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Activation of PLCγ/AKT/IκBα/p65 signaling increases inflammation in mast cells to promote growth of cutaneous neurofibroma

Jing Xu[a], Hongke Zhang[b], Chengbin Li[b], Huicong Du[b], Maoguo Shu[b,*], Jing Jia[b,*]

[a] *Teaching Department, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China*
[b] *Department of Plastic, Cosmetic and Maxillofacial Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi, China*

A R T I C L E I N F O

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A B S T R A C T

Aim: Cutaneous neurofibroma (cNF), a hallmark feature of neurofibromatosis type 1 (NF1), results in psychological and physical damage to patients. Considering the important role of mast cells in neurofibroma development, the aim of this study was to elucidate the underlying mechanism of the interaction between cNF cells and mast cells.

Main methods: SW10 cells with *Nf1* knocked down were used as a cNF cell model. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony formation assays, as well as a mouse xenograft tumor model, were used to assess the cNF tumor growth in vivo and in vitro. ELISAs and IHC were used to examine the inflammatory activity of mast cells.

Key findings: We demonstrated that cNF cells activated mast cells, which in turn promoted the cNF cell growth, while suppression of the inflammatory activity of cNF-associated mast cells reversed their stimulating effect on the growth of cNF cells. Mechanistic studies revealed that SW10 cells upregulated PLCγ/AKT/IκBα/p65 signaling in mast cells, thereby increasing inflammation. Moreover, PLCγ modulated the AKT/IκBα/p65 signaling activity and played a critical role in the interaction of mast cells and cNF cells. Knockdown of *PLCγ* in mast cells diminished their cNF cell-induced inflammatory activity and subsequently reduced the cNF cell growth in vivo and in vitro.

Significance: This study revealed a novel interaction between mast cells and cNF cells, suggesting a potential strategy for treating cNF by targeting the newly recognized signaling pathway.

1. Introduction

Neurofibromatosis type 1 (NF1), with an incidence of 1 in 3000 individuals worldwide [1], is usually associated with a mutation in the *Nf1* gene and shows autosomal dominant inheritance. Despite almost complete penetrance, NF1 patients from the same family can exhibit multiple types of manifestations. NF1 is a tumor-susceptible syndrome, and neurofibroma is the main symptom in NF1 patients, among which cutaneous neurofibroma (cNF) can be found in almost all patients [2]. Although few cNF tumors become malignant, cNF-associated pain, itching, and disfigurement result in psychological and physical damage to NF1 patients [3]. A MEK inhibitor showed an efficacy in both a mouse model and an early clinical study [4,5]. However, effective treatment for NF1 patients is limited to surgical resection, which is not applicable in cases of abundant or enormous neoplasia.

cNF is composed of Schwann cells, endothelial cells, fibroblasts, and inflammatory cells, which were found to play key roles in neurofibroma development [6]. Some evidence also suggested that injury-facilitated inflammation promoted neurofibroma formation in mice [7] and NF1 patients [8]. Mast cells, the largest population of inflammatory cells in neurofibroma, secret molecules capable of remodeling the extracellular matrix and provide growth factors advancing the tumor progression [9]. Accordingly, elimination of mast cells from the tumor microenvironment abrogated initiation of neurofibroma and tumor maintenance in mouse models [10]. Moreover, targeting mast cells and inflammation achieved some success in NF1 patients [11,12]; however, some patients failed to respond to the above treatment. Considering the activated status of mast cells in NF1 patients [13,14], further elucidation of the mechanisms underlying mast cell activity is necessary.

Evidence has shown that mast cells participate in inflammation in tumors, thus modulating tumor development [6,7,15]. Mast cells also show non-immunological activities, including facilitation of angiogenesis, extracellular matrix remodeling, and wound repair [9]. The different roles of mast cells illustrate their flexible nature, which is due to

* Corresponding authors.
E-mail addresses: maoguo_shu@126.com (M. Shu), jiayu0717@126.com (J. Jia).

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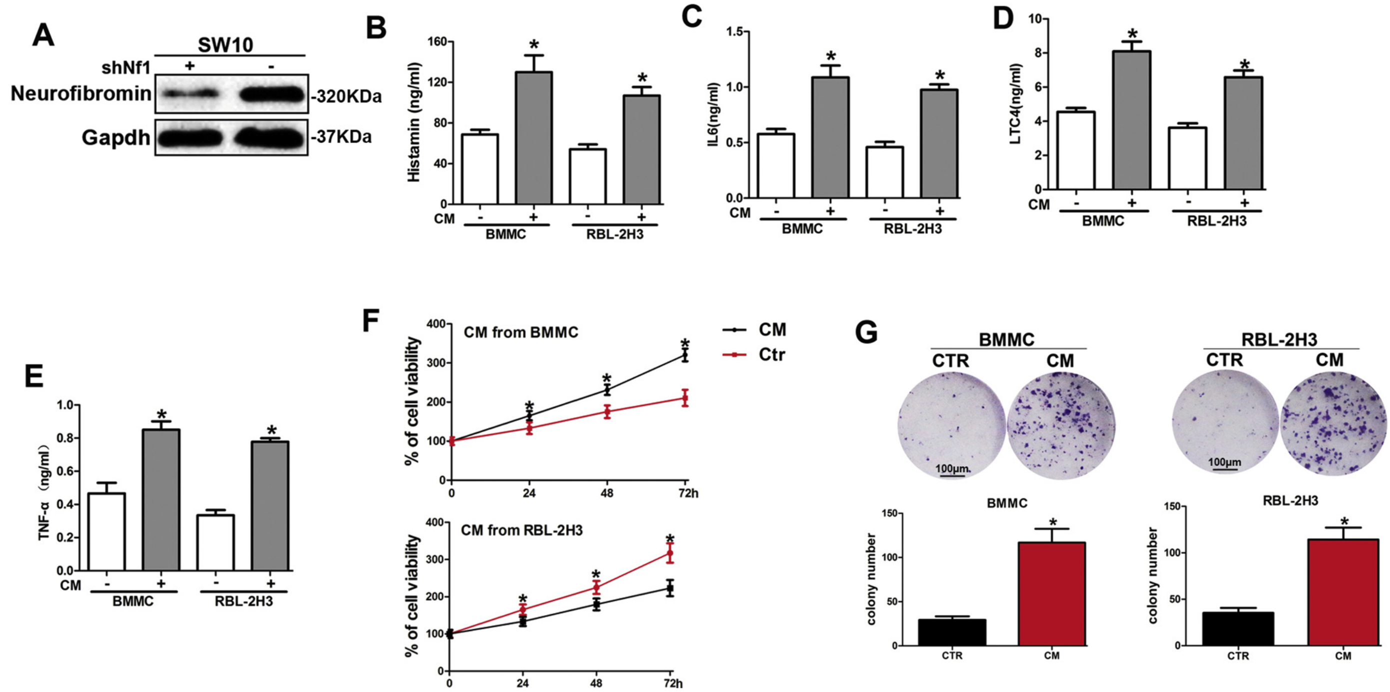

Fig. 1. shNf1-SW10 cells increased inflammatory activity of mast cells, and stimulated mast cells promoted the shNf1-SW10 cell growth. (A) A lentiviral vector with shRNA was used to knock down *Nf1* in SW10 cells. Conditioned medium (CM) from shNf1-SW10 cells was used to treat mast cells. IgE-sensitized BMMCs and RBK-2H3 cells were stimulated with CM, and then the supernatant of mast cells was collected for ELISAs. (B–E) Concentrations of histamine, IL-6, LTC4, and TNF-α in BMMC and RBL-2H3 cell media, with or without CM stimulation. Data are presented as the mean ± SD of four independent experiments. (F) Viability of shNf1-SW10 cells incubated in media from CM-stimulated and non-stimulated mast cells (IgE-sensitized BMMCs and RBK-2H3 cells). Data are expressed as the mean ± SD of five independent experiments. (G) Colony formation by shNf1-SW10 cells incubated in media from differently treated mast cells. Lower panel: colony numbers expressed as the mean ± SD of four independent experiments. $^*P < .05$.

their broad array of bioactive mediators, stored in granules or newly generated and released [16,17]. Localization of mast cells in tissues and their proliferation in distinct tissues is determined by the chemical environment of the tissue. Therefore, elucidation of the underlying mechanism involved in the modulation of the inflammatory activity of cNF-associated mast cells may provide a therapeutic target for inhibiting mast cells in the cNF microenvironment.

