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Isolation of human Fab antibodies specific for the low-affinity IgE receptor (CD23) by selecting a hierarchical antibody library system against B lymphoblastic IM-9 cells

Hyo-jung Choi^a, Suk-yoon Song^b, Jae-bong Yoon^a, Li-kun Liu^a, Kristine Kim^{a,c}, Sang-hoon Cha^{a,b,c,*}

^a Department of Systems Immunology, College of Biomedical Science, Kangwon National University, Chuncheon, Gangwon 200-701, South Korea

^b IG Therapy Co., Rm 202, Biomedical Science A Building, Kangwon National University, Chuncheon, Gangwon 200-701, South Korea

^c Institute of Antibody Research, Kangwon National University, Chuncheon, Gangwon 200-701, South Korea

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ABSTRACT

The development of human antibodies specific for certain B cell markers is required to generate therapeutic antibody leads with improved therapeutic indices against B-cell lymphomas. To meet this demand, we selected a primary human antibody library, HuDV Fab-8L, against human B lymphoblastic IM-9 cells via a 'Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL)' cell panning approach. Six Fab clones that specifically bound to IM-9 cells were successfully isolated. Among these clones, two clones (IM-L6-E and IM-L8-G), were found to be specific for CD23 (FcεRII). Affinity maturation of these Fab clones was then performed in a hierarchical manner by constructing secondary antibody libraries through combining heavy (H) chains of two Fabs with the human kappa L chain sublibrary HuNL-D3 followed by biopanning against the CD23 antigen. Clone IM-L6-5, one of the affinity matured Fab derivatives from IM-L6-E, has a binding affinity of $K_D \approx 30$ nM to soluble CD23. In addition, IM-L6-5 Fab is able to bind to an inducible form of CD23 expressed on U937 cells upon IL-4 stimulation, and inhibits binding of human IgE to CD23. Since the Fab IM-L6-5 is derived from a fully human naïve origin, we believe that IM-L6-5 can be utilized for the development of a therapeutic mAb which may have an improved therapeutic index over lumiliximab, a primatized anti-CD23 mAb, for the treatment of CLL or allergic diseases.

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1. Introduction

Along with chemotherapeutic agents such as fludarabine (FA), cladribine (2-CdA-chlorodeoxyadenosine) and pentostatin (DCF, 20-deoxycoformycin), monoclonal antibodies (mAbs) have dramatically changed the treatment of patients with non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). For example, the antibodies rituximab (Rituxan®) and alemtuzumab (Campath-1H®) have already demonstrated their crucial value in clinics [1,2]. Treatment with rituximab as a single agent has resulted in significant responses in patients with almost every subtype of B-cell lymphoma. This agent induces death of normal and malignant B cells expressing the cell-surface molecule CD20 via antibody-dependent cellular cytotoxicity (ADCC), complement-dependent

cytolysis (CDC) and direct induction of apoptosis [3–5]. The therapeutic effect of rituximab is further improved with a combination of chemotherapy regimens for patients with aggressive B-cell non-Hodgkin lymphomas (NHLs) [1], in which CD20 expression appears to be higher than that of CLL [6]; however, the correlation between the intensity of CD20 expression on the cell surface and clinical response is uncertain [7]. The anti-CD52 antibody alemtuzumab has also shown significant activity in previously untreated and pretreated CLL patient who are refractory to FA. This antibody is superior to rituximab at eliminating minimal residual disease (MRD) in the bone marrow, resulting in an improvement in long-term survival [8].

Although rituximab is an effective agent in the treatment of B-cell lymphomas, approximately 50% of patients with relapsed or refractory CD20⁺ follicular lymphomas do not respond to initial therapy because of innate resistance [9]. In addition, roughly 60% of prior rituximab responding patients will not gain additional benefit from further rituximab treatment, due to resistance required by unknown mechanism [10]. In the case of alemtuzumab, the antibody causes significantly more infusion, hematologic and immune toxicity than rituximab, mainly due to the fact that CD52 is ubiquitously expressed on lymphocytes and monocytes [8]. Therefore, it has been suggested that greater diversity in the number of human

Abbreviations: DVS, dual-vector system; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; pAb, polyclonal antibody; HRPO, horse radish peroxidase.

* Corresponding author at: Department of Systems Immunology, College of Biomedical Science, Kangwon National University, Chuncheon, Gangwon 200-701, South Korea. Tel.: +82 33 250 6485; fax: +82 33 253 6485.

E-mail address: chash@kangwon.ac.kr (S.-h. Cha).

antibodies with selective binding capability to B-cell lymphomas needs to be generated [8] to overcome the differential efficacy and side-effects of rituximab and alemtuzumab. Consequently, new mAbs have recently been explored and have shown promise in treating B-cell lymphomas including CLL [5,11,12]. These antibodies include humanized mAbs targeting diverse antigens such as CD22, CD23, CD40, CD80 or HLA-DR beta chain, and fully human mAbs, such as ofatumumab (HuMaxCD20) against CD20 [13].

Phage-displayed antibody libraries have been used extensively to isolate antibodies with desirable antigen-binding specificities [14,15], and the importance of phage display technology in therapeutic antibody development has been well-acknowledged because of the ability to obtain fully human antibodies [16,17]. In this study, we selected a primary phage-displayed antibody library composed of human naïve Ig repertoires against antigens on IM-9 cells by a cell panning procedure known as 'Biopanning and Rapid Analysis of Selective Interactive Ligands (BRASIL)' [18], and successfully obtained human antibodies specific for surface antigens expressed on human B lymphoblastic cells including CD23, the low-affinity IgE receptor (FcεRII), followed by affinity maturation via light (L) chain shuffling in a hierarchical manner for which is often necessary to make human antibodies of naïve origin suitable for therapeutic applications.

2. Materials and methods

2.1. Cell culture

Human B lymphoblastic IM-9, SKW6.4, Raji and Ramos, and human leukemic monocytic U937 cells were purchased from the Korean Cell Line Bank (KCLB, South Korea) and grown in RPMI supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco Invitrogen, USA) at 37 °C in a CO₂ incubator.

2.2. Selection of phage antibodies

The selection of phage antibodies bound to IM-9 cells was carried out by 'BRASIL' as described previously [18] with slight modification. Briefly, recombinant phage antibodies were prepared from the primary antibody library HuDV Fab-8L (IG Therapy Co., South Korea) using Ex12 helper phage (IG Therapy Co.) [19]. A total of 10¹² cfu of the phage antibodies were pre-incubated with 1 × 10⁶ U937 cells in 1 ml of ice-cold RPMI containing 1% bovine serum albumin (BSA) (Sigma–Aldrich, USA). After 1.5 h of incubation at 4 °C on a rocker, the phage in the supernatant were collected by brief centrifugation, used to resuspend 1 × 10⁶ IM-9 cells and incubated for 2 h at 4 °C on a rocker. The cells were then washed two times with RPMI containing 1% BSA, and 400 µl of the cell-phage suspension was gently transferred to the top of 400 µl of an organic lower phase (dibutyl phthalate:cyclohexane 9:1 [v:v]) and centrifuged at 10,000 × g for 10 min. Each tube was snap frozen in liquid nitrogen and the bottom of the tube was sliced off. The cell-phage pellet was then transferred to a new tube and mixed with 1 ml of *E. coli* TG1 cell culture in log phase, followed by phage amplification and preparation using EX12 helper phage. A total of four rounds of selection were performed.

