Fc Receptor-Like 1 as a Promising Target for Immunotherapeutic Interventions of B-Cell-Related Disorders

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ABSTRACT

BACKGROUND: Human B-cell responses are regulated through synergy between a collection of activation and inhibitory receptors. Fc receptor-like (FCRL) molecules have recently been identified as co-receptors that are preferentially expressed in human B-cells, which may also play an important role in the regulation of human B-cell responses. FCRL1 is a member of the FCRL family molecules with 2 immunoreceptor tyrosine-based activation motifs (ITAMs) in its cytoplasmic tail. This study aimed to investigate the regulatory roles of FCRL1 in human B-cell responses.

MATERIALS AND METHODS: The regulatory potential of FCRL1 in human B-cell through knockdown of FCRL1 expression in the Ramos and Daudi Burkitt lymphoma (BL) cell lines by using the retroviral-based short hairpin ribonucleic acid (shRNA) delivery method. The functional consequences of FCRL1 knockdown were assessed by measuring the proliferation, apoptosis, and the expression levels of *Bcl-2, Bid*, and *Bax* genes as well as phosphoinositide-3 kinase/-serine-threonine kinase AKT (PI3K/p-AKT) pathway in the BL cells, using the quantitative real-time polymerase chain reaction (PCR) and flow cytometry analysis. The NF-κB activity was also measured by the enzyme-linked immunosorbent assay (ELISA).

RESULTS: FCRL1 knockdown significantly decreased cell proliferation and increased apoptotic cell death in the BL cells. There was a significant reduction in the extent of the *Bcl-2* gene expression in the treated BL cells compared with control cells. On the contrary, FCRL1 knockdown increased the expression levels of *Bid* and *Bax* genes in the treated BL cells when compared with control cells. In addition, the extent of the PI3K/p-AKT expression and phosphorylated-p65 NF-κB activity was significantly decreased in the treated BL cells compared with control cells.

CONCLUSIONS: These results suggest that FCRL1 can play a key role in the activation of human B-cell responses and has the potential to serve as a target for immunotherapy of FCRL1 positive B-cell-related disorders.

KEYWORDS: Cell proliferation, apoptosis, B-lymphocytes, regulatory

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Introduction

Immune response modulation is critical to maintaining hemostasis and preventing any destructive responses in the host.[1] To date, a collection of activating and inhibitory co-receptors were identified, which coordinated together to regulate cellular and humoral immune responses.[2]

A large number of known immune co-receptors lack the innate enzymatic activity, and they regulated immune responses through correlation with other adaptor proteins containing immunoreceptor tyrosine-based activation (ITAM) and/or immunoreceptor tyrosine-based inhibition (ITIM) motifs in their cytoplasmic domains. This co-engagement leads to the recruitment of various tyrosine kinases or tyrosine phosphatases, which have modulated downstream signaling mediators and regulated immune responses.[3-5]

An interesting group of Fc receptor (FcR) related molecules with ITAM/ITIM-like components in their cytoplasmic domains has recently been identified, known as Fc receptor-like (FCRL) molecules, IgSF receptor translocation–associated genes (IRTA), Fc receptor homologs (FcRH), B-cell cross-linked by anti-immunoglobulin M–activating sequences (BXMAS), Src homology 2 domain–containing phosphatase anchor proteins (SPAP), and immunoglobulin FcR-gp42–related genes (IFGP).[6-13] However, the unified nomenclature FCRL was chosen to describe these receptors.[14] The FCRL genes are located on chromosome 1q21-23 and encode 2

intracellular proteins (FCRL-A and B) and 6 transmembrane receptors with 3-9 immunoglobulin (Ig-like domains-in extracellular region (FCRL1-6).[7,11,15-17] FCRL1-5 molecules are selectively expressed in the different lineages of human B cell, while the expression of FCRL3 and FCRL6 was also detected in the natural killer (NK) cells and human T-cells.[12,18-25] Preferential expression of FCRL molecules in the different subsets of human B-cell and presence of ITAM/ITIM-like motifs in their cytoplasmic domains suggest that these molecules may be involved in the regulation of human B-cells.[17]

Among the FCRL receptors, FCRL1 has 2 ITAM-like components in the cytoplasmic tail and residual glutamic acids with negative charges its transmembrane region. The expression of FCRL1 started in the pre-B cells changed during B-cell development. Higher levels of FCRL1 expression have been detected in the subpopulations of naive and memory B cells, although very low levels or negative expression of this molecule is identified in the germinal center B-cells and plasma cells.[7,26,27] In addition, numerous studies have reported the aberrant expression of this molecule in the various B-cell-related disorders, including hematological malignancies,[19,27-31] disorders,[32-34] infection diseases.[35,36]

The structural features and expression pattern of FCRL1 in B-lineage cells suggest that it may act as an activation receptor in human B cell or plays a significant role in the pathogenesis of B-cell-related disorders. To date, a great number of studies have been performed about the expression profile of FCRL1 in normal and neoplastic B-cells, whereas few data are available on the functional roles of this molecule in human B-cell.[26-31] This study aimed to investigate the potential roles of FCRL1 in human B-cell responses.