In this study, we tried to investigate the interaction between cNF cells and mast cells. We used shNf1-SW10 cells and mast cells, BMMCs and RBL-2H3 cells, to detect whether cNF cells could stimulate inflammatory activity in mast cells and the efficacy of activated mast cells to cNF cells. Additionally, we explored the mechanisms involved in the upregulating inflammatory activity of mast cells activated by cNF cells. Targeting this newly identified pathway may open a new avenue for cNF treatment.

2. Materials and methods

2.1. Chemicals and reagents

Primary antibodies against neurofibromin (#14623), phospho-(p-)ERK (#4370), ERK (#4695), phospho-(p-)JNK (#4668), JNK (#9252), phospho-(p-)p38 (#4511), p38 (#8690), phospho-(p-)AKT (Ser473; #3787), AKT (#4691), phospho-(p-)IκBα (Ser32; #5209), phospho-(p-)c-Jun (#3270), c-Jun (#9165), c-Kit (#37805), p65 (#8242) phospho-(p-)mTOR (#5536), phospho-(p-)GSK3β (#5558), phospho-(p-)MDM2 (#3521), phospho-(p-)AS160 (#8619), and phospho-(p-)PFKFB2 (#13064) were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-(p-)Fyn (ab182661), Fyn (ab125016), phospho-(p-)Lyn (ab33914), Lyn (ab137338), phospho-(p-)Syk (ab62338), Syk (ab40781), phospho-(p-)LAT (ab4476), LAT (ab2507), phospho-(p-)PLCγ (ab76031), and PLCγ (ab76155) antibodies were purchased from Abcam (Cambridge, MA, USA). U73122, BAY11-7082, BAY61-3606, and LY294002 were purchased from Sigma–Aldrich (St. Louis, MO, USA). The histamine ELISA kit (DEE5800) and TNF-α ELISA kit (JP27194) were purchased from Demeditec Diagnostics GmbH (Kiel, Germany). The IL-6 ELISA kit (ab100712) was from Abcam, and the LTC4 ELISA kit was from Cayman Chemical (Ann Arbor, MI, USA). All reagents were reconstituted and stored following the manufacturers' protocols.

2.2. Cell culture

SW10 cells, a murine Schwann cell line, and human skin fibroblasts, and RBL-2H3 rat basophilic leukemia cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SW10 and fibroblast cells were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone, Logan, Utah, USA). RBL-2H3 were propagated in DMEM containing 10% FBS without IL-3. Bone marrow-derived mast cells (BMMCs) were obtained from the bone marrows of femurs and tibias of C57BL/6J wild-type mice (8–10 weeks old). In brief, mice were sacrificed via cervical dislocation. The femurs and tibias were harvested, and a sterile medium was flushed through the marrow cavity to collect the bone marrow. Bone marrow cells were cultured in RPMI 1640 medium containing 1% glutamine (Thermo Fisher, Waltham, MA, USA), 1.5% HEPES (Thermo Fisher), 2% penicillin/streptomycin (Thermo Fisher), and 10 ng/mL IL-3 (Thermo Fisher). When a flow cytometry assay (PE-cyanine 7 anti-mouse FcεRIα and ef450 anti-mouse c-Kit), purchased from eBioscience (San Diego, CA, USA), indicated that the proportion of c-Kit^{high}FcεRIα^{high} cells reached 95%, cells were used for the experiments. All the above cells were maintained in a humidified atmosphere with 5% CO_2. The animal experiments were approved by the institutional review board of the First Affiliated Hospital of Xi'an Jiaotong University.

A lentiviral vector carrying an *Nf1*-specific short hairpin RNA (shRNA) or a non-specific control (NC) shRNA (GenPharma, Shanghai,

