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GYF-21, an epoxide 2-(2-phenethyl)-chromone derivative, suppresses dysfunction of B cells mainly via inhibiting BAFF activated signaling pathways

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ABSTRACT

Activated B cells targeted to autoantigens proliferate and differentiate into antibody-secreting cells. Overproduced autoantibodies will give rise to autoimmune diseases. In this study, we investigated the inhibitory effects of GYF-21, an epoxide 2-(2-phenethyl)-chromone derivative extracted from Chinese agarwood, on the survival, activation, proliferation, and differentiation of B cells for revealing its potential to treat autoimmune diseases related to B cell dysfunction. The results showed that GYF-21 slightly inhibited the survival, activation and proliferation of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4 while weakly up-regulated differentiation of B cells induced by combination of anti-CD40 and IL-4. In addition, GYF-21 intensively suppressed survival, activation, proliferation, and differentiation of B cells stimulated by B-cell activating factor (BAFF) which plays extremely important roles in autoantibody production and pathogenesis of autoimmune diseases. The mechanism study revealed that GYF-21 slightly down-regulated phosphorylation of NF-κB p65, Akt, STAT3, but up-regulated phosphorylation of Erk1/2 in B cells activated by anti-IgM, anti-CD40, IL-4 or their combinations. However, GYF-21 not only moderately down-regulated phosphorylation of NF-κB p65 and MAPK p38, but also intensively inhibited phosphorylation of Erk1/2 and Akt induced by BAFF. These data suggest the inhibitory effects of GYF-21 on the survival, activation, proliferation, and differentiation of B cells mainly via blocking BAFF activated signaling pathways, and its potential to be developed into therapeutic drug for autoimmune diseases, especially systemic lupus erythematosus (SLE).

1. Introduction

B lymphocytes are the effectors of adaptive immunity, providing defense against pathogens through different functions including antibody production. These antigen-specific antibodies are secreted by plasma cells, which arise from proliferation and differentiation of activated B cells [1]. Although B cells play very important roles in disease defense, autoimmune diseases can result from abnormal B cell recognition of self-antigens followed by production of autoantibodies [2]. In the past decade, improved understanding of the immune response and abnormalities in autoimmune diseases have allowed the recognition of B cells that are crucial to the development of systemic lupus erythematosus (SLE) and other autoimmune diseases [3–6]. Both mice and human subjects with SLE show evidences of over-activated polyclonal B cells which produce a number of autoantibodies against self-antigens [7,8].

Differentiation of B cells and subsequent antibody production are usually induced upon triggering of the B cell receptor (BCR) by antigen and provision of costimulatory signals and cytokines from T helper lymphocytes or B-cell activating factor (BAFF) produced by dendritic cells, macrophages and monocytes [9–12]. BAFF is a crucial factor that regulates maturation, survival, activation, proliferation and differentiation of polyclonal B cells through three receptors, B-cell activating factor receptor (BAFF-R), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B-cell maturation antigen (BCMA) [13,14]. Nevertheless, overproduction of BAFF has been shown to lead to the pathogenesis of autoimmune disorders in animal models, and BAFF concentrations are known to be higher in patients with various autoimmune conditions compared with normal subjects. On March 9, 2011, the Food and Drug Administration approved belimumab, a humanized anti-BAFF monoclonal antibody, as a new treatment for SLE by inhibiting B cell overreaction. However, blockade of

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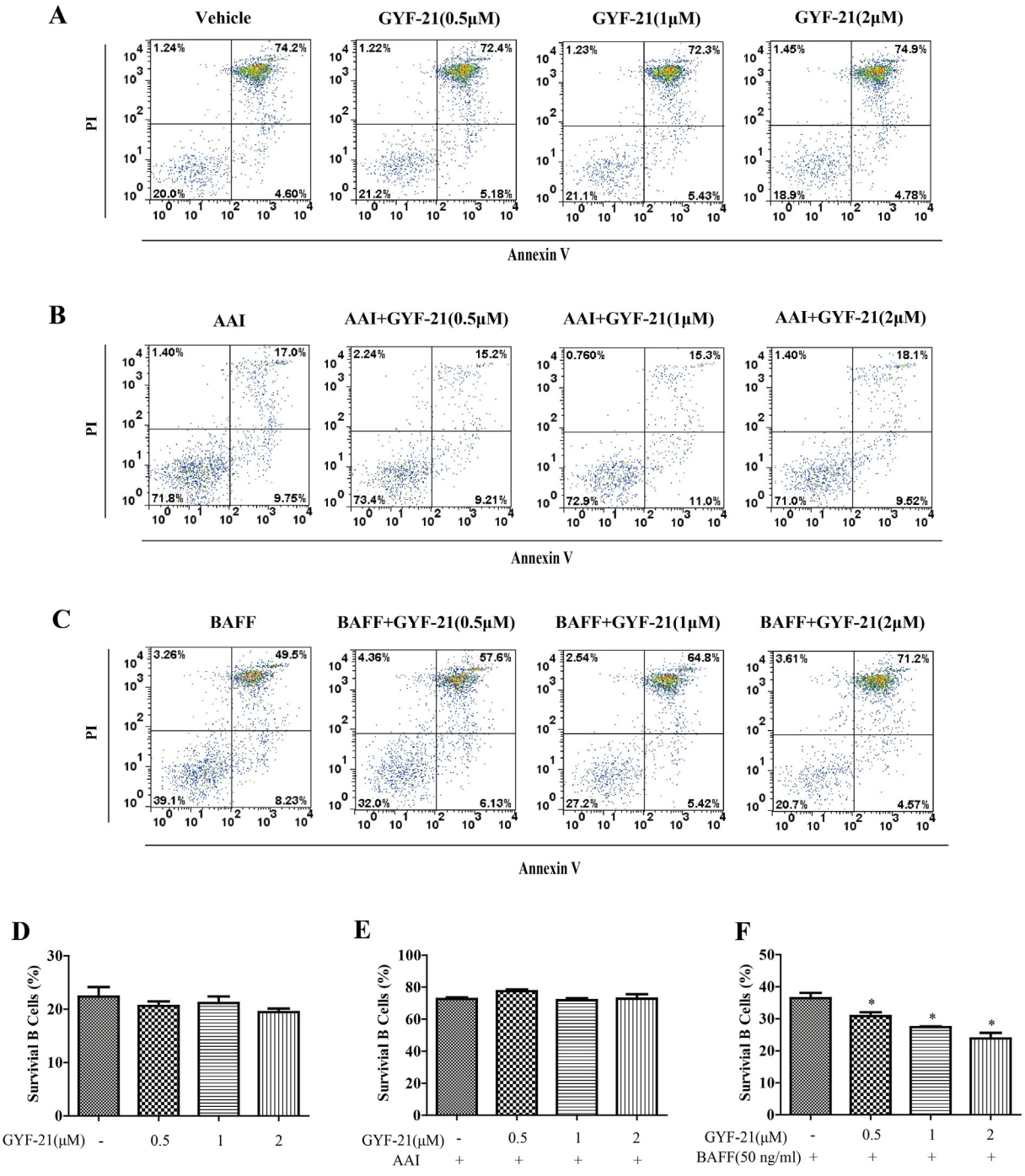

Fig. 1. Effects of GYF-21 on the survival of B cells. Splenocytes were pretreated with various concentrations of GYF-21 for 0.5 h and then stimulated with combination of anti-IgM, anti-CD40 and IL-4 (AAI) or BAFF for 24 h. The cells were collected and stained with anti-B220-APC, Annexin V-FITC, and PI. The percentages of survival B cells ($B220^+$ Annexin V^- PI^-) were analyzed by flow cytometry (A–F). #$P < 0.05$, significantly different from Vehicle group; *$P < 0.05$, significantly different from AAI-treated or BAFF-treated group.