Materials and Methods

Cell culture

Burkitt lymphoma (BL) cell lines Ramos and Daudi were obtained from the Stem Cell Technology Research Center (Tehran, Iran) and grown in the Roswell Park Memorial Institute (RPMI) 1640 culture media (Gibco; Grand Island, NY, USA) supplemented with 100 U/ml penicillin-streptomycin (Sigma-Aldrich; St Louis, MO, USA) and 10% fetal bovine serum (FBS) (Gibco). Platinum-A (Plat-A) cell, a retroviral packaging cell line, was also purchased from the Cell Biolabs (San Diego, CA, USA) and cultured in the Dulbecco's modified Eagle medium (DMEM-high glucose medium (Gibco) containing 2 mM L-Glutamine (Life Technologies; Grand Island, NY, USA), 1× non-essential amino acids (Gibco), antibiotics, 10% FBS, and 10 µg/ml Blasticidin S (Life Technologies). The cells were maintained in the humidified incubator with 5% CO_2 atmosphere at 37°C.

FCRL1 knockdown

FCRL1 knockdown was performed by using the retrovirus-mediated short hairpin RNA (shRNA) delivery method. For this purpose, FCRL1 Human shRNA Plasmid Kit (Locus ID 115350), including 4 unique 29mer shRNA constructs in the retroviral GFP vector pGFP-V-RS against different splice variants of FCRL1 gene, and a scrambled nontargeting shRNA as control were purchased from the OriGene Technologies company (Madison, Alabama, USA). After the chemical transformation of shRNA constructs in the *Escherichia coli* DH5α strain,[37] AccuPrep Plasmid Maxi-Prep DNA Extraction Kit (Bioneer; Daejeon, Korea) was used for the large-scale extraction of each plasmid.

The retrovirus particles were generated following the of Plat-A cells with 80 µg of each FCRL1-targeting DNA or scrambled control DNA in T75-cell culture flasks, using the calcium phosphate (CaPO4) precipitation method.[38] The efficiency of was evaluated based on the GFP signals under the fluorescence microscopy. Afterward, the supernatants were collected after 2 and 3 days of infection procedure, centrifuged (for 10 min at 1000g) to remove cell debris, sterile filtrated using a 0.45 µm syringe filter (Millipore; Billerica, MA, USA), and stored at -80 °C till for infection of the target cells. About 1×10^6 target cells were infected with a combination of 1 ml, 10µg/ml goat f'2 anti-human IgG/IgM (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA), and 10 µg/ml Polybrene (Santa Cruz Biotechnology; Dallas, TX) in 24-well tissue culture plates (Nunc- Nalgene; Rochester, New York USA). Afterward, plates were centrifuged at 2500 90 min at 30 °C and incubated in a CO_2 for 2 to 3 days. The FCRL1 knockdown was determined by using the quantitative real time-polymerase chain reaction (PCR) and flow cytometry assays, after 2 and 3 days of the infection procedure (data are not shown).

Here, the phrases of treated and control cells are used to describe the BL cells that are infected with the retroviral particles harboring FCRL1-targeting DNA or the retroviral particles containing control vector DNA, respectively.

extraction, cDNA synthesis, and quantitative real-time PCR

The total RNA was extracted from the 1×10^6 cells/ml by using the 1 ml RNX-Plus solution (CinnaGen; Tehran, Iran), according to the manufacturer's protocol. The purity and concentration of the extracted RNAs were assessed by the ratio of absorbance at 260/280 nm using a NanoDrop spectrophotometer (Thermo Scientific; Waltham, MA, USA). Afterward, synthesis of the first strand of complementary DNA (cDNA) was conducted by using the one-step SYBR PrimeScript RT Reagent Kit (Takara Bio Inc; Otsu, Shiga, Japan) according to the kit instructions. Then, amplification of the target genes was performed by a Rotor-gene 6000 instrument (Qiagen; Hilden, Germany) and SYBR Green PCR Master Mix (Takara) on the cDNA samples.

Each reaction underwent 45 cycles (*FCRL1*), 30 cycles (*Bcl-2, Bid*), and 35 cycles (*Bax*, phosphoinositide-3 kinase [*PI3K*]) with denaturation at 95°C for 5 s, annealing at 60°C (*FCRL1,*

Table 1. Sequences of specific primers used in quantitative real-time polymerase chain reaction approach.

