ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
December 2017; 16(6):460-470.

Generation and Characterization of Siglec-F-Specific Monoclonal Antibodies

Sima Shahmohammadi-Farid[1,2], Roya Ghods[3,4], Mahmood Jeddi-Tehrani[5], Ali-Ahmad Bayat[5], Nazanin Mojtabavi[6], Alireza Razavi[1], and Amir-Hassan Zarnani[7,8]

[1] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2] Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Oncopathology Research Center, Iran University of Medical Sciences, Tehran, Iran
[4] Department of Molecular Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
[5] Monoclonal Antibody Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran
[6] Department of Immunology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
[7] Reproductive Immunology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran
[8] Immunology Research Center, Iran University of Medical Sciences, Tehran, Iran

Received: 7 February 2017; Received in revised form: 2 September 2017; Accepted: 27 September 2017

ABSTRACT

Siglec-F (SF) is a surface glycoprotein expressed by mouse eosinophils and induces caspase- and mitochondria-dependent apoptosis after engagement with its cognate ligand or specific antibodies. This targeting eosinophils by monoclonal antibodies may help diverse diseases associated with increased frequency of eosinophils including allergy and asthma. In this paper, production of murine and rat monoclonal antibodies (mAbs) against Siglec-F has been addressed.

Balb/c mice were immunized with siglec-F1 (SF1) and siglec-F2 (SF2) synthetic peptides conjugated to a carrier protein. Rats were immunized with Chinese hamster ovary CHO cells overexpressing Siglec-F (CHO-SF) or with Siglec-F-human immunoglobulin FC fusion protein (CHO-SF-Ig). Hybridomas were produced by standard protocol and screened for their reactivity by enzyme-linked immunosorbent assay (ELISA), western blotting (WB), and flow cytometry. In parallel, polyclonal antibodies were generated in New Zealand White rabbits immunized with SF1 and SF2 peptides.

Three mouse and three rat mAbs were generated against synthetic peptides and SF-Ig, respectively. All mouse monoclonal and rabbit polyclonal antibodies reacted well with immunizing molecules in ELISA and detected specific band of Siglec-F in WB. However, they failed to detect native molecule in flow cytometry analysis. Quite the contrary, rat mAbs did not reacted with the denatured protein in WB, instead exhibited significant reactivity with CHO-SF cells in flow cytometry.

Based on the heavily glycosylated nature of Siglec-F, it seems that generation of anti-SF antibodies able to detect native protein needs a properly folded molecule for immunization. Monoclonal antibodies reported here are invaluable tools for studying linear and conformation epitopes of SF and tracing mouse eosinophils.

Keywords: Epitopes; Flow cytometry; Monoclonal antibody; Native protein; Siglec-F; Western blotting

Corresponding Authors: Amir-Hassan Zarnani, DMT, PhD; Reproductive Immunology Research Center, Avicenna Research Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran. Tel: (+98 21) 2243 2020, Fax: (+98 21) 2243 2021, E-mail: zarnania@gmail.com, zarnani@ari.ir

Alireza Razavi, MD, PhD;
Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 4293 3068, Fax: (+98 21) 8895 4913, E-mail: razavial@tums.ac.ir

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are single-pass type I transmembrane proteins[1] Fourteen Siglecs have been identified in humans and nine in mice.[2] Each Siglec contains a N-terminal V-type immunoglobulin domain (Ig domain) in the extracellular part of the molecule which binds sialic acid and variable numbers of C2-set Ig domains, ranging from 1 in CD33 to 16 in sialoadhesin.[1]

The structure of some Siglecs is closely similar to CD33 and such Siglecs are generally referred as CD33-related (CD33r) Siglecs.[3] Most Siglecs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytosolic region.[4] ITIMs down-regulate signaling pathways including phosphorylation induced by immunoreceptor tyrosine-based activation motifs (ITAMs).[5]