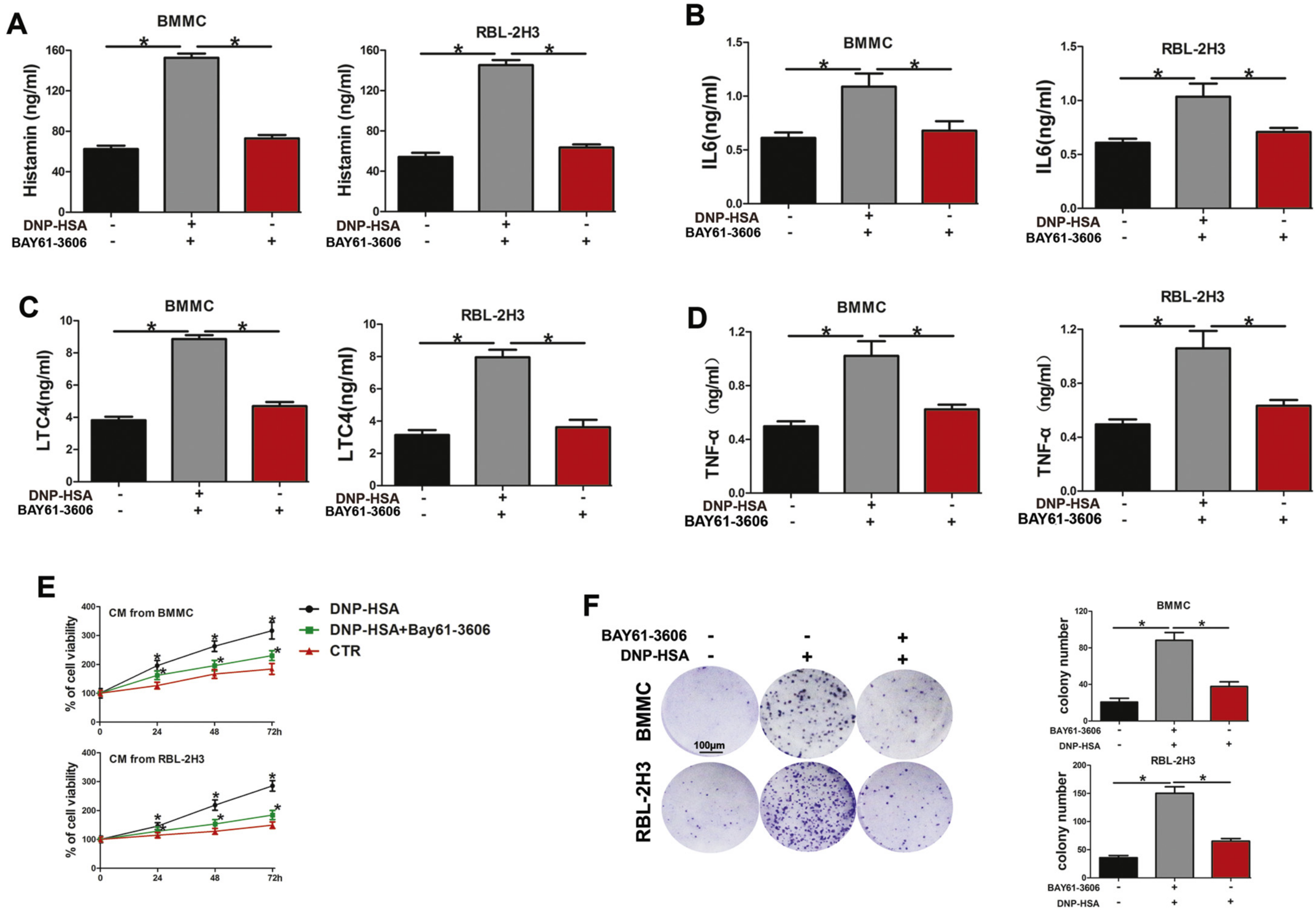

Fig. 2. Upregulated inflammation in mast cells is critical for their ability to promote shNf1-SW10 cell viability. For BAY61-3606 treatment, IgE-sensitized mast cells were incubated with BAY61-3606 (10 μM) for 1 h before stimulation with DNP-HSA (100 ng/mL). IgE-sensitized BMMCs and RBK-2H3 cells without stimulation were used as a control (CTR). (A–D) Concentrations of histamine, IL-6, LTC4, and TNF-α in the media of differently treated mast cells. Data are expressed as the mean ± SD of three independent experiments. (E) Viability of shNf1-SW10 cells incubated in media of BAY61-3606-treated and untreated mast cells. Data are expressed as the mean ± SD of five independent experiments. (F) Colony formation by shNf1-SW10 cells incubated in media of differently treated mast cells. Right panels: colony numbers expressed as the mean ± SD of four independent experiments. *$P < .05$.

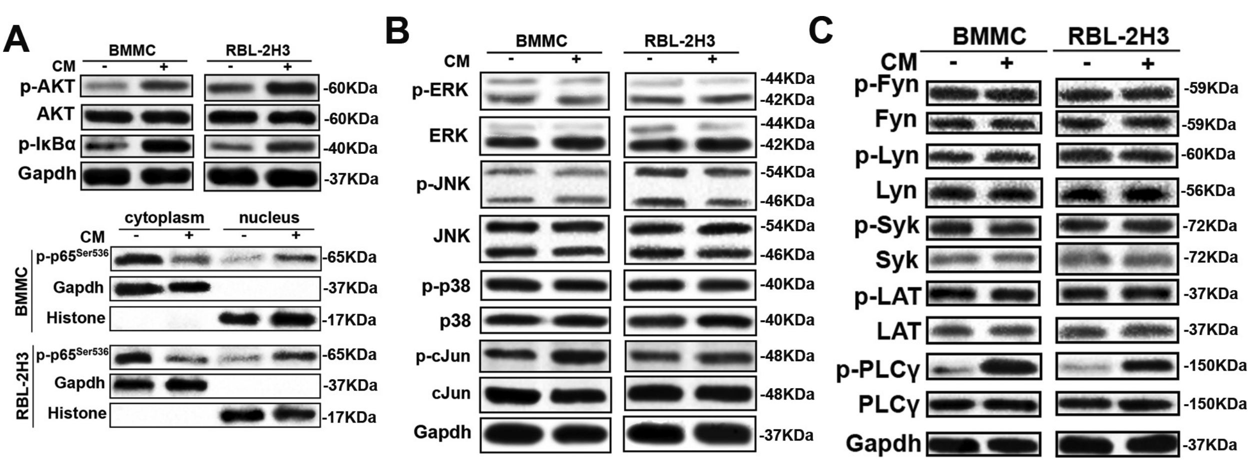

Fig. 3. Stimulation of mast cells with CM of *Nf1* knockdown SW10 cells upregulated AKT/IκBα/p65 signaling and PLCγ in mast cells. (A) Protein levels of p-AKT (Ser473), AKT, p-IκBα (Ser32), and cytoplasmic and nuclear p65 in CM-stimulated mast cells. (B) Expression of p-ERK, ERK, p-JNK, JNK, p-p38, p38, p-c-Jun, and c-Jun in CM-stimulated mast cells. (C) Expression of p-Fyn, Fyn, p-Lyn, Lyn, p-Syk, Syk, p-LAT, LAT, p-PLCγ, and PLCγ in CM-stimulated and control mast cells.

China) was used to knock down *Nf1* in cNF cells, and that carrying a *PLCγ*-specific or an NC shRNA was used to knock down *PLCγ* in cells. In addition, a lentiviral vector encoding PLCγ or NC was used to overexpress PLCγ in cells.

