Bacterial production and biophysical characterization of a hard-to-fold scFv against myeloid leukemia cell surface marker, IL-1RAP

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Abstract

**Background** Interleukin-1 receptor accessory protein (IL-1RAP) is one of the most promising therapeutic targets proposed for myeloid leukemia. Antibodies (Abs) specific to IL-1RAP could be valuable tools for targeted therapy of this lethal malignancy. This study is about the preparation of a difficult-to-produce single-chain variable fragment (scFv) construct against the membrane-bound isoform of human IL-1RAP using *Escherichia coli* (*E. coli*).

**Methods** Different approaches were examined for refolding and characterization of the scFv. Binding activities of antibody fragments were comparatively evaluated using cell-based enzyme-linked immunosorbent assay (ELISA). Homogeneity and secondary structure of selected scFv preparation were analyzed using analytical size exclusion chromatography (SEC) and circular dichroism (CD) spectroscopy, respectively. The activity of the selected preparation was evaluated after long-term storage, repeated freeze-thaw cycles, or following incubation with normal and leukemic serum.

**Results** Strategies for soluble expression of the scFv failed. Even with the help of Trx, ≥98% of proteins were expressed as inclusion bodies (IBs). Among three different refolding methods, the highest recovery rate was obtained from the dilution method (11.2%). Trx-tag substantially enhanced the expression level (18%, considering the molecular weight (MW) differences), recovery rate (>1.6-fold), and binding activity (>2.6-fold increase in absorbance at 450nm). The produced scFv exhibited expected secondary structure as well as acceptable bio-functionality, homogeneity, and stability.

**Conclusion** We were able to produce 21 mg/L culture functional and stable anti-IL-1RAP scFv via recovering IBs by pulse dilution procedure. The produced scFv as a useful targeting agent could be used in scheming new therapeutics or diagnostics for myeloid malignancies.

**Keywords** Myeloid leukemia · IL-1RAP · Antibody characterization · Thioredoxin · Refolding · Cell-based ELISA
Introduction

Acute myeloid leukemia (AML, the list of abbreviations provided in Table 1) is the most prevalent and fatal leukemia in adults [1].

Immunotherapeutic approaches beyond hematopoietic stem cell transplantation (HSCT), especially antibody-dependent cellular cytotoxicity, (ADCC) have not been successful so far. The immunosuppressive quality of bone marrow niches in AML is the greatest impediment to developing immune-dependent therapy [2; 3]. Therefore, immune-independent approaches may be more reasonable.

Gemtuzumab ozogamicin (Mylotarg) is a good example. This drug exerts its anti-leukemic effects not through the immune effector cells but via the delivery of a potent cytotoxic molecule, calicheamicin [4]. The use of a more specific targeting agent may eventuate to better effectiveness.

Interleukin 1 receptor accessory protein (IL-1RAP), has been introduced as an excellent tumor target for targeted therapy of acute and chronic myeloid leukemia (CML). It is present or over-expressed on the surface of nearly all CML leukemic stem cells (LSCs) and most AML cells, while more than 97% of normal HSCs do not express it at all. [5–7]. Antibodies against IL-1RAP could be valuable tools for the targeted delivery of cytotoxic agents to myeloid leukemia cells [5].

A single-chain variable fragment (scFv) is a ~30 kDa artificial biomolecule comprised of only variable regions of heavy (VH domain) and light (VL domain) chains of a monoclonal antibody (mAb) [8]. These two antigen-binding domains are connected through a flexible linker of 15–25 residues, typically (Gly4Ser)n, [8].

ScFvs have prominent advantages over the whole mAbs, including higher tissue/tumor penetration, quicker blood clearance, and lower immunogenicity [9]. Due to the small size and lack of glycosylation, scFvs could be produced in prokaryotic systems that have fast growth rate and inexpensive transformation and cultivation procedures. E. coli, especially, has a high popularity thanks to its well-known genetics and, therefore, availability of various engineered strains and vectors, each designed for a particular purpose [10].