2.3. Construction of a secondary antibody library

Secondary antibody libraries were constructed using DVS-III as previously described [16,20]. Briefly, the 350 bp V_H genes utilized for Fab IM-L6-E and IM-L8-G were obtained by treating the recombinant pHg3f1-2 phagemid vector with *Sfi*I and subsequently subcloning into the pHg3A-3 plasmid of DVS-III. Thereafter, the *E. coli* TG1 cells carrying the recombinant pHg3A-3 plasmid were

transformed with the recombinant pLf1T-3 phagemid from the HuNL-D3 sublibrary [20], followed by cell growth in 20 ml of 2× YT/AT medium for 8 h at 37 °C and phage rescue using the Ex12 helper phage. Finally, the recombinant phage were purified by PEG/NaCl precipitation and conventional biopanning of the library was performed against the recombinant CD23 protein immobilized on MaxiSorb ELISA plates (Nunc, Denmark).

2.4. Preparation of soluble Fab-pIII and Fab molecules

Soluble Fab-pIII molecules were prepared from the *E. coli* TG1 cells housing DVS-II using 0.02% arabinose (Sigma–Aldrich, USA) and 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma–Aldrich) [21], and the periplasmic extracts were prepared from *E. coli* TG1 cells as previously described [22]. Soluble Fab molecules were prepared by subcloning the Fd (V_H+C_{H1}) genes into the pFdx plasmid (IG Therapy Co.), followed by cultivation of *E. coli* TG1 cells in 2× YT/AT supplemented with 0.02% arabinose and 0.1 mM IPTG and purification using CNBr-activated sepharose (Amersham-Pharmacia, Sweden) conjugated with anti-human IgG (Fab')₂ antibody (Pierce, USA) [19].

2.5. Flow cytometry analysis

For analysis of Fab-pIII phage antibody binding to the cells, IM-9 cells (~10⁵) were resuspended in 100 µl of PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.3) containing 0.1% BSA (PBSB) were incubated with 100 µl of 10¹¹ cfu phage antibodies diluted in PBSB for one hour at 4 °C. After being washed with cold PBS, the cells were resuspended in 100 µl of FITC conjugated goat anti-kappa L pAb (Sigma–Aldrich) in PBSB and incubated for 30 min at 4 °C. The cells were then washed with cold PBS and the antibodies bound to the cells were detected and analyzed by flow cytometry using a FACScan (Becton Dickinson, USA). For analysis of the Fab binding to IM-9, SKW6.4, Raji, Ramos, or U937 cells, each cell line was incubated with either soluble Fab-pIII or soluble Fab molecules for one hour at 4 °C. Fab molecules bound to the cells were detected as described above.

2.6. Enzyme-linked immunosorbent assay (ELISA)

All recombinant protein antigens were purchased from R&D Systems, USA. ELISA was performed as previously described [23] using MaxiSorb ELISA plates coated with 10 µg/ml of CD23, Ep-CAM-Fc, c-Met-Fc, CD40-Fc or CD33-Fc in coating buffer (0.1 M NaHCO₃, pH 9.6). BSA was also included as a negative control antigen. Soluble Fab-pIII fusion molecules prepared from the *E. coli* were then added into each well and incubated for 1 h at 37 °C. Incubation with goat anti-human kappa L chain pAb conjugated to horseradish peroxidase (HRPO) (Sigma–Aldrich) and staining with a 3,3',5,5'-tetramethyl benzidine (TMB) substrate (Sigma–Aldrich) were used to analyze the antibody bound to the antigen. Absorbance at 450 nm was measured using an ELISA reader (Biorad, USA).

2.7. Measurement of relative affinity

To measure the relative affinities of the Fab clones, ELISA was performed with a thiocyanate elution procedure as described by Macdonald et al. [24]. This procedure was similar to the aforementioned ELISA procedure, except that ammonium thiocyanate in 0.1 M phosphate buffer, pH 6.0 in concentration ranging from 0 to 2 M was added to the appropriate wells (100 µl/well) in triplicate following incubation of the soluble Fab molecules and washing with PBS containing 0.001% tween (PBST). The plates were then allowed to stand for 20 min at room temperature before washing three times with PBST, and the ELISA signal was analyzed as described above.

2.8. Affinity measurement and epitope mapping with the BIAcore

The kinetic interactions between CD23 and the Fab fragments were measured using a BIAcore X biosensor (BIAcore AB, Sweden) at 25 °C. Briefly, after immobilization of the CD23 protein onto the carboxymethylated dextran surface of a CM5 sensor chip at a level of approximately 9000 response units, 60 μ l of serially diluted IM-L6-5 Fab molecules were injected into the flow cell at a flow rate of 30 μ l/min for 180 s. The surface was regenerated between Fab antibody injections by two 5 μ l injections of 10 mM glycine and 1 M NaCl (pH 2.5) to remove the Fab molecules bound to CD23. Dissociation (k_{off}) and association (k_{on}) rate constants, as well as the dissociation constant (K_D), were then obtained. For analyzing the binding inhibition of ligands (IgE, CD11b/CD18 and CD11c/CD18) to CD23 by the Fab IM-L6-5, 40 μ l of 200 nM Fab molecules were injected into the flow cell at a flow rate of 20 μ l/min for 120 s. And then 100 nM of human IgE (Abcam, USA), CD11b/CD18 (R&D Systems) or CD11c/CD18 (R&D Systems) was injected into the flow cell at a flow rate of 20 μ l/min for 120 s and sensorgrams were obtained.

2.9. Competitive inhibition ELISA

Competitive inhibition ELISA was performed using microtiter plates coated with the recombinant CD23 protein as described above with slight modification. After pre-incubating, different concentrations of mouse anti-CD23 mAb (R&D Systems), IM-L6-5 Fab, EG-L4 Fab, a negative control which has specificity for epidermal growth factor receptor (EGFR) (IG Therapy Co., unpublished) or a pre-determined concentration of human IgE (Abcam, USA) was added into each well of the microtiter plates and incubated for 2 h at 4 °C. Antibody bound to the protein was analyzed following incubation with goat anti-human IgE conjugated to HRPO (BETHYL, USA) and TMB substrate. The absorbance at 450 nm was measured as described above.