GENES	PRIMERS	SEQUENCES (5′–3′)
FCRL1	Forward	CAGAGTTCAGATGCCCAGTTC
	Reverse	TCACATCAGCGACAGGGAC
Bcl2	Forward	GGGATGCGGGAGATGTGG
	Reverse	GTAGCGGCGGGAGAAGTC
Bid	Forward	CATCCGGAATATTGCCAGGC
	Reverse	CCATGTCTCTAGGGTAGGCC
Bax	Forward	AACAAGCTGAGCGAGTGTCT
	Reverse	GTTCTGATCAGTTCCGGCAC
PI3K	Forward	CTTCCTCCACCTCTTTGCCCTG
	Reverse	AGCCACTACTGCCTGTTGTCTTG
β-actin	Forward	GGACTTCGAGCAAGAGATGG
	Reverse	AGCACTGTGTTGGCGTACAG

Bcl-2, and *Bid*) and 61°C (*PI3K* and *Bax*) for 30 s, and extension at 70°C for 30 s. All reactions were performed in triplicate and the human *β-actin* gene expression level was used to normalize the results. The relative expression of target genes was measured by the ratio of threshold cycle (Ct) values of the target genes to the *β-actin* gene, using the Relative Expression Software Tool 2009 (REST 2009).[39] In addition, the statistical significance and relative fold changes of gene expression were calculated by bootstrapping methods and $2^{-\Delta\Delta Ct}$ formula.[40] Primers are listed in Table 1.

Flow cytometry assay

The BL cells were collected and washed twice with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) for the flow cytometry analysis. The cells were then stained with the Phycoerythrin (PE)-conjugated recombinant IgG1 to human antigen FCRL1 (CD307a) (clone: REA440; Entrez Gene ID 115350) from the Miltenyi Biotec company (catalog number: 130-106-448) or PE-conjugated REA control monoclonal antibody (clone: REA 239) from the Miltenyi Biotec company (catalog number: 130-104-612) and incubated for 30 min at 4°C.

In addition, intracellular staining of the cells was performed with the fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody to human antigen Phospho-Akt1 (S473) (clone: SDRNR) from the eBioscience company (catalog number: 11-9715-42) or matched Isotype antibody, after fixation and permeabilization of the cells by the BD Cytofix/Cytoperm Kit (BD Biosciences; San Jose, CA, USA). The Fluorescence Minus One (FMO) controls were also used for proper gating of the cells and determination of fluorescence spread. Data were assessed using the FACSCalibur flow cytometry (BD Biosciences) and analyzed by the FlowJo software (Tree Star Inc; Ashland, OR, USA).

Apoptosis and cell proliferation assay

To investigate the possible effects of FCRL1 on B-cell apoptosis, the extent of the expression of pro-apoptotic *Bcl-2* gene and anti-apoptotic *Bid* and *Bax* genes was evaluated in the BL cells by using the real-time PCR approach, following the knockdown of FCRL1 expression. The percentage of the apoptotic cell death was also measured by using the PE Annexin V apoptosis detection Kit with 7-AAD (BD Biosciences) and analyzed by FACSCalibur flow cytometry (BD Biosciences) on days 2, 3, and 4 of infection procedure.

To determine the effects of FCRL1 knockdown in B-cell proliferation, the BL cells were labeled with cell division tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Biolegend; San Diego, CA, USA) according to the manufacturer's instructions. Briefly, BL cells (1×10^6 cells/mL) were resuspended in PBS and incubated with 0.5 μM CFSE dye (Biolegend) in the dark at room temperature for 8 min. The CFSE-labeled cells were washed 3 times with complete RPMI 1640 medium, infected with appropriate retroviral particles according to the method described above, and cultured in the 24-well cell culture plates (Nunc-Nalgene) for 3 to 5 days. The percentage of cell proliferation was evaluated via fluorescent intensity measurement of CFSE by the FACSCalibur flow cytometry and analyzed using FlowJo software. The labeled cells without any treatment served as negative controls. The non-labeled cells were also used to exclude the auto-fluorescent of the cells. All experiments were performed in triplicate.

Measuring the NF-κB activity

To evaluate FCRL1 knockdown effects on p65 NF-κB activity of B cells, the cells were collected and washed with PBS on day 4 of infection procedure. They were resuspended in the culture media with 0.5% FBS and incubated at 37°C in the presence or absence of 20 ng/mL of tumor necrosis factor-α (TNF-α) for 30 min. Then, the cells were collected and analysis of p65 NF-κB activity was performed by the NF-kB p65 (Total) Multispecies Instant One ELISA Kit (eBioscience; San Diego, CA, USA) based on the kit's protocol.

Statistical analysis

Data analysis was conducted using the SPSS 20 software (SPSS; Chicago, IL, USA) and shown as mean ± standard deviation. The unpaired Student *t* test and Mann-Whitney *U* test were used to compare the groups with normal and nonnormal distribution, respectively. A P value $< .05$ was considered statistically significant. OriginPro v8.6 software (OriginLab Corporation) was used to create the artworks.