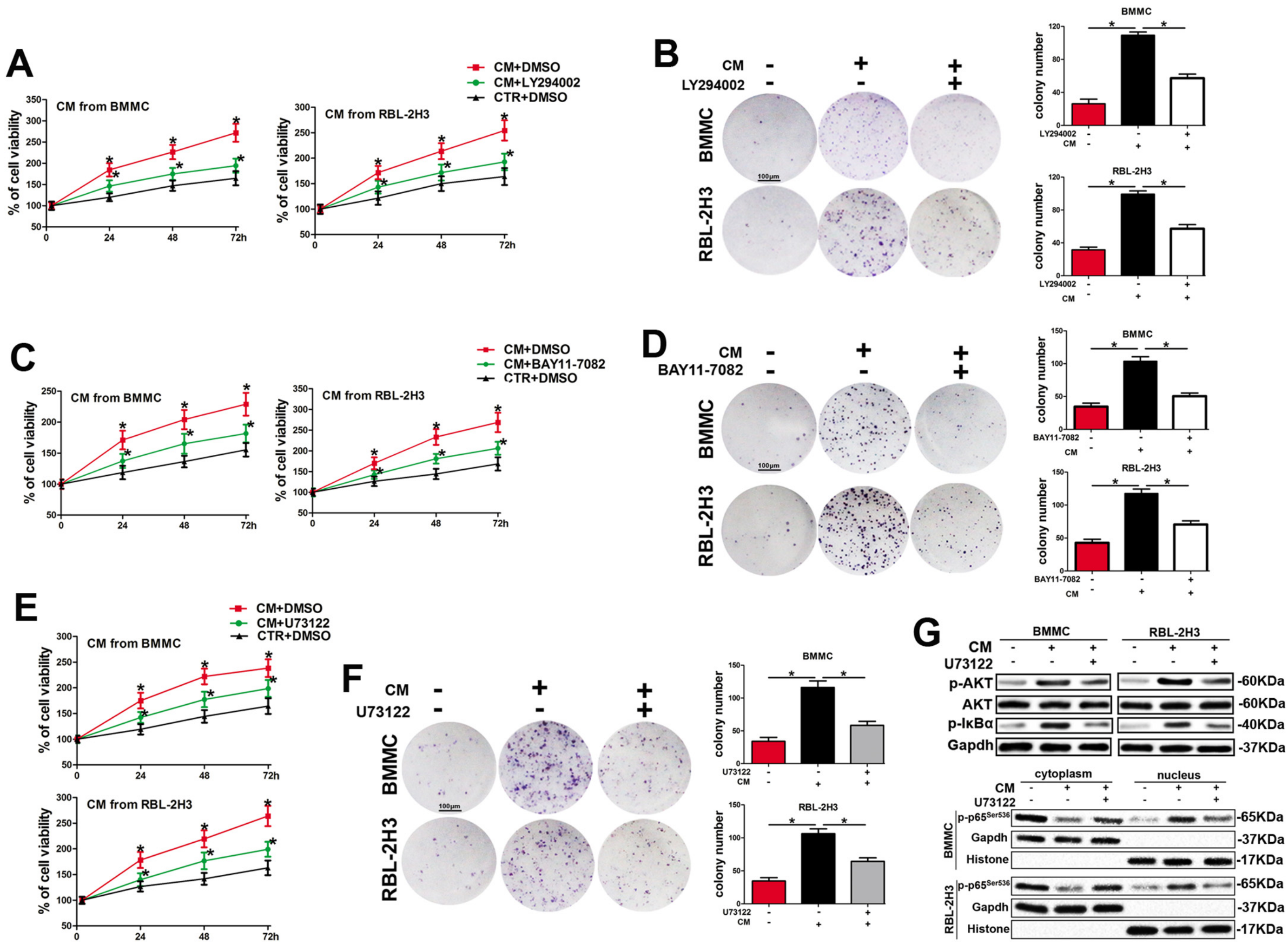

Fig. 4. Inhibition of AKT, NF-κB, or PLCγ in mast cells impaired mast cell-mediated shNf1-SW10 growth. IgE-sensitized mast cells were preincubated with LY294002 (10 μM), BAY11-7082 (10 μM), U73122 (5 μM), or DMSO as a control for 1 h prior to stimulation with CM, and the media were collected to treat shNf1-SW10 cells. (A) Viability of shNf1-SW10 cells incubated with media from LY294002-treated or untreated mast cells. Data are expressed as the mean ± SD of five independent experiments. (B) Colony formation by the above shNf1-SW10 cells. Right panels: colony numbers expressed as the mean ± SD of four independent experiments. (C) Viability of shNf1-SW10 cells incubated with media from BAY11-7082-treated or untreated mast cells. Data are expressed as the mean ± SD of five independent experiments. (D) Colony formation by the above shNf1-SW10 cells. Right panel: colony numbers expressed as the mean ± SD of four independent experiments. (E) Viability of shNf1-SW10 cells incubated with media from U73122-treated or untreated mast cells. Dara are expressed as the mean ± SD of five independent experiments. (F) Colony formation by the above shNf1-SW10 cells. Right panel: colony numbers expressed as the mean ± SD of four independent experiments. (G) Protein levels of p-AKT, AKT, p-IκBα, and cytoplasmic and nuclear p65 in CM-stimulated mast cells, with or without treatment with U73122. *$P < .05$.

2.3. Mast cell activation

BMMCs and RBL-2H3 cells were treated with anti-DNP IgE (500 ng/mL) overnight and then stimulated with a conditioned medium (CM) from shNf1-SW10 cells or shNf1-fibroblast cells for 10 min or with DNP-human serum albumin (HSA; Ag, 100 ng/mL) for 15 min. For inhibitor treatment, mast cells were treated with the indicated inhibitors (including U73122 to inhibit PCLγ, BAY11-7082 to inhibit NF-κB, BAY61-3606 to inhibit Syk and LY294002 to inhibit AKT) for 1 h before stimulation. Afterward, the supernatants were collected for ELISAs, and cell lysates were used for western blotting.

2.4. CM collection and measurement of secreted inflammatory cytokines

Nf1 knockdown SW10 cells (5×10^5) were seeded into a 60-mm culture dish. After the cells attached, they were washed three times with a serum-free medium (SFM) and then cultured in SFM (5 mL) for an additional 24 h. The supernatant was collected and centrifuged to remove cell debris. CM was used immediately or stored at −80 °C.

IgE-sensitized BMMCs and RBL-2H3 cells were treated with the prepared CM for 10 min, and the media of the mast cells were centrifuged to obtain supernatants for ELISAs. Alternatively, IgE-sensitized BMMCs and RBL-2H3 cells were preincubated for 1 h with the indicated inhibitors prior to stimulation with CM or DNP-HSA, and the media were centrifuged to obtain supernatants for ELISAs. Inflammatory cytokines were quantified in the media using ELISAs following the manufacturers' protocols.

2.5. Colony formation assay

Nf1 knockdown cells were seeded in a 6-well plate (1000 cells/well) and incubated in media collected from differently treated mast cells for 2 weeks. The colonies formed were fixed with 4% paraformaldehyde, stained with crystal violet for 10 min at room temperature, and counted.