3. Results and discussion

3.1. Enrichment of phage antibodies bound to IM-9 cells by BRASIL cell panning and identification of the human Fab clones specific for the CD23 antigen

Recombinant phage antibodies were prepared from the primary combinatorial Fab antibody library HuDV Fab-8L which had been created by combining the HuNH-D2 sublibrary (4.5×10^9 diversity of human naïve heavy chains) with eight discrete human naïve kappa light chains (HuL1–HuL8) with undefined antigen-binding specificity [19]. After depletion of phage antibodies with human promonocytic U937 cells to remove non-specific binders, four rounds of cell panning via the 'BRASIL procedure' were carried out to isolate phage antibodies that bound to the human B lymphoblastic IM-9 cells. In BRASIL both selection and sorting of cell-surface-binding phage are based on differential centrifugation of cells with phage through an interface between aqueous and non-miscible organic phases. The successful isolation of antibodies using this approach had been reported, for examples, CX₆C phage peptides specific for vascular endothelial growth factor (VEGF) using human umbilical vein endothelial cells (HUVECs) [18] and phage antibodies against tumor associated antigens using human cancer cells [25]. The colony forming unit (cfu) of eluted phage determined after the cell panning indicated that specific phage enrichment had occurred in the primary antibody library utilizing HuL1, HuL2, HuL6 and HuL8 light chains (data not shown). Ninety-six *E. coli* clones were randomly chosen after the final round of cell panning, and soluble Fab-pIII molecules were prepared. Following flow cytometry analysis using IM-9 and U937 cells indicated that 26 out of the

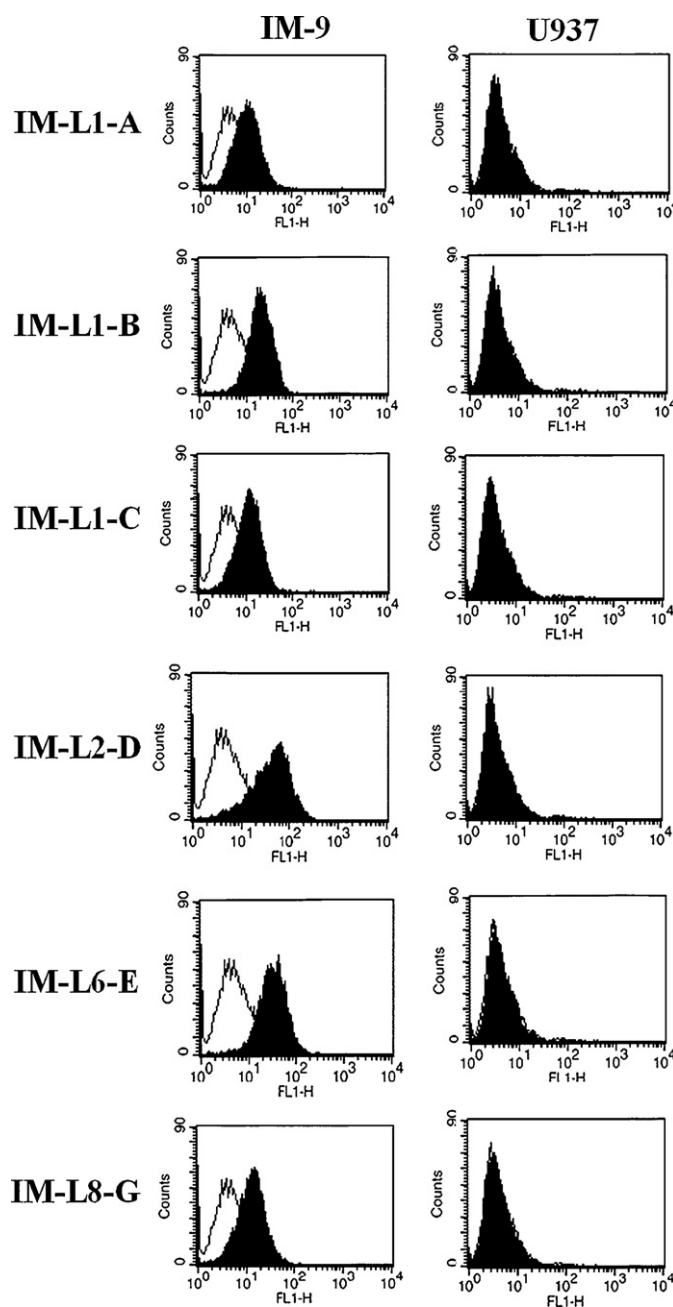


Fig. 1. Flow cytometry analysis of six human Fab clones showing specific binding to IM-9 cells.

IM-9 cells were incubated with soluble Fab-pIII molecules obtained from *E. coli* periplasmic extract, and the binding of soluble Fab-pIII molecules to the cells was detected by using secondary FITC-conjugated goat anti-human kappa (black area). The fluorescent signal produced by the secondary mAb in the absence of Fab-pIII is shown as white area.

96 Fab clones specifically bound to IM-9 but not to U937 cells. The DNA sequences of the V_H genes utilized by these positive Fab clones were determined, and deduced amino acid sequence found six discrete V_H s among 26 Fab clones (data not shown). The Fab clones utilizing one of those V_H s were named IM-L1-A, IM-L1-B, IM-L1-C, IM-L2-D, IM-L6-E and IM-L8-G. In terms of L chain usage, IM-L1-A, IM-L1-B and IM-L1-C utilize HuL1, and IM-L2-D, IM-L6-E and IM-L8-G utilize HuL2, HuL6 and HuL8, respectively. Binding specificity of six Fab clones to IM-9 cells was reconfirmed by flow cytometry analysis and the results were shown in Fig. 1.

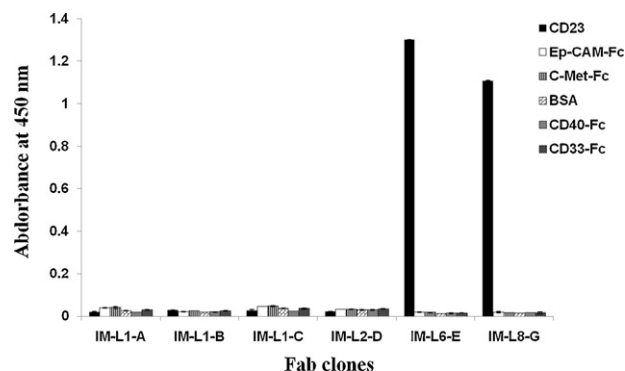


Fig. 2. Determination of antigen-binding specificity of the human Fab clones by ELISA.

Soluble Fab-pIII molecules obtained from *E. coli* periplasmic extract were allowed to bind to the recombinant CD23, Ep-CAM-Fc, c-MET-Fc, CD40-Fc, CD33-Fc or BSA immobilized on the microtiter plates, and Fab-pIII binding to the protein was detected using an HRP-conjugated goat anti-human kappa light chain pAb as a secondary antibody and, TMB as the substrate followed by measuring the OD_{450nm}. The reported data represent the average $\pm$ standard deviation of three independent experiments.