Because of the acidic nature of sialic acid, the active sites of the Siglecs contain a conserved arginine residue which is positively charged at physiological pH. This amino acid forms salt bridges with the carboxyl group of the sugar residue.[1] Sialic acid consists of abundant hydroxyl groups which can be involved in the construction of glycosidic linkages. The specificity of each Siglec is determined by chemical interaction between the sugar ligand and the Siglec amino acids. The position in space of the individual groups on the sugar and the protein amino acids affects the sialic acid linkage to which each Siglec binds.[6]

Cross-linking of some CD33r Siglecs with antibody leads to inhibition of cell proliferation and function, and/or initiation of apoptosis. Most Siglecs are short molecules that do not expand far from the cell surface. This prevents most Siglecs from binding to other cells which are basically covered by sialic acid-containing glycans, so the majority of Siglecs only bind ligands on the surface of the same cell.[7]

Siglec-F (SF) is a CD33r Siglec prominently expressed on mature circulating mouse eosinophils, and on some myeloid precursors in bone marrow.[8] Interaction of SF on circulating eosinophils with its ligand leads to caspase- and mitochondria-dependent apoptosis. SF, as a glycan ligand, preferentially binds 6′- sulfated sialyl-Lewis X. It should be noted that this molecule is mostly found in bronchial mucins.[9]

SF modifies Th2-cell functions; so it can regulate eosinophil production and recruitment which is an essential factor in allergic disorders such as asthma.[10] The pro-inflammatory mediators derived from eosinophil are major contributors to inflammation in asthma, including airway epithelial cell damage and loss, airway dysfunction of cholinergic nerve receptors, airway hyper-responsiveness, mucus hypersecretion, and airway remodeling, characterized by fibrosis and collagen deposition.[11]

Recent studies have showed that the levels of allergen-induced airway eosinophilic inflammation were increased and resolution of airway eosinophilic inflammation following acute allergen challenge was delayed in SF deficient mice.[10]

Administration of SF antibody reduced the number of eosinophils and increased the number of apoptotic eosinophils in lung and bone marrow in a chronic mouse model of asthma and consequently decreased the level of allergen-induced eosinophilic inflammation of airway and hallmarks of airway remodeling particularly subepithelial fibrosis.[10] As such, targeting eosinophils by monoclonal antibodies may help diverse diseases associated with increased frequency of eosinophils including allergy and asthma.[12]

Humanized monoclonal antibodies that block IgE have been approved for the treatment of allergic asthma. Omalizumab (anti-IgE antibody) which is now widely used in the treatment of allergic bronchial asthma, is a recombinant humanized monoclonal antibody (rhuMAb-E25) developed by immunization of mice with human IgE. This antibody recognizes IgE at the same site as does by the high-affinity receptor for IgE (FcεRI).[13]

The aim of this study was to produce specific monoclonal antibodies against SF. Such antibodies could be viewed as invaluable tools to delineate the pathophysiology of allergic asthma and the role that eosinophils serve in this context.

MATERIALS AND METHODS

Cell Culture

Murine myeloma cell lines, SP2/0 and P3X63Ag8.653 (National cell bank, Pasture Institute of Iran) were cultured in RPMI-1640 (Gibco, Invitrogen, CA, USA) supplemented with 100U/mL penicillin, 100μg/mL streptomycin and 10% fetal bovine serum (FBS) (Gibco). Chinese hamster ovary (CHO) cells stably overexpressing surface (CHO-SF) or secreted SF (two N-terminal Ig domains of Siglec-F fused to the Fc region of human IgG (CHO-SF-Ig) [9]generously gifted

by Dr James C. Paulson from the Scripps Research Institute, USA) were cultured in RPMI-1640 with 10% FBS, 2 mM L-Glutamine and 0.5 mg/mL Hygromycin B (Invitrogen, USA). Cultured cells were incubated in humidified atmosphere containing 5% CO2 at 37°C.

Immunogens

Due to heavily-glycosilated pattern of Siglec-F making production of monoclonal antibody difficult, three types of immunogens were used for immunization of animals: Two synthetic peptides, recombinant SF-Ig protein and CHO-SF cells.

