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Cloning, Expression and Characterization of Recombinant Human Fc Receptor Like 1, 2 and 4 Molecules

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Background: The Fc receptor like (FCRL) molecules belong to the immunoglobulin (Ig) superfamily with potentially immunoregulatory function. Among the FCRL family FCRL2 and 4 are predominantly expressed on memory B cells and FCRL1 is a pan- B cell marker. To date, no ligand has been identified for the human FCRL1, 2 and 4 molecules.
Objectives: Cloning, expression, purification and structural analysis of the extracellular domain of human FCRL1, 2 and 4 proteins.
Materials and Methods: In this study, the extracellular part of human FCRL1, 2 and 4 were subcloned into prokaryotic expression vectors pET-28b (+) and transformed into BL21-DE3 *E.coli* strain. Protein expression was optimized by fine adjustments such as induction time, incubation temperature and expression hosts. Recombinant FCRL proteins were purified by metal affinity chromatography using Ni-NTA resin. Purified FCRL proteins were further characterized by SDS-PAGE and immunoblotting using His-tag and FCRL specific polyclonal antibodies.
Results: Our results demonstrated that FCRL1, 2 and 4 were successfully expressed in pET-28b (+) vector. Optimization of the expression procedure showed that IPTG induction at OD600 = 0.9 and overnight incubation at 37˚C resulted in the highest expression levels of FCRL proteins ranging from approximately 15% (FCRL1) to 25% (FCRL2 and 4) of the total bacterial lysate proteins.
Conclusions: These purified recombinant proteins are potentially a valuable tool for investigating the immunoregulatory function of FCRL molecules and the production of specific mAbs for immunotherapeutic interventions.

Keywords: FCRL; His-tag; Polyclonal Antibody; Protein Expression; Purification

1. Background

Signaling through the B-cell Receptor (BCR) can induce B cell activation, proliferation, differentiation or apoptosis. Thus, regulation of the BCR signaling is crucial for B cell development and function (1). Balancing the competing activation and inhibitory signals is critical in regulating a BCR-mediated response to an antigenic stimulation. In addition to classical BCR negative regulators (FcγRIIb, CD22 and CD72) and positive regulators (CD19 and CD86), it has been recently shown that Fc receptor like (FCRL) molecules also have the potential to be important regulators of BCR signaling (2-4).

The human FCRL family is composed of six homologous members which are exclusively expressed in immune cells, particularly B cells. Each member possesses three to nine Ig domains in its extracellular region, a cytoplasmic tail together with consensus immunoreceptor tyrosine-based activating (ITAM) and/or inhibitory (ITIM) motifs (2, 5). FCRL1, 2 and 4, which are the subject of this study, have been found only on B lineage cells (3), with FCRL2 and 4 being predominantly expressed in memory B cells (3, 4), whereas FCRL1 is a pan B cell marker (2). Due to their unique expression on B lymphocytes, FCRLs have been introduced as suitable targets for immunotherapy of B-cell malignancies such as chronic lymphocytic leukemia, hairy cell leukemia and B-cell non-Hodgkin's lymphoma (6, 7). FCRL1 has 2 ITAM-like motifs, a glutamic acid residue in its transmembrane region, and 3 extracellular Ig-like domains (2). FCRL2 has four extracellular Ig domains, an uncharged transmembrane segment and a cytoplasmic tail that contains a potential ITAM and two consensus ITIMs (3). FCRL4, on the other hand, has 3 ITIM-like motifs, three acidic glutamic acid residues in its trans-

Implication for health policy/practice/research/medical education:
The optimized condition for the production and purification of these recombinant proteins will be useful for other scientists working in this field.

membrane region and 4 extracellular Ig-like domains (4, 8). Thus, FCRL1 and FCRL4 could operate as either activating or inhibitory receptors, whereas FCRL2 may play as a bifunctional receptor due to possessing both ITAM and ITIM motifs.

2. Objectives

To date, no ligand has been identified for any of the human FCRL molecules, except FCRL6 which has recently been shown to bind to HLA-DR, an MHC class II molecule (9). Search for functionality of these molecules without known ligand(s) needs extensive investigations using recombinant FCRL molecules and specific polyclonal and monoclonal antibodies (mAbs). In this study, we present data on the cloning, expression and purification of extracellular part of human recombinant FCRL1, 2 and 4 molecules. The optimization of the expression protocol resulted in a high expression level in a prokaryotic expression system with a potential implication for antibody production and functional and structural analyses of FCRL molecules.

3. Materials and Methods

3.1. FCRL Plasmids Construction

The FCRL1-pCMV6-XL5 (SC123097, NM_052938, 2657bp), FCRL2-pCMV6-XL5 (TC305281, NM_030764, 1600bp) and FCRL4-pCMV6-XL5 (SC305326, NM_031282, 1600bp) DNA ready to transfection have been purchased from ORIGENE company (ORIGENE, MD, USA). These constructs were used as template for subcloning the extracellular portion of FCRL molecules into pET-28b(+) vector containing 2 His-tags in the up and down stream of the multiple cloning site (Novagen, Madison, WI, USA). The extracellular region of FCRL1, 2 and 4 were amplified with FCRL specific primers harboring restriction enzymes cutting sites (Table 1). Briefly, PCR amplification was performed with pfu taq DNA polymerase (Promega, Madison, WI, USA) as follows: heating for 5 min at 95 °C, 35 cycles of denaturation for 30 s at 95 °C, an annealing process for 30 s at 57 °C, and an extension for 2 min at 72 °C. The amplified genes were electerophoresed on a 1.5% Low Electroendosmosis (LE) agarose gel, extracted and purified by QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In the next step, seven thymidine bases were added to the 5' end of FCRL specific primers (defined as TT-FCRL primers) used as docking site for restriction enzymes. Then the purified PCR products were used as template for amplification using the new set of TT-FCRL primers following the same PCR protocol described above. The new PCR products were subsequently extracted and digested with the corresponding restriction enzymes. After re-purification, the FCRL amplicons were ligated into digested pET-28b(+) vector using T4 DNA ligase (Promega, Madison, WI, USA) according to the manufacturer's instruction. The inserted genes were verified by restriction enzyme digestion and DNA sequencing methods using universal T7 promoter and terminator primers (Applied Biosystems 3130 genetic analyzer, Foster City, CA, USA).

