



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

JSI-124 inhibits IgE production in an IgE B cell line

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ARTICLE INFO

Article history:

Received 1 December 2016

Accepted 12 December 2016

Available online xxx

Keywords:

Atopic diseases

JSI-124

IgE

ABSTRACT

IgE is a key effector molecule in atopic diseases; however, the regulation mechanisms of IgE production in IgE B cells remain poorly understood. In the present study, we demonstrate that JSI-124 (cucurbitacin I), a selective STAT3 inhibitor, selectively inhibits production of IgE by a human IgE B cell line, CRL-8033 cells, while does not affect the IgG production by IgG B cell lines. In the aspect of molecular mechanism, we found that *Igλ*, but not *Ighe*, gene expression was suppressed by JSI-124. The above effects of JSI-124 were not mediated by affecting cellular proliferation or apoptosis. Furthermore, multiple B cell differentiation-related genes expression was not significantly affected by JSI-124. Taken together, we demonstrate a potential strategy of therapeutically suppressing IgE production without affecting IgG production in atopic patients.

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1. Introduction

IgE is the key effector molecule in atopic diseases [1]. IgE is produced by terminally differentiated IgE B cells. IgE binds to FcεRI, the high affinity receptor expressed by mast cells and basophils, leading to the release of inflammatory mediators in asthma and other anaphylactic diseases [2–4]. However, current therapeutic strategies for controlling IgE level in atopic patients are limited. Most studies on IgE regulation focused on B cell differentiation into IgE-producing plasma cells [5], in parallel with the differentiation of IgG-producing plasma cells. However, these studies did not address the clinical needs of selectively controlling IgE level in atopic patients, while preserving IgG production, which is indispensable for host immunity.

JSI-124 (cucurbitacin I), a small molecule known to inhibit the JAK/STAT3 signaling pathway, may exert anti-tumor activity [6–9]. To date, whether JSI-124 plays a role in atopic diseases remains unknown. Considering that STAT3 mutation has been related to

hyper-IgE syndrome, we wondered if interference of STAT3 may have effects on IgE production [10]. Here, we report that JSI-124 selectively suppresses IgE production in a human IgE B cell line, CRL-8033 cells [11], but not IgG production in IgG-producing hybridomas, and does not interfere with B cell differentiation related genes expression program. Our findings demonstrate a potential therapeutic strategy for selectively controlling IgE level in atopic patients without affecting IgG production required for host immunity.

2. Materials and methods

2.1. Cell line and culture

CRL-8033 cells (SKO-007 cells [11]; from Zhangjiang Biotechnology), Raji cells (from Shanghai Cell Bank), and hybridoma cells (3H4 and 6A10, produced in house) secreting anti-human IgE protein, were cultured and maintained in RPMI 1640 media (Hyclone, Logan, UT, USA), which was supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), penicillin (100 IU/mL) and streptomycin (100 µg/mL) in a humidified incubator aerated with 5% CO₂ at 37 °C. The cells were dispersed 2–3 times per week.

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2.2. ELISA

CRL-8033 cells were seeded in 12-well plates (2×10^4 cells/well). Cell supernatants were collected after being incubated with 100 nM JSI-124 (Sigma, Germany) or 1% DMSO for 24, 36, 48 or 72 h. Levels of Human IgE were determined by using ELISA Kit (Dakewe, Shenzhen, China) according to the manufacturer's instructions.

2.3. Cell viability assay

CRL-8033 cells after being incubated with different amounts of JSI-124 for 48 h were collected to be evaluated for viability with trypan blue. Briefly, 10 μ L of cell suspension from each culture was mixed with an equal volume of trypan blue dye. The mixture was loaded onto a hemocytometer and cells were counted under a microscope.