BAFF with anti-BAFF antibody resulted in significant but only small beneficial clinical effects in the treatment of SLE [15], probably because a proliferation-inducing ligand (APRIL), a similar cytokine with BAFF, hadn't been blocked. Currently, there is a fusion protein of TACI-immunoglobulin which is named as atacicept and under development to treat SLE by blocking both BAFF and APRIL to binding to their receptors. In a phase IIb clinical study, atacicept showed promising results in treatment of SLE and could be an encouraging therapeutic agent for SLE [7,16]. In addition to biotherapy, we propose that blocking signaling pathways induced by BAFF and APRIL with small molecule compound is another promising choice for SLE therapy.

In our previous study, we isolated and purified dozens of 2-(2-phenethyl)-chromone derivatives from ethyl acetate extract and petroleum ether extract of Chinese agarwood. In the immunomodulatory activity screening, we identified that several 2-(2-phenethyl)-chromone derivatives remarkably suppressed inflammation response, suggesting that these 2-(2-phenethyl)-chromone derivatives may have potential immunosuppressive activity. Among these active 2-(2-phenethyl)-chromone derivatives, the compound, (1a*S*, 2*S*, 3*S*, 7b*R*)-2, 3-dihydroxy-5-(4-methoxyphenethyl) -2, 3-dihydro-1a*H*-oxireno [2, 3-

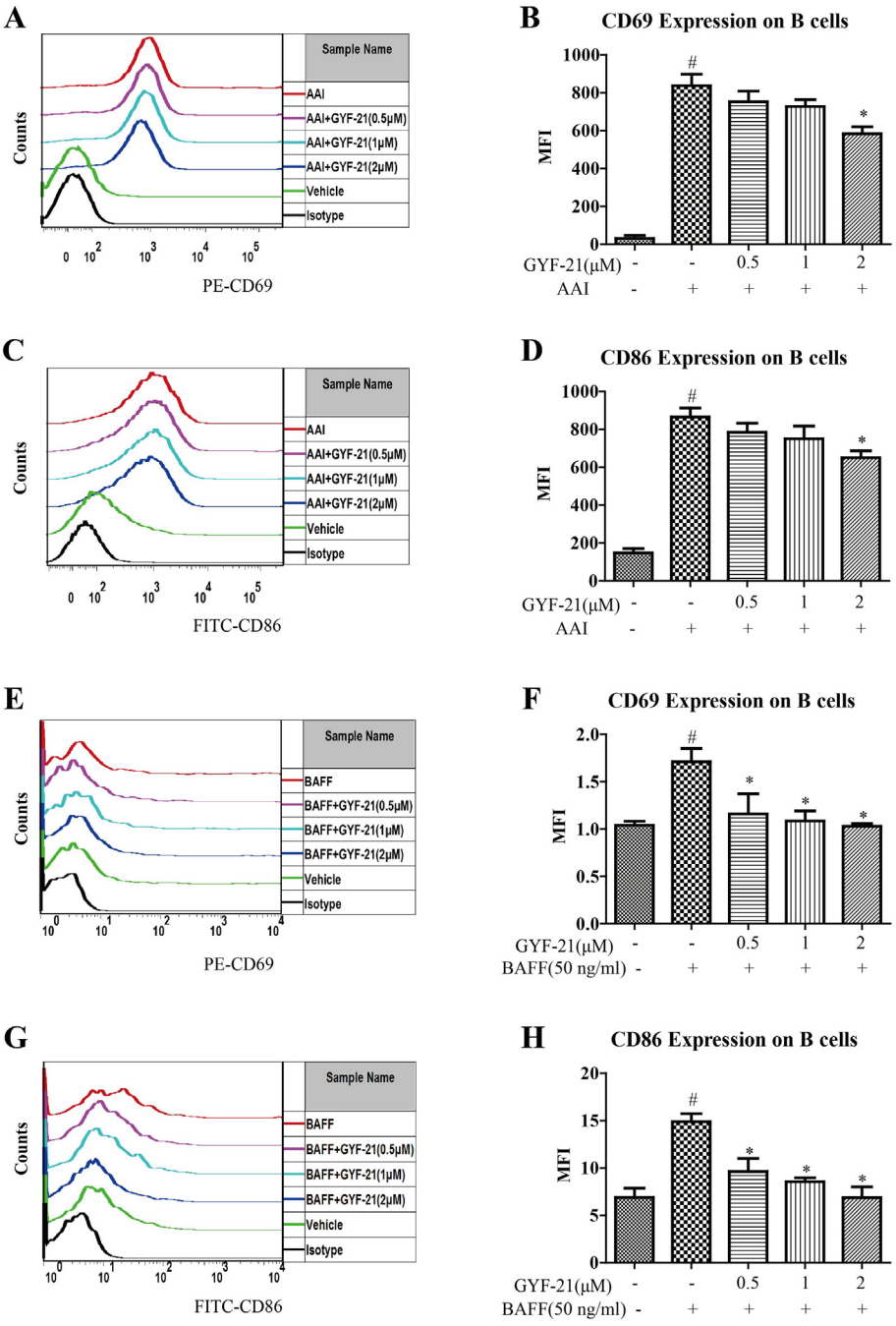

Fig. 2. Effects of GYF-21 on the activation of B cells. Splenocytes were pretreated with various concentrations of GYF-21 for 0.5 h and then stimulated with combination of anti-IgM, anti-CD40 and IL-4 (AAI) or BAFF for 24 h. The expressions of CD69 (A, B, E, and F) and CD86 (C, D, G, and H) on B cells were determined by flow cytometry. $\#P < 0.05$, significantly different from Vehicle group; $*P < 0.05$, significantly different from AAI-treated or BAFF-treated group.

f] chromen-7 (7b*H*)-one (GYF-21), which was firstly reported by Wu et al. [17], showed the most excellent activity [18]. In the present study, we further investigated the inhibitory activity of GYF-21 on the survival, activation, proliferation, differentiation of B cells induced by multiple stimulants, especially BAFF. Then the underlying mechanisms that GYF-21 exerts effects and it's potential to treat SLE and other autoimmune disease were revealed.