Table 1. Specific Primers Employed for Subcloning of the Extracellular Region of FCRL1, 2 and 4

Primer Name	Sequence[a]	Amplicon Size, bp
EcoR I -FCRL1 forward	GAATTCGATGCTGCCGAGGCTGTTGCTG	936
Not I-FCRL1 reverse	GCGGCCGCTGAGGTAAGATGATTGCTTCTGGCC	936
Nco I -FCRL2 forward	CCATGGATATGCTGCTGTGGTCATTGCTGGTCAT	1216
Not I -FCRL2 reverse	GCGGCCGCTCCAGCTGTCATGAGGTCTCTTCTA	1216
Sal I-FCRL4 forward	GTCGACATGCTGCTGTGGGCGTCCTTG	1172
Not I-FCRL4 reverse	GCGGCCGCGCCATCTCTGTTGCCTGGGGTC	1172

[a] restriction sites are underlined.

3.2. Expression of Recombinant FCRL Proteins

The competent BL21-DE3 *Escherichia coli* strain (Novagen, Madison, WI, USA) was transformed with FCRL-pET-28b (+) constructs by heat shock method. Briefly, 50 μL of competent BL21-DE3 glycerol stock was thawed on ice and mixed gently. Then 1 μL of FCRL-pET28b (+) construct was added directly to the bacterial suspension, stirred gently and incubated on ice for 5 min. The mixture was incubated for exactly 70 s in a 42°C water bath and quickly placed on ice for 2 min. For recovery, 100 μL of regular Luria–Bertani (LB) broth (10 g bacto-tryptone (Sigma, St. Louis, USA), 5 g bacto-yeast extract (Sigma, St. Louis, USA), 5 g NaCl (Roche, Germany), pH 7.0 was added on the transformed cells and incubated at 37 °C for 1 h without shaking. The transformed bacteria were cultured on LB agar plates containing Kanamycin and selected by colony PCR using T7 universal promoter primer (Novagen, Madison, WI, USA) as forward and Not I-FCRL1, 2 and 4 as reverse primer (Table 1). Single transformed colony was inoculated into 5 mL pre-heated LB broth medium supplemented with 50 μg.mL $^{-1}$ Kanamycin while being shaked at 250 rpm at 37 °C. Three milliliters of the overnight starter culture were added to 500 mL of LB media and let OD600 nm to reach the desired point. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) (Sigma, St. Louis, USA) and cells were grown for a further 14 - 16 h in shaker incubator at 250 rpm at 37 °C. One milliliter of

cultured induced bacteria was collected and centrifuged at 4000 × g for 10 min. The pellet was dissolved in SDS-PAGE sample buffer 6X (Tris-base 0.27 M, pH = 6.8, SDS 6%, bromophenol blue 0.06%, glycerol 60%, DTT 0.1 M) and heated in boiling water bath for 3 min. After centrifugation at 13000 rpm for 5 min, 20 μL of supernatant were separated by 10% SDS-PAGE and stained by coomassie brilliant blue. Furthermore, the expression of recombinant FCRL proteins in bacteria was confirmed by immunoblotting technique.

3.3. Purification of Recombinant FCRL Proteins

After the confirmation of the recombinant FCRL expression, the remaining amount of cultured bacteria was centrifuged and the pellet was washed twice with wash buffer (NaH_2PO_4 100 mM and Tris-Hcl 30 mM) and stored at 70 °C overnight. For cell lysing a ratio of 10 mL of cold lysis buffer (pH = 8 containing NaH_2PO_4 100 mM, Tris-Hcl 30 mM and NaCl 100 mM) to 1 g of wet weight bacteria was applied and mixture was incubated for 1 h on ice. Cells were lysed completely by sonication with 15 cycles (60 s on at 25% power, 60 s off, at 0°C) (HD2070 BANDELIN Sonopuls, Berlin). The lysate was centrifuged at 10000 rpm for 10 min at 4°C and the inclusion bodies in the pellet were dissolved in buffer A (NaH_2PO_4 100 mM, Tris-base 10 mM, urea 8 M, NaCl 100 mM, pH = 8) containing imidazole 30 mM and centrifuged at 12000 rpm for 10 min. Ni–NTA agarose resin (Qiagen, Hilden, Germany) was equilibrated with buffer A. The resin was then mixed with transparent filtered lysate at a ratio of 1 mL of resin (50% slurry) with 1 mL of lysate and the mixture was shaken for 30 - 45 min on a rotator platform. After coupling the FCRL-His-tag recombinant proteins to Ni-NTA resin, unbound fraction was washed and removed with buffer A supplemented with 30 mM imidazole. Bound proteins were subsequently eluted by gradual increasing of imidazole molarity (80, 300 and 1000 mM) in buffer A. In order to remove urea, the eluted recombinant proteins were dialyzed against phosphate buffer saline (PBS). In addition, protein purity was determined by analyzing coomassie-stained SDS-PAGE as well as immunoblotting.

3.4. Production of Polyclonal Antibodies to FCRL Peptides

The specific peptides related to extracellular part of human FCRL1, 2 and 4 were designed (Table 2), purchased from Thermo Electron Corporation (Thermo Electron Corporation, GmbH, Ulm, Germany) and conjugated to Keyhole Limpet Hemocyanin (KLH) (Sigma, St. Louis, USA) according to standard protocol. Briefly, 1 mg of Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) in 200 μL of Dimethyl Formamide (DMF) was added to the KLH solution (5 mg in 1 mL deionized water) and incubated at room temperature for 2 h with gentle stirring. The MBS activated KLH was dialyzed against large volumes of PBS overnight and added to the FCRL peptide solution (5 mg in 1 mL PBS) and the mixture was incubated for 4 h at room temperature. After overnight dialysis against PBS, the FCRL-KLH conjugates were stored at -20 °C for later use. Six-month-old New Zealand white rabbits were immunized subcutaneously with the FCRL peptide-KLH in Freund's adjuvant five times on weeks 0, 4, 7, 9, 11. The first immunization was performed using an emulsion of peptide-KLH (250 μg) and Freund's complete adjuvant (Sigma, St. Louis, USA) and the subsequent ones with peptide-KLH (125 μg) and incomplete Freund's adjuvant (Sigma St. Louis, USA). Blood samples were taken before every immunization for evaluation of anti-FCRL peptide antibody titration by ELISA. Sera were obtained from the immunized rabbits 2 weeks after the last immunization. The rabbits' IgG were purified with affinity purification using protein-G Sepharose 4B columns (GE Healthcare, Uppsala, Sweden) and their purity was checked by SDS-PAGE.

Table 2. Peptides Designed From the Extracellular Domains of FCRL1, 2 and 4 Molecules for Polyclonal Antibody Production

Peptide Name[a]	Peptide Sequence	Amino Acid Position	Gene ID [NCBI]
FCRL1-1	QINVHRVPVADVGKKC[b]	102 - 113	115350
FCRL1-2	QPPGGQVMEGDRLVLIGKKC	118 - 133	115350
FCRL2-1	CQGEQNWKIQKMA	38 - 50	79368
FCRL2-2	CKAETVTHRIRKQ	177 - 189	79368
FCRL4-1	IQELFPHPELGKKC	186 - 195	83417
FCRL4-2	LRAELELPAIRQGKKC	340 - 351	83417

[a] Two peptides were designed for each FCRL molecule.
[b] Underlined sequences were used for conjugation to KLH.

