

Anti-Inflammatory Regulatory Role of Signal Transducer and Activator of Transcription 3 Phosphorylation in Regulating Hypersensitivity Responses to *Echinococcus granulosus* Hydatid Cyst Fluid

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Objective: This study aims to investigate the anti-inflammatory regulatory function of signal transducer and activator of transcription 3 (STAT3) phosphorylation in hypersensitivity responses triggered by *Echinococcus granulosus* hydatid cyst fluid through in vitro RBL-2H3 cell culture.

Methods: RBL-2H3 cells were cultured in vitro and sensitized with immunoglobulin E (IgE), followed by intervention with STAT3 inhibitors Stattic and JSI-124. Cells were subsequently exposed to crude *Echinococcus granulosus* hydatid cyst fluid to induce an allergic reaction. β -Hexosaminidase (HEX) release in the cell supernatant was measured to evaluate degranulation. Apoptosis was detected using flow cytometry, and changes in phosphorylated protein levels were determined via Western Blot analysis.

Results: Analysis of β -HEX release in the *Echinococcus*-induced IgE-mediated RBL-2H3 cell degranulation model revealed that both inhibitors effectively inhibited mast cell degranulation ($P < 0.01$). Apoptosis assays revealed that both inhibitors caused varying degrees of cell damage ($P < 0.01$), potentially leading to late-stage apoptosis.

Conclusion: Immunoblotting analyses confirmed that treatment with the two inhibitors reduced STAT3 phosphorylation levels at the S727 and Y705 sites, thereby inhibiting cell degranulation and alleviating immune responses.

Keywords: allergic reaction, *Echinococcus granulosus* hydatid cyst fluid, RBL-2H3 cells, STAT3 phosphorylation

Introduction

Cystic Echinococcosis (CE) is a zoonotic parasitic disease with a high prevalence in the northwestern regions of China, including Xinjiang, Gansu, Ningxia, Qinghai, and Inner Mongolia.^{1,2} This condition is primarily caused by the ingestion of *Echinococcus granulosus* tapeworm eggs of canine origin. Leading to the development of hydatid cysts within intermediate hosts that include humans, artiodactyl herbivores, and rodents.^{3–5} Clinically, trauma, medical procedures, and spontaneous rupture of cysts can lead to severe allergic reactions.⁶ These allergic responses, categorized as immediate hypersensitivity responses, are pathological processes regulated by a complex network of immune cells.⁷

Among all immune cells involved in allergic reactions, mast cells play a dominant role in immune regulation by releasing immune mediators via degranulation.⁸ The cellular signal transduction initiating mast cell degranulation is

triggered by antigens binding to fragment crystallizable (Fc) receptors on the cell membrane.⁹ Mast cell-mediated allergic reactions are mainly induced when antigens re-enter the body, causing the aggregation of high-affinity IgE receptors (FcεRI) on the surface of mast cells. The formation of antigen-IgE complexes promotes the cross-linking and clustering of FcεRI on the cell surface, initially activating the protein kinase Lyn. This activation leads to the acidification of immunoreceptor tyrosine-based activation motif (ITAM) residues on FcεRI, which in turn stimulates the phosphorylation and activation of protein kinase Syk.⁴ Activated Lyn and Syk further phosphorylate docking proteins LAT, NTAL, and Ras kinase, as well as phospholipase C-gamma (PLC-γ) and the phosphatidylinositol-3-kinase (PI3K) signaling pathways. PLC-γ plays a dual regulatory role by activating protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) signaling pathway via diacylglycerol (DAG) produced within mast cells, while concurrently regulating intracellular calcium flux by controlling the production of inositol-1,4,5-trisphosphate (IP3). Sphingosine-1-phosphate (S1P) regulates intracellular calcium flux and mast cell degranulation through PKC- and IP3-independent pathways, facilitating cytoskeleton rearrangement or enhancing degranulation through engaging with S1P1 or S1P2 cell membrane receptors. In addition to activating Lyn, FcεRI aggregation can also activate Fyn, another kinase of the Src family, which phosphorylates the docking protein Gab2 and subsequently activates the PI3K pathway, ultimately influencing the AKT/IKK/NF-κB signaling pathway, which regulates inflammatory cytokine and chemokine production, thus modulating mast cell functions.⁵

Given the central role of phosphorylation within this complex signaling network, targeting reversible phosphorylation presents a potential therapeutic approach for the treatment of mast cell degranulation and anaphylactic shock induced by hydatid cyst fluid leakage.