2. Materials and methods

2.1. Materials

GYF-21 was isolated from Chinese agarwood (purity > 94%) and the isolation method was described previously [18]. GYF-21 was prepared in dimethyl sulfoxide (DMSO) as a stock solution at the concentration of 10 mM. Carboxyfluorescein diacetate *N*-succinimidyl ester (CFDA-SE), 5-bromo-2′-deoxyuridine (BrdU), and deoxyribonuclease I (DNase I) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-B220-APC (RA3-6B2), anti-CD86-PE (GL1), anti-CD69-PE (H1.2F3), anti-CD138-PE (281-2), and 7-aminoactinomycin D (7-AAD) were purchased from Becton Dickinson (San Diego, CA, USA). Anti-BrdU-FITC (3D4) was from Biolegend (San Diego, CA, USA). Magnetic bead isolation kit for mouse B cells was obtained from Miltenyl Biotec (Bergisch Gladbach, Germany). Purified anti-IgM F(ab′)$_2$ was purchased from Jackson ImmunoResearch (Soham, Cambridgeshire, UK). Purified anti-CD40 (FGK4.5) was from Bio X cell (West Lebanon, NH, USA).

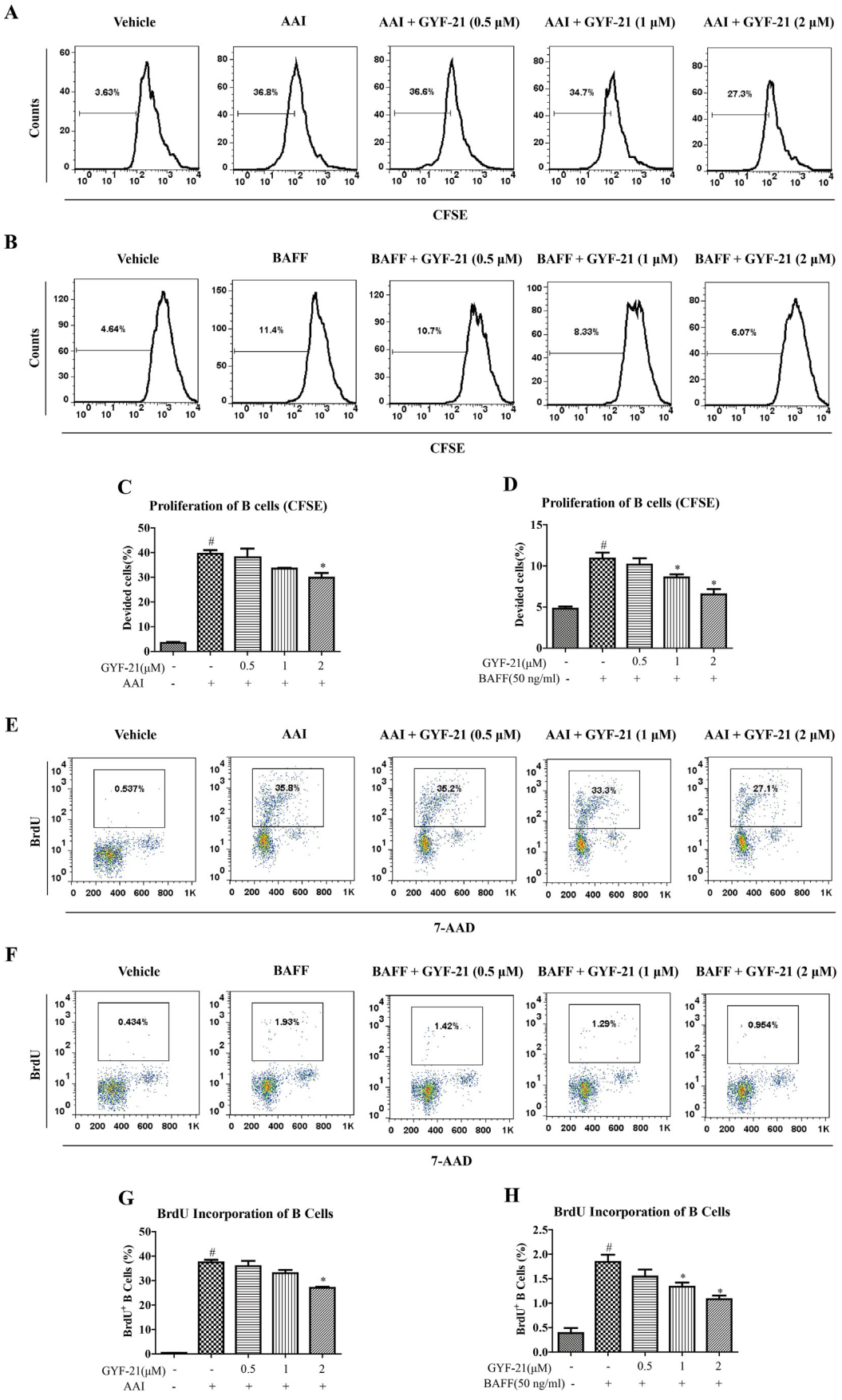

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Fig. 3. Effects of GYF-21 on the proliferation of B cells. Splenocytes were stained by CFSE and seeded in 24-well plate. Then the cells were pretreated with various concentrations of GYF-21 for 0.5 h and then stimulated with combination of anti-IgM, anti-CD40 and IL-4 (AAI) or BAFF for 72 h. The division of B cells was analyzed by CFSE quantitation with flow cytometry (A, B, C and D). In some experiments, splenocytes were stimulated with AAI or BAFF for 36 h and BrdU were added into each well for last 12 h. After surface staining with anti-B220-APC, the cells were fixed, permeabilized, and stained with anti-BrdU-FITC and 7-AAD. BrdU incorporated B cells were analyzed with flow cytometry. #$P < 0.05$, significantly different from Vehicle group; *$P < 0.05$, significantly different from AAI-treated or BAFF-treated group.