2.4. Measurement of human IgG antibody (3H4 and 6A10) by ELSIA

96-well plates were coated with 100 ng/mL mouse anti-human IgE antibody (produced by our lab) in 50 mM carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C. The plates were blocked with 5% non-fat milk at 37 °C for 1 h before washing, and then incubated with 100 nM JSI-124 or equal volume of DMSO 48hr-treated 3H4 or 6A10 hybridoma cell culture supernatants. Anti-human IgE antibody (Abcam, ab65866) was used as standards. Incubation took 1 h at room temperature before washing. The horseradish peroxidase conjugated goat anti-mouse IgG antibody (Proteintech, SA00001-1) diluted at 1:6000 was added and incubated for 1 h at room temperature before washing. After incubation, tetramethylbenzidine (Beijing 4A Biotech, Beijing, China) was added, and the plate was incubated for 20 min at room temperature. The reaction was stopped with 1 M sulphuric acid, and absorbance was measured with an ELISA reader at 450 nm. Experiments were performed in triplicate.

2.5. Conventional PCR

Total RNAs from 100 nM JSI-124 or 1% DMSO-treated CRL-8033 cells for 48 h were purified with the RNA extraction kit according to the manufacturer's instructions (Takara, Dalian, China). RNA (0.5–1 μ g) was reversely transcribed to cDNA using PrimeScript™ RT Master Mix Kit (Takara, Dalian, China), and PCR was performed with Premix Taq™ (Takara, Dalian, China). The primers were synthesized by Invitrogen (Shanghai, China). Their sequences were as follows: IgE heavy chain (*Ighe*), forward primer 5'-ATCAGCTTGCTGACCGTCTC-3', reverse primer 5'-TAA-GATCTTCACGGTGGGCG-3'; λ chain (*Ig λ*), forward primer 5'-GGC CACACTGGTGTGTCTCATAA -3', reverse primer 5'-CCTTGAC GGGGCTGCTATCT-3'; *Bcl-6*, forward primer, 5'-TCATTGTGTGAG CCGTGAGCAGTT-3', reverse primer 5'-ACAACATGCTCCATCT GCAGG-3'; *Blimp-1*, forward primer 5'-TCGGGTGCTTTACCCCATC-3', reverse primer 5'-CACAGCGCTCAGGCCATTA-3'; *Xbp-1*, forward primer 5'-ACGCACCTGAGCCCGAGGAGAA-3', reverse primer 5'-GGGAGATGTTCTGGAGGGGTGACAA-3'; *AID*, forward primer 5'-GACCCTGGCCGCTGCTACC-3', reverse primer 5'-CAAAAG-GATGCGCCGAAGCTGTCTGGAG-3'; *IRF4*, forward primer TCCCA-CAGAGCCAAGCATAAGGT-3', reverse primer 5'-AGGGAGC GGCCGTGGTGAGCA-3'; *Ezh2*, forward primer 5'-TACTTGTG-GAGCCGCTGAC-3', reverse primer 5'-CTGCCACGTGATGGTG-3'. The β -actin primer sequences were used as the internal gene as described previously [12]. PCR was carried out at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. After amplification, products were resolved on a 1.2% agarose gel. Gel with amplification fragments

was visualized and photographed with a gel documentation system (Dolphin-DOC PLUS). Data are representative of two independent experiments.

2.6. Apoptosis detection assay

100 nM JSI-124 or 1% DMSO 48h-treated CRL-8033 cells were assessed for apoptosis using an Annexin-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (TransGen Biotech, Beijing, China) according to the manufacturer's instruction. Data was analyzed with FlowJo version 7.6.1 software (TreeStar, Ashland, OR, USA). Experiments were repeated three times.

2.7. Cell proliferation assay

The proliferation of 100 nM JSI-124 and 1% DMSO 48h-treated CRL-8033 cells was evaluated by the 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution evaluated by flow cytometry. Data was analyzed by FlowJo version 7.6.1 software (TreeStar, Ashland, OR, USA). Experiments were performed in triplicate.