Correct folding of scFvs in bacterial cytosol is usually challenging due to their mammalian origin and non-natural entity. More notably, the proper formation of two structurally important intra-domain disulfide bridges in scFvs needs a proficient folding system under oxidizing environment [11]. So, these biomolecules mostly become misfolded and

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### Table 1 List of abbreviations/symbols used in this paper (including supplementary material)

| CD       | Circular dichroism               | MW | Molecular weight |
|----------|----------------------------------|----|-----------------|
| HSCT     | Hematopoietic stem cell transplantation | FBS | Fetal bovine serum |
| SA-X     | Streptavidin fused with TAT-EED peptide | kDa | Kilodalton |
| scFv     | Single-chain variable fragment | RT | Room temperature |
| VH       | Heavy chain variable domain      | ON | Overnight |
| VL       | Light chain variable domain      | PI | Isoelectric point |
| Ab       | Antibody                        | ε  | Extinction coefficient |
| mAb      | Monoclonal antibody              | LB | Luria–Bertani |
| IB       | Inclusion body                   | BSA | Bovine serum albumin |
| ELISA    | Enzyme-linked immunosorbent assay | Tris | 2-amino-2-(hydroxyl methyl)-1,3-propanediol |
| rpm      | Revolutions per minute           | Ni-NTA | Nickel-nitriopictastic acid |
| Trx      | Thioredoxin                      | RPMI | Roswell park memorial institute |
| ADCC     | antibody-dependent cellular cytotoxicity | HPLC | High-performance liquid chromatography |
| IL-1RAP  | Interleukin-1 receptor accessory protein | SEC | Size exclusion chromatography |
| E. coli  | Escherichia coli                 | DAB | 3, 3'-diaminobenzidine |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis | DSSP | Dictionary of Secondary Structure of Proteins |
| PEI      | Polyethyleneimine                | Abs600nm | Absorbance at 600 nm wavelength |
| WB       | Western blot                     | Abs450nm | Absorbance at 450 nm wavelength |
| AML      | Acute myeloid leukemia           | Abs280nm | Absorbance at 280 nm wavelength |
| CML      | Chronic myeloid leukemia         | IPTG | Isopropyl-b-D thiogalactopyranoside |
| LSCs     | Leukemic stem cells              | L-Arg | L-arginine |
| TCFS     | Total cell fractions             | dH2O | Distilled water |
| HBSS     | Hank’s balanced salt solution    | ns  | Not significant |
| 2ME      | 2-Mercaptoethanol                | h   | Hours |
| PBS      | Phosphate buffered saline        | min | minutes |
| TMB      | 3,3’,5,5’-Tetramethylbenzidine   | mg  | Milligram |
| HRP      | Horseradish peroxidase           | EDTA | Ethylenediaminetetraacetic acid |
form dense aggregates (inclusion bodies, IBs) after overexpression in the cytoplasm [12].

Here, we focused on the refolding of an E. coli codon-optimized scFv construct specific to the membrane-bound isoform of IL-1RAP. Our previous efforts to the soluble expression of full-length scFv from this construct repeatedly failed. Trying different strategies, including periplasmic expression (with or without sucrose supplementation), co-expression of chaperons (GroEL, GroES, and Tig), fusion with two solubility partners (Trx and eukaryotic initiation factor 2, IF-2 in Origami B context) and use of SHuffle strains, all were associated with no or very low yield.

The success in in vitro refolding mainly depends on the refolding method employed [13] and the types and balance of components in refolding buffer [12]. In the present study, three different refolding methods were comparatively investigated and then, detailed analyses of the selected scFv preparation were performed to evaluate its folding accuracy, homogeneity, binding activity, and stability.

Materials and methods

Human cell lines and serums

K-562 (for overexpression of IL-1RAP) and KG-1 (low expression of IL-1RAP) cell lines were purchased from Iran National Cell Bank (INCB, Iran). Cells were cultured and maintained in Roswell Park Memorial Institute (RPMI) 1640 medium plus glutamine (BioIdea, Iran), 10% v/v fetal bovine serum (FBS) (Gibco, USA), and 1% Penicillin/Streptomycin (100 U/mL and 100 µg/mL, respectively) in a humidified incubator, at 37 °C with 5% CO2.

Normal serum was voluntarily obtained from one of the authors and leukemic serum from a 59 years old male patient with relapsed AML and high leukemic burden before treatment initiation.