Materials and Methods

Cell Line

RBL-2H3 cells were purchased from Wuhan Pricella Biotechnology Co., Ltd.

Primary Reagents

The bicinchoninic acid (BCA) protein assay kit, loading buffer, SDS-PAGE gel preparation kit, and colored pre-stained protein marker were sourced from Beijing Solarbio Science & Technology Co., Ltd. The STAT3 inhibitors Stattic and JSI-124 were procured from Shanghai Haoyuan Chemexpress Co., Ltd. Antibodies for STAT3, phosphorylated p-STAT3 (S727), p-STAT3 (Y705), and ACTIN, as well as secondary antibodies, were purchased from Wuhan ABclonal Biotechnology Co., Ltd. High-resolution rapid electrophoresis liquid and ice-free rapid transfer liquid were obtained from Wuhan Servicebio Technology Co., Ltd. Immunoglobulin E (IgE) was purchased from Sigma-Aldrich, USA.

Main Equipment

The equipment used in this study included a cell culture incubator (Thermo Fisher Scientific, USA), an inverted microscope (Leica, Germany), a multifunctional microplate reader (Thermo Fisher Scientific, USA), a chemiluminescence imaging instrument (Cytiva Life Sciences), a flow cytometer (Bestbio), and an electrophoresis apparatus (Bio-Rad Laboratories).

Experimental Methods

Cell Culture

RBL-2H3 cells were routinely revived and inoculated in 25T cell culture flasks containing DMEM medium supplemented with 15% fetal bovine serum. Culturing was performed in a 37 °C incubator with 5% CO₂, and cells were subcultured at a 1:3 ratio every 2 to 3 days. Cells in the logarithmic growth phase were selected for further experimentation.

Preparation and Storage of *Echinococcus granulosus* Hydatid Cyst Fluid

Fresh sheep livers infected with *Echinococcus granulosus* were obtained from local slaughterhouses. The liver surfaces were disinfected with alcohol for three minutes, and the cyst fluid was aspirated into sterile centrifuge tubes using

a sterile syringe. The samples were centrifuged at 4 °C at 2500 rpm for 10 minutes. The supernatant was collected, and the pellet was discarded. Approximately 10 mL of cyst fluid supernatant was transferred to a sterile culture dish, sealed with plastic wrap, and subjected to freeze-drying for 24 hours. The resulting product was reconstituted by dissolving in sterile PBS solution, and protein content was determined using a BCA protein assay kit. The cyst fluid was diluted to the desired concentration with PBS. The remaining cyst fluid was aliquoted and stored at -80 °C for future use.

Effect of STAT3 Phosphorylation Inhibitors on β -HEX Release from Mast Cells

RBL-2H3 cells in optimal growth condition during the logarithmic phase were selected from the thermostatic incubator, digested, and centrifuged to prepare a cell suspension with a density of 2×10^4 /mL. Cells were evenly seeded into 24-well plates at 1 mL of cell suspension per well. After mixing in a figure-eight motion, the plate was transferred to a thermostatic incubator at 37 °C with 5% CO₂ and cultured for 4 to 6 hours overnight to achieve complete adherence.

The adherent cells were divided into the following experimental groups: the blank control group (group A), the IgE intervention group (normal intervention group, or group C), the IgE + inhibitor intervention group (with Stattic concentrations of 3 nM/mL [group D] and 6 nM/mL [group E], and JSI124 concentrations of 150 nM/mL [group F] and 300 nM/mL [group G]), and the positive drug group treated with C48/80 at 50 μ L/mL (group B).

Following adherence, all groups were sensitized overnight with DNP-IgE at a concentration of 0.125 μ g/mL. On the next day, cells were washed with PBS and treated with STAT3 inhibitors: Stattic at a final concentration of 3 nM/mL and 6 nM/mL, JSI124 at a final concentration of 150 nM/mL and 300 nM/mL for 2 hours. This was followed by stimulation with 3 mg/mL of an *Echinococcus granulosus* cyst fluid (EgCF) mixture. The reaction was halted by placing the samples on ice for 10 minutes, and supernatants from all groups were collected.