2.8. Protein extraction and western blot analysis

CRL-8033 cells were plated in cell culture flask (1×10^5 cells/ml). Briefly, after being treated with 100 nM JSI-124 (Sigma, Germany) or 1% DMSO for 48 h, cells were collected for protein extraction. The concentration of proteins in lysates was quantified by the BCA Protein Assay Kit (Beyotime, Shanghai, China), and 80 μ g protein was loaded in each lane. Samples were electrophoresed with SDS-PAGE (12%) and blotted onto nitrocellulose filter (NC) membranes. The membranes were blocked with 5% non-fat milk (Sangon Biotech, Shanghai, China) at room temperature for 1 h. Blots were incubated with mouse anti-human IgE monoclonal antibody (prepared by our Lab) and anti- β -actin antibody (Santa Cruz biotechnology) overnight at 4 °C, and then with the horse-radish peroxidase conjugated-secondary antibodies (Proteintech, Wuhan, China) for 40 min at room temperature. The proteins were visualized using an enhanced chemiluminescence detection kit (Millipore, Darmstadt, Germany) and analyzed by Amersham Imager 600 (GE Healthcare Life Sciences, Tokyo, Japan).

2.9. Statistical analysis

All statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA). Figures were displayed as means $\pm$ SEM. Statistical comparisons between groups were determined using the Student's *t*-test to calculate the two-tailed *p* values: ****p* < 0.001, ***p* < 0.01, **p* < 0.05.

3. Results

3.1. JSI-124 selectively suppresses IgE, but not IgG production

In order to investigate whether JSI-124 affects IgE production by IgE B cells, we treated a human IgE B cell line, CRL-8033 cells, with a STAT3 inhibitor, JSI-124, for 48 h. We found that JSI-124 suppresses IgE production in CRL-8033 cells by a dose-dependent manner (Fig. 1A). Such effects were not caused by cell death, since the viability of CRL-8033 cells were not affected by the dose of JSI-124 used in the experiments, as assessed by trypan blue staining at 48 h (Fig. 1B). Furthermore, the suppression of IgE concentrations in the cell culture was observed from 24 to 72 h after adding JSI-124 to cell culture (Fig. 1C). These data demonstrate that IgE production by IgE B cells is suppressed by JSI-124.

We wondered whether JSI-124 also affects IgG production in IgG

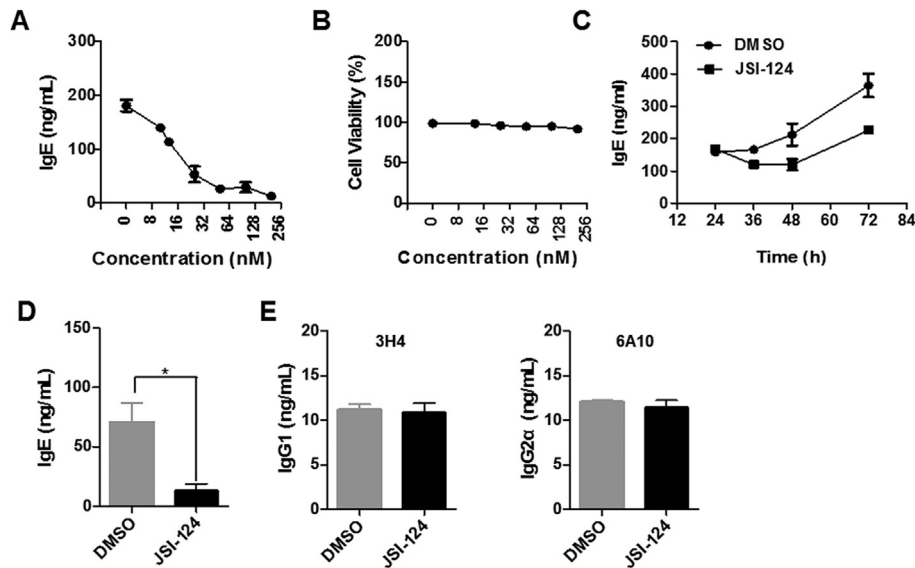


Fig. 1. JSI-124 selectively inhibits IgE production by CRL-8033 cells, but not IgG production by IgG B cells. (A) The inhibitory effect on IgE production by CRL-8033 cells at different concentrations of JSI-124. (B) Cell viability of CRL-8033 cells treated with different concentrations of JSI-124. (C) CRL-8033 cells were cultured with DMSO or 100 nM JSI-124. IgE concentrations in the culture supernatant at different time points were determined. (D and E) CRL-8033 cell line (D) and two IgG secreting hybridoma cell lines (E) were treated with 100 nM JSI-124 or DMSO for 48 h. IgE (D) or IgG (E) concentrations in the culture supernatant were determined by ELISA. (A–E) Data are representative of at least three independent experiments and are represented as the mean $\pm$ SEM.