3.5. Immunoblotting of Recombinant FCRL Proteins Under Reduced and Non-Reduced Conditions

Purified FCRL recombinant proteins were suspended in sample buffer, separated on a 10% SDS-PAGE under reduced and non-reduced conditions and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, MA, USA). The membrane was blocked with 5% skim milk in PBS containing 0.05% Tween 20 (Sigma, St. Louis, USA) (PBST) at 4 °C overnight and subsequently immunoblotted with primary antibodies [polyclonal rabbit an-

ti-His-tag (abcam, Cambridge, UK) at 1: 1000 dilution, polyclonal anti-FCRL1 and FCRL4 peptides produced in our lab at 10 - 15 μg.mL^{-1} concentration] for 1.5 h at room temperature on shaker platform. After extensive washing with PBST, the membrane was next incubated with the appropriate dilution of peroxidase-labeled sheep anti-rabbit Ig (produced in our lab) for 45 min. The bands were visualized using ECL chemiluminescence detection system (GE Healthcare, Uppsala, Sweden) and exposed by ECL Hyperfilm.

3.6. Transient Transfection of CHO Cell Lines

The transfection reagent, jetPEI (Polyplus, Paris, France), was used to transfect the CHO cell line (National Cell Bank of Iran, Tehran, Iran) based on the manufacturer's recommendation. The CHO cells were seeded at 3×10^5 cells/well in a 6-well plate a day before transfection. On the transfection day, 6 μL of jetPEI and 3 μg of constructs were diluted with 100 μL of 150 mM NaCl separately and mixed gently. The DNA solution was then added to the jetPEI solution, and after 20 min of incubation at room temperature, 200 μL of the jetPEI/constructs mixture were added drop-wise onto the cells grown in the serum containing medium. The mixture was homogenized by gently swirling the plate and the cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere. Transfection experiments were analyzed after 48 h by flow cytometry.

3.7. Inhibition of Binding of Anti-FCRL mAbs to FCRL Transfected Cells by Recombinant FCRL Proteins

In this experiment 10 μg.mL^{-1} of commercial mAbs to FCRL1 (1F9, human IgG4), FCRL2 (7G7, mouse IgG1), FCRL4 (1A3, mouse IgG2a) (generous gift from Prof. Polson, Genentech Inc., CA, USA), mAbs against recombinant FCRL2 (3D8-G8, mouse IgG1) and FCRL4 (1A5-C10 mouse IgG2b) (produced in our lab, data not presented) were separately adsorbed to 10 and 40 μg.mL^{-1} of the corresponding purified recombinant FCRL1, 2 and 4 proteins at room temperature overnight. Adsorbed and non-adsorbed mAbs were used to stain CHO cells transiently transfected with the corresponding FCRL genes. Stained cells were subjected to flow cytometry.

The transient CHO-FCRL and CHO-pCMV6-Neo transfected cell lines were washed 2 times with wash buffer (PBS containing 1% BSA) and stained with 5 μg.mL^{-1} of commercial mAbs (adsorbed and non-adsorbed) and 10 μg.mL^{-1} of our FCRL2 and 4 mAbs (adsorbed and non-adsorbed) for 1 h at 4°C. Cells were then washed 2 times with wash buffer and stained with appropriate dilution of FITC-conjugated goat anti-mouse Ig (Sigma, St. Louis, USA) or FITC-conjugated goat anti human polyvalent Ig. Stained cells were subsequently analyzed by flow cytometry (FacsScan flow cytometer, Becton-Dickinson, San Jose, CA, USA).

4. Results

4.1. FCRL Plasmids Construction

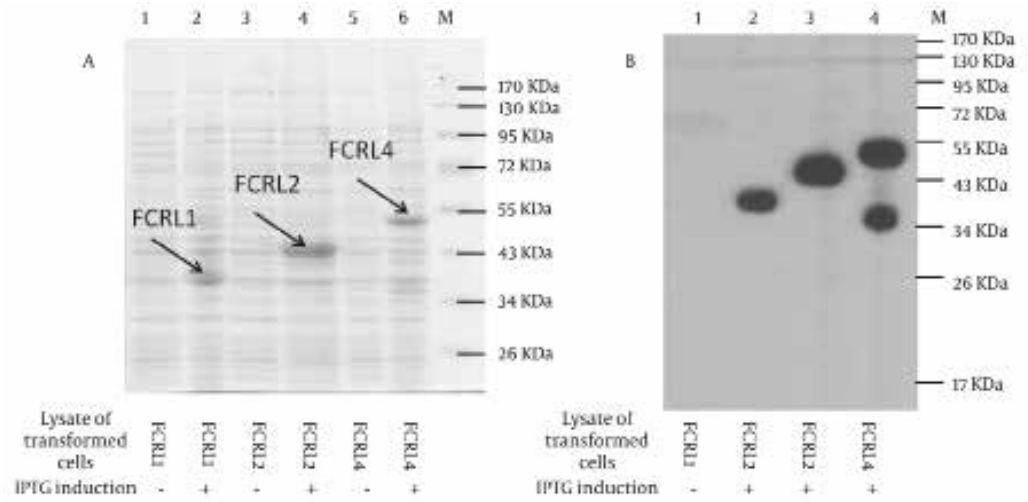
The extracellular region of FCRL1, 2 and 4 molecules was amplified from the full-length FCRL1-, FCRL2- and FCRL4-pCMV6-XL5 constructs using PCR. Electrophoresis of the PCR products verified the amplification of the target genes. The size of purified FCRL1, 2 and 4 amplicons was 936 bp, 1216 bp and 1172 bp, respectively (data not presented). The amplification was repeated on the purified products using specific TT-FCRL primers and the amplified products were directly digested. The DNA fragments encoding FCRL1, 2 and 4 were then cloned into prokaryotic expression plasmid pET-28 b(+), followed by restriction site analysis and DNA sequencing for final approval. DNA sequencing analysis showed that the sequences of FCRL1, 2 and 4 were consistent with those deposited at NCBI (FCRL1 GenBank accession no: NM_052938, FCRL2 GenBank accession no: NM_030764 and FCRL4 GenBank accession no: NM_031282). These results clearly indicate that the expression plasmids were constructed successfully with no mutation, frame shift or stop codons.