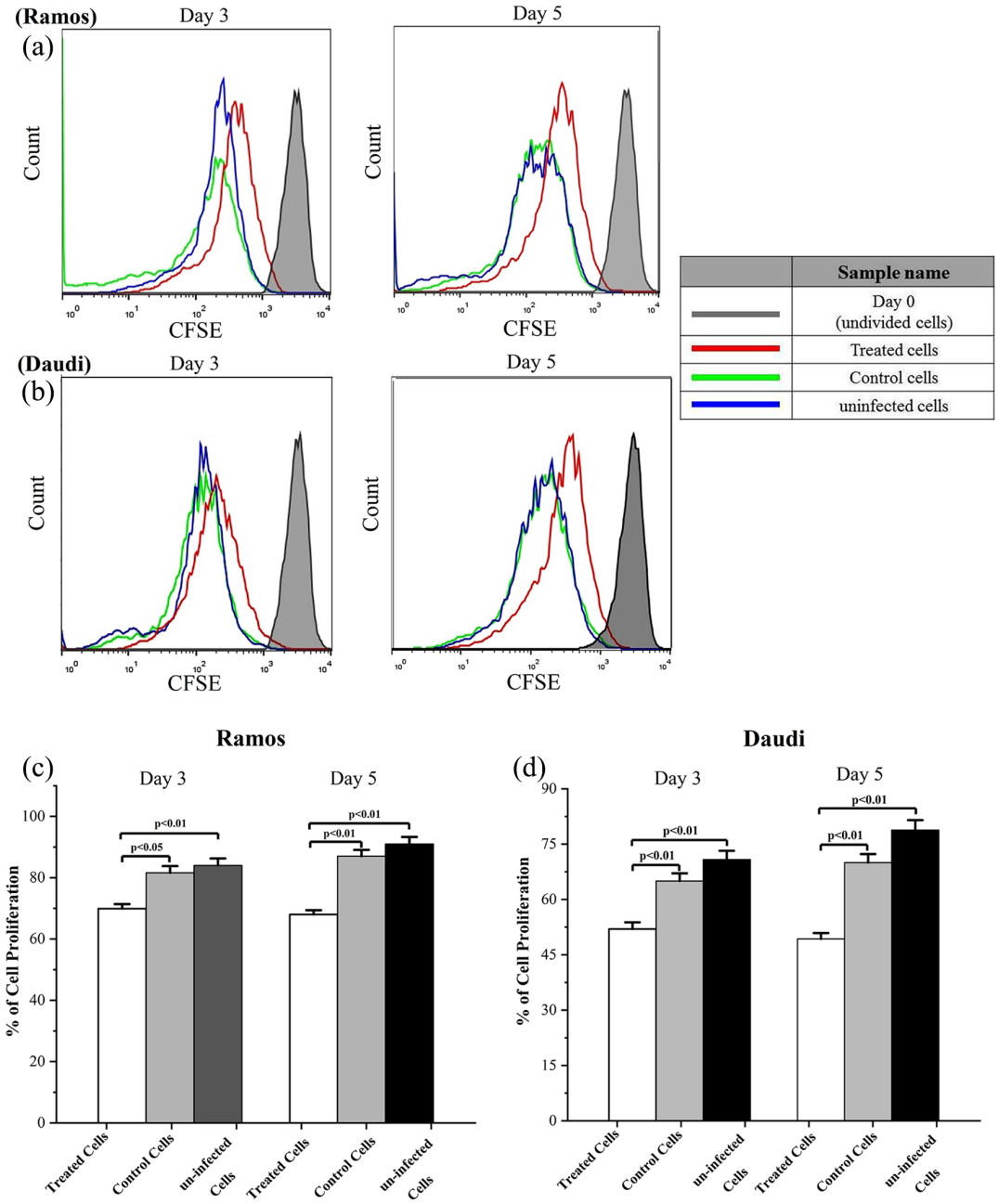

Figure 1. The effect of FCRL1 knockdown on the proliferation of Ramos and Daudi cells. The cells were collected and the proliferation of the CFSE-labeled BL cells was assessed by (A and B) flow cytometry assay and then analyzed by (C and D) FlowJo software, after 3 and 5 days of infection procedure. Data represented as mean ± standard deviation.
BL indicates Burkitt lymphoma; CFSE, carboxyfluorescein diacetate succinimidyl ester; FCRL, Fc receptor-like.

Results

The effect of FCRL1 knockdown on the proliferation of the BL cell lines

Since the B-cell receptor (BCR) signaling plays a critical role in driving proliferation, the effect of FCRL1 knockdown was investigated in the proliferation of the BL cell lines by a CFSE labeling assay. The CFSE assay is a superior tool for the quantitative analysis of cell proliferation ability and could be detected about 8 rounds of cell proliferation.[41,42] Our flow cytometry results revealed that FCRL1 knockdown significantly decreased the proliferation of treated Ramos (Figure 1) and Daudi (Figure 1) cells compared with control cells on day 3 of infection procedure ($P < .01$–$.05$). The same results were also observed after 5 days of incubation ($P < .01$, Figure 1a).