B cells. With the dose and treatment time (48 h) of JSI-124 used in this study which reduced IgE production by CRL-8033 cells (Fig. 1D), we found that, strikingly, IgG production by IgG-secreting hybridoma cells was not affected (Fig. 1E). These results show that JSI-124 selectively suppresses the production of IgE, but not IgG.

3.2. JSI-124 suppresses IgE production by regulating *Ig λ* gene expression

We next sought to find out how IgE production was suppressed by JSI-124. We treated CRL-8033 cells with JSI-124, and found that total amounts of IgE or IgE heavy chain protein only modestly decreased, as compared with CRL-8033 cells treated with DMSO (Fig. 2A). The mRNA level of *Ighe* was not affected by JSI-124 either (Fig. 2B). These indicated that the expression of IgE heavy chain in CRL-8033 cells was not significantly affected by JSI-124. On the other hand, however, the mRNA level of *Ig λ* was decreased by JSI-124 (Fig. 2B). Thus, these data demonstrated that JSI-124 suppresses IgE production possibly by negatively regulating *Ig λ* gene expression, therefore abrogating the formation of the complete IgE complex in CRL-8033 cells.

3.3. JSI-124 suppresses IgE production without affecting apoptosis or proliferation

A high dose of JSI-124 was reported to cause apoptosis and cell cycle arrest [13]. However, with the dose used throughout this study, we observed a significant suppression of IgE production (Fig. 1A) with only a modest decrease in numbers of JSI-124-treated CRL-8033 cells, compared with that of DMSO treated (Fig. 3A), and we did not detect significant levels of cell apoptosis (Fig. 3B). Cell proliferation, as assessed by CFSE dilution, was not significantly affected by JSI-124 (Fig. 3C). These data demonstrated that suppression of IgE production by JSI-124 is not due to significant levels of apoptosis, or significantly inhibited cell proliferation.

3.4. JSI-124 does not regulate B cell differentiation related genes expression

Current studies on regulation of IgE production mainly focused on B cell differentiation. Here, we assessed the possible effects of JSI-124 on the expression of B cell differentiation-related genes (Fig. 4), including B-cell lymphoma-6 (*Bcl-6*) [14], B lymphocyte-

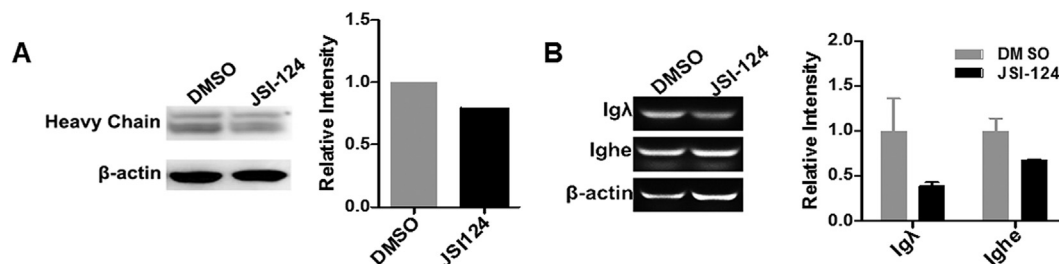


Fig. 2. The inhibitory effect of JSI-124 on IgE production by CRL-8033 cells. (A) Western blot analysis of the effect of JSI-124 on IgE heavy chain protein expression in CRL-8033 cells treated with JSI-124 100 nM or DMSO for 48 h. (B) *Ighe* and *Ig λ* gene expression levels in CRL-8033 were determined by PCR. The histogram showed the relative expression levels of each gene after normalization to that of β -actin. (A and B) Data are representative of three independent experiments.