For the normal group, 0.5% Triton X-100 (200 μ L) was added, and the cells were lysed on ice for 30 minutes, followed by centrifugation at 1000 \times g for 8 to 10 minutes. Each experimental condition was replicated in triplicate. A volume of 50 μ L of the supernatant was taken and added to a 96-well plate, followed by the addition of 50 μ L of color reagent. After sealing the plate and incubating at 37 °C for 2 hours, 200 μ L of a stop solution was added and mixed thoroughly. The optical density (OD) value of each group was measured at 405 nm using a microplate reader. The β -HEX release rate of cells was calculated using the formula release rate of β -HEX (%) = (experimental group OD - control group OD value) / (total lysate OD value - control group OD value) \times 100%.

Effect of STAT3 Phosphorylation Inhibitors on Mast Cell Apoptosis

Cells in the logarithmic growth phase were obtained from the incubator, and cell suspensions were prepared as described in Section 2.4. The cell concentration was adjusted to 1×10^6 /mL, and the suspension was seeded into 6-well plates. Following incubation at 37 °C in a 5% CO₂ atmosphere for 4 to 6 hours to achieve complete adherence, cells in each well were sensitized overnight with DNP-IgE culture medium at a final concentration of 0.125 μ g/mL.

On the next day, the cells were rinsed with PBS buffer, and STAT3 inhibitors, that is, Stattic (final concentrations of 3 nM/mL and 6 nM/mL) and JSI124 (final concentrations of 150 nM/mL and 300 nM/mL) were added for a 2-hour intervention. Cells were then incubated with 3 mg/mL of EgCF buffer for 1 hour. Following this, the cells were digested using a 0.25% trypsin solution without EDTA, and the cell suspensions were transferred into flow cytometry tubes and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were washed twice with pre-cooled PBS buffer and centrifuged again. To each tube, 400 μ L of 1X Annexin V binding buffer was added to gently resuspend the cells. After this, 5 μ L of Annexin V-FITC staining solution was added, mixed evenly, and incubated at 4 °C in the dark for 15 minutes. Subsequently, 10 μ L of PI staining solution was added, and after gentle mixing, the samples were incubated at 4 °C for 5 minutes before being subjected to flow cytometry analysis.

Extraction and Quantification of Cellular Protein Samples Following STAT3 Phosphorylation Inhibitor Intervention

Cellular samples were prepared as described in Section 2.3, and proteins were extracted using a phosphorylated protein extraction kit, following the manufacturer's instructions. Protein concentration was measured using the Thermo Fisher

BCA protein assay kit. A mixture of 4× protein loading buffer and protein solution was prepared, with 10 µL of protein mixture containing 15 ng of protein. The mixture was subjected to metal bath heating for 15 minutes to ensure protein denaturation. The denatured protein solution was aliquoted into appropriate volumes, and the excess was stored at −80 °C.

Immunoblotting Experiment

An 8% separating gel and a 5% stacking gel were freshly prepared according to the manufacturer's protocols. For each sample, 10 µL of protein mixture was loaded into the gel wells, with 5 µL of protein marker added on either side of the sample lanes. The electrophoresis tank was filled with running buffer, and the voltage was initially set to 80 V, then increased to 200 V for the separating gel, adjusted according to the position of the marker. The gel was excised at the marker location and placed in transfer buffer.

Six pieces of absorbent filter paper and the PVDF membranes were activated by immersion in ethanol for 30 seconds and equilibrated in transfer buffer. The transfer unit was assembled sequentially as follows: 1 layer of filter sponge, 3 layers of absorbent filter paper, gel block, PVDF membrane, 3 layers of absorbent filter paper, and 1 layer of filter sponge. The layers were aligned and gently pressed to eliminate bubbles using a glass rod.