4.2. Optimization of Expression of the FCRL Constructs

The pET-28b (+)-FCRL1, 2 and 4 constructs were transformed into BL21-DE3 *E.coli* strains and cultured on LB agar medium containing Kanamycin (50 μg.mL^{-1}). Some single growing colonies were checked by PCR using T7 promoter as forward and Not I-FCRL1, 2 and 4 as reverse primers (Table 1). Positive colonies were selected and protein expression was induced by IPTG (1 mM) when OD600 reached a 0.6 absorbance value and then were cultured at 25 °C overnight. Interestingly, SDS–PAGE analysis clearly indicated that all FCRL molecules were successfully expressed in BL21-DE3 transformed cells (Figure 1 A). Theoretically, the molecular weight of extracellular recombinant FCRL1 (354aa, PI: 6.43), FCRL2 (413aa, PI: 6.5) and FCRL4 (437aa, PI: 6.8) proteins was determined to be 38.5 KDa, 45.1 KDa and 48.8 KDa, respectively. The expressed FCRL1 and 4 molecules contain two His-tags at their C- and N-terminals, whereas FCRL2 contains one His-tag in its C-terminal. In order to clone FCRL2, the Nco I restriction site was chosen in order to sublcone it into pET-28 b(+), upstream of the N-terminal His-tag. Thus, the expressed FCRL2 contains only one His-tag in its C-terminus. Immunoblotting of the transformed BL21-DE3-FCRL extracts with polyclonal anti-His-tag antibody showed that all FCRL proteins were strongly expressed and exhibiting the expected molecular weight (Figure 1 B). The results obtained from our extensive optimization experiments, using a variety of bacterial strains, including [BL21(DE3)-pLysS (Novagen, Madison, WI, USA), BL21-CodonPlus-DE3-RIL (Stratagene,

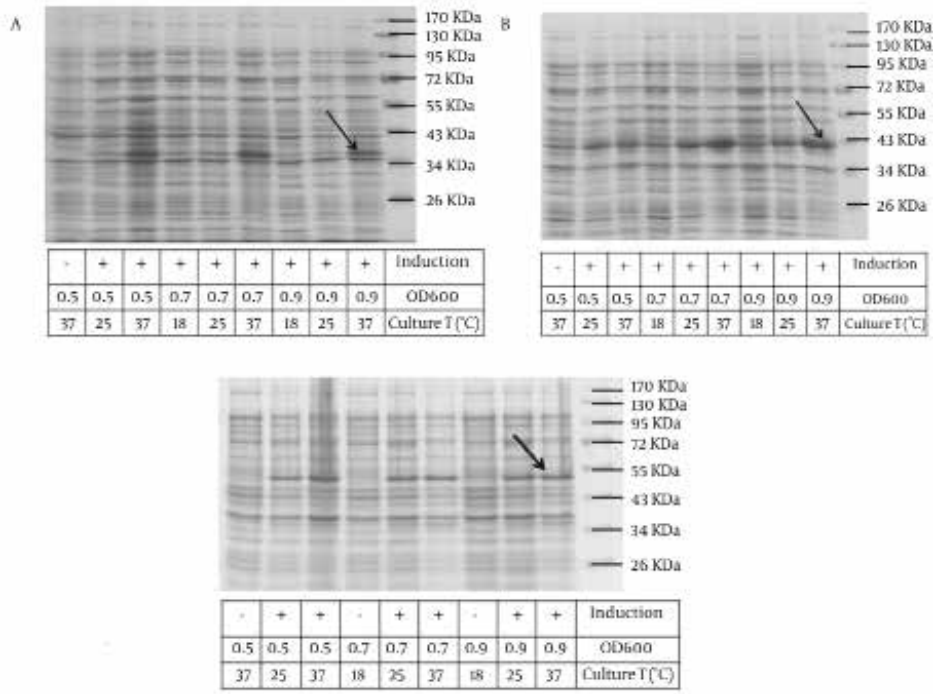
La. Jolla, CA, USA), NovaBlue-DE3 (Novagen, Madison, WI, USA), Origami™B-DE3-pLacI (Novagen, Madison, WI, USA), Rosetta-gami™-DE3-pLysS (Novagen, Madison, WI, USA) and JM109 (Takara, Kyoto, Japan)] (data not presented), and different induction times (1, 2, 3, 4, 5, 6, and 14 h), incubation temperatures (37°C, 25°C and 18 °C) and induction points (OD600 : 0.5, 0.7, 0.9 and 1.1) showed that induction at OD600 = 0.9 in BL21-DE3 incubated at 37 °C are the optimum conditions for all of the FCRL genes expression. Representative results are illustrated in Figure 2. The densitometry of the expressed protein bands in comparison to that of the total cell extract in each lane showed expression efficiency of approximately 15%, 25% and 25% for FCRL1, FCRL2 and FCRL4, respectively.

Figure 1. Expression of FCRL1, 2 and 4 Proteins in BL21-DE3 Transformed With pET-28b(+)-FCRL Constructs



(A) SDS–PAGE profile of the bacterial extract before and after induction; (B) Western blot analysis of the bacterial extract using polyclonal anti-His-tag antibody.

Figure 2. Representative Results Obtained From the Optimization of FCRL1

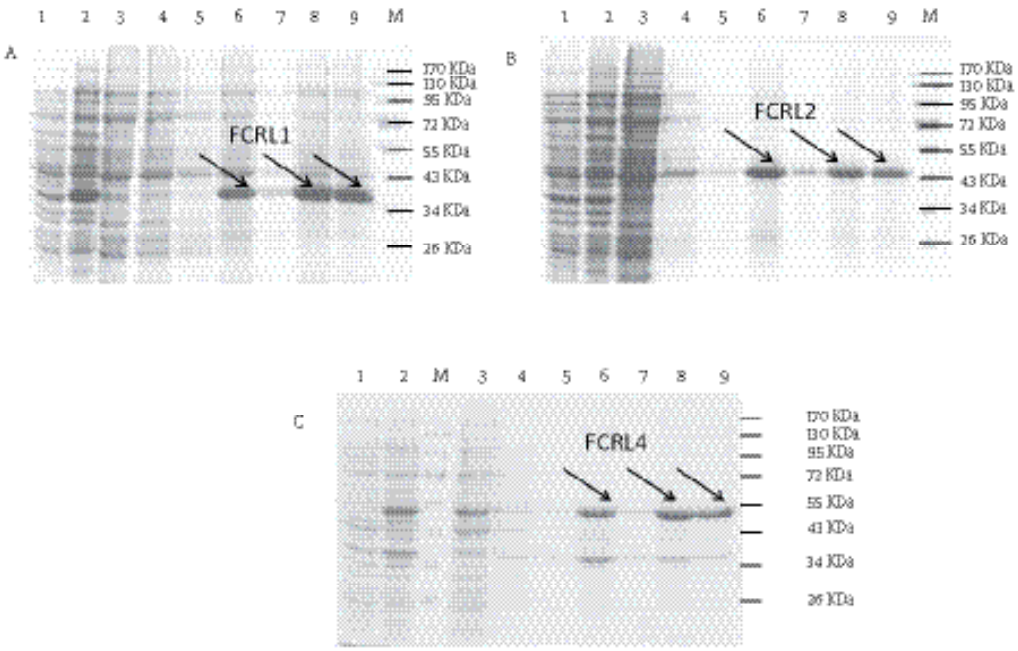


(A), FCRL2 (B) and FCRL4 (C) Expression of pET-28b (+) Vector in BL21-DE3 Cells
Induction at OD600=0.9 in FCRL transformed BL21-DE3 and culture at 37 °C are the optimum conditions for expression of all FCRL genes. Arrowhead represents the recombinant FCRL protein.