Epitope Selection

Forecasting of continuous B-cell epitopes for Siglec-F was performed according to the protocol published recently[14] using such web servers as Bcepred, BCPREDS, COBEpro, BepiPred and ABCpred. Where convenient, the specificity was set to 90%. Epitopes with highest scores predicted by most of the prediction tools were selected. To ensure unique nature of the selected epitope, BLAST analysis was performed by NCBI BLAST software. Finally, two 12-mer peptides, LSWERPTQKPFQ (SF1) from Ig-like domain 2 corresponding to amino acids 275-286 and SPVATNDPQRSV (SF2) from v-set domain corresponding to amino acids 66-77 were selected as immunogens. A cysteine residue was added to the C-terminus end of peptides to facilitate the conjugation to carrier protein.

Immunogen Preparation

Synthetic peptides were conjugated separately with Imject Maleimide-activated Keyhole Limpet Hemocyanin (Thermo Scientific, Rockford, USA) and used for immunization. For evaluation of conjugation efficacy, peptides were also conjugated with Imject Maleimide-activated bovine serum albumin (BSA) (Thermo Scientific) and analyzed by SDS-PAGE. In parallel, recombinant protein secreted from CHO-SF-Ig cells (SF-Ig) into medium was purified by chromatography column containing nickel-agarose matrix and used as immunogen. Indeed, CHO-SF cells were also used for immunization in some settings.

Immunization Protocol

Ethical committees of the Avicenna Research Institute and Tehran University of Medical Sciences approved all experiments with animals conducted in this study (No. 16302). For production of monoclonal antibodies, female Balb/c mice, 8–10 weeks old and 2-3 month old rats were immunized as mentioned below. Six month old New Zealand White rabbits were used for production of polyclonal antibodies. All animals were purchased from animal facility of Pasteur Institute of Iran (Tehran, Iran).

For each peptide two mice were immunized with peptide-KLH. Briefly, immunization of each mouse was performed intraperitoneally with 50 μg SF1-KLH or SF2-KLH, five times every 3 weeks. The first immunization was carried out with complete Freund's adjuvant. Following immunizations were performed with incomplete Freund's adjuvant. One week after the last immunization, using a vertical incision of the tail vein, blood was collected and antibody titer was detected by ELISA. Finally, three days before the cell fusion, 20 μg SF1-KLH or SF2-KLH (without any adjuvant) were injected intravenously.

Rats were immunized with 400 μg SF-Ig recombinant protein intraperitoneally. The second immunization was performed three weeks later and the rest three injections were done with two- week intervals. Rats were further intravenously immunized with 100 μg antigen three days before cell fusion.

For immunization with CHO-SF cells, rats were immunized intraperitoneally with 1 x 10^7 cells thoroughly admixed with complete Freund's adjuvant. All subsequent steps for immunization (five injections) were carried out in three-week intervals and one week after the last immunization, antibody titer was detected by ELISA.

For polyclonal antibody production, IMMACCEL (Pickcell Laboratories, Netherlands) was used as adjuvant. Rabbits were first immunized subcutaneously with 80 μg SF1-KLH or SF2-KLH mixed with 0.25mL IMMACCEL and equal volume of complete Freund's adjuvant. The second and third immunizations were performed with one-week interval using 400 μg and 800 μg immunogen mixed with IMMACCEL and incomplete Freund's adjuvant, respectively. Three weeks after the third immunization, 125 μg SF1-KLH or SF2-KLH admixed with incomplete Freund's adjuvant was used as a booster.

