the C3a and C5a content in serum, compared with the IgG IC-induced group. Meanwhile, the mRNA expression levels of C3aR and C5aR were prominently increased in the lung tissues induced by IgG-IC, and XFBD-L and XFBD-H suppressed the levels of C3aR and C5aR (Fig. 2C). Similarly, western blotting analysis showed that the protein levels of C3a/C3aR, C5a/C5aR in lung tissues remarkably increased in IgG-IC group, while XFBD-L and XFBD-H treatment significantly decreased these protein expressions (Fig. 2D). All of the above results together implied the potent suppressive effects of XFBD on the interaction between C3a and C3R, C5a and C5aR.

**XFBD inhibited the production of pro-inflammatory mediators in IgG IC-injured lungs**

A key driver of IgG-IC-induced acute lung injury is the over-production of a variety of pro-inflammatory mediators, so we explored the effect of XFBD on the production of pro-inflammatory factors induced by IgG-IC. As shown in Fig. 3A, there were dramatically
augments of the concentrations of pro-inflammatory mediators including IL-6, IL-1β, TNF-α and MCP-1 in the lung tissues induced by IgG-IC. However, XFBD-H treatment results in significantly reduced the production of these mediators. Furthermore, as shown in Fig. 3B, the mRNA levels of the pro-inflammatory mediators including IL-6, IL-1β, IL-18 and MCP-1 were all notably increased in the lung tissues induced by IgG-IC, while XFBD-L and XFBD-H administration decreased the levels of these pro-inflammatory factors significantly. Meanwhile, western blotting analysis indicated that the changes in protein expressions of IL-18 and IL-1β were consistent with the changes in mRNA levels (Fig. 3C).

Figure 2: XFBD attenuated C3a and C5a generation and the expressions of C3aR and C5aR. (A) Immunohistochemistry staining of C3a, C5a, C3aR and C5aR in lung tissues induced by IgG-IC. The nuclei were counter stained by Hematoxylin (magnification, ×200). (B) The contents of C3a and C5a in serum was measured by ELISA assay. (C) RT-PCR analysis of C3aR and C5aR in the lung tissues of different groups. (D) Western blot result of the expression of C3a, C5a, C3aR and C5aR in the lung tissues stimulated by IgG-IC. n=6, *p<0.05 compared with the control group; †p<0.05 compared with the anti-BSA group. ¥p<0.05 compared with the IgG-IC induced group.

Previous studies have shown that STAT3 and NF-κB play essential roles in the pathogenesis of IgG-IC-induced ALI (Lentsch et al., 1998; Tang et al., 2011). To investigate whether the inhibition effects of XFBD on the production of pro-inflammatory mediators were related to the regulation of the JAK2/STAT3 and NF-κB signaling pathways, we measured p-JAK2, total JAK2, SOCS3, p-STAT3, total STAT3, p-IKKα/β, total IKKα/β, p-NF-κB p65, total NF-κB protein expressions by western blot in lung tissues. As shown in Fig. 4A, the phosphorylation levels of
JAK2, STAT3 and protein level of SOCS3 were elevated in the mice which receiving airway administration of anti-BSA IgG alone, compared with the control group. Meanwhile, the expressions of these proteins were significantly increased in mice induced by IgG-IC, compared with the anti-BSA group, while XFBD-L and XFBD-H administration absolutely decreased these protein levels.

Besides, results from Fig. 4B demonstrated that the phosphorylation levels of IKK\(\alpha/\beta\) and NF-\(\kappa\)B p65 were up-regulated in lung tissues after airway deposition of IgG-IC, compared with the anti-BSA group. Moreover, XFBD-L and XFBD-H treatment significantly rescued the enhancement of the phosphorylation levels of IKK\(\alpha/\beta\) and NF-\(\kappa\)B p65, compared with the IgG-IC induced group.

**XFBD reduced the expressions of C3aR and C5aR in IgG IC-induced alveolar macrophages**

It is well known that IgG-IC triggered complement activation results in generation of C3a and C5a during ALI. C3a activates C3aR, whilst C5a acts through two C5a receptors, C5aR1 and C5aR2, to regulate the immune cell function. After MH-S were pretreatment with IgG-IC for 1h and then treated with XFBDs for 24 h. The immunofluorescence staining indicated that the expressions of C3aR and C5aR were notably increased by IgG-IC treatment and they were significantly decreased by 10% XFBDs treatment compared with the corresponding CONs (Fig. 5A). As shown in Fig. 5B-C, the mRNA and protein levels of C3aR and C5aR were all elevated by IgG-IC stimulation, while they were obviously down-regulated by 10% XFBDs administration compared with 10% CONs.