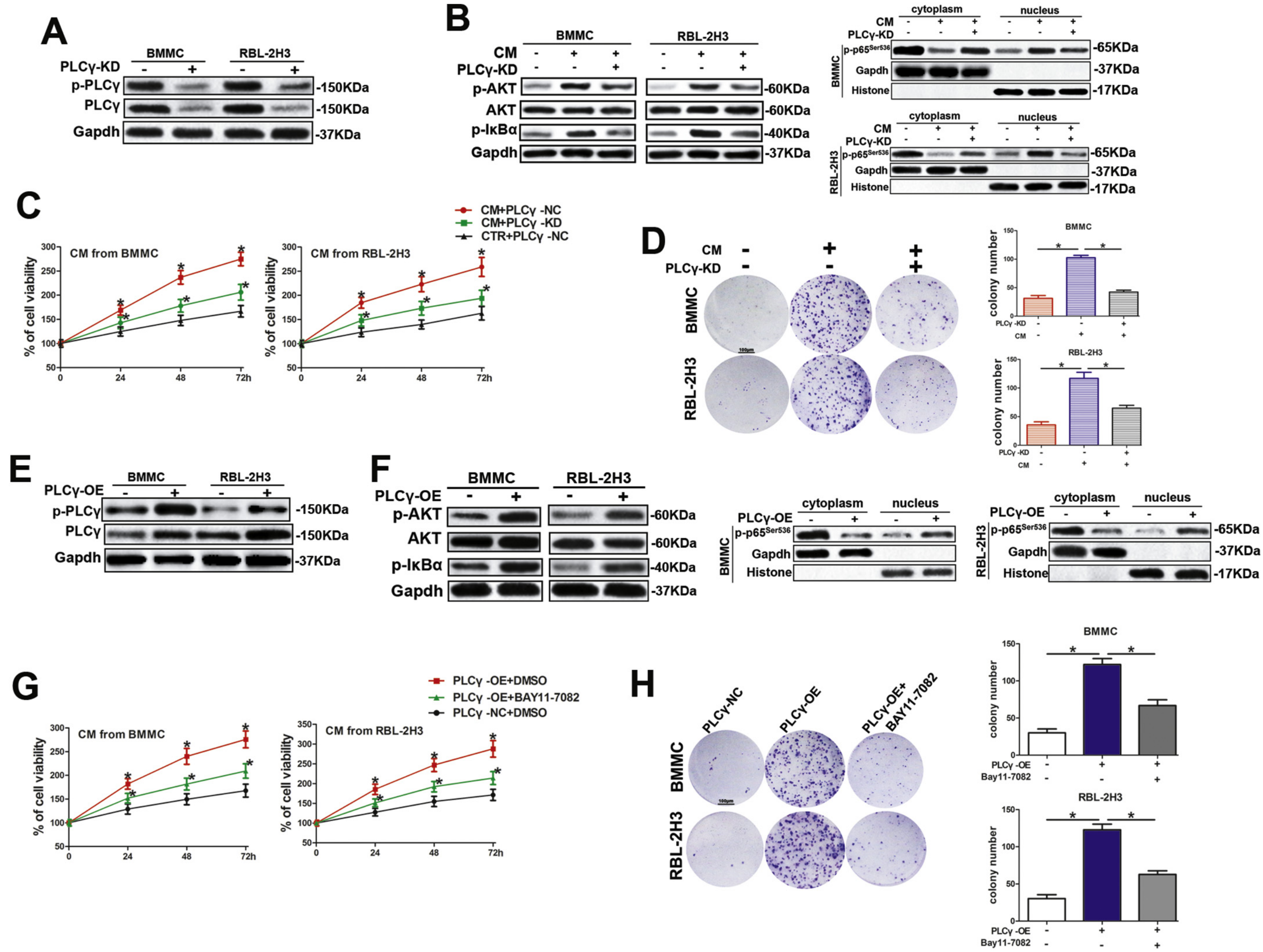

Fig. 5. PLCγ played a critical role in the promotion of shNf1-SW10 cell growth by mast cells via regulation of AKT/NF-κB signaling. (A) A lentiviral vector carrying a *PLC*γ-targeting shRNA was used to transfect BMMCs and RBL-2H3 cells, and p-PLCγ and PLCγ expression was analyzed in these cells using western blotting. (B) Protein levels of p-AKT, AKT, p-IκBα, and cytoplasmic and nuclear p65 in mast cells with or without *PLC*γ knocked down. (C) Viability of shNf1-SW10 cells incubated with media of *PLC*γ knockdown and negative control mast cells. Data are expressed as the mean ± SD of five independent experiments. (D) Colony formation by the above shNf1-SW10 cells. Right panels: colony numbers expressed as the mean ± SD of four independent experiments. (E) Protein levels of PLCγ and p-PLCγ in PLCγ-overexpressing BMMCs and RBL-2H3 cells (PLCγ-OE cells). (F) Expression of p-AKT, AKT, p-IκBα, and cytoplasmic and nuclear p65 in PLCγ-overexpressing cells. (G) Viability of shNf1-SW10 cells incubated with media of PLCγ-OE mast cells preincubated with/without BAY11-7082 for 1 h before stimulation with CM. Data are expressed as the mean ± SD of five independent experiments. (H) Colony formation by the above shNf1-SW10 cells. Right panel: colony numbers expressed as the mean ± SD of four independent experiments. *$P < .05$.

2.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability and the growth rate were measured using the MTT assay. Briefly, 5×10^3 transfected cells were seeded in 96-well culture plates, followed by incubation with media from differently treated mast cells. Subsequently, the cells were washed and incubated with MTT (0.5 mg/mL) for 4 h at 37 °C. The medium was carefully removed, and 150 μL of DMSO was added to dissolve the formazan crystals formed. Finally, absorbance was measured in each well at a wavelength of 490 nm using a microplate reader (BioTek, Winooski, VT, USA). The experiment was repeated five times.

2.7. Mouse xenograft model

Eighteen nude mice (4 weeks old) were randomly allocated into three groups ($n = 6$ per group) and were subcutaneously injected into the right femoral region with 2×10^6 SW10 cells, 2×10^5 RBL-2H3 cells, and a mixture of both cell lines, respectively. The body weights of the mice were measured every 7 days. Four weeks later, the mice were sacrificed, and tumor tissues were surgically excised.

The animal experiments were approved by the institutional review board of the First Affiliated Hospital of Xi'an Jiaotong University.

2.8. Immunohistochemistry

Tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, and then cut into 5-μm-thick sections. The prepared sections were deparaffinized and rehydrated, and antigen retrieval was carried out with heat. H_2O_2 (3%) mixed with methanol was used to block the activity of alkaline phosphatase and endogenous peroxidase. Immunohistochemical staining was performed using a DAKO Autostainer Plus system. The sections were incubated with primary antibodies against IL-6 (dilution 1:200), TNF-α (dilution 1:200), and Ki-67 (dilution 1:250) for 16 h at 4 °C. Afterward, the sections were washed three times with PBS and incubated with the Envision secondary

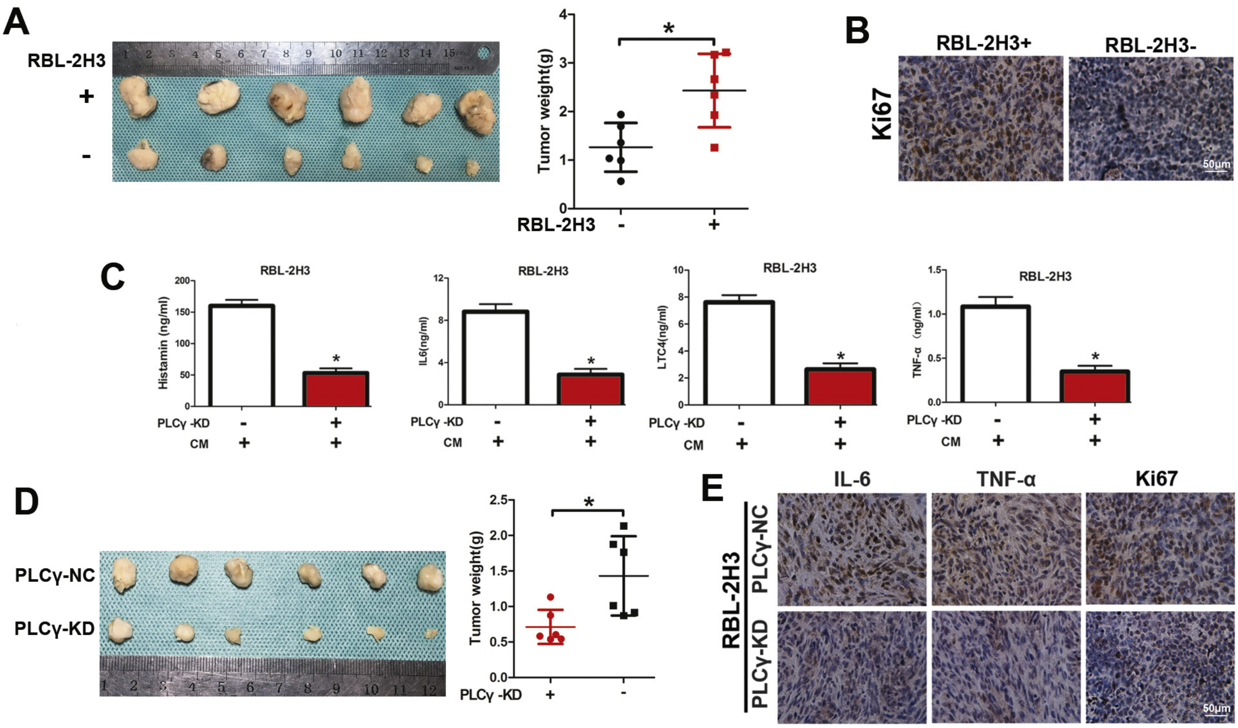

Fig. 6. shNf1-SW10 cell-stimulated mast cells, with upregulated inflammatory activity, promoted the shNf1-SW10 cell growth in vivo. (A) Xenograft tumors developed in mice at 4 weeks post-injection with shNf1-SW10 cells and/or RBL-2H3 cells. Right panel: tumor weights expressed as the mean ± SD. (B) IHC staining of Ki-67 in tumors induced by shNf1-SW10 cells with or without RBL-2H3 cells. (C) Concentrations of histamine, IL-6, LTC4, and TNF-α in CM-stimulated RBL-2H3 cells with or without *PLCγ* knocked down. Data are expressed as the mean ± SD of three independent experiments. (D) Xenograft tumors developed in mice at 4 weeks post-injection with a mixture of shNf1-SW10 cells with *PLCγ* knockdown or negative control RBL-2H3 cells. Right panel: tumor weights expressed as the mean ± SD. (E) Representative IHC staining of xenograft tumor tissues with antibodies against IL-6 and TNF-α. $^*P < .05$.

antibody for 30 min at room temperature. The stained sections were treated with diaminobenzidine buffer and counterstained with hematoxylin. One pathologist examined each section under a high-power field (×400) in a double-blinded manner.

2.9. Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic proteins were extracted using the NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Briefly, cells were washed with cold PBS and centrifuged for 3 min at 500 ×*g*. The cell pellet was resuspended in cytoplasmic extraction reagent I (200 μL) with vortexing. The suspension was incubated on ice for 10 min, followed by the addition of cytoplasmic extraction reagent II (11 μL), vortexing, incubation for 1 min on ice, and centrifugation (5 min, 16,000 ×*g*). The supernatant fraction (cytoplasmic extract) was transferred to a cold tube. The pellet fraction was resuspended in the nuclear extraction reagent (100 μL), vortexed for 15 s, and incubated on ice for 10 min to obtain nuclear proteins. Finally, the suspension was centrifuged (10 min, 16,000 ×*g*), and the supernatant was carefully collected.