4.3. Purification of Recombinant FCRL Proteins

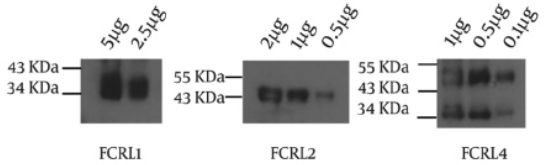
Since all the expressed proteins were obtained from pellets of sonicated lysates after centrifugation as inclusion bodies, so purification was performed under denaturation condition using 8M urea. Large amounts of purified recombinant FCRL proteins were collected following elution of Ni-NTA resin with a buffer containing 300 mM and 1 M imidazole. Representative results obtained for FCRL1, 2 and 4 purification are shown in Figure 3. The purity of FCRL2 and FCRL4 was higher than that of FCRL1 protein. The purified FCRL proteins were detected with polyclonal anti-His-tag antibody by immunoblotting (Figure 4). Although FCRL1 and 2 displayed a single band on their SDS-PAGE, FCRL4 transformed BL21-DE3 bacteria expressed FCRL4 in two different sizes (~47 and 36 KDa) (Figures 1, 3 and 4). The FCRL4 predicted size is 47 KDa, however, the smaller band was seen both in SDS-PAGE and western blotting with polyclonal anti-His-tag antibody (Figure 1). This band could also be detected with polyclonal anti FCRL4-2 peptide (Figure 5 F) and the commercial anti FCRL4 mAbs (1A3) (data not presented). These results confirmed that both bands belong to FCRL4. FCRL4 protein has 4 Ig domains in its extracellular segment. The polyclonal antibody to FCRL4-2 peptide was designed to a segment of the 4th Ig domain of FCRL4 proximal to membrane. Thus, it seems that the small molecule represents the extracellular FCRL4 protein truncated at the N-terminal. This truncated protein might have been generated due to enzymatic digestion. The smaller fragment of FCRL4 has no effect on mAbs production against this recombinant protein, because it is a part of FCRL4. Further investigations should be performed to identify whether it corresponds to the N-terminal or C-terminal of the native protein and whether it displays any functional activity. Basically, the untruncated 47 KDa FCRL4 protein could be electro-eluted from the SDS-PAGE gel for functional and structural studies.

Figure 3. Purification of FCRL1 (A), FCRL2 (B) and FCRL4 (C) Recombinant Proteins by Ni-NTA Chromatography



Lane 1: total cell extract before induction, lane 2: total cell extract after induction, lane 3: Ni-NTA unbound fraction, lane 4: elution with 80 mM imidazole, lane 5: washing with 30 mM imidazole, lane 6: elution with 300 mM imidazole, lane 7: washing with 30 mM imidazole, lane 8: elution with 1 M imidazole, lane 9: re-elution with 1M imidazole, M: PageRuler prestained protein ladder. FCRL2 and FCRL4 were purer than FCRL1 recombinant protein. Arrowhead represents the purified recombinant FCRL protein.

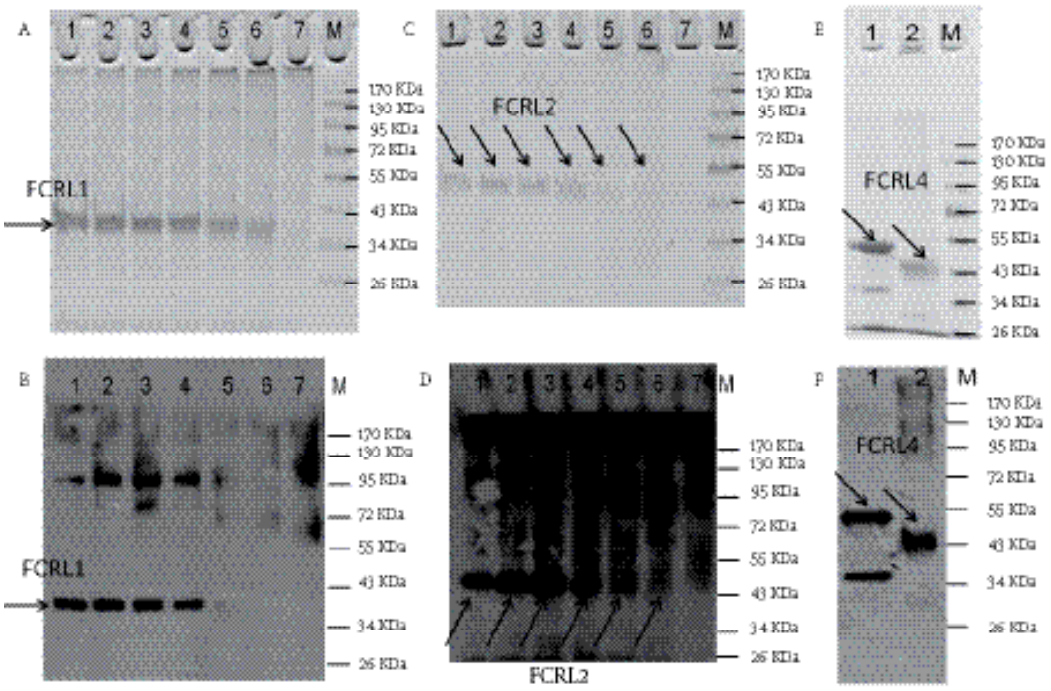
Figure 4. Western Blot Analysis of Purified Recombinant FCRL Proteins Using Polyclonal Anti-His Antibody