**XFBD-containing serum prevented IgG IC-induced inflammatory responses in alveolar macrophages**

It is clearly that alveolar macrophages are actively involved during IgG-IC–induced ALI. To further investigate the effects of XFBD in vitro experiments, we measured whether XFBD suppressed the inflammatory responses of IgG-IC-induced macrophages. MH-S were pretreated with IgG-IC for 1 h and then incubated with different proportions of CONs or XFBDs for 24 h. Firstly, we detected the influence of XFBDs on IgG-IC-stimulated pro-inflammatory mediators’ generation. As shown in Fig. 6A, the secretions of IL-1\(\beta\), IL-6, TNF-\(\alpha\) and MCP-1, were greatly elevated by IgG-IC stimulation, while these pro-inflammatory mediators
were blocked by 10% XFBDs compared with 10% CONs. Which was coincident with the in vivo data. The RT-PCR results showed that IL-6, IL-1β, IL-18 and MCP-1 were upregulated by IgG-IC induced, and they were all notably decreased by 10% XFBDs compared with 10% CONs (Fig. 6B). Consistent with the mRNA expression results, 10% XFBDs administration significantly downregulated the protein levels of IL-18 and IL-1β induced by IgG-IC compared with 10% CONs (Fig. 6 C).

**XFBD blocked the signaling pathway of JAK2/STAT3/SOCS3 and NF-κB in IgG-IC-stimulated alveolar macrophages**

As STAT3 and NF-κB play essential roles in regulating IgG-IC-induced inflammation. We evaluated the protein levels of components of the JAK2/STAT3/SOCS3 and NF-κB pathway. As expected, the phosphorylation levels of JAK2, STAT3, IKKα/β, NF-κB p65 and the level of SOCS3 were elevated by IgG-IC stimulation compared with control, 10% XFBDs treatment significantly reduced the expression of these proteins compared with 10% CONs (Fig. 7A-B).

**XFBD blocked the signaling pathway of JAK2/STAT3/SOCS3 and NF-κB by suppressing C5a-C5aR axis in macrophages**

To further explore the possible mechanisms of XFBDs on the regulation of pro-inflammatory mediators’ reduction, MH-S cells were pretreated with CAF (STAT3 activator) or C5a prior to IgG-IC stimulation, and then incubated with 10% CONs or XFBDs for 24 h. On pretreatment with the CAF or C5a, western blot analysis indicated that the expression of p-STAT3, IL-18 and IL-1β was increased significantly, and XFBDs treatment partially eliminated the effects of CAF and C5a compared with CONs treatment (Fig. 8 A&B). These results demonstrated that XFBDs prevented IgG-IC-induced inflammatory responses partly through inhibiting STAT3 pathway.

In addition, the levels of C5aR, p-NF-κB p65 were both elevated in C5a pretreated MH-S cells, however, CAF pretreatment had no influence on the expressions of C5aR and p- NF-κB p65, except increased p-STAT3 protein expression (Fig. 8A&B). These data illustrated that STAT3 and NF-κB are two different inflammatory signaling pathways in IgG-IC induced macrophages, which were regulated by C5a-C5aR axis. Simultaneously, these results show that XFBDs inhibited complement activation and decreased cytokine production by inhibiting JAK2/STAT3/SOCS3 and NF-κB signaling pathway.

**Discussion**

At present, the epidemic situation of COVID-19 is spreading worldwide, which brings great threat to the life safety and health of people all
over the world, and continues to expand and deepen the impact on world development (Yassin and Saleh, 2021). TCM has played a huge positive role in the struggle against COVID-19 in China. XFBD is one of the TCM which comes from four classical prescriptions: Maxing Shigan Decoction, Maxing Yigan Decoction, Qianjin Weijing Decoction and Tingli Dazao Xiefei Decoction and integrates 13 classical herbs including Ephedra Herba, Amygdalus Communis Vas, Gypsum Fibrosum, licorice and so on (Yan et al., 2021). XFBD has a good effect in blocking the development of the disease and improving symptoms, especially in shortening the course of the disease (Huang et al., 2021). The network pharmacology suggested that XFBD ameliorates cytokine storm in the treatment of COVID-19 by regulating key targets such as IL-6, TNF-α, IL-1β, IL-18, MCP-1 (Wang et al., 2020a). However, the underlying mechanism of XFBD remains unclear.