The effect of FCRL1 knockdown on the extent of the Bcl-2, Bid and Bax of BL cell lines

The regulation of human B-cell apoptosis is critical to maintaining hemostasis and cell tolerance. Previous studies have reported that the Bcl-2 family proteins play a fundamental role in the regulation of apoptotic cell death. They contain various numbers of anti-apoptotic and pro-apoptotic proteins.[43] To investigate the effect of FCRL1 knockdown on the B-cell

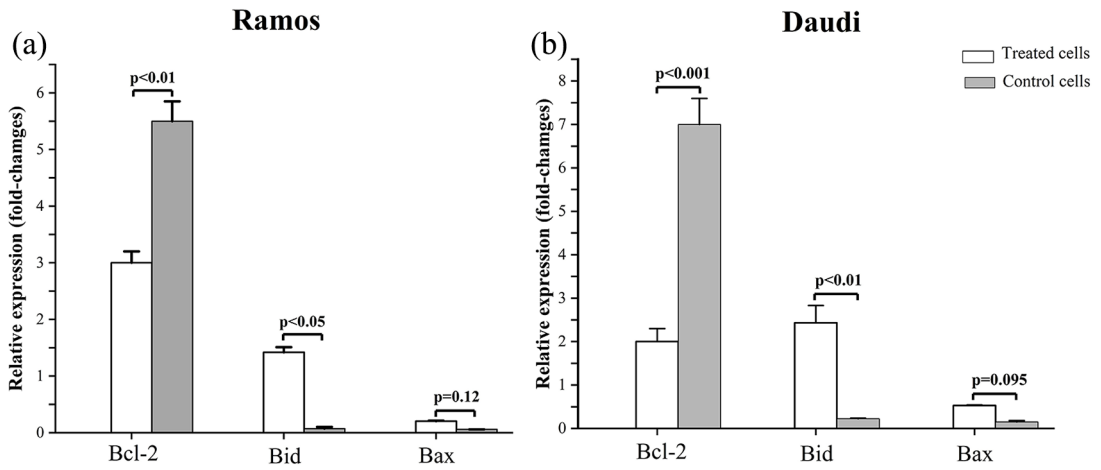

Figure 2. The effect of FCRL1 knockdown on the extent of the expression of anti-apoptotic *Bcl-2* and pro-apoptotic *Bid* and *Bax* genes in the (a) Ramos and (b) Daudi cells after 2 days of infection procedure. All data show mean ± standard deviation.
FCRL indicates Fc receptor-like.

apoptosis, the extent of the expression of anti-apoptotic *Bcl-2* and pro-apoptotic *Bid* and *Bax* genes was measured in the BL cells by using the real-time PCR on day 2 of infection procedure. The results showed that the *Bcl-2* mRNA level significantly decreased in the treated Ramos cells (median 2.5-fold change, $P < .01$) and treated Daudi cells (median 5-fold change, $P < 001$) compared with control cells. There was a significant increase in the extent of the *Bid* gene expression in the treated Ramos (median 1.5-fold change, $P < .05$) and Daudi cells (median 2.21-fold change, $P < .001$) compared with control cells. However, no significant difference was observed in the extent of the *Bax* gene expression in both treated Ramos and Daudi cells compared with control cells (Figure 2a and b).

Staining of the BL cells with a combination of PE Annexin V and 7-AAD and flow cytometry analysis were then performed to further confirm the effect of FCRL1 knockdown in B-cell apoptosis. The results revealed that FCRL1 knockdown significantly increased the apoptosis of treated Ramos ($P < .05$) and Daudi cells ($P < .01$) compared with control cells, after 2 days of infection procedure. Also, the percentage of apoptotic cells increased over time in both treated Ramos and Daudi cells compared with control cells ($P < .001$–$.05$) (Figure 3a).

The effect of FCRL1 knockdown on the PI3K/p-AKT expression and p65 NF-κB activation of BL cells

These data suggest that FCRL1 may be involved in the regulation of human B-cell responses. Thus, the understanding of the FCRL1 signaling pathways might be helpful to clarify the mechanisms underlying FCRL1 during B-cell activation and its relation to the various B-cell-related disorders. Phosphoinositide-3 kinase signaling plays an important role in the proliferation of human B cell. In addition, PI3K signaling activated different serine/threonine kinases, including AKT.[44-46] Previous studies have also indicated that PI3K/p-AKT pathway is a fundamental mediator of TNF-α-mediated NF-κB activation in human B cell. AKT phosphorylated and activated p65 subunit of NF-κB after degradation of IκB inhibitor.[47,48] The cross-talk between key transcription factor NF-κB and PI3K/phospho-serine-threonine kinase AKT (p-AKT) led to the activation of several cell survival mediators and apoptosis regulating genes such as *Bcl-2* family genes.[44] Given this evidence, we tested the possible effects of FCRL1 knockdown on the *PI3K*/p-AKT expression and p65 NF-κB activation in the BL cells. There was a significant reduction in the extent of the expression of *PI3K* gene in the treated Ramos (median 2.28-fold change, $P < 01$) treated Daudi cells (median 2.07-fold change, $P < 01$) with control cells, after 3 days of cell infection (Figure 4a). Our flow cytometry results also revealed that the expression level of p-AKT (S473) protein was significantly decreased in both treated Ramos ($P < .001$) and Daudi cells ($P < .01$) compared with control cells on day 4 of the infection procedure (Figure 4b and c).