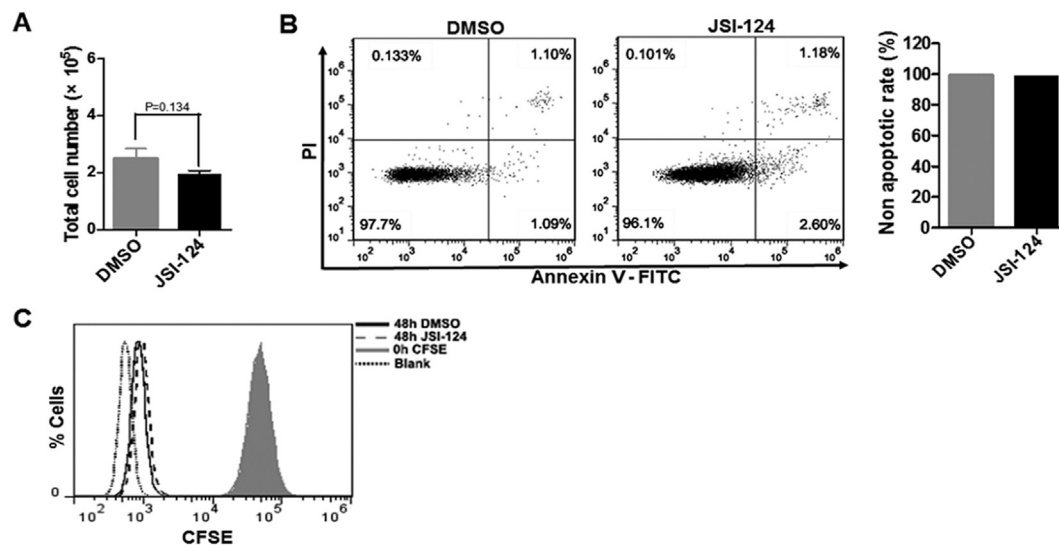


Fig. 3. The effect of JSI-124 on cell apoptosis and proliferation of CRL-8033 cells. (A) Total cell number of CRL-8033 cells was shown after incubation with DMSO or JSI-124 for 48 h. (B) Level of apoptosis in DMSO or JSI-124-treated CRL-8033 cells as in (A) was assessed by Annexin V-7AAD staining using flow cytometry. (C) Level of proliferation in DMSO or JSI-124-treated CRL-8033 cells as in (A) was assessed by CFSE dilution using flow cytometry. (A–C) Data are representative of three independent experiments.

induced maturation protein-1 (*Blimp-1*), X-box binding protein 1 (*XPB-1*), interferon regulatory factor 4 (*IRF4*) [15], activation-induced cytidine deaminase (*AID*) [16], and enhancer of zeste homolog 2 (*Ezh2*) [17]. Neither in an undifferentiated human B cell line, Raji, nor in the differentiated IgE B cell line, CRL-8033, were the above B cell differentiation-related genes' expression significantly affected by JSI-124 (Fig. 4), except that *AID* was not detected in CRL-8033 cells (Fig. 4). These suggest that IgE-suppressive JSI-124 might not affect the gene expression program that directs B cell differentiation into IgE-secreting plasma cells.

4. Discussion

While most studies on IgE regulation focused on B cell differentiation into IgE-producing plasma cells [5], investigation of IgE production in terminally differentiated IgE B cells was difficult due to the lack of an IgE B cell line. CRL-8033 cells, a terminally differentiated IgE producing human B cell line, derived from the U266

myeloma cell line [11], is a good model for studying the mechanisms underlying IgE production independent of the B cell differentiation process.

JSI-124 was known as a small molecule inhibitor of JAK/STAT3 pathway for tumor growth suppression both in vitro and in vivo [6] and [7]. However, with the dose used, we didn't observe significant cell death (Fig. 1B), apoptosis (Fig. 3B) or proliferation inhibition (Fig. 3A and C) in the presence of JSI-124. This suggests that JSI-124 might have yet unknown effects through a JAK/STAT3-dependent but proliferation/apoptosis-independent pathway.