The assembled transfer unit was inserted into an electrotransfer tank filled with buffer, and transfer was performed using the Bio-Rad system at 400 mA for 30 minutes. Following transfer, PVDF membranes were washed in 1× TBST buffer for 15 minutes on a shaker, blocked in 5% skim milk for 1 hour, and rinsed with TBST for 30 minutes. Primary antibodies against STAT3, S727, and Y705 were diluted at a 1:1000 ratio, while actin and secondary antibodies were diluted at a ratio of 1:10000. The blocked PVDF membranes were placed in the primary antibody incubation box and incubated overnight at 4 °C on a shaker. The PVDF membranes were then washed with TBST for 30 minutes, and the diluted secondary antibodies were added and incubated at room temperature in the dark for 1 hour. Following a final TBST wash for 30 minutes, the membrane was developed with luminescent solution for exposure.

Statistical Analysis

Data analysis was performed using SPSS 22.0, and GraphPad Prism 8.3 software was used for graphical representation. Experimental results were expressed as the mean ± standard deviation ($\bar{x} \pm s$). Independent sample *t*-tests were used for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups. A *P* value of < 0.05 was considered statistically significant.

Results

Effect of STAT3 Phosphorylation Inhibitors on Mast Cell β-HEX Release Rate

During the experimental period, the β-HEX release rate in the blank control group cells (group A) was minimal, while the positive control C48/80-treated group (group B) demonstrated a β-HEX release rate approaching 90%, indicating strong cell degranulation function. Compared with the control group (group A), sensitized cells and inhibitor intervention groups showed a significantly increased β-HEX release rate ($P < 0.001$). In comparison with the normal intervention group (group C), there was a significantly reduced release rate in cells treated with Stattic 3 µM (group D) and Stattic 6 µM (group E) ($P < 0.001$). There was also a significant decrease in β-HEX release rates in cells treated with JSI-124 150 nM (group F) and JSI-124 300 nM (group G), compared with the normal intervention group (group C) ($P < 0.001$) (Figure 1).

Effect of STAT3 Phosphorylation Inhibitors on Mast Cell Apoptosis

Compared with the blank control group (group A), the apoptosis rate in cells treated with Stattic 3 µM (group D) and Stattic 6 µM (group E) significantly increased, with a nearly equal ratio of early and late apoptosis, though the ratio of early apoptosis was slightly higher. The JSI-124 150 nM (group F) and the JSI-124 300 nM groups (group G) also showed a significant increase in apoptosis rate compared with the blank control group (group A), but the proportion of necrosis and late apoptosis was significantly higher than that of early apoptosis in these groups (Figure 2).

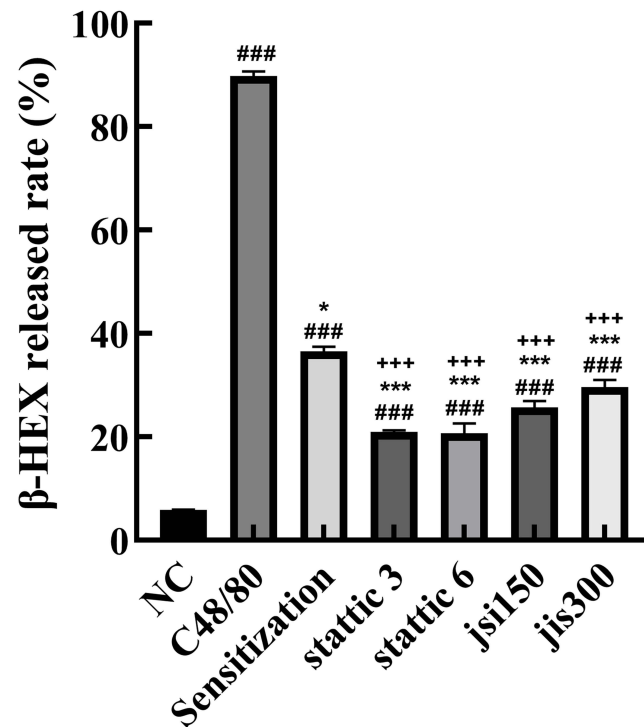


Figure 1 The effect of different concentrations of inhibitors on β-HEX release rates.

Notes: Group A, blank control group; Group B, C48/80 group; Group C, normal intervention group; Group D, Static 3 μM group; Group E, Static 6 μM group; Group F, JSI-124 150 nM group; Group G, JSI-124 300 nM group. # compared with the blank control group, ### for $P < 0.001$; * compared with the C48/80 group, *** for $P < 0.001$; + compared with the normal intervention group, +++ for $P < 0.001$.