2.10. Western blotting

RIPA lysis buffer, containing protease inhibitors, was used to lyse cells, and the protein concentration in the lysates was measured using the Bradford method. Protein samples (30 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific binding sites on the membranes were blocked with 5% nonfat milk for 1 h at room temperature. The blots were incubated with primary antibodies for 16 h at 4 °C, followed by incubation with secondary antibodies anti-rabbit or anti-mouse antibody for 1 h at room temperature. Protein bands were photographed using a ChemiDoc XRS molecular imager system (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as the loading control.

2.11. Statistical analysis

A Student's *t*-test was used to analyze differences between two groups with the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). For comparison of three groups, we used one-way ANOVA and Fisher's least-significant difference test with the SPSS for Windows 10.0 statistical software. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. SW10 cells stimulated inflammatory activity of mast cells, and activated mast cells promoted SW10 cell growth

First, we constructed a cNF cell model by transfecting murine Schwann SW10 cells with an *Nf1*-specific shRNA (shNf1) (Fig. 1A), as it is known that a loss-of-function mutation of *Nf1* is critical for cNF development [18]. Considering their activating status in cNF, we focused on the inflammatory activity of mast cells. A previous study has reported that Schwann cells enhance degranulation of mast cells to promote development of neurofibroma [19]. During activation, mast cells can release histamine-containing granules and generate inflammatory mediators, including IL-6, TNF-α, and LTC4 [20]. Therefore, we collected CM from shNf1-SW10 cells to treat BMMCs and RBL-2H3 cells, two mast cell models used in this study. The ELISA results revealed that CM of shNf1-SW10 cells elevated levels of histamine, IL-6, LTC4, and TNF-α in culture media of mast cells (Fig. 1B–E). Considering the important role of mast cells in neurofibroma development, we incubated shNf1-SW10 cells in media from mast cells and found that the media

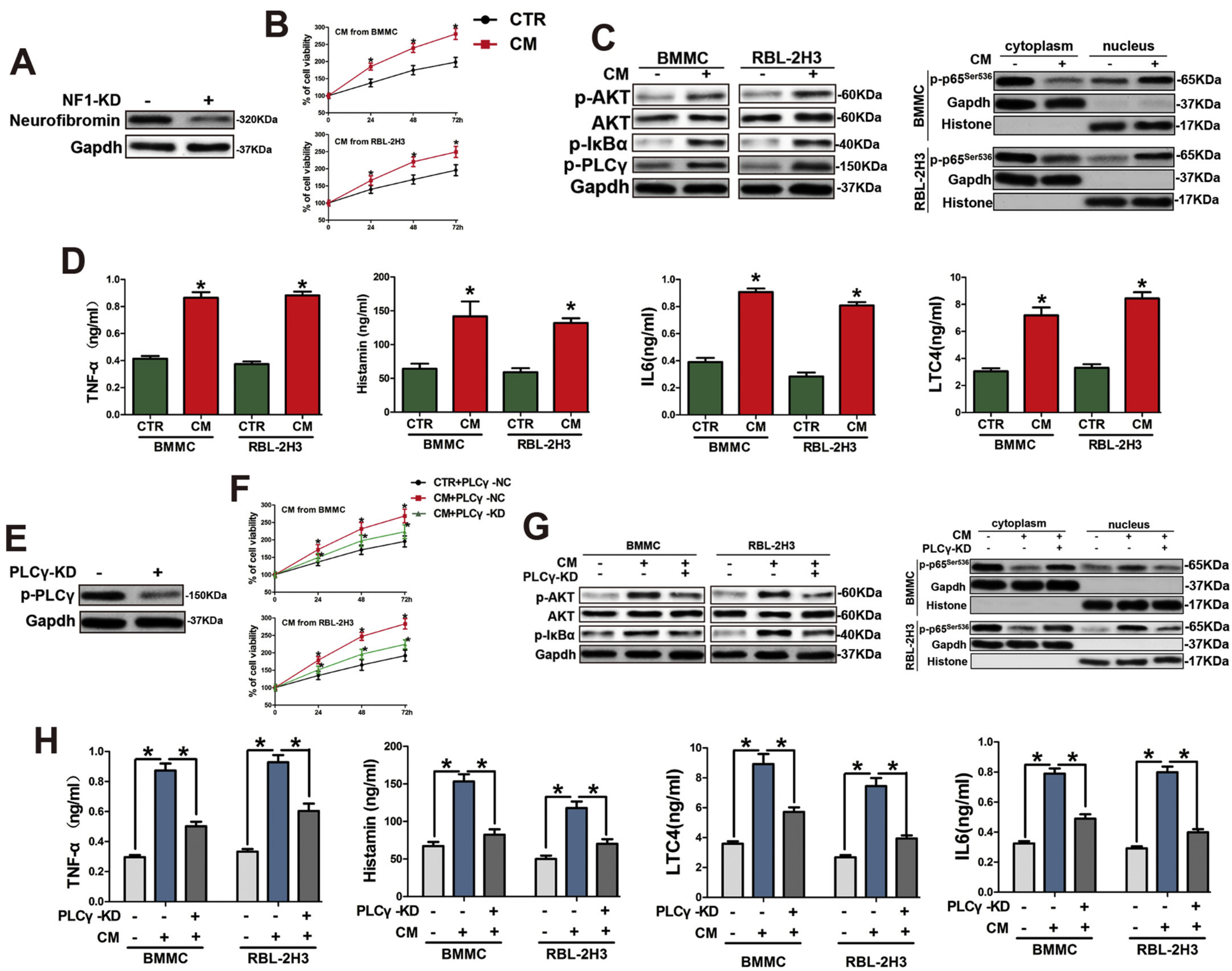

Fig. 7. PLCγ/AKT/IκBα/p65 signaling was important for the inflammatory activity of shNf1-fibroblast cell-stimulated mast cells. (A) Protein levels of neurofibromin in fibroblast cells with or without transfection with a lentivirus containing an *Nf1*-targeting shRNA. (B) Viability of shNf1-fibroblast cells incubated with the medium of shNf1-fibroblast CM-stimulated mast cells. Data are expressed as the mean ± SD of five independent experiments. (C) Protein levels of p-PLCγ, p-AKT, p-IκBα, and nuclear p65 in lysates of CM-stimulated and unstimulated mast cells. (D) Levels of histamine, IL-6, LTC4, and TNF-α secretion by CM-stimulated and unstimulated mast cells. Data are expressed as the mean ± SD of three independent experiments. (E) Protein levels of p-PLCγ in shNf1-fibroblast cells with and without *PLCγ* knocked down. (F) Viability of shNf1-fibroblast cells incubated with media of CM-stimulated and unstimulated mast cells with and without *PLCγ* knocked down. Data are expressed as the mean ± SD of five independent experiments. (G) Protein levels of p-PLCγ, p-AKT, p-IκBα, and nuclear p65 in the above mast cells. (H) Concentrations of histamine, IL-6, LTC4, and TNF-α in the media of the above mast cells. Data are expressed as the mean ± SD of three independent experiments. $^{*}P < .05$.

from CM-activated mast cells promoted the shNf1-SW10 cell growth (Fig. 1F). Consistently, CM-activated mast cells also enhanced the colony formation ability of shNf1-SW10 cells (Fig. 1G). Taken together, these results demonstrated that shNf1-SW10 cells stimulated inflammatory activity of mast cells and activated mast cells in turn promoted the shNf1-SW10 cell growth.