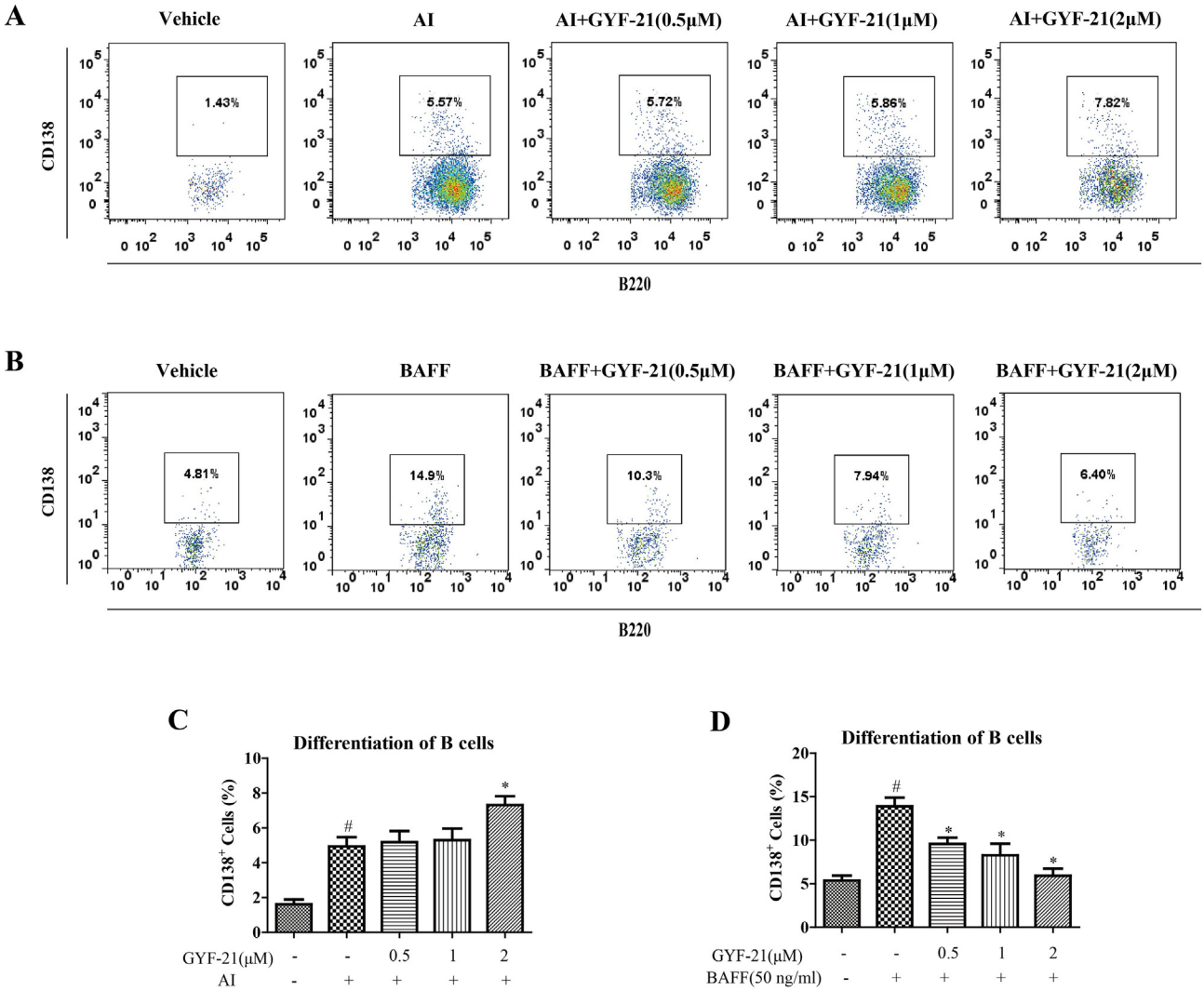

Fig. 4. Effects of GYF-21 on the differentiation of B cells. Splenocytes were seeded in 24-well plate and pretreated with various concentrations of GYF-21 for 0.5 h. Then the cells were stimulated with combination of anti-CD40 and IL-4 (AI) or BAFF for 72 h. The percentage of 7-AAD$^-$ B220$^+$ CD138$^+$ cells was analyzed by flow cytometry (A, B, C and D). #$P < 0.05$, significantly different from Vehicle group; *$P < 0.05$, significantly different from AI-treated or BAFF-treated group.

Recombinant mouse interleukin-4 (IL-4) was obtained from PeproTech (Rocky Hill, NJ, USA). Recombinant mouse BAFF and enzyme-linked immunosorbent assay (ELISA) kits for mouse IgM and IgG were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies to β-actin, p38, Erk1/2, p65, Akt, STAT3, STAT6, phopho-p38 (Thr180/Tyr182), phopho-Erk1/2 (Thr202/Tyr204), phospho-p65 (Ser536), phospho-Akt (Thr308), phospho-Akt (Ser473), phospho-STAT3 (Tyr705) and phospho-STAT6 (Tyr641) were purchased from Cell signaling Technology (Beverly, MA, USA).

2.2. Animals

Male BALB/c mice (8–10 weeks old) were from Charles River Laboratory China (Beijing, China) and housed under specific pathogen-free and climate-controlled conditions. The study was approved by the Ethical Committee on Animal Research in Beijing University of Chinese Medicine. All experimental procedures conformed to the guidelines in Beijing University of Chinese Medicine (Beijing, China).

2.3. Cell isolation and culture

Splenocytes were prepared from the spleens of BALB/c mice as described [18]. In brief, spleen was cut, homogenized, and filtered through a 70 μm cell strainer to generate single-cell suspension. Red blood cells were depleted by osmotic lysis. The remaining splenocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. To purify B cells, splenocytes were resuspended in 1 ml magnetic activated cell-sorting (MACS) buffer (PBS, 2 mM EDTA, 0.5% BSA) and isolated with negative magnetic bead separation kit of B cells according to the manufacturer's protocols. Cell purity was analyzed using flow cytometry (> 93%).

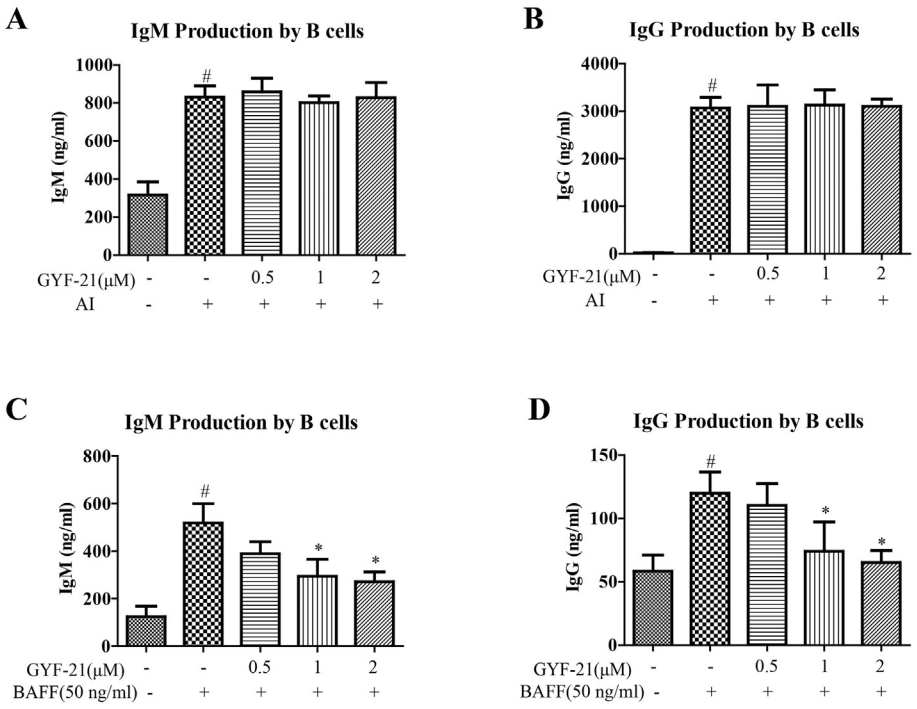

Fig. 5. Effects of GYF-21 on the antibody production during B cell differentiation. In differentiation analysis of B cells, the levels of mouse IgM and IgG in the medium were evaluated with ELISA kits according to the manufacturer's protocols (A, B, C and D). #$P < 0.05$, significantly different from Vehicle group; *$P < 0.05$, significantly different from AI-treated or BAFF-treated group.