ELISA was then performed to determine whether soluble Fab-pIII molecules isolated from cell panning exhibit any specific reactivity to recombinant antigens that were readily available in our laboratory, such as CD23, Ep-CAM-Fc, c-MET-Fc, CD40-Fc, and CD33-Fc proteins, and BSA was also used as a negative control (Fig. 2). Four Fab clones (IM-L1-A, IM-L1-B, IM-L1-C and IM-L2-D) did not recognize the antigens tested, implying that they were not specific for those antigens. Unexpectedly, however, other two Fab clones, IM-L6-E and IM-L8-G, were specifically reactive to the CD23 antigen. To further confirm their specific binding reactivity to CD23, flow cytometry analysis was performed using SKW 6.4 cells and Raji cells, which express a high and low level of surface CD23, respectively [26], as well as the CD23-negative Ramos cells (Fig. 3). The black area in Fig. 3 indicates fluorescent signals produced by

control mouse anti-CD23 mAb, IM-L6-E and IM-L8-G Fab antibodies, and the white area indicates fluorescent signals produced by the secondary antibody (goat anti-human kappa pAb-FITC conjugated) only. As expected, mouse anti-CD23 mAb, a positive control antibody, produced a strong and a weak fluorescent signal on SKW 6.4 and Raji cells, respectively, without positive binding on Ramos cells. Soluble IM-L6-E and IM-L8-G Fabs also produced the same staining patterns, suggesting that both IM-L6-E and IM-L8-G Fabs have binding specificity for membrane-expressed CD23. The relatively weaker fluorescent signals compared to the control mouse anti-CD23 mAb was probably due to either low affinity or low concentration of the soluble Fab-pIII molecules. Cell panning enables selection of diverse antibody clones specific for the native form of membrane proteins expressed on the cells, but there is a preferential selection of binders specific for the membrane proteins that are over-expressed on the cell surface. Therefore, isolation of anti-CD23 Fab clones by our panning approach is not surprising because IM-9 cells are known to constitutively express high levels of CD23 (CD23a) [27], and the identification of the putative target antigens recognized by the other four Fab clones (IM-L1-A, IM-L1-B, IM-L1-C and IM-L2-D) is currently under investigation by immunoprecipitation and mass spectrometry.

The CD23 has been known as a good target antigen for B-CLL and served as a surface marker with significant prognostic importance in this disease because of its high expression on B lymphocytes from the patients [28]. Indeed, lumiliximab (IDEC-152), a primatized anti-CD23 chimeric antibody [29,30], has been recognized as one of the most promising new therapeutic antibodies for treating CLL as both a single agent and combination therapy by inducing ADCC, CDC and apoptosis in CD23-positive B-cell lines [31–34].

3.2. Affinity maturation of IM-L6-E and IM-L8-G Fab clones by L chain shuffling

The Fabs isolated from an antibody library composed of naïve Ig repertoires generally have weak affinities (in the range of

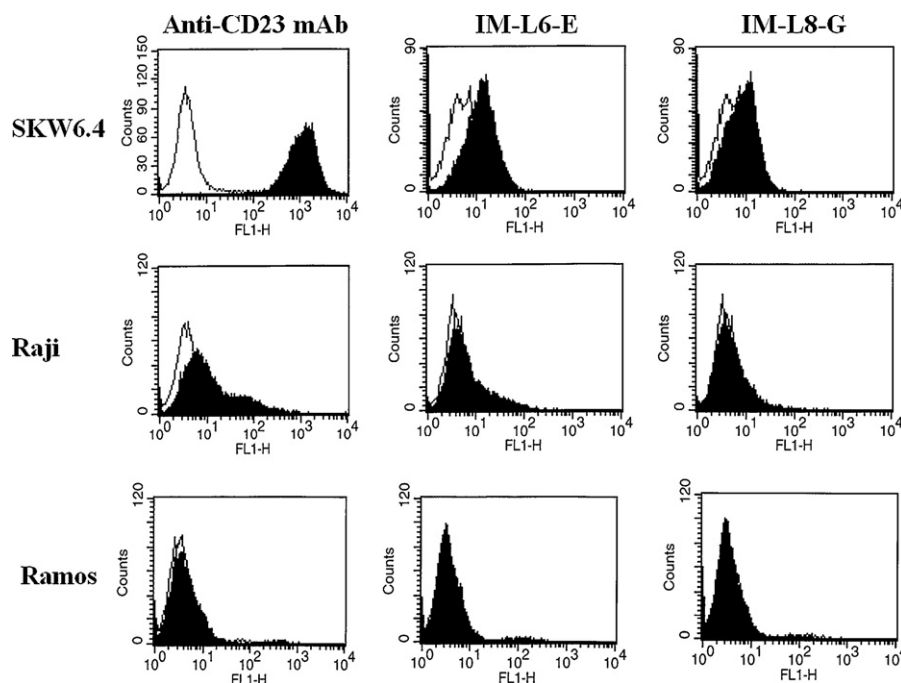


Fig. 3. Flow cytometry analysis of IM-L6-E and IM-L8-G Fab clones showing specific binding to B lymphoblastic cell lines.

SKW 6.4, Raji and Ramos were incubated with soluble IM-L6-E or IM-L8-G Fab pIII molecules, and binding signals produced by the Fab clones were detected with an anti-human kappa-FITC-conjugated mAb (black area). The fluorescent signals produced by the goat anti-human kappa-FITC-conjugated mAb alone (white area) show the lack of binding to the cell lines tested. Experiments were performed twice, with the same results.

Table 1Classification of IM-L6-E and IM-L8-G Fab variants based on their amino acid sequence and IC₅₀ determined by a thiocyanate elution method.

Fab clone	Human Y _H subgroup	Homologous germline Y _H	L chain variants	Human V _L subgroup	Homologous germline V _L	IC ₅₀ (M) ^a
IM-L6-E	II	IGHV4-34	L6	I	IGKV1-6	0.99
			IM-L6-1	II	IGKV3-NL5	0.46
			IM-L6-3	I	IGKV1-6	0.84
			IM-L6-5	III	IGKV3-NL5	<2
			IM-L6-9	III	IGKV3-NL5	0.43
			IM-L6-13	III	IGKV3-NL5	0.2
			IM-L6-14	III	IGKV3-15	0.6
			IM-L6-18	III	IGKV3-NL5	0.48
			IM-L6-19	II	IGKV1-6	0.35
IM-L8-G	I	IGHV1-f	L8	I	IGKV1-33	0.47
			IM-L8-14	III	IGKV3-NL5	0.66
			IM-L8-20	III	IGKV3-NL5	0.4
			IM-L8-23	I	IGKV3-33	0.35

^a IC₅₀ determined as the molar concentration of NH₄SCN that resulted in a 50% binding reduction of the antibody fragment to the CD23 protein.