As the expression of IgE heavy chain in CRL-8033 cells was not significantly affected at either protein or mRNA level (Fig. 2A and B), and *Igλ* gene expression was significantly decreased (Fig. 2B) in the JSI-124-treated samples, it seems that the JSI-124 suppress IgE production mainly by down-regulating *Igλ* expression. The analysis of the expression of B cell differentiation-related genes (Fig. 4) revealed that in either Raji or CRL-8033 cells, B cell differentiation-related genes expression was not significantly affected by JSI-124,

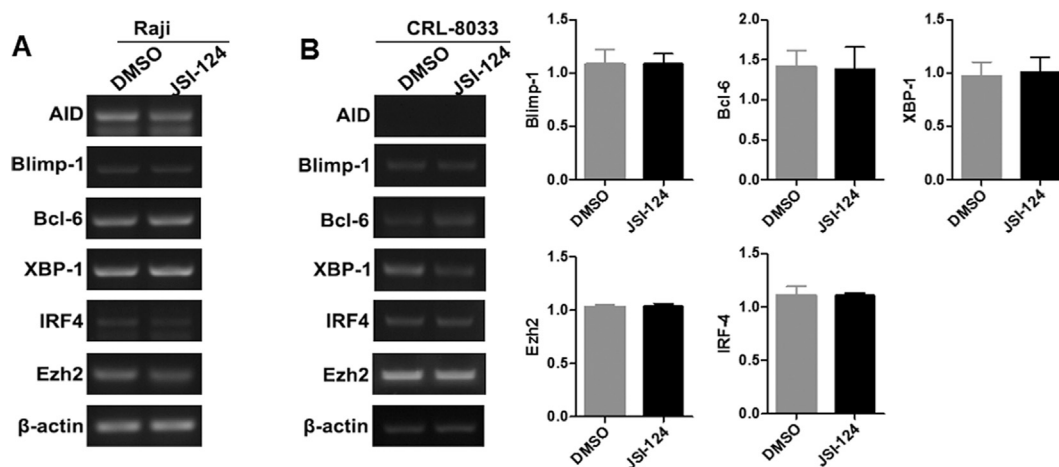


Fig. 4. B cell differentiation related genes expression in CRL8033 cells. (A) Expression of *Bcl-6*, *Blimp*, *XPB-1*, *IRF4*, *AID*, and *Ezh2* genes in Raji treated with DMSO or JSI-124 for 48 h. (B) Expression of *Bcl-6*, *Blimp*, *XPB-1*, *IRF4*, *AID*, and *Ezh2* genes in CRL-8033 cells in the presence of DMSO or JSI-124 for 48 h was assessed by PCR. The level of these genes were normalized to that of β -actin. Data are representative of two independent experiments.

further suggesting that the effects of JSI-124 on IgE production was caused by a suppression of IgE production.

In conclusion, our study provides evidence that JSI-124 can selectively suppress IgE production in human IgE B cells, without affecting IgG production, which represents a potential therapeutic strategy for controlling IgE level in atopic patients.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgments

We thank Zhangjiang Biotechnology for providing the CRL-8033 cells. This work was supported by the Natural Science Foundation of China (#81373112 to W.X., #81373162 to Y.G., and #81501355 to B.J.), the Science and Technology Project of Guangdong (2013A022100037), the Science and Technology Innovation Fund of Shenzhen (JCYJ20130401113257009 to W.X., JSGG20160229202150023 to W.X., and JCYJ20150521094519472 and JCYJ20150630114942288 to B.J.), Shenzhen Peacock Next-generation Monoclonal Antibody Drug research and development program (KQTD201210), the Leading Talents Introduction Special Funds of the Fourth Year in Guangdong (Office of Talents in Guangdong [2014] No.1), and the Shenzhen Laboratory of Antibody Engineering (Development and Reform Commission in Shenzhen [2014] No.1782 to W.X.).

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2016.12.085>.

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