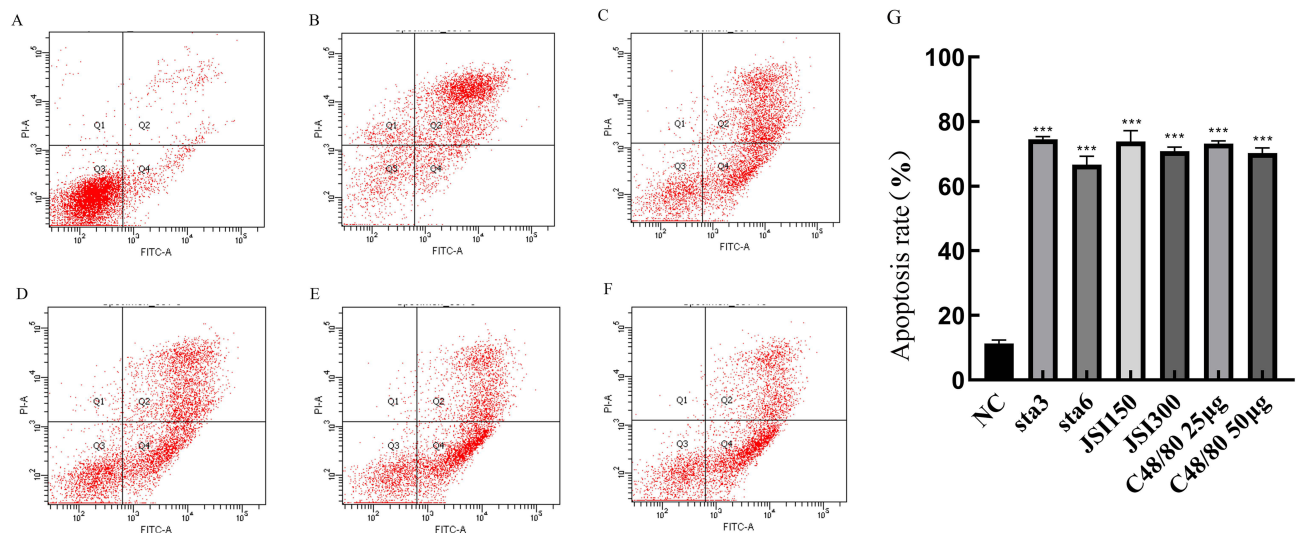


Figure 2 The effect of different concentrations of inhibitors (Static 3 nM / mL, 6 nM / mL and JSI-124 150 nM / mL, 300 nM / mL) on mast cell apoptosis.

Notes: Group A, control group; Group B, C48/80 group; Group C, Static 3 μM group; Group D, Static 6 μM group; Group E, JSI-124 150 nM group; Group F, JSI-124 300 nM group; G represents the statistical histogram of the apoptosis rate of each group compared with the control group. The apoptosis rate is the sum of early apoptosis (Q3 region) and late apoptosis (Q2 region). * Compared with the control group, *** indicates $p < 0.001$.

Inhibitory Effect of Different Concentrations of Inhibitors on STAT3 Phosphorylation

Western Blot was utilized to detect the changes of STAT3 phosphorylation at the S727 and Y705 sites in RBL-2H3 cells after treatment with various concentrations of the STAT3 phosphorylation receptor inhibitors Static and JSI-124. The