Sequences, constructions, and bioinformatics

Sequence extraction, design, gene synthesis, and cloning procedures

The E. coli codon-optimized sequence of the anti-IL-1RAP scFv (fused to fusion format of streptavidin (SA-X), Gen-Bank accession number: OM642337) in pET28a (Fig. S1a) was already present in our lab. The sequence of the scFv had been formerly extracted from a patent (PCT/US2013/077323 [14]: SEQ ID NO 13 for VH and SEQ ID NO 14 for VL) (Fig. S1b). The scFv was constructed as VH-(Gly4Ser)5-VL-Gly4-6xHis (Fig. S1c) by removal of downstream sequences and substitution with a Gly4 coding oligo (Fig. S1d).

Secondary structure prediction

We utilized two web servers, 2Struc [15] and PSIPRED [16], for secondary structure predictions. The scFv constructs were modeled using I-TASSER [17]. Model 1 from each protein was employed for analysis by 2Struc. For PSIPRED, the amino acid (AA) sequences were submitted.

Exploration of aggregation hotspots

Potential aggregation regions were investigated in silico by five different web servers (inspired from [18]). For AGGRESCAN [19], Tango [20], Waltz [21], and FoldAmyloid [22], the AA sequences of the scFv-6xHis in FASTA format were inputted. For the AGGRESCAN3D (A3D) predictor [23], I-TASSER generated 3D model of the molecule was uploaded as an input. All predictions were accomplished with the default input parameters settings of tools.

Preliminary expression of scFv

The scFv-6xHis construct was transformed to BL21 (DE3), and a single transformant was cultured overnight (ON) in a 5 ml Luria-Bertani (LB) medium at 37 °C. The ON culture was refreshed and induced at Abs600nm of ~0.5 (after obtaining before induction (BI) sample) with 0.5 mM IPTG and continued for 4 h at 37 °C, 150 rpm.

Analysis of expression samples

For analysis of expression in total cell fractions (TCFs), cellular pellets of before and after induction (AI) samples were resuspended in dH2O. To study the soluble and insoluble fractions, the pellets of un-induced and induced (4 h, ON) cultures from three different temperature conditions were resuspended in phosphate-buffered saline (PBS) and lysed with sonication. After centrifugation (~11,000 g for 10 min), supernatants were collected as soluble fractions. The sediments were washed with PBS, dissolved in 0.5 ml of 8 M urea, and centrifuged. The supernatants of the second step were collected as insoluble fractions. All samples were mixed with an appropriate volume of Laemmli buffer (with 2% 2-mercaptoethanol (2ME)) and boiled for ~10 min before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
We compared three different methods for refolding scFv from IBs produced in BL21 (DE3). In all, 2.5 mg denatured IB (denatured in solubilization buffer (Table 2)) with ≥75% purity was introduced, and all the procedures were done in the same buffer condition (50 mM Tris-HCl, 150 mM NaCl, pH 8). Denatured protein samples in all practices were prepared from the stock solution (10 mg/ml protein in solubilization buffer plus 10 mM 2ME). We used 2 mM cysteine and 0.4 mM cystine in all methods but at different urea concentration points. After refolding and before purification, aggregates were removed using centrifugation (≥10,000 g for 30 min). The refolded protein samples were buffer exchanged to PBS and concentrated using a 10 kDa cut-off Amicon™ Ultra-0.5 Centrifugal Filter (Merck). The final concentrations were determined using a UV spectrophotometer (Abs\textsubscript{280nm} at the related extinction coefficient (Table S2)). The recovery rates were calculated using the formula [1].

$$\text{Recovery (\%)} = \frac{\text{mg of purified refolded scFv}}{\text{mg of denatured scFv introduced in the refolding process}} \times 100$$  \hspace{1cm} (1)

### Western blot (WB) analysis

**WB on expressed scFv**

Samples were subjected to SDS-PAGE using 12% polyacrylamide gel. After electrophoresis, protein bands were transferred to the nitrocellulose membrane by the semi-dry method. The membrane was blocked (3% w/v skim milk in PBS) ON at 4 °C and after 4 times washing the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-His-tag Ab (Sigma-Aldrich, USA). The membrane was then washed five times, and His-tagged scFv bands were developed by 3, 3’-diaminobenzidine (DAB) substrate solution.