3.2. CM-stimulated mast cells promoted SW10 cell growth via elevated inflammation

According to the important role of inflammation in neurofibromas, we examined whether upregulated inflammatory activity played an important role in mast cell-mediated shNf1-SW10 cell growth. For this purpose, we activated inflammatory activity of mast cells using DNP-HSA (Ag), with or without pretreatment with BAY61-3606, a Syk inhibitor. A medium from IgE-sensitized mast cells without any stimulation was used as a control. As shown in Fig. 2A–D, DNP-HSA elevated the secretion of histamine, IL-6, LTC4, and TNF-α by mast cells, while BAY61-3606 successfully reduced these effects. Similar to the inflammatory activity, the medium from DNP-HSA-stimulated mast cells increased shNf1-SW10 cell viability and colony formation ability, while preincubation with BAY61-3606 attenuated the mast cell-mediated shNf1-SW10 cell growth (Fig. 2E, F). These results indicated that the suppression of inflammation in mast cells could reduce the shNf1-SW10 cell growth and suggested that inflammation was critical for the promotion of shNf1-SW10 cell growth by CM-stimulated mast cells.

3.3. CM stimulation upregulated AKT/IκBα/p65 signaling and p-PLCγ in mast cells

Because active PI3K/AKT and MAPK pathways are essential features of neurofibroma [21] and mast cell activity [22], we determined the activity of these pathways in mast cells. Only phospho-AKT, phospho-IκBα (Fig. 2F), and nuclear p65, a subunit of NF-κB, were found to

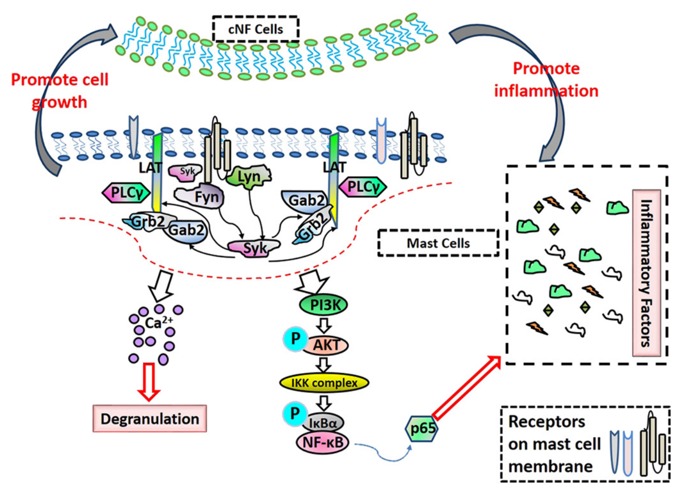

Fig. 8. Schematic representation of the mechanism underlying the interaction of cNF cells and mast cells. cNF cells promoted inflammatory activity of mast cells via the activation of PLCγ/AKT/IκBα/p65 signaling, and inflammatory mast cells facilitated the cNF cell growth.

increase, while no significant alterations were found in protein levels of p-mTOR, p-GSK3β, p-MDM2, p-AS160, and p-PFKFB2 (Fig. 3A and Supplementary Fig. S1), as well as in those of phospho-ERK, phospho-JNK, phospho-p38, and phospho-c-Jun (Fig. 3B). Consistently, inhibition of ERK, p38, or JNK failed to reverse the increase of inflammation by CM (Supplementary Fig. S2A–C), while inhibition of AKT or p65 attenuated the increase (Supplementary Fig. S3A, B). These results indicated that CM stimulation promoted inflammation in mast cells via the activation of AKT/IκBα/p65 signaling.

In mast cell activation, tyrosine kinases of the Syk and Src families, including Lyn and Fyn, are responsible for the initiation of inflammation-associated signaling [23]. Activated Syk can further activate multiple molecules, including the linker for activated T cells (LAT) and the signaling enzyme PLCγ [23], thus facilitating inflammatory signaling. The results presented in Fig. 3C show that CM stimulation upregulated the level of p-PLCγ, whereas no changes were found in the expression levels of other molecules. Moreover, inhibition of Syk did not reduce CM-upregulated inflammation (Supplementary Fig. S2D), while inhibition of PLCγ could successfully temper the upregulation (Supplementary Fig. S3C).

Collectively, these results suggested that CM of shNf1-SW10 cells stimulated AKT/IκBα/p65 signaling and upregulated p-PLCγ in mast cells.

3.4. Inhibition of AKT, NF-κB, or PLCγ in mast cells impeded the mast cell-mediated shNf1-SW10 cell growth

Because of the enhanced PLCγ and AKT/IκBα/p65 signaling in CM-stimulated mast cells, we next explored the role of this signaling in mast cell-mediated shNf1-SW10 cell growth by preincubating BMMCs and RBL-2H3 cells with LY294002, an AKT inhibitor, before stimulation with CM. The MTT and colony formation assays indicated that LY294002-treated mast cells showed a reduced ability to promote the shNf1-SW10 cell growth (Fig. 4A, B). Likewise, an inhibitor of NF-κB signaling, BAY11-7082, reduced the enhancing effect of CM-stimulated mast cells on the viability of shNf1-SW10 cells (Fig. 4C, D). Furthermore, treatment of CM-stimulated BMMCs and RBL-2H3 cells with U73122, a PLCγ inhibitor, reduced their ability to promote the shNf1-SW10 growth (Fig. 4E, F). In addition, U73122 treatment decreased the protein levels of p-AKT, p-IκBα, and nuclear p65 in CM-stimulated mast cells, which indicated that inhibition of PLCγ could reduce AKT/IκBα/p65 signaling (Fig. 4G).

Taken together, these results suggested that CM-stimulated mast cells promoted the shNf1-SW10 cell growth through PLCγ/AKT/IκBα/p65 signaling. Combined with the results shown in Supplementary Figs. S3 and S4, these findings further demonstrated that increased inflammation might be responsible for the promotion of shNf1-SW10 cell growth.

3.5. PLCγ modulated AKT/IκBα/p65 signaling during interaction of mast cells and shNf1-SW10 cells

Considering that PLCγ plays an important role in regulation of inflammatory activity of mast cells [24,25], we explored the role of PLCγ in the CM-stimulated activity of mast cells and subsequent stimulation of shNf1-SW10 cell growth. We transfected BMMCs and RBL-2H3 cells with a lentivirus containing a PLCγ-targeting shRNA to obtain *PLCγ* knockdown mast cells (Fig. 5A). Under stimulation with CM, PLCγ-deficient mast cells showed reduced p-AKT, p-IκBα, and nuclear p65 expression (Fig. 5B). Consistently, PLCγ-deficient mast cells showed a weakened ability to promote the shNf1-SW10 growth (Fig. 5D).

Furthermore, we overexpressed PLCγ in BMMCs and RBL-2H3 cells via lentiviral transfection (Fig. 5E), which resulted in upregulation of p-AKT, p-IκBα, and nuclear p65 (Fig. 5F). To investigate whether PLCγ regulates the activation of mast cells through AKT/IκBα/p65 signaling, we used BAY11-7082, an inhibitor of NF-κB signaling, to treat PLCγ-overexpressing (PLCγ-OE) mast cells. CM-stimulated PLCγ-OE mast cells showed an increased ability to promote the shNf1-SW10 cell growth, while BAY11-7082 treatment significantly reduced this effect (Fig. 5G, H).

Taken together, these results suggested that PLCγ played a critical role in the promotion of shNf1-SW10 cell growth by CM-stimulated mast cells via the modulation of AKT/IκBα/p65 signaling.