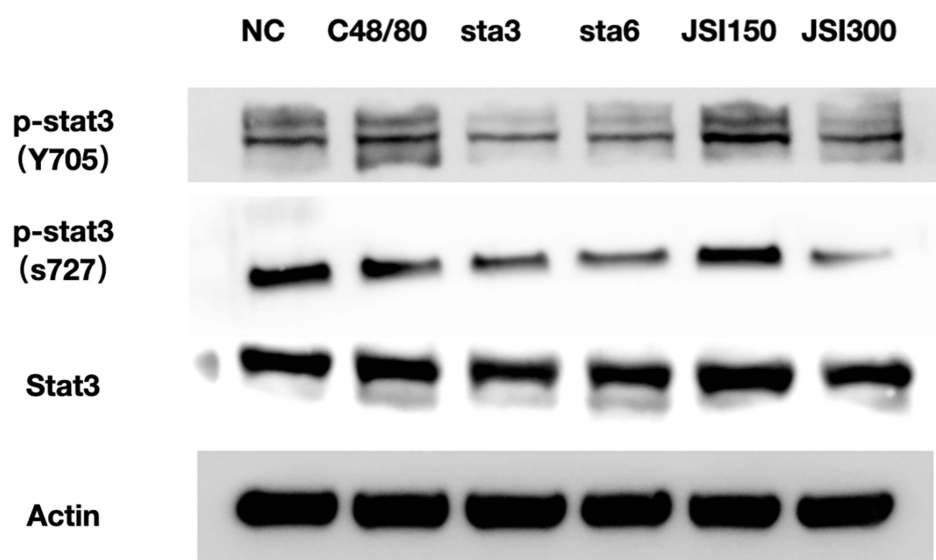


Figure 3 The inhibitory effect of different concentrations of inhibitors on STAT3 phosphorylation.

results showed that both inhibitors effectively suppressed STAT3 phosphorylation at the S727 and Y705 sites to varying degrees during mast cell degranulation (Figure 3).

Discussion

Mast cells play a key role in allergic reactions as effector cells. Normal mast cells are few in number, round or oval in shape, with numerous granules filled with bioactive substances.¹⁰ These cells are strategically located in regions that are potential entry points for pathogens, such as the skin, oral and nasal mucosa, and the gastrointestinal tract. Like dendritic cells, they can recognize antigens and release inflammatory factors upon degranulation, as well as recruit various immune cells to target tissue sites, becoming a frontline defense against pathogens and other harmful stimuli.¹¹ Mast cell activation of mast cells primarily occurs via the antigen-specific IgE binding to high-affinity Fc receptors on the cell membrane, activating a cascade of signal transduction pathways that culminates in mast cell degranulation.

However, IgE is not the only component that activates mast cells; various other ligands, including Toll-like receptors, IgG, cytokines, and neuropeptides, can also trigger mast cell degranulation.¹² Compound C48/80, a well-known mast cell deactivator, can also stimulate cell degranulation; neither an antigen nor a hapten, it directly activates G protein-coupled pathways without requiring immune system involvement.⁵

The ultimate result of mast cell activation is the release of an array of inflammatory mediators and vasoactive substances into the body. In the initial stage, these pre-stored granules containing inflammatory factors such as tumor necrosis factor- α (TNF- α) and biogenic amines like histamine are rapidly expelled. In later stages of the immune response, new mediators, including chemokines and prostaglandins, are synthesized to recruit various cytokines and chemokines, thereby perpetuating the immune response.^{13,14}

Basophils share many similarities with mast cells, especially in immune function and cellular morphological characteristics. One of their defining features is the high expression of Fc receptors on their cell membranes, which facilitates degranulation and the release of immune mediators.¹⁵ In the context of in vitro studies of allergic reactions, the RBL-2H3 cell line, derived from basophils, is widely used in various studies due to its stable phenotype, reliable passage characteristics, and ease of cultivation. These features make RBL-2H3 cells a suitable model for studying degranulation processes.

In this study, the initial plan was to use peritoneal mast cells from *Echinococcus*-infected mice as the experimental model, but the extended time required to model *Echinococcus* infection, combined with challenges in extracting and purifying sufficient quantities of peritoneal mast cells, posed significant practical limitations. Consequently, after an

extensive review of the literature, RBL-2H3 cells were chosen as they offer functional and mechanistic similarity to primary mast cells while providing a more accessible and consistent in vitro model.