4.4. Analysis of the Recombinant FCRL Proteins in Reduced and Non-Reduced Conditions

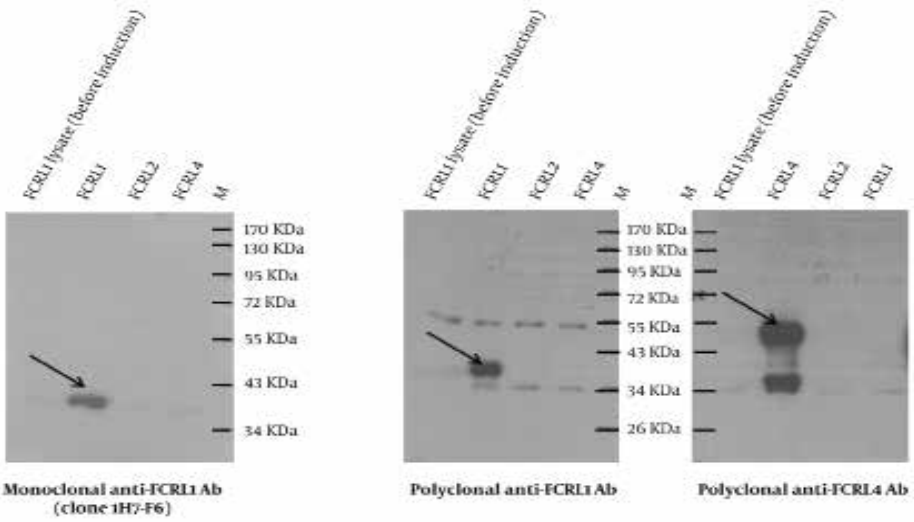
To demonstrate the presence of disulphide bridges in the recombinant FCRL proteins, all purified recombinant FCRL proteins were electrophoresed under reduced and non-reduced conditions. When the recombinant FCRL1 and 2 proteins were run in non-reduced conditions in SDS-PAGE, no band could be detected after staining the

Figure 5. SDS-PAGE and Immunoblotting Profile of Recombinant FCRL1, 2 and 4 Proteins in Reduced and Non-Reduced Conditions



(A) SDS-PAGE and (B) immunoblotting: FCRL1 recombinant protein; (C) SDS-PAGE and (D) immunoblotting: FCRL2 recombinant protein. Lanes 1-6: reduced with different concentration of DTT (lane 1: 100 mM, lane 2: 50 mM, lane 3: 12.5 mM, lane 4: 3.12 mM, lane 5: 0.78 mM, lane 6: 0.19 mM), lane 7: non-reduced, lane M: PageRuler prestained protein ladder. Immunoblotting was performed using polyclonal anti-His-tag antibody. E) SDS-PAGE and (F) immunoblotting profiles of recombinant FCRL4 protein. Lane1: reduced, lane 2: non-reduced, M: PageRuler prestained protein ladder. The polymerized FCRL1 and 2 recombinant proteins were completely reduced to monomer structure by increasing the concentration of DTT. Immunoblotting was performed using polyclonal anti-FCRL4-2 peptide antibody. The reduced monomer band of FCRL4 seems to have a molecular weight higher than the non-reduced monomer, indicating linearization of the reduced FCRL4 molecule results in a shorter migrated distance in the gel.

Figure 6. Detection of Recombinant FCRL1 and FCRL4 Proteins with Monoclonal and Polyclonal anti FCRL Antibodies by Immunoblotting



M: PageRuler prestained protein ladder, arrowhead represents the expressed recombinant FCRL proteins in transformed bacteria.

gel with coomassie blue. Both proteins were polymerized and mostly retained in stacking gel and stacking-resolving gels interface (Figure 5 A and C). Immunoblotting with polyclonal anti-His-tag antibody showed a faint band in the stacking and resolving gels interface (data not presented). This finding implied that the polymerized FCRL1 and 2 recombinant proteins were not transferred from gel to the PVDF membrane because of their big size. To confirm this assumption, the recombinant proteins were reduced with different concentration of DTT and run in a 8% resolving gel without a stacking gel. These experiments showed that, by increasing the concentration of DTT, the polymerized FCRL1 and 2 recombinant proteins were completely reduced to monomer structure (Figure 5 A-D). These results indicate that the cysteine residues in FCRL1 and 2 proteins bind to each other to form highly polymerized molecules.

Similar results were obtained for FCRL4 recombinant protein, except that this protein was not totally polymerized in non-reduced conditions, and a band corresponding to monomer FCRL4 protein with approximately 36 KDa molecular weight, could be observed in non-reduced conditions (Figure 5 E). Thus we decided to use only one concentration of DTT for its reduction. The SDS-PAGE and WB results showed that the reduced monomer band seems to have a molecular weight higher than the non-reduced monomer, indicating linearization of the reduced FCRL molecule resulting in a shorter migrated distance in the gel (Figure 5 E and F). The difference between the upper bands of Figure 5 E and Figure 5 F could be related to the difference of electrophoresis running times for the two experiments. The Coomassie Blue stained proteins shown in Figure 5 E were not transferred and blotted directly. This gel was used just to show the SDS-PAGE pattern. We did run the samples again at the same conditions for the purpose of immunoblotting. Obviously, there will be some variations between the two electrophoresis patterns, despite the fact that the same conditions were applied.

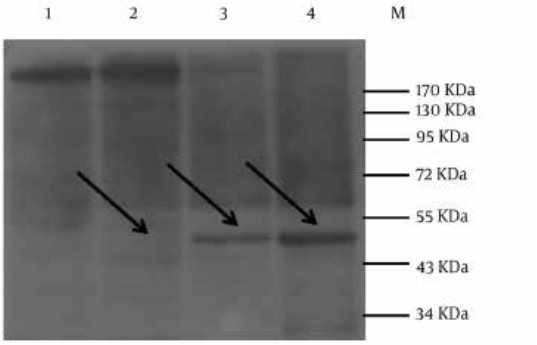
4.5. Identification of Purified Recombinant FCRL Proteins by FCRL-Specific Polyclonal Antibodies

To further characterize the purified FCRL proteins, we separately raised polyclonal antibodies to 6 specific peptides spanning two different parts of the extracellular region of each FCRL molecule (Table 2). Sera collected from all 6 immunized rabbits, each immunized with a single peptide, reacted with the immunizing peptides at dilutions higher than 1/2500 by ELISA. However, only two of the antisera produced to FCRL1-1 and FCRL4-2 peptides could specifically recognize the target proteins by western blot (WB) technique (results not presented). IgG fractions purified from serum of these two rabbits together with a mAb to specific for FCRL1 (clone 1H7-F6) (produced in our lab) were employed to perform immunoblotting on un-purified FCRL proteins. The results showed specific reactivity of these antibodies with their corresponding FCRL proteins without any cross-reactivity with other FCRL proteins or other proteins from the lysate of non-transformed BL21-DE3 bacteria (Figure 6).