10⁻⁶ M and 10⁻⁸ M), and subsequent affinity maturation is generally required to generate antibodies with decent affinity (in the range of 10⁻⁹ M) by using one of several methodologies including L chain shuffling [35,36]. One can easily conceive that the L chains utilized by IM-L6-E and IM-L8-G Fab clones are not optimal because they were isolated from the primary antibody library HuDV Fab-8L, which contains only eight human naïve kappa L chains. Therefore, we attempted affinity maturation of IM-L6-E and IM-L8-G Fab clones in a hierarchical manner to identify optimal L chains for these Fab clones.

We previously reported the DVS-III vector system for L chain shuffling and the human naïve kappa light chain sublibrary HuNL-D3 which possesses 1.4×10^8 diversity of human naïve kappa L repertoire [19,20]. To generate improved derivatives of IM-L6-E and IM-L8-G Fab clones through L chain shuffling two secondary antibody libraries were constructed by combining the H chain of IM-L6-E with HuNL-D3 and the H chain of IM-L8-G with HuNL-D3, respectively, and two rounds of biopanning were carried out against the recombinant CD23 protein immobilized on microtiter plates. Conventional panning on solid matrix was employed at this time because it is more efficient than cell panning in enriching target-specific binders. Twenty-four *E. coli* clones were randomly picked from each library after the final selection, soluble Fab-pIII molecules were prepared from *E. coli* periplasmic extract, and ELISA was performed using microtiter plates coated with CD23 or BSA to identify the Fab clones specific for CD23 at the monoclonal level (Fig. 4). The results showed that twenty-two Fab clones derived from IM-L6-E and sixteen Fab clones derived from IM-L8-G had specific binding to CD23, while negligible binding was observed for BSA. The DNA sequence of the V_L genes utilized by these positive Fab clones revealed eight discrete V_L sequences that paired with the V_H of IM-L6-E and three discrete V_L sequences that paired with the V_H of IM-L8-G. The resulting eight Fab clones derived from IM-L6-E were named IM-L6-1, IM-L6-3, IM-L6-5, IM-L6-9, IM-L6-13, IM-L6-14, IM-L6-18 and IM-L6-19, and the three Fab clones derived from IM-L8-G were named IM-L8-14, IM-L8-20 and IM-L8-23. Their human V subgroups and germline counterparts showing the highest homology are listed in Table 1. Some discrepancies between human V subgroup and corresponding germline counterparts are observed as a result of the H chain repertoires in the HuDV Fab-8L antibody library, which had been created by shuffling naïve HCDR1-HCDR2 and naïve HCDR3, while the L chain repertoires in the HuNL-D3 L chain library was created by shuffling naïve kappa L-CDR1 and naïve L-CDR2-3 [20].

The binding affinities of these Fab variants were then compared by ELISA using a thiocyanate elution procedure in which the relative affinity indices were determined as the molar concentration of NH₄SCN that caused a 50% reduction in binding of the antibody fragments to the CD23 protein (IC₅₀) (Table 1). The affinity indices

of the parents, IM-L6-E and IM-L8-G, were 0.99 M and 0.47 M, respectively. Among the IM-L6-E variants IM-L6-5 (IC₅₀ < 2 M) seemed to achieve higher affinity than that of the parent Fab. Similarly, an affinity-improved Fab clone, IM-L8-14 (IC₅₀ < 0.66 M) was identified among the IM-L8-G variants, although the degree of improvement seemed only marginal (Table 1). Since IM-L6-5 showed the highest IC₅₀ value among all Fab derivatives after L chain shuffling, it was chosen for a subsequent affinity study by SPR using BIAcore to determine the binding affinity by flowing purified IM-L6-5 Fab over the CD23-immobilized on a CM5 sensor chip. To confirm the affinity improvement by our selection procedure the affinity of the parent IM-L6-E also measured at the same experimental setup. The result indicated that the parent IM-L6-E bound to CD23 at k_{on} of $2.38 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and k_{off} of $1.45 \times 10^{-3} \text{ s}^{-1}$ with dissociate constant (K_D) of 600 nM and IM-L6-5 Fab bound to the antigen at k_{on} of $4.45 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and k_{off} of $1.34 \times 10^{-4} \text{ s}^{-1}$ with dissociate constant (K_D) of 30 nM, suggesting a 20-fold increase in affinity by L chain shuffling. K_D value of IM-L6-5 Fab also suggests that the Fab binds to CD23 about 3–30-folds stronger than IgE which known to have affinity for CD23 in the range of 10⁶ to 10⁷ M⁻¹ [28]. In general, conversion of a Fab to IgG form would result in up to 10-fold increase of affinity constant because of the bivalency of IgG. For example, the K_D value of cetuximab-derived C225 Fab fragment is approximately 2.7 nM while the cetuximab in (IgG form) has the K_D value of 0.25 nM [37]. Thus, the conversion of IM-L6-5 Fab to IgG could have affinity at low nanomolar range, which would be sufficient for therapeutic antibody application [38].

3.3. Binding characteristics of the Fab IM-L6-5

Because L chain shuffling can alter the original binding specificity of an antibody, we performed the same flow cytometry analysis and ELISA as in Figs. 1, 2 and 3 and obtained identical results (data not shown), and confirmed that IM-L6-5 maintains the same antigen binding specificity of the parent. Next, we determined the binding reactivity of the Fab IM-L6-5 to the inducible form of CD23 (CD23b) by flow cytometry analysis using the U937 cell line, which is known to be CD23-negative but acquires CD23 expression through IL-4 treatment [39,40]. As expected, U937 cells without IL-4 treatment were not stained at all by IM-L6-5 Fab (Fig. 5 left panel, black area) and the staining pattern was identical to the staining obtained by the secondary antibody only (white area). In contrast, the cells treated with IL-4 were stained by the Fab (Fig. 5 left panel, black area), indicating that IM-L6-5 also binds to CD23b. The control mouse anti-CD23 mAb also produced an identical staining pattern (data not shown).