3.6. Promotion of the shNf1-SW10 cell growth depended on the inflammatory activity of mast cells in an in vivo model

The in vitro data suggested that PLCγ/AKT/IκBα/p65 signaling participated in cNF-associated mast cell inflammation and mast cell-stimulated shNf1-SW10 cell growth, indicating that targeting this signaling may be a strategy to treat cNF. We then generated a subcutaneous tumor xenograft model to study in vivo effects of cNF and mast cell interaction. After 4 weeks of growth, the weight of the tumors induced by shNf1-SW10 cells mixed with RBL-2H3 cells was upregulated compared with that of shNf1-SW10 cell-derived tumors (Fig. 6A), while RBL-2H3 cells alone failed to form tumors (data not shown). Meanwhile, a higher level of Ki-67 was detected in the tumors induced by shNf1-SW10 cells in combination with RBL-2H3 cells, which further confirmed that mast cells accelerated the shNf1-SW10 cell growth in vivo (Fig. 6B). However, no significant difference was found in the body weight between the two groups of mice (Supplementary Fig. S4A).

Next, we evaluated whether the cNF cell growth promotion by mast cells was dependent on the level of inflammation. Considering that antitumor activity cannot be ruled out for inflammation-suppressing drugs, we knocked down *PLCγ* to inhibit the inflammatory activity of RBL-2H3 cells. ELISAs showed diminished concentrations of histamine, IL-6, LTC4, and TNF-α in the medium of *PLCγ* knockdown (PLCγ-KD) RBL-2H3 cells (Fig. 6C). Moreover, the subcutaneous xenograft tumors generated using a mixture of shNf1-SW10 cells and PLCγ-KD or PLCγ-NC RBL-2H3 cells showed that PLCγ-KD mast cells had an impaired ability to promote the shNf1-SW10 growth in vivo (Fig. 6D). Similarly, no significant difference was found in the body weight between the two groups (Supplementary Fig. S4B). An IHC assay showed reduced levels of IL-6 and TNF-α, accompanied by a diminished Ki-67 level, in the tumors containing PLCγ-KD RBL-2H3 cells, which indicated that the repression of inflammatory activity of mast cells through targeting PLCγ could successfully weaken the cNF growth (Fig. 6E).

Taken together, these results suggested that mast cells could accelerate the shNf1-SW10 cell growth in vivo, while inhibition of inflammation through targeting PLCγ inhibited the mast cell-mediated cNF growth.

3.7. PLCγ/AKT/IκBα/p65 signaling played an important role in the interaction of shNf1-fibroblasts and mast cells

Fibroblast cells are another important component in cNF and can be used as a cell model of cNF. Thus, we employed fibroblast cells to further confirm the interaction of cNF cells and mast cells. First, we knocked *Nf1* down in fibroblasts (Fig. 7A) and collected CM from a shNf1-fibroblast cells. Consistent with the previously discussed data, the viability of shNf1-fibroblast cells increased with medium from CM-treated mast cells (Fig. 7B). Mast cells stimulated with CM from shNf1-fibroblast cells also showed upregulation of p-AKT, p-IκBα, p-PLCγ, and nuclear p65 (Fig. 7C). Moreover, CM from shNf1-fibroblast cells increased the secretion of histamine, IL-6, LTC4, and TNF-α by mast cells (Fig. 7D).

To clarify the role of PLCγ in the interaction of cNF cells and mast cells, we knocked down *PLCγ* in shNf1-fibroblasts (Fig. 7E). The data showed that *PLCγ* knockdown reduced the ability of shNf1-fibroblast-stimulated mast cells to promote shNf1-fibroblast cell viability (Fig. 7F) and also reversed the upregulation of p-AKT, p-IκBα, p-PLCγ, and nuclear p65 in CM-induced mast cells (Fig. 7G). Moreover, downregulation of PLCγ weakened the CM-caused increases in the levels of histamine, IL-6, LTC4, and TNF-α in the mast cell medium (Fig. 7H).

Taken together, these results further demonstrated that cNF cells promoted the inflammatory activity of mast cells via PLCγ/AKT/IκBα/p65 signaling and mast cells in turn promoted the cNF cell growth.

4. Discussion

Previous studies demonstrated that a microenvironment containing mast cells is required for cNF formation [6,10,26]. Dislodgement of mast cells induced shrinkage and impaired the development of neurofibroma [6,10], while CM from *Nf1*$^{-/-}$ Schwann cells enhanced mast cell degranulation [19] and recruitment [27]. Clinical observations showed that cNF occurred after tissue injury, which involved inflammation for tissue repair. Contribution of inflammation to neoplasia has been recognized and associated with the abundant production of cytokines and growth factors by inflammatory cells [28,29]. Long-term usage of ketotifen, a drug with antihistamine and anti-inflammatory activities, was successful in relieving human cNF [12]. In this context, our study further demonstrated that cNF cell-stimulated mast cells showed strong inflammation, which in turn contributed to cNF development. Moreover, mast cells with reduced inflammatory activity showed a reduced ability to promote cNF cell growth.

Infiltrating mast cells, widely observed in neurofibromas [13,30], remodel the microenvironment via chemokines such as CCL2, CCL5, and CXCL8 and cytokines such as IL-3, IL-6, and GM-CSF, thereby promoting tumorigenesis [31]. In neurofibroma, upregulated levels of both serum IgE and local histamine suggest the activated status of neurofibroma-associated mast cells [14,30]. Similarly, macrophages in neurofibroma showed a tendency of M1 polarization [32], which indicated their inflammatory status. Previous studies have suggested an important role for PI3K/AKT/SCF/KIT signaling in mediating neurofibroma-associated degranulation and recruitment of mast cells [10,27,33], and a KIT inhibitor showed certain effects in neurofibroma clinical therapy. However, some patients failed to respond to either KIT inhibition or to ketotifen [11]. Hence, SCF/KIT signaling may be an important but not the only pathway modulating mast cells, suggesting that other mechanisms are involved in the regulation of mast cell activity. In our study, we found that PI3K/AKT/p-IκBα/p65 signaling was critical for SW10 cell-stimulated mast cell inflammation, and its targeting could reduce the inflammatory activity of mast cells and subsequent cNF cell growth.

The functional flexibility and phenotypic plasticity of mast cells are due to differential expression of multiple receptors, including Fc, trimeric G protein-coupled receptors, and tyrosine kinases [16]. Most of these receptors can act in concert with the IgE receptor to modulate the inflammatory response in mast cells [34]. The main pattern of inflammatory activity starts from the FcεRI-induced activation of spleen tyrosine kinase (Syk) and phosphorylation of LAT and is followed by the activation of PLCγ [35]. In our study, we found that PLCγ played a critical role in the interaction of SW10 cells and mast cells. Inhibition of PLCγ significantly repressed inflammatory activity of mast cells and the subsequent growth of cNF cells. The activated upstream pathways initiated downstream signaling, such as MAPK, PI3K/AKT, and NF-κB signaling pathways. Dysregulation of the transcription factor NF-κB is related to some inflammatory diseases [36] and is a target of several anti-inflammatory drugs [37]. In our study, we demonstrated that the increased inflammatory activity of CM-stimulated mast cells was modulated via PLCγ/AKT/p-IκBα/p65 signaling, which is known to involve translocation of p65 to the nucleus to activate various inflammatory genes [38].

5. Conclusion

In conclusion, cNF cells could stimulate inflammation in mast cells, and enhanced inflammation played a key role in the promotion of cNF cell growth by activated mast cells in vivo and in vitro. Mechanistically, phosphorylated PLCγ modulated AKT/p-IκBα/p65 signaling in mast cells to regulate their inflammatory activity. The newly identified pathway may provide an effective target for cNF clinical therapy (Fig. 8).

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Author contributions

JJ. and JX. designed the experiments, and JJ. received funding for the study. MS performed the animal experiments and provided assistance in other assays. HZ, CL, and HD performed the cell experiments. JX. analyzed the data. JJ. and MS wrote the manuscript. All the authors have read and revised the manuscript and approved its submission.

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Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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