Production of Monoclonal and Polyclonal Antibodies

Monoclonal antibodies were generated using hybridoma technology as described elsewhere.[15] In

brief, three days after of the last immunization, spleen cells of the immunized mice were fused with mouse myeloma SP2/0 cell line at 5:1 ratio. In rats, P3X63Ag8.653 cell line was used as fusion partner. Then, pre-warmed polyethylene glycol (PEG) 1500 (Sigma, USA) was gently added to the cell pellet and resulting hybridoma cells were cultured in RPMI supplemented with 20% FBS and HAT selective medium (Sigma, USA). Supernatants of hybridomas were screened by indirect ELISA as below. In rats, screening was performed by indirect ELISA and flow cytometry. Positive hybridomas were cloned four times by limiting dilution process. The isotype of each mAb was checked by mouse monoclonal antibody isotyping kit (IsoStrip, Roche, Ind, USA). Ascites fluid was obtained after intraperitoneal injection of anti-Siglec-F1 and anti-Siglec-F2 hybridoma cells. MAbs were purified by HiTrap protein G affinity chromatography column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instruction.

In case of polyclonal antibodies, 50 mL blood was collected one week after the last injection and serum was clarified by centrifugation. Collected sera were then diluted 1:5 with PBS, pH: 7.2 and antibody fraction was isolated by affinity chromatography using SF1-KLH or SF2-KLH peptides-coupled with CNBr-activated Sepharose 4B (GE Healthcare, United Kingdom) and dialyzed overnight against PBS. Purity of purified antibodies was evaluated by SDS-PAGE.

Enzyme-Linked Immunosorbent Assay

Indirect ELISA was employed for antibody titration and screening of hybridomas. In brief, 96 well plates (Greiner, Germany) were coated with either 5 µg/mL SF1or SF2 peptides or 10 µg/mL SF-Ig recombinant protein dissolved in phosphate buffered saline (PBS, 0.15 M pH=7.2) and incubated at 37°C for 1 hr followed by overnight incubation at 4°C. Then, blocking was performed with 3% skim milk in PBS at 37°C for 2 h. Hybridoma supernatants, serial dilutions of mouse, rat and rabbit sera or purified antibodies were added subsequently for 1.5 h. After washing three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T), signals were developed by successive addition of HRP-conjugated sheep anti-mouse (for mouse sera and monoclonals), human-Ig-adsorbed anti-mouse (for rat sera and monoclonals) or anti-rabbit Ig (for rabbit polyclonals) for 1 h (all from Sina Biotech, Tehran, Iran) and tetramethylbenzidine (TMB) for 15 min. Optical density (OD) of the wells was then measured at 450 nm using a microplate reader (Anthos 2020, UK).

In case of rat monoclonals and based on the nature of immunogen which was SF-Ig, reactivity of generated monoclonal antibodies with human IgG as ELISA coating layer was also tested. In brief, 96 well plates were coated with 10 µg/mL human IgG followed by sequential addition of titrating concentrations of rat anti-SF monoclonal antibodies starting from 10 µg/mL and human Ig-adsorbed sheep anti-mouse Ig. Signals were then developed as above.

To determine whether or not the generated rat clones originating from the same well during cloning (2H9C7 and 2H9G11) have different clonality, competitive ELISA was performed. To this end, purified 2H9C7 antibody was first conjugated to biotin using Sulfo-NHS-Biotin (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Wells were coated with SF-Ig as above followed by a pre-incubation period of 30 min with different concentrations of purified 2H9G11 rat anti-SF antibody. After washing, biotin-conjugated 2H9C7 antibody (250 ng/mL was added to the wells for 1 h Wells were washed three times with PBS-T. HRP-conjugated streptavidin (Thermo Fisher Scientific, USA) (1:30000) was then added to the wells for 30 min. Wells were then processed for signal development exactly as mentioned above.