2.4. Survival and activation analysis of B cells

Splenocytes were inoculated in 24-well plate (1×10^6 cells/well). The cells were pretreated with or without series concentrations of GYF-21 (the final concentration of DMSO was 0.02% and added into all treatment and control groups) for 0.5 h and stimulated with combination of anti-IgM (10 μg/ml), anti-CD40 (10 μg/ml) and IL-4 (10 ng/ml) or BAFF (50 ng/ml) for 24 h. In survival analysis of B cells, the cells were collected and stained with anti-B220, Annexin V-FITC and PI, then percentage of survival B cells ($B220^+$ Annexin V^- PI^-) were determined by flow cytometry on BD FACSCanto II (BD Biosciences, San Diego, CA, USA). In the activation analysis of B cells, the expressions of CD69 and CD86 on 7-AAD^- $B220^+$ B cells were also evaluated by flow cytometry.

2.5. Proliferation analysis of B cells with CFSE

Splenocytes were stained by CFDA-SE (5 μM) for 10 min at room temperature and protected from light, washed and seeded in 24-well plate (1×10^6 cells/well). The cells were pretreated with or without GYF-21 for 0.5 h and then induced with combination of anti-IgM, anti-CD40 and IL-4 or BAFF for 72 h. The division of 7-AAD^- $B220^+$ B cells was analyzed by fluorescence quantitation of CFDA-SE product with flow cytometry.

2.6. Proliferation analysis of B cells with BrdU

In some experiments, splenocytes were seeded in 24-well plate (1×10^6 cells/well). The cells were treated with or without GYF-21 for 0.5 h and then stimulated with combination of anti-IgM, anti-CD40 and IL-4 or BAFF for 36 h. Then BrdU was added into each well with a final concentration of 10 μM for last 12 h. The cells were collected and stained anti-B220 on the surface. Subsequently, the cells were fixed with 1% paraformaldehyde for 30 min, and permeabilized with PBS including 0.25% of Triton X-100 for 15 min. After repeated washing, the cells were treated with DNase I at the concentration of 25 μg/ml at 37 °C for 1 h and stained with anti-BrdU-FITC and 7-AAD according to the manufacturer's protocol. Then BrdU incorporated B cells were analyzed with flow cytometry.

2.7. Differentiation analysis of B cells

Splenocytes were seeded in 24-well plate (1×10^6 cells/well). The cells were treated with or without GYF-21 for 0.5 h and then stimulated with combination of anti-CD40 (10 μg/ml) and IL-4 (10 ng/ml) or BAFF (50 ng/ml) for 72 h. Then the percentage of 7-AAD^- $B220^+$ $CD138^+$ plasma cells or plasmablasts in 7-AAD^- $B220^+$ cells was detected by flow cytometry.

2.8. Enzyme linked immunosorbent assay

In differentiation analysis of B cells, the levels of mouse IgM and IgG in the medium were evaluated with ELISA kits according to the manufacturer's protocols.

2.9. Western blot analysis

Purified B cells were inoculated in 6-well plate (4×10^6 cells/well). The cells were treated with or without GYF-21 for 0.5 h and then activated with anti-IgM, anti-CD40, IL-4 or their combinations for 3 h. In some experiments, the cells were stimulated with BAFF for 1 h. After stimulation, the cells were collected and total cell proteins were extracted with RIPA lysis buffer. Then proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes. The membranes were blocked in 5% skim milk for 1 h and incubated overnight at 4 °C with monoclonal antibodies against β-actin, p38, Erk1/2, p65, Akt, STAT3, STAT6 and their phosphorylated forms. Membranes were rinsed and incubated with horseradish peroxide-conjugated secondary antibodies for 2 h at room temperature, then rinsed again. Blot images were acquired with enhanced chemiluminescence reagents on ImageQuant™ LAS3000 (GE Healthcare Bio-Sciences, Pittsburgh, PA).

2.10. Statistical analysis

All qualitative data were represented by one typical experiment and quantitative data were expressed as mean ± standard error (SEM) of values from three or more independent experiments. Statistical analyses