4.6. Reactivity of Anti-FCRL Peptide Polyclonal Antibodies with Mammalian FCRL Proteins

To test the reactivity of the polyclonal anti FCRL peptide antibodies with the native FCRL proteins, anti-FCRL1-1 and FCRL4-2 antibodies were applied in WB using cell extracts from peripheral blood or tonsil; as well as a Burkitt's lymphoma B-cell line (Ramos) known to endogenously express FCRL1 protein. The polyclonal anti-FCRL1-1 recognized native FCRL1 protein in normal PBMCs, tonsil mononuclear cells (MNCs) and Ramos B-cell line, however, the enriched T cells did not react with this antibody (Figure 7). The polyclonal anti-FCRL4-2 could not detect any band related to FCRL4 protein in PBMCs or tonsil MNCs (data not shown). The restricted expression of FCRL4 on memory B cells could explain these findings. Thus, the anti-FCRL4-2 peptide polyclonal antibody was applied in WB on FCRL4 transfected CHO lysate. However, no specific band was detected (data not presented). Altogether, our results indicate that with the exception of anti-FCRL1-1 peptide antibody, the polyclonal anti–FCRL4-2 peptide antibody does not react with the native FCRL4 protein expressed in mammalian cells.

Figure 7. Detection of Native FCRL1 Protein with Polyclonal Anti-FCRL1-1 Peptide Antibody by Immunoblotting.



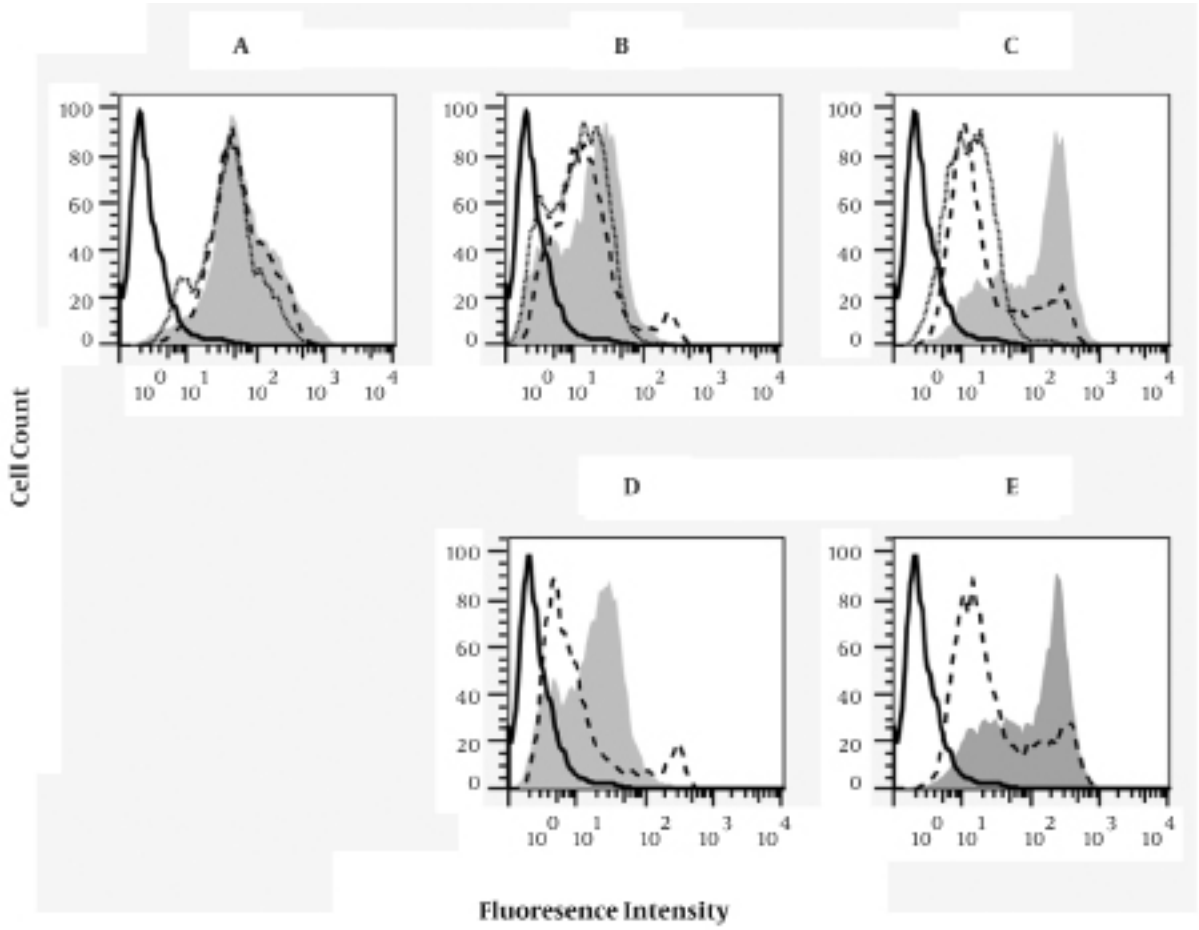
Cell extracts were prepared from T cells (lane 1), tonsil mononuclear cells (lane 2), peripheral blood mononuclear cells (lane 3) and Ramos B cell line (lane 4). M: size marker. Arrowhead represents the location of FCRL protein.

4.7. Inhibition of Binding of Anti-FCRL mAbs to FCRL Transfected Cells by Recombinant FCRL Proteins

To confirm the activity of our recombinant FCRL proteins, we investigated whether these proteins could inhibit the

binding of FCRL-specific mAbs to FCRL-transfected cells. Our results showed that the commercial FCRL4 specific mAb adsorbed with FCRL4 recombinant protein could no longer bind to the native FCRL4 protein expressed on the surface of transfected CHO cells (Figure 8 C). However, no inhibition was observed for the adsorbed FCRL1 and FCRL2 specific commercial mAbs (Figure 8 A and Figure 8 B). Interestingly, our anti FCRL2 and 4 mAbs were blocked with recombinant FCRL2 and 4, respectively (Figure 8 D and E). This finding implied that the FCRL4 specific mAb recognizes an epitope on the FCRL protein expressed in transfected mammalian cells. However, the epitopes recognized by the commercial FCRL1 and 2 specific mAbs were either not expressed on the prokaryotic recombinant FCRL proteins or lost due to denaturation of these proteins by 8M urea. It is worth noting that the commercial mAbs against FCRL1, 2 and 4 were produced against recombinant FCRL proteins expressed in eukaryotic cells (10).

Figure 8. Flow Cytometry Results of Reactivity of Monoclonal Anti-FCRL Antibodies Adsorbed with the Corresponding Prokaryotic Recombinant FCRL Proteins



CHO cells transiently transfected with FCRL1 (A), FCRL2 (B, D) and FCRL4 (C, E) were stained with the corresponding mAbs: A (commercial mAb to FCRL1 1F9), B (commercial mAb to FCRL2 7G7), C (commercial mAb to FCRL4 1A3), D (our mAb to FCRL2 3D8-G8) and E (our mAb to FCRL4 1A5-C10).Solid black: CHO-pCMV6 stained with isotype matched mAb as negative control, filed gray: non-adsorbed mAb to FCRL, dotted line: mAb adsorbed with 10 μg.mL-1 recombinant protein, dashed line: mAb adsorbed with 40 μg.mL-1 recombinant protein.