The inhibitory effect of IM-L6-E and IM-L6-5 Fabs on interaction between CD23 and human IgE was then measured by competitive ELISA and SPR analysis (Fig. 6A and B). The competitive ELISA

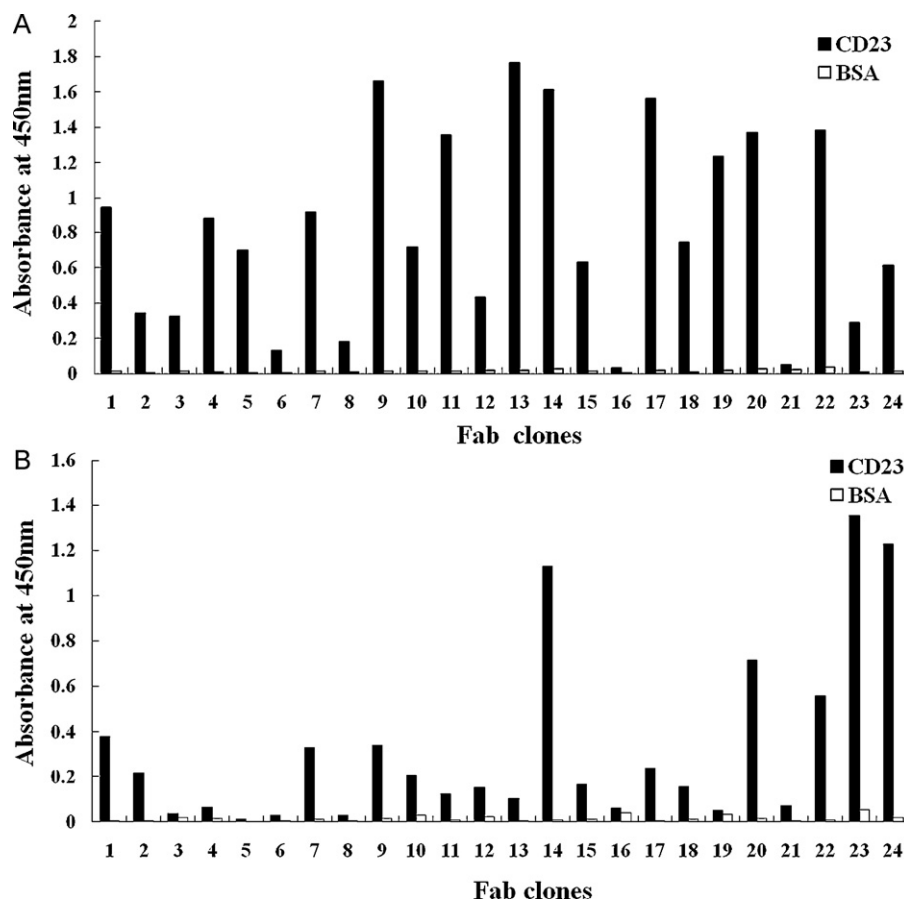


Fig. 4. Identification of the Fab derivatives showing specific binding reactivity to CD23 by ELISA.

Soluble Fab-pIII molecules obtained from the *E. coli* clones after the L chain shuffling were incubated with the immobilized recombinant CD23 protein or BSA on microtiter plates. HRPO-conjugated goat anti-human kappa light chain pAb was used as the secondary antibody, and the binding signals were visualized with TMB substrate, followed by measuring the OD_{450nm}. The reported data represent the average $\pm$ standard deviation of three independent experiments.

results showed that the purified IM-L6-E Fab substantially inhibits binding of human IgE to CD23 starting from 50 nM, and IM-L6-5 Fab showed inhibitory effect starting from 25 nM of concentration in the Fab concentration-dependent pattern, probably due to the difference in binding affinity to CD23 (Fig. 6A). The mouse anti-CD23 mAb also strongly inhibits binding of human IgE to CD23 even at 12.5 nM concentration probably due to its high affinity to CD23 which was determined to be $K_D \approx 85$ pM (data not shown). The inhibitory effect of IM-L6-5 on CD23 and IgE interaction was further demonstrated by SPR analysis in that the presence of 200 nM of IM-L6-5 Fab molecules profoundly inhibits binding of IgE to CD23 immobilized on a CM5 sensor chip (Fig. 6B), indi-

cating that IM-L6-5 Fab molecules bind to the epitope which is located close to at the IgE binding site on CD23. It had been elucidated that there are four antigenic epitopes (A–D) on CD23 of which A and D appear to lie outside the IgE binding site, and B and C seem to be located within or close to the IgE binding site [41]. Although the exact function of CD23 remains poorly understood, CD23 plays a role in the regulation of IgE production and allergy-induced immune responses through enhancing IgE antigen complex presentation, regulating IgE synthesis and releasing pre-formed and *de novo* synthesized pro-inflammatory mediators from monocytes and eosinophils. These roles suggest an importance for an anti-CD23 mAb in clinical usages [41]. Lumiliximab,

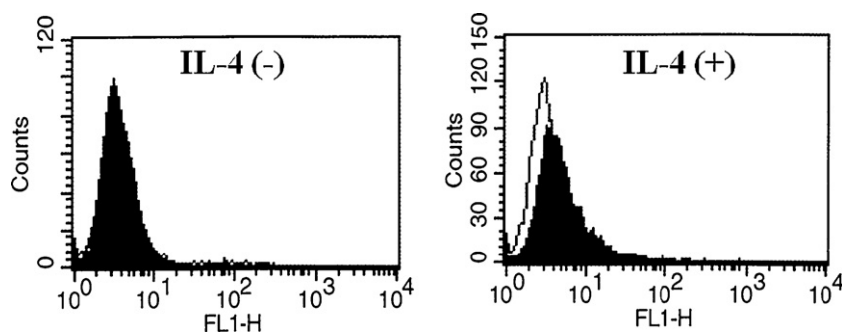


Fig. 5. Flow cytometry analysis showing detection of the inducible form of CD23 (CD23b) expressed on U937 cells by IM-L6-5 Fab.

U-937 cells were cultured for 2 days in the presence or absence of IL-4 (400 U/ml). Thereafter, the cells were harvested and incubated with soluble IM-L6-5 Fab molecules in FACS buffer (PBS with 2% FBS) (black area) or FACS buffer only (white area), for 30 min at 4 °C. Subsequently, the cells were washed and incubated in the dark with goat anti-human kappa L FITC-conjugate antibody and analyzed on a FACScan.

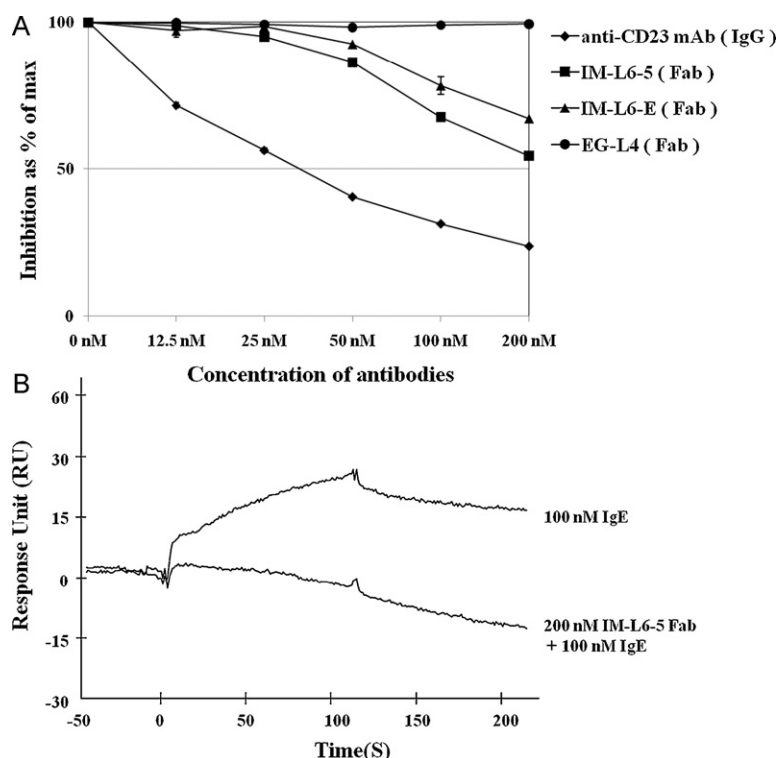


Fig. 6. Determination of the inhibitory effect of IM-L6-5 Fab on the binding of human IgE to CD23.