**WB on lysates of leukemic cells**

K-562 and KG-1 cells were harvested (400 g, 5 min) and lysed by resuspension in minimum volumes of lysis buffer (Table S1). Total protein contents were estimated using Bradford assay, and equal protein quantities (~40 µg) from each sample were loaded on 8% polyacrylamide gel and subjected to SDS-PAGE. The protein bands were transferred to the nitrocellulose membrane. The membrane was blocked, washed, and then incubated with the anti-IL-RAP scFv solution (~5 µg scFv per 1 ml PBS). The membrane was then washed and incubated with HRP-conjugated anti-His-tag Ab, and the experiment was continued as mentioned above.

### Isolation of IBs for refolding

A single colony of transformant was cultured and induced with 0.2 mM IPTG at 37 °C, ON. After harvesting the culture (~4000 g, 15 min) the cellular pellet was resuspended in 2 ml lysis/wash buffer (Table S1) plus ~5 mg lysozyme and incubated for about 1 h, at RT with occasional shaking. The suspension was volumized to 30 ml with the same buffer and lysed using two rounds of sonication (10 s pulses/20 s rest intervals for 20 times at 40% amplitude). IBs were separated by centrifugation, resuspended in the buffer mentioned above, and, then sonicated. Washed IBs were isolated using centrifugation and then washed with PBS to remove residual ethylenediaminetetraacetic acid (EDTA) and detergent. High-quality IBs were stored at -20°C until solubilization.

### Table 2 The buffer components used in refolding procedures (inspired by [8] and [12])

| Components* | Tris-HCl | NaCl | L-Arg | Urea | L-cystine | L-cysteine | Imidazole | 2ME | pH |
|-------------|---------|------|-------|------|----------|-----------|-----------|------|----|
| Solubilization buffer | 50 | 150 | - | 8000 | - | - | 25 | - | 8 |
| Refolding buffer A | 50 | 150 | 500 | 500 | 0.4 | 2 | 25 | 1 | 8 |
| Refolding buffer B | 50 | 150 | - | - | 0 or 0.4 | 0 or 2 | 25 | 1 | 8 |

*Concentrations in mM
Size exclusion chromatography

In order to analyze the homogeneity of dilution-refolded scFv preparation, size exclusion chromatography (SEC) was carried out according to the following method. 100 µL of purified Ab (0.37 µg/µl) was injected into a TSK-gel G2000 SWXL column (7.8 × 300 mm, Tosoh Bioscience, Tokyo, Japan) connected to a Shimadzu HPLC system (Kyoto, Japan). The sample was eluted with an isocratic mobile phase (Table S1) with a 0.5 ml/min flow rate (25 ºC).

Analysis of secondary structure by CD

The secondary structures of three Ab preparations were figured out by far-UV CD spectroscopy (Jasco J-810 Spectropolarimeter, Japan). The ellipticity (θ) of samples was measured at 190 to 240 nm wavelength. The samples’ buffer was extensively exchanged to dH₂O using a 10 kDa cut-off Amicon™ Ultra-0.5 Centrifugal Filter prior to the measurements. The analyses were performed at RT by injection of ~300 µl of each sample into a 1 mm quartz cuvette.

Statistical analysis

The statistical analysis of data was accomplished using GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla, CA, USA). The significance level between the groups was analyzed using one-way ANOVA.

Results

Cytoplasmic expression of scFv in BL21 (DE3)

A preliminary expression experiment with the scFv-6xHis construct showed an apparent 28 kDa band absent in the “before induction” sample (Fig. 1a). We repeated the experiment for a 200 ml LB culture (0.2 mM IPTG, 37 ºC, 150 rpm, ON). The cells were harvested and lysed, and the clarified lysate was subjected to Ni-NTA purification to capture any soluble scFv. However, no protein was detected in the elution sample.

Next, we tested the experiment in different conditions, including soluble favorable conditions, and analyzed the induced samples by SDS-PAGE. No expression was observed in soluble fractions (Fig. 1b), and the protein was expressed entirely as IB in all conditions (Fig. 1c).