In addition, the extent of the expression of p65 NF-κB was measured in the BL cells by ELISA method in the presence or absence of TNF-α. The level of phosphorylated p65 NF-κB was significantly decreased in the treated BL cells compared with control cells in the presence of TNF-α after 4 days of cell infection ($P < .05$). Also, there was a reduction in the total p65 NF-κB levels in the treated BL cells compared with control cells in the absence of TNF-α, although it was not significant (Figure 5a).

Discussion

The regulatory potential of FCRL1 in human B-cell responses is not completely clear and previous studies have focused on the expression profile of FCRL1 in normal B cell and various B-cell-related disorders.[6,7,9,27,29-32,34-36,49] In this study, we used BL cell lines Ramos and Daudi as cell models to investigate the potential mechanisms underlying FCRL1 in human B cell. Given the fundamental role of proliferation and apoptosis in the fate of a cell, we examined the potential effects of FCRL1

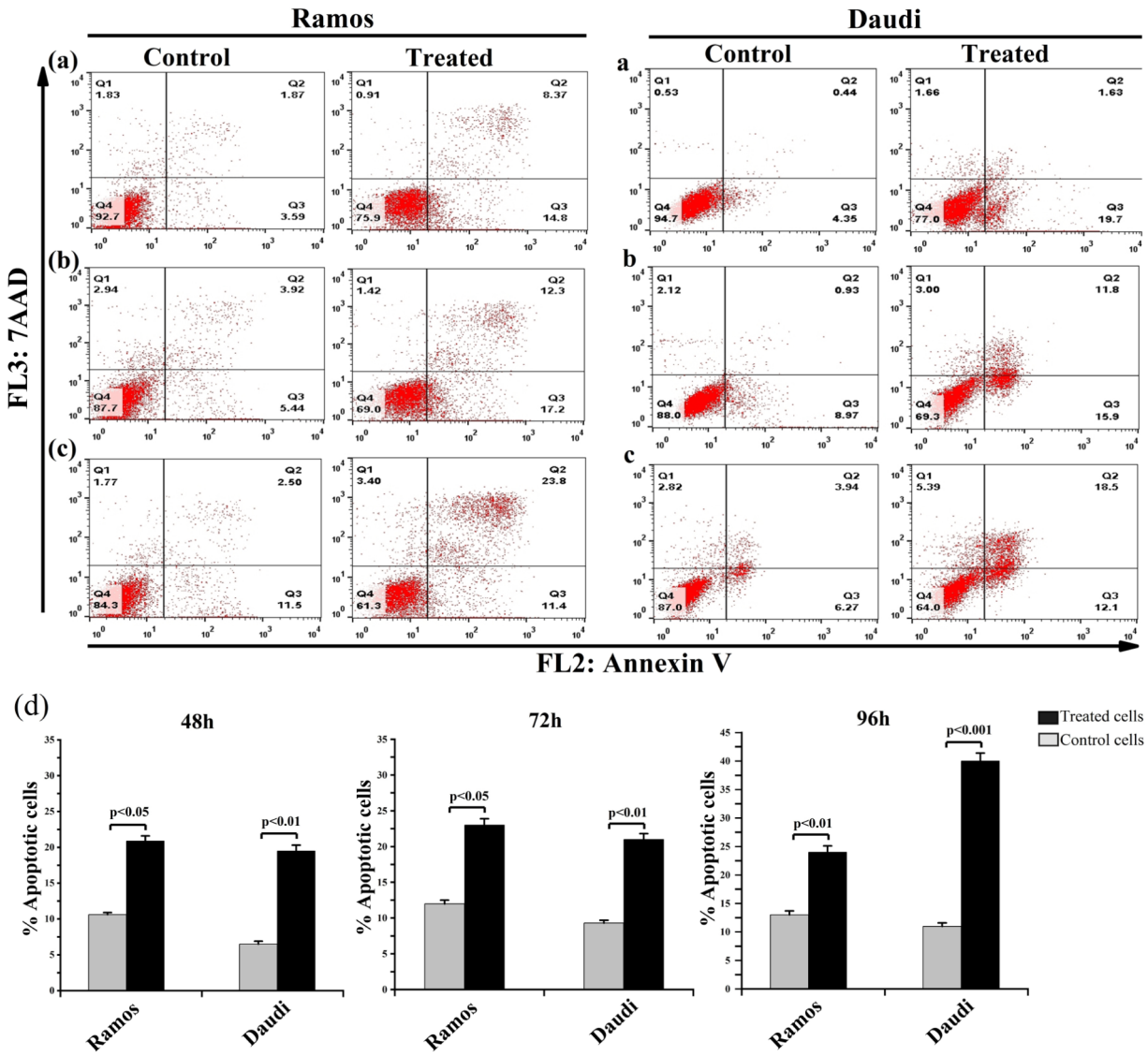

Figure 3. The effect of FCRL1 knockdown on the apoptosis of BL cells. The percentage of the BL cells undergoing early apoptosis (Annexin V(+), 7-AAD (–)) or late apoptosis (Annexin V(+), 7-AAD (+)) is shown in each group after (A) 48 h, (B) 72 h, and (C) 96 h of infection procedure. Data were assessed by using the (a, b, and c) (d) then analyzed by FlowJo. The results indicated as mean ± standard deviation.
BL indicates Burkitt lymphoma; FCRL, Fc receptor-like; 7-AAD, 7-amino-actinomycin D.