The classical pathway of complement activation is activated by IgG-IC which first binds to C1q and activates other complements sequentially, resulting in the generation of C3a and C5a. Recent researches have shown that complement anaphylatoxins C3a, C5a, as well as cytokines and chemokines(IL-6, IL-1β, IL-18, TNF-α and MCP-1) are significantly upregulated in COVID-19 patients (Hamed et al., 2021; Liu et al., 2020). In this study, our data showed that IgG-IC significantly induced the excessive activation of completement C3a and C5a and the production of pro-inflammatory mediators including IL-6, IL-1β, IL-18, TNF-α and MCP-1. Therefore, the inflammatory responses process of lung tissue induced by IgG-IC is similar to that of infected by SARS-CoV-2.

Furthermore, the previous study indicated that anti-C5a neutralizing antibodies protective in sepsis-induced ARDS of non-human primates (Stevens et al., 1986). Zetoune et al. indicated that silencing C5aR in lung epithelial cells markedly reduced albumin leakage, diminished neutrophils accumulation, and decreased the production of proinflammatory mediators in IgG-IC-induced ALI (Sun et al., 2009). In addition, both C3 antagonist AMY-1 or C5 activation inhibitor eculizumab afforded significant therapeutic benefits in COVID-19 (Mastellos et al., 2020). Beside anaphylatoxin, targeting the anaphylotoxin receptors C3aR and C5aR resulted in a prominent anti-inflammatory

Fig. 5. XFBD reduced the expression of C3aR and C5aR in macrophages induced by IgG-IC. MH-S were pretreated with IgG-IC for 1 h and then incubated with 5% or 10% CONs or XFBDs respectively, for 24 h. (A) Immunofluorescence analysis of C3aR and C5aR. (B) RT-PCR analysis of C3aR and C5aR. (C) Western blot analysis of C3aR and C5aR. Data are shown as the mean ±SD of at least three independent experiments. *p<0.05 vs. control; &p<0.05 vs. the 5% CONs group. #p<0.05 vs. the 10% CONs group.
response in the treatment of COVID-19 (Carvelli et al., 2020; Posch et al., 2021). Recent studies have indicated that XFBD inhibited the inflammation of LPS-induced ALI (Wang et al., 2022b; Zhao et al., 2021). In this study, we found that XFBD significantly reduced histopathological changes and decreased the production of proinflammatory mediators, including IL-6, IL-1β, IL-18, TNF-α and MCP-1 in ALI mice induced by IgG-IC. Of note, for the first time, our study showed that XFBD markedly reduced the levels of C3a and C5a in serum and lung tissues of IgG-IC-induced ALI mice. Moreover, XFBD significantly decreased the expression of C3aR and C5aR. Therefore, we speculated that the efficacy of XFBD in the treatment of COVID-19 patients is most likely due to regulation of the complement system.

During IgG-IC-induced ALI, a series transcription factors, such as NF-κB and STAT3, are active and initiate a strong proinflammatory cascade. They have been suggested to be involved in the proinflammatory responses after deposition of IgG-IC (Lentsch et al., 1998; Tang et al., 2011). In addition, SOCS3 acts as a negative feedback inhibitor of JAK2/STAT3 signaling pathway. It was shown that SOCS3 is also activated in lung following IgG-IC stimulation (Gao et al., 2006a). Furthermore, it has been suggested that SARS-CoV-2-induced hyperactivation of proinflammatory mediators is achieved through the NF-κB and JAK/STAT3 signaling pathways (Kumar and Zhou, 2022). In this study, we showed that XFBD suppressed the activation of JAK2/-STAT3/SOCS3 and NF-κB signaling pathway in IgG-IC-injured lungs.