(A) ELISA was performed using microtiter plates coated with the recombinant CD23 protein. Various concentration of mouse anti-CD23 mAb (positive control), IM-L6-E Fab, IM-L6-5 Fab or a negative control anti-EGFR EG-L4 Fab were added to the microtiter plates, followed by incubation at 4 °C for 6 h. The plates were washed and incubated with 500 ng/ml of human IgE for over night at 4 °C. Goat anti-human IgE-HRP conjugated mAb was used as a secondary antibody and binding signals were visualized with TMB substrate and analyzed at OD_{450nm}. The data represent the average $\pm$ standard deviation of three independent experiments. (B) After immobilization of the CD23 protein onto the carboxymethylated dextran surface of a CM5 sensor chip at a level of approximately 9000 response units, 40 μ l of 200 nM IM-L6-5 Fab molecules were injected into the flow cell at a flow rate of 20 μ l/min for 120 s, followed by injection of 100 nM of human IgE. The kinetic interactions were then measured at 25 °C using a Biacore X biosensor.

a macaque human chimeric anti-CD23 mAb, has been known to reduce allergen-induced PBMC proliferation and T_H2-type immune responses which may lead to clinical benefit for treating allergic diseases [42]. Lumiliximab has thus been recognized to be a promising therapeutic candidate for the future treatment of allergic diseases [43,44]. Since IM-L6-5 appears to recognize the B or C epitopes, we assumed that IM-L6-5 is able to inhibit IgE biosynthesis induced by IL-4 in B cells [45]. In this regard, it would be interesting to compare an epitope recognition pattern between IM-L6-5 and lumiliximab and to investigate whether IM-L6-5 has the same beneficial effect as lumiliximab for treating allergic diseases.

In addition to IgE, CD23 has other natural ligands such as CD11b/CD18 ($\alpha_M\beta_2$ -integrin), CD11c/CD18 ($\alpha_X\beta_2$ -integrin) and CD21 (CR2) [46]. Therefore, we performed SPR analysis to measure the inhibitory effect of IM-L6-5 Fab on the interaction between CD23 and its natural ligands. We observed that the IM-L6-5 Fab also inhibits the interaction between CD23 and CD11b/CD18 as well as between CD23 and CD11c/CD18, but to a much lesser degree than the inhibition of the IgE-CD23 interaction (data not shown). This observation further supports that the epitope recognized by IM-L6-5 Fab is located closer to the IgE binding site of CD23. Unfortunately, we were not able to determine the inhibitory effect of the Fab on the interaction between CD23 and CD21 because, for unknown reasons, CD21 did not bind to the CD23-immobilized on the CM5 sensor chip in our experimental setup.

In this study we successfully generated the Fab IM-L6-5 which, to the best of our knowledge, is the first report of a fully human anti-CD23 Fab antibody derived from human naïve origin with reasonable binding affinity. The conversion of the Fab IM-L6-5 to full IgG1 is currently in progress to analyze the biological and therapeutic functions of the antibody. In addition, we believe that the

determination of antigen-binding specificity and characterization of the four other Fab clones (IM-L1-A, IM-L1-B, IM-L1-C and IM-L2-D) would be useful for finding potential new target antigens or developing new therapeutic antibodies for the treatment of B cell lymphomas.

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References

- [1] Coiffier B. Treatment of non-Hodgkin's lymphoma: a look over the past decade. *Clin Lymphoma Myeloma* 2006;7:S7–13.
- [2] Hallek M. State-of-the-art treatment of chronic lymphocytic leukemia. *Hematol Am Soc Hematol Educ Program* 2009;440–9.
- [3] Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD 59 regulate complement-mediated cell lysis. *Blood* 2000;95:3900–8.
- [4] Pedersen IM, Buhl AM, Klausen P, Geisler CH, Jurlander J. The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein-kinase dependent mechanism. *Blood* 2002;99:1314–9.
- [5] Robak T. Recent progress in the management of chronic lymphocytic leukemia. *Cancer Treat Rev* 2007;33:710–28.
- [6] Keating MJ, O'Brien S, Kontoyannis D, Plunkett W, Koller C, Beran M, et al. Results of first salvage therapy for patients refractory to a fludarabine regimen in chronic lymphocytic leukemia. *Leuk Lymphoma* 2002;43:1755–62.
- [7] ManShouri T, DoKA, Wang X, Giles FJ, O'Brien SM, Safer H, et al. Circulating CD20 is detectable in the plasma of patients with chronic lymphocytic leukemia and is of prognostic significance. *Blood* 2003;101:2507–13.