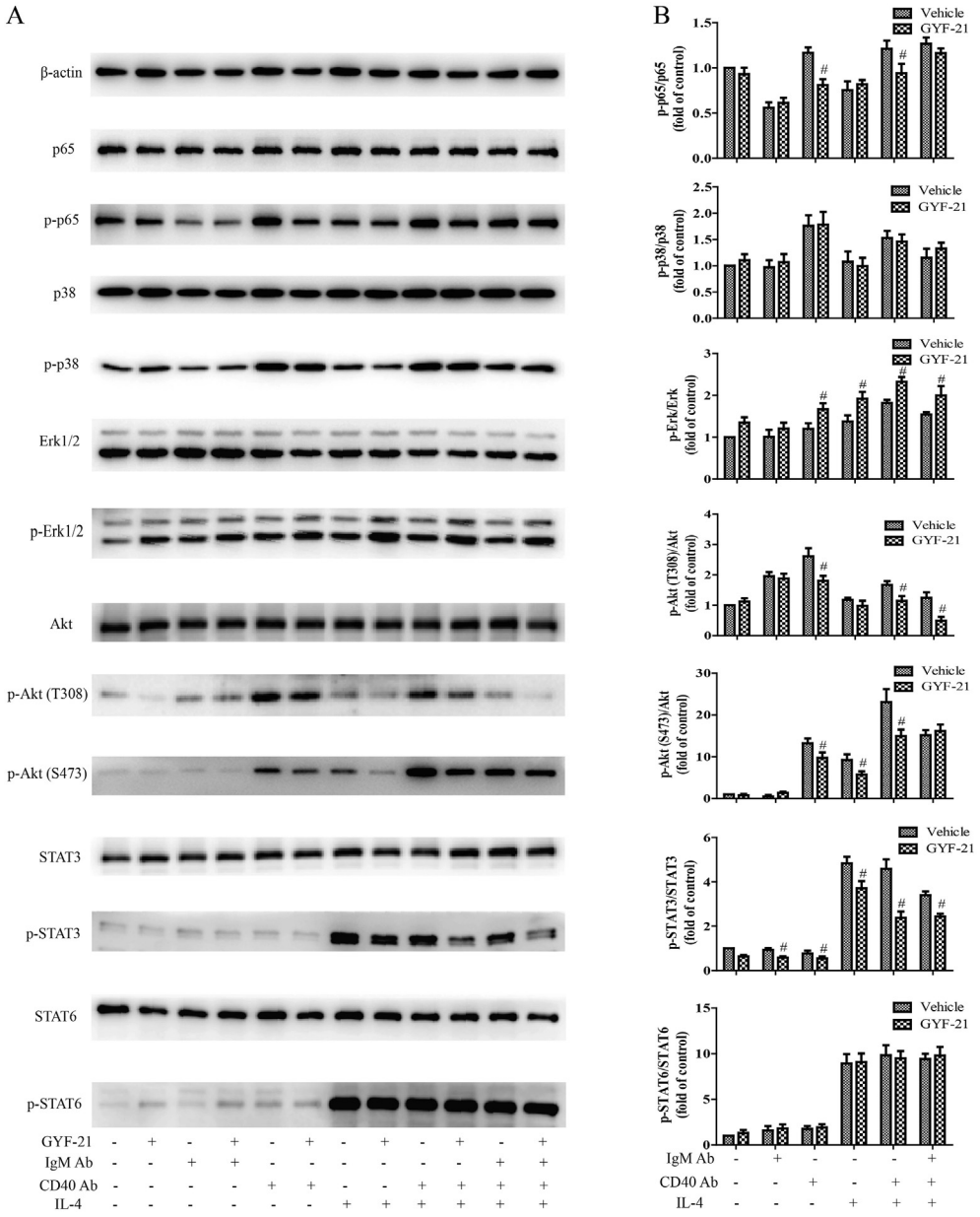

Fig. 6. Effects of GYF-21 on the activation of signaling pathways in B cells stimulated by anti-IgM, anti-CD40, IL-4, or their combinations. Splenocytes were seeded in 6-well plate and pretreated with GYF-21 (2 μM) for 0.5 h. Then the cells were stimulated with anti-IgM, anti-CD40, IL-4 or their combinations for 3 h. The phosphorylation of p65, p38, Erk1/2, Akt, STAT3 and STAT6 were analyzed by western blot (A). The level of each protein was quantified using Quantity One software and normalized to β-actin (B). $\#P < 0.05$, significantly different from Vehicle group.

were performed using GraphPad Prism 5.0. One-way ANOVA followed by Dunnett's post hoc test was used to analyze statistical significance. The significance level was set at 0.05.

3. Results

3.1. Effects of GYF-21 on the survival of B cells

Because BAFF plays important roles in promoting survival of B cells, we firstly investigated the effects of GYF-21 on the survival of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4 or BAFF. As shown in Fig. 1, combination of anti-IgM, anti-CD40 and IL-4 or BAFF significantly increased the survival of B cells. Under doses without cytotoxicity, GYF-21 intensively inhibited survival of B cells induced by BAFF. On the other hand, GYF-21 only slightly suppressed survival of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4.

3.2. Effects of GYF-21 on the activation of B cells

In order to evaluate the effects of GYF-21 on the activation of B cells, we investigated the effects of GYF-21 on activated B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4 or BAFF. As shown in Fig. 2, combination of anti-IgM, anti-CD40 and IL-4 or BAFF significantly stimulated up-regulation of CD69 and CD86 on B cells. However, GYF-21 inhibited the up-regulation of CD69 and CD86 resulted from stimulations by combination of anti-IgM, anti-CD40 and IL-4 or BAFF. Especially, GYF-21 almost completely reversed up-regulation of CD69 and CD86 on BAFF stimulated B cells.

3.3. Effects of GYF-21 on the proliferation of B cells

After the inhibitory effects of GYF-21 on the activation of B cells were discovered, we furtherly investigated the effects of GYF-21 on the