Alveolar macrophages (AMs) are resident lung macrophages. They are the initiators, control the process and severity of inflammatory responses, and play crucial roles in IgG-IC-induced ALI (Gao et al., 2006b). During ALI, activation of AMs leads to the hypersecretion of various inflammatory cytokines and chemokines, thereby recruiting neutrophils to migrate to the injury sites (Tsushima et al., 2009). In turn, neutrophil recruitment also affects alveolar macrophage activity (Chopra et al., 2009). For these reasons, alveolar macrophages have attracted interest in studies on the mechanisms of ALI. In the present study, a serum pharmacological method was applied to investigate the effects of XFBD on the alveolar macrophages’ inflammation induced by IgG-IC. However, in this study, we didn’t measure the serum concentration of the XFBD, which deserves further investigation. Recent study have demonstrated that 42 chemical components were identified in XFBD granules, 27 of which were identified in rat plasma after intragastric administration (Wang et al., 2022a). These results can give us some
In addition, IgG-ICs triggered complement activation results in generation of C5a during ALI. More recent work has demonstrated that C5a can directly activate macrophages for chemokine production (Ricklin et al., 2010). C5aR signaling is required for C5a to elicit the proinflammatory responses. It has been shown that C5aR was markedly upregulated in alveolar macrophages in ALI mice (Hu et al., 2014). Furthermore, previously studies demonstrated that inhibiting the activities of transcription factors such as NF-κB and STAT3 in macrophages could obviously attenuate the IgG-IC-induced inflammatory responses (Lentsch et al., 1998; Tang et al., 2011). Therefore, we also perform in vitro experiments in macrophages. In this study, we established an IgG-IC-induced macrophage inflammation model in vitro and observed that the results are consistent with the in vivo data. XFBD significantly reduced the levels of IL-6, IL-1β, IL-18, TNF-α, MCP-1 as well as C3aR and C5aR in IgG-IC stimulated macrophages. Furthermore, XFBD markedly suppressed JAK2/STAT3/SOCS3 and NF-κB signaling pathway on IgG-IC stimulation. C5a has a higher biological activity than C3a. In order to elucidate the relationship between C5a and STAT3 and the effects of them on the production of pro-inflammatory mediators in IgG-IC stimulation, we pretreated CAF (STAT3 activator) or C5a prior to IgG-IC stimulation, and then incubated with 10% CONs or XFBDs for 24 h. The results indicated that the levels of pro-inflammatory mediators including IL-18 and IL-1β were notably elevated in both CAF and C5a pretreated cells, and co-treatment of XFBDs decreased the upregulated of IL-18 and IL-1β stimulated by CAF or C5a. However, CAF treatment had no influence on the expression of C5aR. In contrast, the protein levels of p-STAT3 and p- NF-κB p65 were elevated by C5a treatment. These results suggested that C5a had regulatory effect on STAT3 and NF-κB signaling pathway, and XFBD could inhibit the overactivated complement system and reduce the inflammation responses of lung mediated by STAT3 and NF-κB signaling pathway.

Conclusions

In summary, the present study used IgG-IC-induced lung injury in vivo and in vitro to mimic the pathological process of virus attacking the lung and illustrated the protective effect and potential mechanism of XFBD. The results suggested that XFBD could modulate complement system to ameliorate lung injury. It blocked the production of C3a and C5a, as well as corresponding receptors (C3aR and C5aR), and reduced the inflammation responses by inhibiting JAK2/STAT3/SOCS3 and NF-κB signaling pathway. Therefore, our studies revealed a crucial...
molecular mechanism of XFBD for treating ALI, which provides a basis for further research to elucidate the targets of TCM in coping with COVID-19.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Statement

The animal study was reviewed and approved by the Animal Ethics Committee of Tianjin Nankai Hospital (NKYY-DWLL-2022-011).

Authors’ Contributions

LC, CL and ZS designed the study. LC, CL, ZY, ZL, GQ, ZZ and TZ performed the experiments including the IgG-IC induced ALI model in vivo and in vitro. HZ provided guidance to use Xuanfei Baidu prescription. SR and WY performed the micro-CT experiments. LC drafted the manuscript. ZJ, LY and SZ revised the manuscript.

Fig. 8. XFBD reduced inflammation responses due to attenuated activation STAT3 and blocked C5a-C5aR axis in IgG-IC-induced macrophages. MH-S cells were pretreated with CAF (STAT3 activator) or C5a prior to IgG-IC stimulation, and then incubated with 10% CONs or XFBDs for 24 h. (A) Western blot analysis of C5aR, p-STAT3, IL-18 and IL-1β. (B) Western blot analysis of C5aR, p-STAT3, p-NF-κB p65, IL-18 and IL-1β. Data are shown as the mean±SD of at least three independent experiments. *p<0.05 vs. the CONs group; #p<0.05 vs. the CAF+ CONs group (A) or the C5a+ CONs group (B); &p<0.05 vs. the XFBDs group.

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CRediT authorship contribution statement

Caixia Li: Conceptualization, Validation, Data curation, Writing – original draft, Investigation. Yuhong Li: Methodology, Writing – review & editing. Han Zhang: Funding acquisition. Yuzhen Zhuo: Methodology. Lanqiu Zhang: Formal analysis. Lei Yang: Funding acquisition. Qiaoying Gao: Methodology. Zhengwei Tu: Formal analysis. Rui Shao: Data curation. Yu Wang: Funding acquisition. Junhua Zhang: Funding acquisition. Lihua Cui: Visualization, Writing – review & editing. Shukun Zhang: Investigation, Resources, Writing – review & editing.
Declaration of Competing Interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

Supplementary materials
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