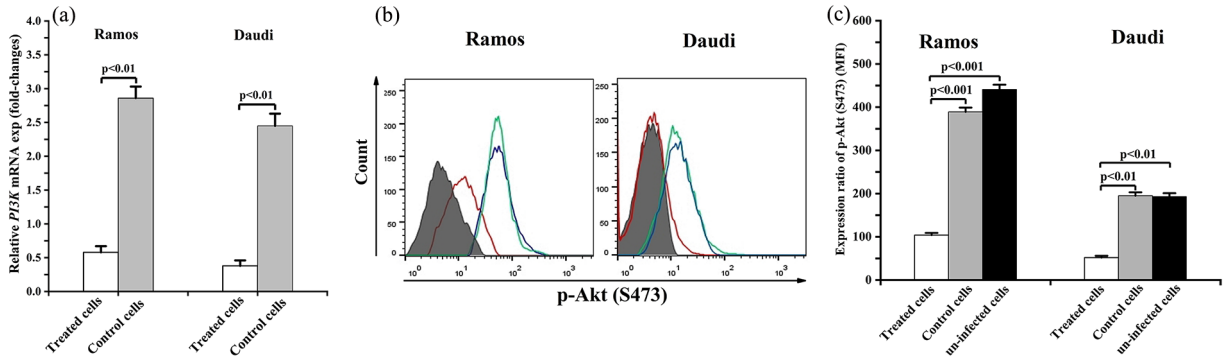

Figure 4. The effect of FCRL1 knockdown the extent of the expression of *PI3K*/p-AKT pathway in the Ramos and Daudi cells. (a) The real-time PCR approach revealed a significant decrease in the extent of the *PI3K gene expression* in the BL cells after 3 days of the infection procedure. The extent of the expression of the p-AKT protein was measured by (b) cytometry and (c) using the FlowJo software on day 4 of infection procedure. Shaded-matched represents matched isotype control antibody, blue represents uninfected cells, green represents control cells, and red represents treated cells. Data are shown as mean ± standard deviation.
BL indicates Burkitt lymphoma; FCRL, Fc receptor-like; PCR, polymerase chain reaction.

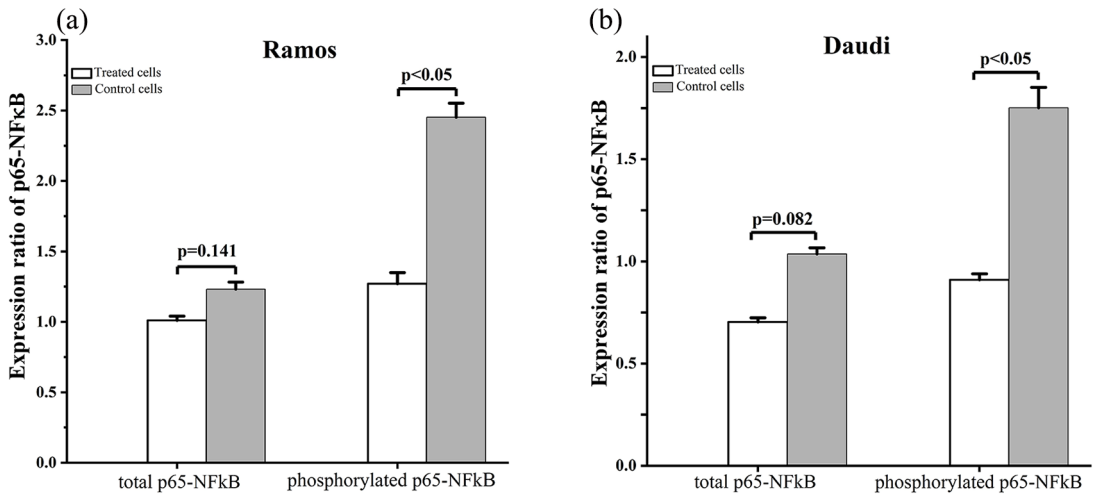

Figure 5. The effect of FCRL1 on the knockdown on the p65 NF-kB activity of BL cells. The expression of p65 subunit of the NF-kB was measured in the (a) Ramos and (b) Daudi cells by ELISA method in the presence (phosphorylated p65-NF-kB) or absence (total p65-NF-kB) of TNF-α, after 4 days of infection procedure. Data indicated as mean ± standard deviation.
BL indicates Burkitt lymphoma; FCRL, Fc receptor-like; ELISA, enzyme-linked immunosorbent assay.

in proliferation and apoptosis of BL cells through knockdown of FCRL1 expression. The effects of FCRL1 knockdown in the extent of the expression of key transcription factor NF-κB and important survival signaling pathway PI3K/p-AKT were also investigated in this study.