Western Blot

Protein concentration of purified SF-Ig protein was measured by a BCA kit (Thermo Scientific). Five µg SF-Ig was boiled for 5 minutes in SDS sample buffer (0.5 M Tris, pH 6.8, 16% (v/v) glycerol, 3%(v/v) SDS, 8%(v/v) 2-mercaptoethanol, and 2% bromophenol blue), electrophoresed in 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Millipore Corporation, Billerica, Massachusetts, USA). Membranes were washed with PBS-T and blocked overnight at 4°C in the same buffer containing 5% skim milk. Membranes were next incubated with purified polyclonal antibodies [anti-SF1(2 µg/mL, anti-SF2(5µg/mL], monoclonal antibodies against SF-1 [5E8C9(10 µg/mL], SF-2 [3A2H3(5 µg/mL; or 3A3H8(10 µg/mL], or SF-Ig [2H9C7(10 µg/mL, 2H9G11(10 µg/mL or 13G2B10 (0.5 µg/mL] mAbs for 1.5 h at room temperature. After washing, membranes

were incubated for 45 min with HRP-conjugated sheep anti-rabbit (1:5000) or human Ig-adsorbed HRP-conjugated sheep anti-mouse Ig (Sina Biotech, Tehran, Iran) (1:2500) at room temperature followed by washing and developing with ECL chemiluminescence detection system (GE Healthcare). As negative controls, non-immune rabbit, rat or mouse Igs were used as primary antibody. In parallel, some lanes were also probed with HRP-labeled anti-His6 tag (Roche, Germany) as positive control.

Flow Cytometry

Un-transfected CHO or CHO-SF cells were harvested with 0.05% EDTA in PBS, washed twice with cold PBS and blocked with 5% sheep serum for 20 min. Cells were then incubated with 100 µL supernatant of positive clones or 10 µg/mL purified anti-Siglec-F monoclonal antibodies for 45 min at 4° C. Cell culture medium or non-immune Igs was used as negative controls. After 3 times washing steps with cold PBS, 1:100 dilutions of PE-conjugated goat anti-rat IgG (Santa Cruz, USA) or FITC-conjugated sheep anti-mouse Ig (Sina Biotech) was added to the cells and incubation was continued for 30 min in the dark at 4° C. Cells were washed and analyzed by a flow cytometer (Partec, Nuremberg, Germany) using Flowmax software. Commercial PE-conjugated anti-Siglec-F antibody (BD Pharminge, USA, Cat No: 552126, Clone: E50-2440) was used as positive control.

RESULTS

Preparation of Immunogens

Peptides spanning from amino acid 275 to 286 (SF1) and 66 to 77 (SF2) (Figure 1a) were selected based on their overall high specificity and antigenicity scores for generation of monoclonal antibodies. High antigenicity prediction scores and unique blast pattern of the selected peptides indicated that they might be suitable for production of highly specific and efficient antibodies against Siglec-F. Electrophoresis pattern of BSA-conjugate peptides showed smear pattern of electrophoretic movement of conjugates implying successful conjugation (Figure 1b).

Recombinant protein secreted from CHO-SF-Ig cells was purified by chromatography column containing nickel-agarose matrix. Western blot analysis using anti-His6 tag antibody revealed three distinct bands of about 43, 85 and 127 kD (Figure 1c).

Production of Anti-Siglec-F Antibodies

In immunized mice and rats, indirect ELISA was performed to detect the presence of specific antibodies against peptides and SF-Ig protein in mice and rats sera, respectively. Mice and rats with higher titer of specific antibody were selected for mAb production. After four consecutive clonings, one final stable clone against SF1 (5E8C9, IgG1/k) and two clones against SF2 (3A2H3 and 3A3H8 both IgG1/k) were achieved. In rats immunized with SF-Ig recombinant protein, three stable clones namely (2H9C7), (2H9G11) and (13G2B10) were selected. The isotype of all were found to be IgG1/k. Ascites fluids from mouse clones including 5E8C9, 3A2H3 and 3A3H8 were prepared and purified by protein G column yielding 0.814, 1.38 and 0.849 mg per 1 mL of ascites fluid, respectively. In rats immunized with CHO-SF cells, no stable colons were obtained.

Monoclonal antibodies from rat hybridoma cell culture supernatants were also purified with the same method yielding 0.336, 0.910 and 1.06 mg per 100 mL of cell culture supernatants. Purified polyclonal (Figure 2a) and monoclonal antibodies were shown to be pure as judged by SDS-PAGE analysis (Figure 2b).