Recovery of soluble scFv from IBs

Failures in the soluble expression of intact scFv in reasonable purity or quantity (data not presented) led us to subclone...
protein was confirmed by WB (Fig. S3b). We compared three different methods to recover soluble Abs from IBs: dialysis, dilution, and on-column refolding. The dilution method showed the highest recovery rate (Fig. 2a). The recovery rates were calculated after concentration and buffer exchange of elution samples and the actual values might be higher than those reported because of probable protein loss during Amicon filtration. For comparison, we repeated the dilution method on the insoluble fraction of both scFv-6xHis and Trx-fusion Ab. The results showed that the recovery rate was remarkably reproducible (11.2% in both experiments, Fig. 2a and b). The recovery value obtained from Trx-6xHis-scFv fusion was considerably higher than scFv-6xHis (Fig. 2b and c), perhaps due to the in vitro solubility enhancement effect of the Trx-tag.

Fig. 1 SDS-PAGE of expression experiments with BL21(DE3) transformant. a) M: Protein marker, 1: After induction, 2: before induction. b, c) Soluble (b) and insoluble (c) fractions from cultures induced in different time/temperature conditions; arrows show scFv bands (28 kDa). BI: before induction, h: hours, ON: overnight

Fig. 2 Recovery rates and productivity levels. (a) Recovery rates of three different methods used to refold scFv-6xHis. (b) Comparison of recovery rate obtained from dilution refolding of Trx-tagged and Trx-free scFv molecules. (c) Comparison of productivity levels and recovery yields (milligram per liter culture) of Trx-fused scFv and Trx-free scFv constructs. The graphs were plotted using GraphPad Prism version 8.0.
Fig. 3 Physical characterization of produced antibody fragments. SDS-PAGE of scFv preparations; 1: protein marker, 2: solubilized IB (reduced/boiled), 3: scFv refolded by dialysis (reduced/boiled), 4: scFv refolded by dialysis (non-reduced/un-boiled), 5: scFv refolded by dilution (reduced/boiled), 6: scFv refolded by dilution (non-reduced/un-boiled). b) 1: protein maker, 2: Trx-6xHis-scFv refolded by dilution (reduced/boiled), 3: Trx-6xHis-scFv refolded by dilution (non-reduced/unboiled). c) SEC-HPLC chromatogram of dilution-refolded scFv-6xHis. d) Far-UV spectra of two different antibody preparations. e) Predicted aggregation hotspots and CDR regions of scFv-6xHis molecule obtained from different predictor servers.

Physical characterizations of produced scFv

We used SDS-PAGE and SEC-HPLC to analyze our Abs’ purity, aggregation, and monomer/multimer status. All preparations (except for on-column refolded preparation) were subjected to SDS-PAGE in two different conditions: reduced/boiled and non-reduced/unboiled. The expected band size for Trx-free and Trx-fused preparations in both conditions is ~28 kDa and ~43.6 kDa respectively (Table S2). The results showed that the dialysis-refolded scFv-6xHis and the dilution-refolded Trx-6xHis-scFv preparations had the highest and the lowest homogeneity, respectively. There seemed to be some multimeric species in dilution preparations (Fig. 3a and b).

We then further analyzed the selected preparation (dilution-refolded scFv-6xHis) by analytical SEC. The result was in agreement with the SDS-PAGE data. Based on the chromatogram analysis, the main peak (monomeric form) encompassed ~60% of the protein content (Fig. 3c).

Table 3 Secondary structure proportions of selected scFv preparation; comparison between predicted data (2Struc (DSSP* method) or PSIPRED webserver) and experimental data

| Protein     | Structure | CD data | 2Struc | PSIPRED |
|-------------|-----------|---------|--------|---------|
| ScFv-6xHis  | α-helix%  | 28.6    | 5.59   | 2.23    |
|             | β-sheet%  | 43.0    | 30.59  | 43.65   |
|             | Others%   | 28.4    | 63.80  | 57.10   |
| Trx-6xHis-scFv | α-helix% | 18.5    | 5.6    | 15.25   |
|             | β-sheet%  | 24.8    | 30.6   | 34.8    |
|             | Others%   | 56.7    | 63.8   | 49.95   |

* DSSP: Dictionary of Secondary Structure of Proteins
The structural analysis of the dilution-refolded scFv-6xHis was accomplished using the CD. We also analyzed the dilution-refolded Trx-6xHis-scFv preparation for comparison. The results (Fig. 3d; Table 3) showed that the protein was mainly composed of β-sheet structures (43%) while in the Trx-6xHis-scFv molecule, coils, and turns were the dominant structures. This observation was in accordance with previous reports [8, 25] and in silico predictions (Table 3).