The FCRL1 knockdown was performed by using the several nonviral gene delivery methods, including Lipofectamine 3000, Polyethylenimine (PEI), Ca_2PO_4 precipitation method, and Electroporation. Regarding nonadherent cells, such as human B cells are resistant to the prevalent nonviral gene delivery methods, our results showed a low rate of transfection efficiency and high levels of cell death in the BL cells. We used a combination of retroviral-based shRNA transfer technology, centrifugation, and optimal concentrations of Polybrene to reduce these limitations and effective knockdown of FCRL1 expression in the BL cells.

This study revealed that blocking of FCRL1 expression resulted in the decreased proliferation of treated BL cells compared with control cells. This finding is consistent with the data of a similar research about the regulatory function of FCRL1 indicating the important role of FCRL1 in B-cell proliferation.[26] Our survey also showed that FCRL1 knockdown increased the expression levels of pro-apoptotic *Bid* and *Bax* genes and decreased the extent of the expression of anti-apoptotic *Bcl2* gene in treated BL cells compared with the control cells. In addition, investigation of the effect of FCRL1 knockdown on the B-cell apoptosis revealed a significant increase in the percentage of apoptotic cell death in the treated BL cells compared with control cells. The observed effects of FCRL1 knockdown in human B-cell apoptosis might be mediated by the different mechanisms involved in the cell cycle progression and thus regulate apoptotic cell death. A growing body of evidence revealed that Bcl-2 family proteins play an important role in the regulation of proliferation, apoptosis, and cell cycle progression. It appears that downregulation of anti-apoptotic Bcl-2 and upregulation of pro-apoptotic proteins such as Bid and Bax inhibited cell cycle progression and thereby enhanced cell apoptosis by induction of mitochondrial membrane permeabilization.[50-53]

In addition, in an in vitro study performed by Leu et al,[26] it has been indicated that FCRL1 ligation results in the enhancement of Ca^{2+} flux in human B cell. Ca^{2+} signaling contributes in the regulation of proliferation and cell apoptosis by control of the cell cycle progression.[54,55] Also, previous studies have shown that increasing the Ca^{2+} flux induced cell cycle progression, which in turn inhibited cell apoptosis.[54-56] These findings may explain the potential effects of FCRL1 knockdown in human B-cell apoptosis. However, our findings on the FCRL1 knockdown effects on B-cell apoptosis are in contrast with the above-mentioned study, which was performed by using the Fab-fragments of anti-FCRL1 monoclonal antibodies to investigate FCRL1 function in human B-cell responses. It has been revealed that FCRL1 had no effect on the human B-cell apoptosis and expression levels of anti-apoptotic proteins Bcl2, Bcl-xL, Mcl-1, and pro-apoptotic protein Bax.[26] This observed discrepancy may be due to the differences in the sensitivity of applied methods in each study. Therefore, additional studies are required to evaluate the regulatory functions of FCRL1 in the apoptosis and cell cycle progression of human B cells and determine the mechanisms involved in these events.

In the next step, we investigated the FCRL1 signaling in BL cells. Interactions between the PI3K/p-AKT signaling pathway and transcription factor NF-κB have resulted in the inhibition of apoptosis and increased cell proliferation.[48] The aberrant expression of PI3K/p-AKT and constitutive activation of NF-κB have been found in a number of hematological malignancies, including BL and diffuse large B-cell lymphoma. Several studies have indicated that these activated signaling pathways play major roles in the pathogenesis and development of various cancers.[44,57,58] Regarding the important effects

of FCRL1 and signaling pathways *PI3K*/p-AKT as well as NF-κB in the B-cell activation and pathogenesis of different B-cell-derived malignancies, the relation between FCRL1 and the extent of the expression of *PI3K*/p-AKT and the p65 subunit of NF-κB was examined in the BL cells. The results revealed that FCRL1 knockdown led to a significant reduction in the levels of *PI3K*/p-AKT pathway and decreased p65 NF-κB activity in both treated BL cells compared with control cells. These findings suggest FCRL1 is a remarkable candidate for immunotherapeutic interventions of various FCRL1 positive B-cell-related disorders.

It should be indicated that the presented study had some limitations: The extent of the PI3K and p-AKT expression was not evaluated by Western blotting. In addition, the effect of FCRL1 on the other downstream components involved in the B-cell responses was not evaluated, which should be examined in further studies.

Conclusions

This study shows the activation potential of FCRL1 in human B-cell responses and the correlation between this receptor with important activated signaling pathways, including PI3K/p-AKT pathway and NF-κB. However, more studies are required to understand this correlation and determination of the physiological roles of the FCRL1 in human B cell.

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Author Contributions

ZY assisted with study design, performed the flow cytometry and real-time polymerase chain reaction assays, analyzed the data, interpreted the results, and drafted the article. SSH and NE conceived and designed the study, and contributed to data interpretation and drafting of the article. VYA and ARA helped with study design, performed the enzyme-linked immunosorbent assay, assisted with data analysis, and critically reviewed the article. All authors read and approved the final article. NE supervised and approved the final version.

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