- [8] Moreton P, Kennedy B, Lucas G, Leach M, Rassam S, Haynes A, et al. Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival. *J Clin Oncol* 2005;23:2971–9.
- [9] McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998;16:2825–33.
- [10] Davis TA, Grillo-Lopez AJ, White CA, McLaughlin P, Czuczman MS, Link BK, et al. Rituximab anti-CD20 monoclonal antibody therapy in non-Hodkin's lymphoma: safety and efficacy of retreatment. *J Clin Oncol* 2000;18:3135–43.
- [11] Robak T, Jamrozik K, Robak P. Current and emerging treatments for chronic lymphocytic leukaemia. *Drugs* 2009;69:2415–49.
- [12] Robak T. Novel monoclonal antibodies for the treatment of chronic lymphocytic leukemia. *Curr Cancer Drug Targets* 2008;8:156–71.
- [13] Celeste Bello, Eduardo M, Sotomayer. Monoclonal antibodies for B-cell lymphomas: rituximab and beyond. *Hematology* 2007;115:233–42.
- [14] Azzazy HM, Highsmith Jr WE. Phage display technology: clinical applications and recent innovations. *Clin Biochem* 2002;35:425–45.
- [15] Marks JD, Bradbury A. Selection of human antibodies from phage display libraries. *Methods Mol Biol* 2004;248:161–76.
- [16] Hoogenboom HR, Chames P. Natural and designer binding sites made by phage display technology. *Immunol Today* 2000;21:371–8.
- [17] Aires da Silva F, Corte-Real S, Goncalves J. Recombinant antibodies as therapeutic agents: pathways for modeling new biodrugs. *BioDrugs* 2008;22:301–14.
- [18] Giordano RJ, Cardó-Vila M, Lahdenranta K, Pasqualini R, Arap W. Biopanning and rapid analysis of selective interactive ligands. *Nat Med* 2001;7:1249–53.
- [19] Hur B, Yoon J, Liu L, Cha S. Isolation of a human anti-epidermal growth factor receptor Fab antibody EG-19-11, with subnanomolar affinity from naïve immunoglobulin repertoires using a hierarchical antibody library system. *Immunol Lett* 2010;134:55–61.
- [20] Hur B, Choi H, Song S, Yoon J, Liu L, Cha S. Development of the dual-vector system-III (DVS-III), which facilitates affinity maturation of a Fab antibody via light chain shuffling. *Immunol Lett* 2010;132:24–30.
- [21] Joo H, Hur B, Lee K, Song S, Cha S. Establishment of a reliable dual-vector system for the phage display of antibody fragments. *J Immunol Methods* 2008;333:24–7.
- [22] Brian KK, Jill W, John MC. Phage display of peptides and proteins, a laboratory manual. San Diego: Academic Press, P107.
- [23] Song S, Hur B, Lee K, Choi H, Kim S, Kang G, et al. Successful application of the dual-vector system II in creating reliable phage-displayed combinatorial Fab library. *Mol Cells* 2009;3:313–9.
- [24] Macdonald RA, Hosking CS, Jones CL. The measurement of relative antibody affinity by ELISA using thiocyanate elution. *J Immunol Methods* 1988;106:191–4.
- [25] Akahori Y, Kurosawa G, Sumitomo M, Morita M, Muramatsu C, Eguchi K, et al. Isolation of antigen/antibody complexes through organic solvent (ICOS) method. *Biochem Biophys Res Commun* 2009;378:832–5.
- [26] Munoz O, Brignone C, Grenier-Brossette N, Bonnefoy J, Cousini J. Binding of anti-CD23 monoclonal antibody to the leucine zipper motif of FcεRII/CD23 on B cell membrane promotes its proteolytic cleavage: evidence for an effect on the oligomer/monomer equilibrium. *J Biol Chem* 2005;273:31795–800.
- [27] Bonnefoy J, Aubry J, Peronne C, Wijdenes J, Banchereau A. Production and characterization of a monoclonal antibody specific for the human lymphocyte low affinity receptor for IgE: CD 23 is a low affinity receptor for IgE. *J Immunol* 1987;138:2970–8.
- [28] Fournier S, Delespesse G, Rubio M, Biron G, Sarfati M. CD23 antigen regulation and signaling in chronic lymphocytic leukemia. *J Clin Invest* 1992;89:1312–21.
- [29] Nakamura T, Kloetzer WS, Brams P, Hariharanb K, Chamatb S, Cao X, et al. In vitro IgE inhibition in B cells by anti-CD23 monoclonal antibodies is functionally dependent on the immunoglobulin Fc domain. *Int J Immunopharmacol* 2000;22:131–41.
- [30] Chassagne S, Laffly E, Drouet E, Hérodin F, Lefranc M, Thullier P. A high-affinity macaque antibody Fab with human-like framework regions obtained from a small phage display immune library. *Mol Immunol* 2004;41:539–46.
- [31] Byrd JC, O'Brien S, Flinn IW, Kipps TJ, Weiss M, Rai K, et al. Phase 1 study of lumiliximab with detailed pharmacokinetic and pharmacodynamic measurements in patients with relapsed or refractory chronic lymphocytic leukemia. *Clin Cancer Res* 2007;13:4448–55.
- [32] Pathan NI, Chu P, Hariharan K, Cheney C, Molina A, Byrd J. Mediation of apoptosis by and antitumor activity of lumiliximab in chronic lymphocytic leukemia cells and CD23⁺ lymphoma cell lines. *Blood* 2008;111:1594–602.
- [33] Smolej L. Modern concepts in the treatment of chronic lymphocytic leukemia. *Hematology* 2009;14:249–54.
- [34] Byrd JC, Kipps TJ, Flinn IW, Castro J, Lin TS, Wierda W, et al. Phase 1/2 study of lumiliximab combined with fludarabine, cyclophosphamide, and rituximab in patients with relapsed or refractory chronic lymphocytic leukemia. *Blood* 2010;115:489–95.
- [35] Marks JD, Griffiths AD, Malmqvist M, Clackson TP, Bye JM, Winter G. By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (NY)* 1992;10:779–83.
- [36] Chowdhury PS, Pastan I. Improving antibody affinity by mimicking somatic hypermutation in vitro. *Nat Biotechnol* 1999;17:568–72.
- [37] Kamat V, Donaldson JM, Kari C, Quadros MRD, Lelkes PI, Chaiken I, et al. Inhibition and distinct epitope recognition by EGFR antagonistic mAbs C225 and 425. *Cancer Biol Ther* 2008;7:726–33.
- [38] Kubetzko S, Balic E, Waibel R, Zangemeister-Wittke U, Plückthun A. PEGylation and multimerization of the anti-p185HER-2 single chain Fv fragment 4D5: effects on tumor targeting. *J Biol Chem* 2006;281:35186–201.
- [39] Ouaz F, Paul-Eugène N, Arock M, Merle-Béral H, Huerta JMM, Debré P, et al. Maturation of human myelomonocytic leukemia cells following ligation of the low affinity receptor for IgE (FcεRII/CD23). *Int Immunol* 1993;5:1251–7.
- [40] So E, Kim S, Cho B, Park H, Lee C. Corticosteroid inhibits IL-4 signaling through down-regulation of IL-4 receptor and STAT6 activity. *FEBS Lett* 2002;518:53–9.
- [41] Zhang M, Murphy RF, Agrawal DK. Decoding IgE Fc receptors. *Immunol Res* 2007;37:1–16.
- [42] Poole JA, Meng J, Reff M, Spellman MC, Rosenwasser LJ. Anti-CD23 monoclonal antibody, lumiliximab, inhibited allergen-induced responses in antigen-presenting cells and T cells from atopic subjects. *J Allergy Clin Immunol* 2005;116:780–8.
- [43] Rosenwasser LJ, Busse WW, Lizambri RG, Olejnik TA, Totoritis MC. Allergic asthma and an anti-CD23 mAb (IDEC-152): results of a phase I, single-dose, dose-escalating clinical trial. *J Allergy Clin Immunol* 2003;112:563–70.
- [44] Rosenwasser LJ, Meng J. Anti-CD23. *Clin Rev Allergy Immunol* 2005;29:61–72.
- [45] Wakai M, Pasley P, Sthoeger ZM, Posnett DN, Brooks R, Hashimoto S, et al. Anti-CD23 monoclonal antibodies: comparisons of epitope specificities and modulating capacities for IgE binding and production. *Hybridoma* 1993;12:25–43.
- [46] Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* 2008;8:205–17.