Reactivity Assessment of Purified Antibodies by ELISA

Reactivity of purified antibodies including two polyclonal and six monoclonal antibodies directed against two synthetic peptides and one recombinant protein (Ig-CHO-SF) were tested by indirect ELISA using immunizing peptides or recombinant protein as coating layer. The results are summarized in Figure 3. In case of rabbit polyclonal antibodies, both anti-SF1 and anti-SF2 antibodies showed excellent reactivity against corresponding immunizing peptides with optical densities reaching to the plateau at antibody concentrations of about 32 ng/mL.

Reactivity of rabbit anti-SF1 was found to be superior over that of anti- SF2 antibody (Figure 3a). Two hybridomas against SF2, namely 3A3H8 and 3A2H3, and one clone against SF1 (5E8C9) were produced in mice. Anti-SF2 mouse clones were found to be highly sensitive in detecting the immunizing peptide. Both clones similarly yielded OD over 3 at concentrations above 250 ng/mL. Reactivity of anti-SF1 mouse monoclonal antibody was found to be lower to some extent compared to the antibodies produced against SF2 (Figure 3b).

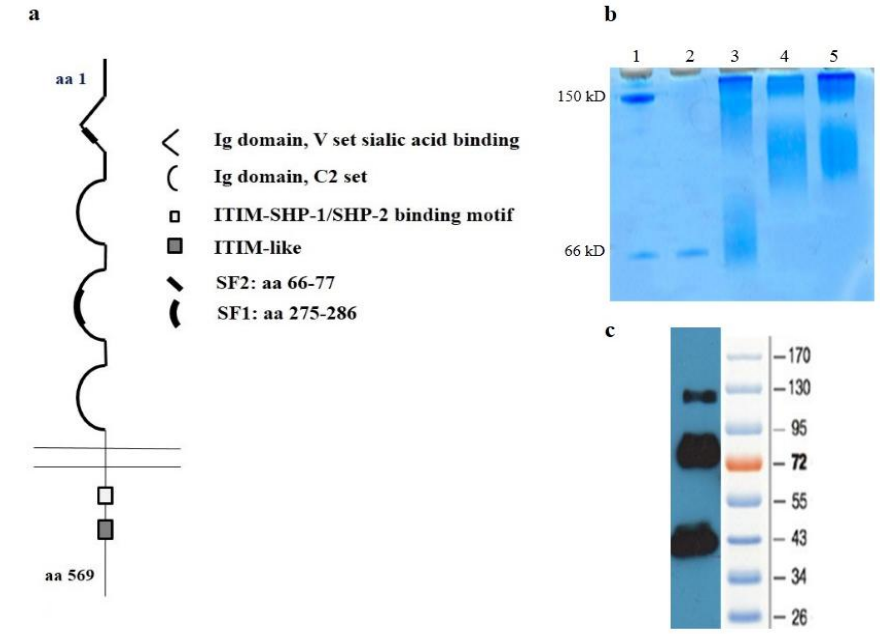

Figure 1. Characteristics of Siglec-F1 and Siglec-F2 peptides used for immunization. a) Schematic diagram of Siglec-F showing the location of peptides selected for immunizations. b) Assessment of peptide conjugation by SDS-PAGE analysis: Lane 1: hIgG and BSA, Lane 2: BSA, Lane 3: BSA-linker, Lane 4: BSA-conjugated Siglec-F1, Lane 5: BSA-conjugated Siglec-F2. The smear pattern of electrophoresis movement of conjugates and the absence of free peptide show successful conjugation. c) Western blot analysis of Siglec-F-Fc recombinant protein by HRP-conjugated anti-His6 tag antibody. Three distinct bands of about 43, 85 and 127 kD are seen. Siglec-F, sialic acid binding Ig-like lectin F; hIgG, human Immunoglobulin G; BSA, Bovine serum albumin; anti-His6 tag , anti histidine-tag.