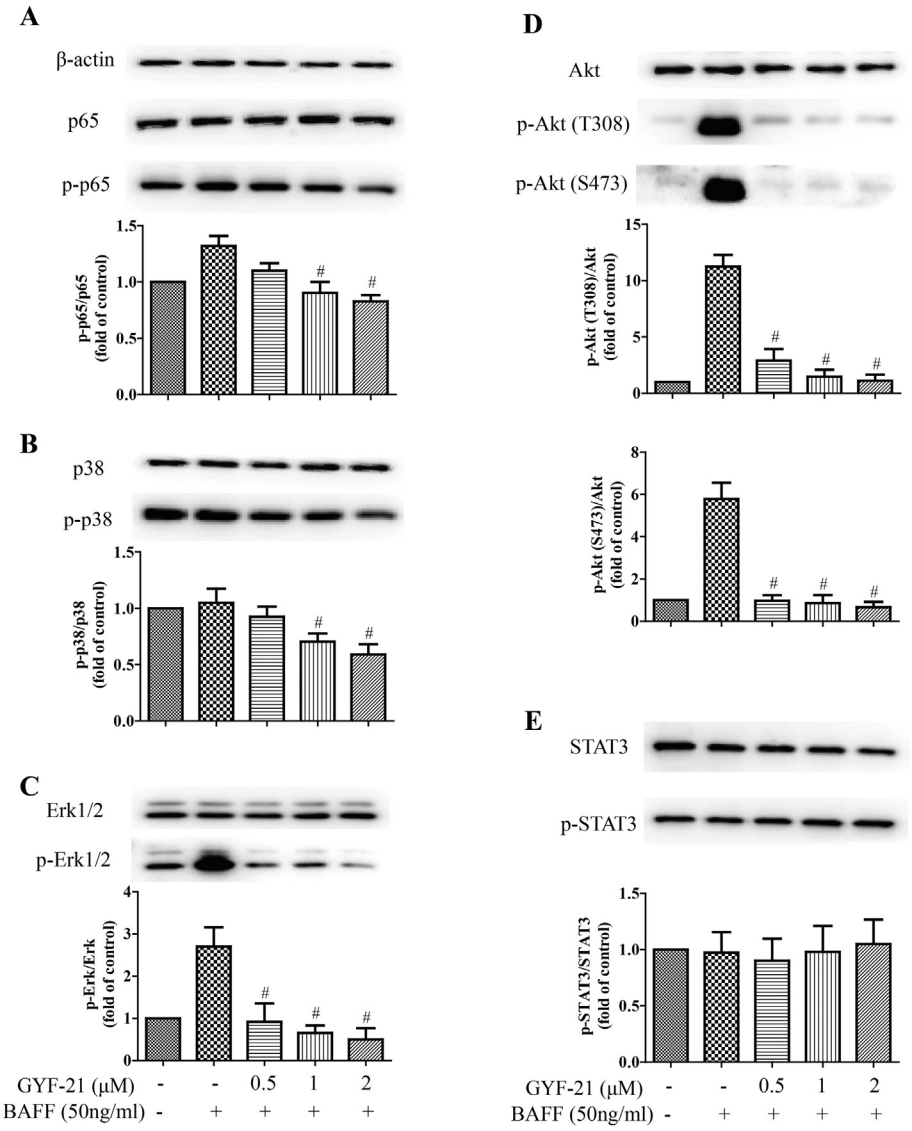

Fig. 7. Effects of GYF-21 on the activation of signaling pathways in B cells stimulated by BAFF. Splenocytes were seeded in 6-well plate and pretreated with various concentrations of GYF-21 for 0.5 h. Then the cells were stimulated with BAFF for 1 h. The phosphorylation of p65 (A), p38 (B), Erk1/2 (C), Akt (D) and STAT3 (E) were analyzed by western blot. The level of each protein was quantified using Quantity One software and normalized to β-actin. $\#P < 0.05$, significantly different from BAFF-treated group.

proliferation of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4 or BAFF. Firstly, we determined B cell proliferation by CFSE labeling. The results showed that B cells were stimulated to divide by combination of anti-IgM, anti-CD40 and IL-4 or BAFF. GYF-21 slightly inhibited division of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4, and moderately suppressed division of B cells stimulated by BAFF. Secondly, we evaluated B cell proliferation by BrdU incorporation analysis. The results showed that GYF-21 weakly reduced BrdU incorporation of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4, but moderately inhibited BrdU incorporation of B cells stimulated by BAFF (Fig. 3).

3.4. Effects of GYF-21 on the differentiation of B cells

The differentiation of B cells into antibody-secreting cells (ASC) is a crucial component of the immune response. In the present study, we further investigated the effects of GYF-21 on the differentiation of B cells. As shown in Fig. 4, combination of anti-CD40 and IL-4 or BAFF significantly stimulated formation of 7-AAD$^-$ B220$^+$ CD138$^+$ plasma cells or plasmablasts. GYF-21 didn't inhibit production of 7-AAD$^-$ B220$^+$ CD138$^+$ cells stimulated by combination of anti-CD40 and IL-4, and even increased the percentage of 7-AAD$^-$ B220$^+$ CD138$^+$ cells at the highest dose (Fig. 4A and C). However, production of 7-AAD$^-$ B220$^+$ CD138$^+$ cells stimulated by BAFF was intensively suppressed by GYF-21 (Fig. 4B and D). Notably, the expression of CD138 on CD138$^+$ B cells stimulated by BAFF was weaker than that induced by combination of anti-CD40 and IL-4. So the gate of CD138$^+$ cells stimulated by BAFF was lower than that induced by anti-CD40 and IL-4, and the percentage of 7-AAD$^-$ B220$^+$ CD138$^+$ cells of vehicle in BAFF-treated experiment was correspondingly higher than that in experiment treated with anti-CD40 and IL-4.

3.5. Effects of GYF-21 on the antibody production during B cell differentiation

As the inhibitory effects of GYF-21 on the differentiation of B cells were demonstrated, we further investigated the effects of GYF-21 on the antibody production during the B cell differentiation. As shown in Fig. 5, GYF-21 didn't inhibit antibody production stimulated by combination anti-CD40 and IL-4, but intensively inhibited production of

both IgM and IgG stimulated by BAFF.

3.6. Effects of GYF-21 on the activation of signaling pathways in B cells stimulated by anti-IgM, anti-CD40, IL-4 or their combinations

Anti-IgM, anti-CD40, IL-4 or their combinations mainly stimulate survival, activation, proliferation and differentiation of B cells through activating NF-κB, MAPK, Akt and STATs signaling pathways. We further investigated the effects of GYF-21 on signaling pathways involved in survival, activation, proliferation and differentiation of B cells induced by anti-IgM, anti-CD40, IL-4 or their combinations. The results (Fig. 6) showed that phosphorylation of NF-κB p65, MAPK p38, MAPK Erk1/2, Akt (T308), Akt (S473), STAT3, and STAT6 was induced by anti-IgM, anti-CD40, IL-4 or their combinations. Interestingly, GYF-21 inhibited phosphorylation of p65, Akt, and STAT3, but promoted phosphorylation of Erk1/2.

3.7. Effects of GYF-21 on the activation of signaling pathways in B cells stimulated by BAFF

As a cytokine produced by activated innate immune cells, especially dendritic cells, BAFF can directly stimulate survival, activation, proliferation and differentiation of B cells. We also further investigated the underlying mechanisms by activation analysis of multiple signaling pathways. The results (Fig. 7) showed that GYF-21 moderately down-regulated phosphorylation of NF-κB p65 and MAPK p38, and intensively inhibited phosphorylation of both Erk1/2 and Akt.

4. Discussion

Signal transduction through the B-cell antigen receptor, costimulatory signals and cytokines from T helper lymphocytes and other cytokines from innate immune cells, regulates multiple biologic functions of B cells such as survival, activation, proliferation, and differentiation. In the absence of survival stimulus, B cells rapidly enter apoptotic process. In the present study, we imitated specific stimulation of Th2 cells on B cells with combination of anti-IgM, anti-CD40 and IL-4 through BCR, CD40 and IL-4 receptor and nonspecific polyclonal B cell stimulation with BAFF alone. After the B cells were treated by the two stimulants, enhanced B cell survivals were observed. Under doses without cytotoxicity, GYF-21 exerted intensive inhibitory effects on B cell survival induced by BAFF while showed no suppressive effects on B cell survival stimulated by combination of anti-IgM, anti-CD40 and IL-4, suggesting that GYF-21 probably inhibits BAFF-activated signaling in B cells with high specificity.

Activation of B cells results in up-regulation of many transmembrane proteins. CD69 is an early activation marker for lymphocytes which is associated with a guanosine triphosphate (GTP)-binding protein and constitutively phosphorylated. The expression of CD69 on lymphocytes stimulated in vitro via a number of different pathways is well described [19]. CD80 and CD86 are members of the immunoglobulin supergene family and expressed on B cells and other antigen-presenting cells. CD80 and CD86 can provide costimulatory signals by engaging CD28 on T cells and their expressions on B cells can be induced by multiple stimulants. Generally, CD80 has higher constitutive expression than CD86 according to previous study [20]. In the activation analysis of B cells, we selected CD69 and CD86 as activation markers for B cells. The results showed that combination of anti-IgM, anti-CD40 and IL-4 induced up-regulation of CD69 and CD86 with higher amplitudes than that BAFF did, suggesting up-regulation of CD69 and CD86 more dependent on signaling pathways activated by combination of anti-IgM, anti-CD40 and IL-4 compared to those pathways induced by BAFF. However, GYF-21 only slightly inhibited the up-regulation of CD69 and CD86 resulted from stimulation by combination of anti-IgM, anti-CD40 and IL-4 (about 20% inhibiting rate at highest dose), but almost completely reversed up-regulation of CD69 and CD86 on BAFF stimulated B cells. The results indicate that GYF-21 exerts inhibitory effects on the activation of B cells and might have higher selective inhibitory effects on the signaling pathways in B cells activated by BAFF than that induced by Th2 cells.