We also further extend the structural characterization of scFv-6xHis construct via in silico analysis of potential aggregation hotspots using different web servers (Fig. 3e).

**Binding assessments of produced ab fragments**

We next, compared the binding activity of four Ab preparations to leukemia cells (K-562 and KG-1) using cell-based ELISA. ScFv from on-column refolding was not analyzed due to insufficient quantity. The highest activity belonged to dilution-refolded preparations (Abs450nm up to 2.5, Fig. 4a). It seemed that the binding activities of refolded Abs were proportional to their recovery rates.

The binding activity of scFv to IL-1RAP was further investigated by WB analysis on leukemia cell lysates (K-562 lysate as test and KG-1 lysate as control). The WB data (Fig. 4b) showed that the produced scFv can successfully recognize the membrane-bound isoform of IL-1RAP (MW: ~66 kDa). As expected the band intensity in the lane pertained to KG1 lysate was weaker.

**Stability analysis**

The decrease in binding activity of dilution-refolded scFv-6xHis was investigated following three freeze-thaw cycles or after one to ten days of incubation at 37 °C. The freeze-thaw process decreased the binding activity by 37% (Fig. 4c). Likewise, incubation at 37 °C decreased the binding activity until day 4 (by ~50% compared to control). Interestingly, the activity seemed to recover since day 4, reaching 76% of its original activity on day 10. Overall, the dilution-refolded scFv exhibited good stability against long-term incubation in body temperature.

**Investigation of binding activity of dilution-refolded scFv-6xHis in serum environment**

To evaluate the binding ability in the serum environment, ~80 µl of dilution-refolded scFv-6xHis (0.260 mg/ml) was added to 320 µl of PBS, normal or leukemic serum. After mixing, the samples were incubated at 37 °C for ~3 h and then ON at RT. The samples were then used for ELISA on K-562 cells seeded on 96 well plate (~67,000 cells/well).

Our produced scFv was fully active in normal human serum (Fig. 4d). However, we surprisingly observed that the ELISA signal was significantly decreased after exposure of the produced scFv to AML serum. Therefore, the AML serum has a strong inhibitory effect on the binding (see discussion).

**Discussion**

Designing a new delivery system for myeloid leukemia targeting IL-1RAP as an LSCs-specific marker is a promising therapeutic approach. Due to its small size, we chose the scFv format of an anti-IL-1RAP mAb and utilized *E. coli* as a suitable host for the inexpensive production of our scFv.

We first focused on different soluble expression strategies, which all were disappointing. Almost all the scFv molecules formed IB when expressed cytoplasmically. Even with the help of Trx, more than 98% of recombinant protein was expressed as IB, emphasizing our scFv molecule’s aggregation tendency.

The high aggregation propensity of the scFv made us explore the aggregation hotspots of the construct in silico. We found several areas inside and outside of hydrophobic complementary determining regions (CDRs) that may be responsible for the low solubility of our hard-to-fold scFv (Fig. 3e). Accordingly, it may be possible to get higher levels of soluble scFv through soluble expression strategies by rational mutagenesis of aggregation-prone regions in this construct.