The critical regulatory role of STAT3 in mast cell degranulation has been demonstrated in multiple studies, indicating that it is a key component of the signaling network responsible for this process.^{16,17} Specifically, mitochondrial STAT3 phosphorylation can enhance oxidative phosphorylation (OXPHOS) activity, further regulating the degranulation responses of primary mast cells and the RBL-2H3 cell line.¹⁸ Additionally, blocking or inhibiting STAT3 phosphorylation can lead to disruption of downstream signal pathways mediated by Fc receptors, thereby modulating the extent of mast cell degranulation.¹⁹ Based on this understanding from our literature review, two inhibitors known for their effects on suppressing STAT3 phosphorylation—Stattic and JSI-124—were selected. Stattic is a classic inhibitor of p-STAT3, and JSI-124 is a novel inhibitor that prevents STAT3 binding to DNA and affects STAT3-mediated gene transcription. Both target STAT3 but exhibit differing mechanisms of action. JSI-124 at a concentration of 150 nM has been reported to induce cell apoptosis by inhibiting STAT3 phosphorylation at the S727 site, thus blocking the STAT3 signaling pathway.^{20,21}

In order to determine the applicability of Stattic and JSI-124 in the context of a mast cell degranulation model induced by *Echinococcus granulosus* hydatid cyst fluid, the release of β -HEX, a marker of mast cell degranulation, was evaluated in this study. Based on a literature review and pre-experimental data, two concentrations for each inhibitor were selected for intervention in sensitized mast cells. When compared with the model group, all four concentrations of the two inhibitors demonstrated a significant impact on the release of β -HEX, indicating the effectiveness of these two inhibitors in modulating mast cell degranulation. Specifically, Stattic at both concentrations (3 nM/mL and 6 nM/mL) reduced degranulation by approximately 50% compared with the model group, whereas JSI-124 at both concentrations (150 nM/mL and 300 nM/mL) had a less pronounced inhibitory effect on β -HEX release. This suggests that JSI-124 may inhibit degranulation through mechanisms not solely reliant on β -HEX modulation.

The results of flow cytometry (Annexin V and PI staining), used to detect cell apoptosis, revealed that JSI-124 at both concentrations significantly increased apoptosis rates compared with the control group, encompassing both early and late apoptotic stages. These experimental results further prove that JSI-124 can induce cell apoptosis, thereby blocking the normal signal transduction of mast cells.

Immunoblotting was used to further validate the inhibitory effect of these two inhibitors on STAT3 phosphorylation during mast cell degranulation. Results confirmed that, after treatment with the two inhibitors, the total STAT3 protein content in mast cells stimulated by *Echinococcus granulosus* hydatid cyst fluid remained constant, but phosphorylated protein levels, mainly at the S727 and Y705 sites, decreased.

Conclusion

In summary, in the mast cell degranulation model induced by *Echinococcus granulosus* hydatid cyst fluid, both inhibitors Stattic (at concentrations of 3 nM/mL and 6 nM/mL) and JSI-124 (at concentrations of 150 nM/mL and 300 nM/mL) effectively reduced STAT3 phosphorylation levels at S727 and Y705 sites, thereby inhibiting mast cell degranulation. This suggests a promising avenue for targeting STAT3 to modulate immune responses mediated by mast cells.

Abbreviations

IgE, Immunoglobulin E; β -HEX, β -Hexosaminidase; CE, Cystic echinococcosis; Fc ϵ RI, High affinity IgE receptor; ITAM, Immunoreceptor tyrosine-based activation motif; PLC- γ , phospholipase C-gamma; PI3K, phosphatidylinositol-3-kinase; DAG, Diacylglycerol; PKC, protein kinase C; MAPK, Schistogen-activated protein kinase; IP3, Inositol triphosphate; S1P, Sphingosine-1-phosphate; PBS, Phosphate Buffered Saline; OD, Optical Density; EgCF, *Echinococcus granulosus* cyst fluid; DNP-IgE, Anti-Dinitrophenol-Immunoglobulin E; EDTA, Ethylene Diamine Tetraacetic Acid; PVDF, polyvinylidene fluoride; TBST, Tris-Borate-Sodium Tween-20; IgG, Immunoglobulin G; C48/80, Compound 48/80; TNF- α , tumor necrosis factor- α ; OXPHOS, Oxidative phosphorylation; DNA, DeoxyriboNucleic Acid.

Data Sharing Statement

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All experiments were evaluated and approved by Animal Ethics Committee of First Affiliated Hospital of Xinjiang Medical University (No.IACUC-20200318-04) and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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Disclosure

The authors declare that they have no conflict of interest regarding this work.

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