Subsequently, we further investigated the effects of GY-21 on the proliferation of B cells. CFSE-DA is a cell permeable fluorescent dye, can enter cells by diffusion and is cleaved by intracellular esterase enzymes to form an amine-reactive product, caboxyfluorescein succinimidyl ester (CFSE). CFSE produces detectable fluorescence and covalently reacts with intracellular lysine residues and other amine sources. If a stained cell divides, the dye is divided equally into two daughter cells. So CFSE-DA is usually used to label cells and analyze cell division and proliferation. BrdU, as an analog of the DNA precursor thymidine, can be incorporated into newly synthesized DNA by proliferating cells. The immunofluorescent staining of incorporated BrdU and flow cytometric analysis provide a sensitive method to determine the frequency of proliferating cells that have synthesized DNA [21]. In this study, we employed CFDA-SE labeling and BrdU incorporation simultaneously to analyze the proliferation of B cells. The results showed that combination of anti-IgM, anti-CD40 and IL-4 stimulated stronger proliferation of B cells than BAFF in both CFDA-SE and BrdU assays. In the flow cytometry with CFDA-SE, GYF-21 slightly inhibited division of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4, and moderately suppressed division of B cells stimulated by BAFF. Consistently, in the DNA synthesis analysis with BrdU, GYF-21 weakly decreased DNA synthesis of B cells stimulated by combination of anti-IgM, anti-CD40 and IL-4, but more strongly reduced DNA synthesis of B cells induced by BAFF. These results suggest that GYF-21 exhibits stronger inhibitory effects on the proliferation of B cells stimulated by BAFF than that induced by combination of anti-IgM, anti-CD40 and IL-4.

The differentiation of B cells into antibody-secreting cells (ASCs) is a crucial procedure for immune response. ASCs, comprising both mature plasma cells and less mature plasmablasts, can easily be identified as mononuclear cells with higher surface expression of CD138 which is up-regulated over a 1000-fold during ASC differentiation [9,10,22–24]. In this study, we imitated specific stimulation of Th2 cells on B cell differentiation with combination of anti-CD40 and IL-4 in the absence of anti-IgM, because strong BCR stimulation inhibits differentiation of B cells. The results showed that both combination of anti-CD40 and IL-4, and BAFF significantly induced differentiation of B cells into 7-AAD$^-$ B220$^+$ CD138$^+$ plasma cells or plasmablasts. However, it was interesting that GYF-21 didn't inhibit production of 7-AAD$^-$ B220$^+$ CD138$^+$ cells stimulated by combination of anti-CD40 and IL-4, and even increased the percentage of 7-AAD$^-$ B220$^+$ CD138$^+$ cells at the highest dose, but intensively suppressed production of 7-AAD$^-$ B220$^+$ CD138$^+$ cells stimulated by BAFF. Furthermore, GYF-21 didn't inhibit production of IgM and IgG stimulated by combination of anti-CD40 and IL-4, but significantly suppressed production of IgM and IgG induced by BAFF. These results suggest that GYF-21 selectively inhibits differentiation of B cells stimulated by BAFF.

NF-κB, MAPK, Akt and STATs signaling pathways play key roles in the immune responses that B cells involve in. Stimulation of B cells through BCR, CD40, IL-4 receptor and BAFF receptor results in activation of NF-κB, MAPK, Akt, STATs signaling pathways [25–29]. The activation of MAPK p38 and MAPK Erk1/2 is important in the activation and proliferation of B cells, since specific inhibition of this kinase markedly reduced cyclin D3 expression and proliferation of B cells [30]. Akt signaling pathway contributes to the survival and proliferation of activated B cells. The involvement of Akt in cellular survival and proliferation is due largely to its phosphorylation-dependent inactivation of the Forkhead Box (Foxo) family of transcription factors, which results in the silencing of Foxo target proteins that promote cell-cycle arrest and apoptosis [31–34]. Activation of NF-κB signaling pathway and following upregulation of IRF4 which is a key transcription factor promotes survival and differentiation of B cells [10,12]. Stimulation by IL-4 results in activation of STAT6 and STAT3 and overexpression of

Blimp-1 which is required for the unique physiological ability of plasma cells that enable the secretion of antibody [29,35]. After revealed the inhibitory effects of GYF-21 on the survival, activation, proliferation, and differentiation of B cells, we investigated the underlying mechanisms by monitoring the activation of NF-κB, MAPK, Akt, STAT3 and STAT6 signaling pathways in B cells induced by anti-IgM, anti-CD40, IL-4, combination of anti-IgM, anti-CD40 and IL-4, combination of anti-CD40 and IL-4, or BAFF. The results showed that GYF-21 moderately reduced phosphorylation of p65, Akt (T308), Akt (S473), and STAT3, but slightly promoted phosphorylation of Erk1/2 in B cells stimulated by anti-IgM, anti-CD40, IL-4, or their combinations. More importantly, GYF-21 not only moderately downregulated phosphorylation of p65 and p38, but also intensively inhibited phosphorylation of Erk1/2, Akt (T308), and Akt (S473) in B cells stimulated by BAFF. These results reveal the reasons why GYF-21 exerts stronger inhibitory effects on the survival, activation, proliferation, and differentiation of B cells stimulated by BAFF than that induced by combination of anti-IgM, anti-CD40, and IL-4 or combination of anti-CD40, and IL-4. Furthermore, the results further suggest that there are great differences in the upstream signaling pathways of Erk1/2 and Akt in B cells activated by Th2 cells and BAFF.

Collectively, the present study suggests that GYF-21, an epoxide 2-(2-phenethyl)-chromone derivative isolated from Chinese agarwood, can significantly suppress survival, activation, proliferation and differentiation of B cells probably via blocking signaling transduction of Erk1/2 and Akt induced by BAFF and exhibits great potential to be developed into therapeutic agent for autoimmune diseases, especially SLE. Meanwhile, the therapeutic applications of GYF-21 in vivo and upstream signaling transduction of Erk1/2 and Akt in B cells induced by different stimulants need further study.

Author contributions

R.G., Y.Q.L. and S.S.L. performed experiments, analyzed data and drafted portions of the manuscript. J.L. and Y.F.G. were involved in compound isolation. X.L.G. provided technical support. Z.X.Z. designed experiments, analyzed data and wrote the manuscript. P.F.T. supervised the study team and revised the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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