For IB denaturation, high concentrations of chaotropic agents are usually used. A reducing agent is also needed in the denaturation step of proteins with disulfide bonds to reduce all S-S bridges that may have been formed in vivo or during cell lysis [13]. Unfortunately, high concentrations of these agents can deteriorate the Ni-NTA resin by reducing Ni²⁺ ions and leaving brownish precipitates in the column. To prepare the denatured protein solution, we first dissolved IBs in a solubilization buffer containing 10 mM 2ME and then diluted this stock solution in a 2ME-free solubilization buffer just before the refolding. We also performed the purification step after the refolding. Through these strategies, we get the advantage of reducing power of 2ME while keeping away from the deteriorating effect on nickel resin. We also used 1 mM 2ME in refolding buffers in dialysis and dilution methods. 2ME in such a low concentration does not affect the nickel resin and has been reported to improve the refolding yield significantly, perhaps through its anti-aggregation activity [12].

We used cysteine in excess and employed a slightly alkaline pH for all refolding procedures, a well-known condition for fast disulfide exchange reactions [27].
Pulse dilution refolding was found to be the most effective refolding method for our Ab. The main benefit of this method is its cost-effectiveness because it does not need massive amounts of salt and urea (in contrast to the dialysis method).

Numerous reports have successfully employed the on-column method with relatively high refolding yields (for example, [25]). However, except for rare reports [25; 28] in the case of scFvs, the recoveries have generally not been satisfactory [24; 29; 30]. The recovery rate from the on-column method in our experience was similarly disappointing (2.66%, the smallest value among the tested methods, Table S3).

Pulse dilution refolding was found to be the most effective refolding method for our Ab. The main benefit of this method is its cost-effectiveness because it does not need massive amounts of salt and urea (in contrast to the dialysis method).

**Fig. 4** Bio-functionality analyses of the selected antibody preparation (scFv-6xHis). (a) Comparative binding assessment of four different antibody preparations (50 µg/ml) using cell-based ELISA on K-562 (IL-1RAP overexpression) and KG-1 (low expression of IL-1RAP) cell lines in triplicate. (b) Western blot on K-562 (lane 2) and KG-1 (lane 3) lysates with dilution-refolded scFv-6xHis as primary antibody; arrows refer to the membrane-bound isoform of IL-1RAP (~66 kDa) recognized by the produced scFv. (c) Binding activity of the scFv after overnight incubation with normal and AML serum. (d) Binding activity of the scFv (25 µg/ml concentration or 1.25 µg/well) to K-562 cells after repeated freeze-thaw or long-term incubation at 37 °C. Data analyses were performed, and the graph was plotted using GraphPad Prism version 8.0. ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
The serum level of membrane isoform (or an unknown structurally similar soluble factor(s)) is increased in this malignancy. The exact identity of the unknown factor(s) can be elucidated by two-dimensional western blot (2D WB) analysis of AML serum with the produced scFv and sequencing of the positive dot(s) using mass spectrometry. Anyway, this is beyond the scope of this study.

From the clinical perspective, the results of the serum interference experiment demonstrated that this antibody is not a suitable anti-leukemic agent for use in high leukemic burden. On the other hand, the proliferation inhibition assay showed that the described scFv cannot prevent leukemia progression through blocking of IL-1 signaling (data not shown). Instead, its conjugated or fusion formats with cytotoxic molecules may offer therapeutic potential in eradicating minimum residual disease (MRD) during complete remission. We’re now designing a new gene delivery system using this targeting agent in our lab.

It is worth noting that alternative approaches for soluble expression may also be helpful, such as the utilization of other chaperons (e.g., RNA-based chaperons) or other solubility tags (e.g., MBP or SUMO) and the use of different prokaryotic (e.g., Bacillus strains) or eukaryotic (mammalian or yeast) hosts.

**Conclusion**

Briefly, in this study, we produced and characterized an scFv against human IL-1RAP. A comparison of various approaches showed that recovering IBs through dilution refolding is the most fruitful method for bacterial production of the molecule. The productivity value reported here (21 mg/L culture in LB medium) is sufficient for preclinical studies and can be improved by further optimizations.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07972-3.

**Ethical statement** The authors are responsible for the correctness of the statements provided in the manuscript. All procedures performed
in studies involving human participants were in accordance with the 1964 Helsinki Declaration and its later amendments and with the ethical standards of the Paster Institute of Iran’s research ethics committee (code of ethics: IR.PII.REC.1401.032). Written informed consent was obtained from the patient volunteer. This article does not contain any studies with animals.

Conflict of interest The authors declare that they have no conflict of interest.

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