5. Discussion

The discovery of the FCRL molecules has considerably expanded the network of lymphocyte coreceptors and unraveled an unexpected layer of biological complexity (11). The human FCRL1–6 encode type I transmembrane glycoproteins with 3 - 9 extracellular Ig domains and cytoplasmic tails containing ITAM and/or ITIM (5, 11). FCRL immunoregulatory potential is implicated by the presence of consensus ITAM or ITIM in their cytoplasmic tails. All of them except FCRL6, are expressed on B cells at different stages of differentiation (5). Although, FCRL

molecules have homology with classical immunoglobulin Fc receptors, but none of the FCRL family members have been shown to bind Igs (3). Up to now just murine FCRL5 and human FCRL6 ligands have been identified (9, 12). Schreeder et al. showed that human FCRL6 which is selectively expressed on cytotoxic T and NK cells directly binds to HLA-DR, an MHC class II molecule (9). Campbell et al. reported that the orthopoxvirus MHC class I-like protein encoded by monkeypoxvirus and cowpoxvirus is the first known virally encoded ligand of FCRL5 (12). Due to the lack of natural ligand(s) for FCRL molecules, direct inspection of the signaling capacity of these molecules has not so far been possible. However, a model of co-ligation of a chimeric FcγRIIb/FCRL (consisting of extracellular domain of FCγRIIb and cytoplasmic domain of FCRL) and the BCR has recently been employed which allows pairing of the chimeric FCRL with the ITAM- and/or ITIM-containing Igα/β of the BCR complex (3). Production of recombinant proteins from different domains of the FCRL molecules will pave the way for more functional and structural analyses of these molecules. In this study, we constructed the pET-28b (+) - FCRL1, 2 and 4 plasmids and successfully expressed His-tagged FCRL proteins in BL21-DE3. The extracellular parts of FCRL1, 2 and 4 have 3, 4 and 4 Ig-like domains, respectively. The digestion and DNA sequencing results showed that the cloned genes in pET vector had the correct size and sequence as predicted for FCRL1 (936 bp), FCRL2 (1216 bp), and FCRL4 (1172 bp). The constructs were transformed into BL21-DE3 *E. coli* strain and the expressed recombinant FCRL proteins containing His-tag were purified successfully using conventional Ni-NTA system. The extracellular portions of FCRL1, 2 and 4 proteins were predicted to have 354, 413 and 437 amino acids, respectively. The predicted amino acids content matched the size of the purified FCRL1 (38.5 kDa), FCRL2 (45.1 KDa) and FCRL4 (48.8 KDa) detected by SDS-PAGE and immunoblotting techniques. The recombinant FCRL proteins were purified in denaturing condition using urea 8M and gradual increasing in imidazole's molarity. After solubilization of a protein with high concentrations of denaturing agents, in our case 8M urea, refolding should be performed by controlled gradual removal of excess denaturant. Thus, the purified proteins need to be equilibrated under a continuous and step-wise decreasing concentration of urea to allow gradual removal of the reducing agent. The recombinant FCRL proteins were produced as inclusion bodies and thus were solubilized in denaturing condition using 8M urea. Renaturation of such proteins requires gradual and step-wise removal of excess urea during Ni-NTA purification process using a continuous gradient mixer. However, since this process might result in significant decrease of protein recovery, in the current study we did not use this approach and the recombinant proteins were dialyzed directly against PBS 1X (0.14 M). At this condition cysteine residues are exposed leading to extensive polymerization of proteins like FCRL. This limitation regarding the process of purification and dialysis of the purified recombinant proteins usually leads to protein aggregation and polymerization. Our results presented in figure 5 may explain this observation. In addition to our study, production of recombinant FCRL protein in prokaryotic system has been reported in two other studies (13, 14). Falini et al. cloned extracellular portion of FCRL4 into pGEX 3X vector and produced the recombinant protein in BL21 *E.coli* strain. They used purified FCRL4 for mAb production in their study (13). Won et al. generated mAb against FCRL3 using His-tagged recombinant protein consisting of D1-D2 and D3-D4 extracellular Ig domains of FCRL3 cloned into pET24b (14). In some studies the recombinant FCRL proteins were also expressed in eukaryotic cells mainly for production of mAbs (2, 6, 10, 12, 15, 16). However, detailed data regarding the process of cloning, expression and purification of FCRL molecules are not available and have not been reported in any of these studies. Our results showed that the human FCRL1, 2 and 4 proteins could be produced with pET-28b (+) in BL21-DE3 *E.coli* strain system. Compatible with this finding, it has been reported that the combination of the bacteriophage T7 promoter with BL21-DE3 host cells appears to be the most frequently used expression system (17, 18). However, it should be kept in mind that the prokaryotic expression system has some advantages and some drawbacks. Recombinant proteins produced in bacteria are often insoluble and inactive and need burdensome refolding procedure, as opposed to the eukaryotic system which yields bioactive soluble proteins (19). Indeed, our results as presented in figure 8, indicate that some epitopes expressed in native FCRL molecules might be lost due to the denaturation of the recombinant FCRL proteins. The other advantage of eukaryotic system is post-translational modification (N- and O-linked glycosylation, fatty acid acylation, phosphorylation) which is not available in prokaryotic systems (19, 20). On the other hand, protein production in eukaryotic host systems is a cumbersome procedure and frequently results in low protein yields. In this regard, gene expression in bacteria is straightforward and has the potential to produce large quantities of recombinant proteins (17).

In summary, human FCRL1, 2 and 4 genes were cloned and expressed in BL21-DE3 *E.coli* strain. The recombinant proteins were purified using Ni-NTA and characterized using His and FCRL specific polyclonal antibodies. These recombinant proteins are potentially useful tools for the identification of the natural ligand(s) of FCRL molecules and also production of a panel of mAbs recognizing different domains of each FCRL molecule for signaling studies and targeted immunotherapeutic interventions. Searching for ligands of these molecules needs appropriately folded proteins. Thus our purified recombinant FCRL proteins need to have their native structure to achieve this purpose.

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Authors' Contributions

MS performed the experiments, analyzed data and wrote the manuscript. AH, MZ, and JK performed experiments, MJT, HR and ZA provided consultation and supervised the study, FS designed and supervised the study, analyzed data and wrote the manuscript.

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The authors declare that they have no competing interests to disclose.

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