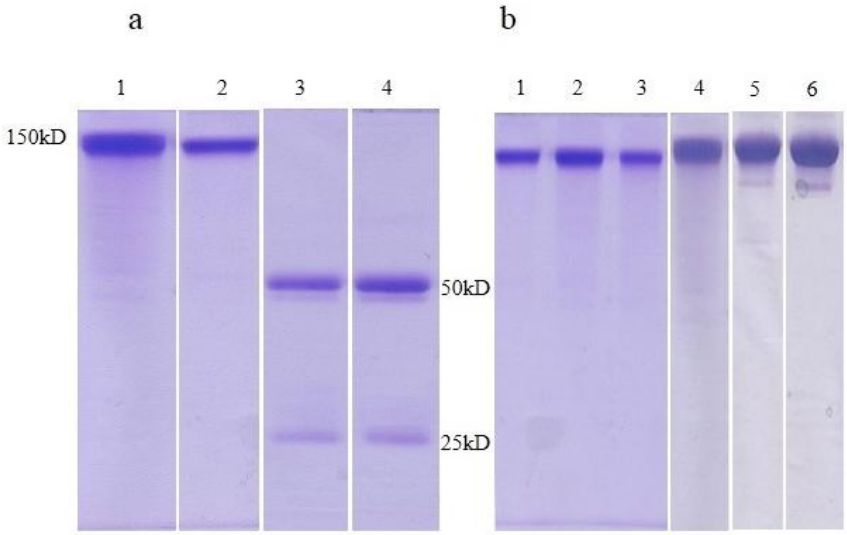

Figure 2. SDS-PAGE analysis of purified anti-SF1 and anti-SF2 antibodies. a) Polyclonal anti-SF1 and anti-SF2 antibodies purified over peptide affinity columns. Lane 1: Rabbit anti-SF1, Lane 2: Rabbit anti-SF2, Lane 3 and 4: Rabbit anti-SF1 and SF2 at reducing condition, respectively. b) Monoclonal anti-SF1 and anti-SF2 antibodies. Lane 1: Anti-SF2; clone 3A3H8, Lane 2: Anti-SF2; clone 3A2H3, Lane 3: Anti-SF1; clone 5E8C9, Lane 4-6: Anti-SF-Ig; clones 2H9C7, 2H9G11 and 13G2B10, respectively. Anti-SF1, anti- Siglec-F1; anti-SF2,anti- Siglec-F2; SF-Ig, secreted Siglec-F composed of Ig domains of Siglec-F fused to the Fc region of human IgG.

Murine anti-peptide antibodies failed to react with recombinant Siglec-F protein (data not shown). Almost similar reactivity was observed in case of rat monoclonals, 2H9C7 and 2H9G11, while 13G2B10 showed relatively lower reactivity (Figure 3c). The results of competitive ELISA showed that reactivity of 2H9C7 with SF-Ig was inhibited by different concentrations of 2H9G11 (Figure 3d). None of the rat monoclonals reacted with human IgG indicating that generated rat mAbs were directed against SF portion of the SF-Ig fusion protein (Fig.3e). This finding was expectable, as selection of the clones in flow ctytometry was based on the reactivity with CHO-SF and not with un-transfected CHO cells.

Western Blot Analysis

In order to confirm the reactivity of purified antibodies with whole Siglec F protein, Western blot analysis was performed using SF-Ig recombinant protein as loading antigen. Besides using purified polyclonal and monoclonal antibodies as primaries, reactivity was also checked with anti-His6 antibody. Anti-His6 tag antibody recognized three distinct bands of about 43, 127 and 85 kD. In parallel, three bands with the same molecular weights were also detected when the membranes were probed with protein G-purified rabbit polyclonal and mouse monoclonal antibodies against SF-Ig (Figure 4). None of the rat monoclonal antibodies was found to be reactive with SF-Ig in this experiment (Figure 4).

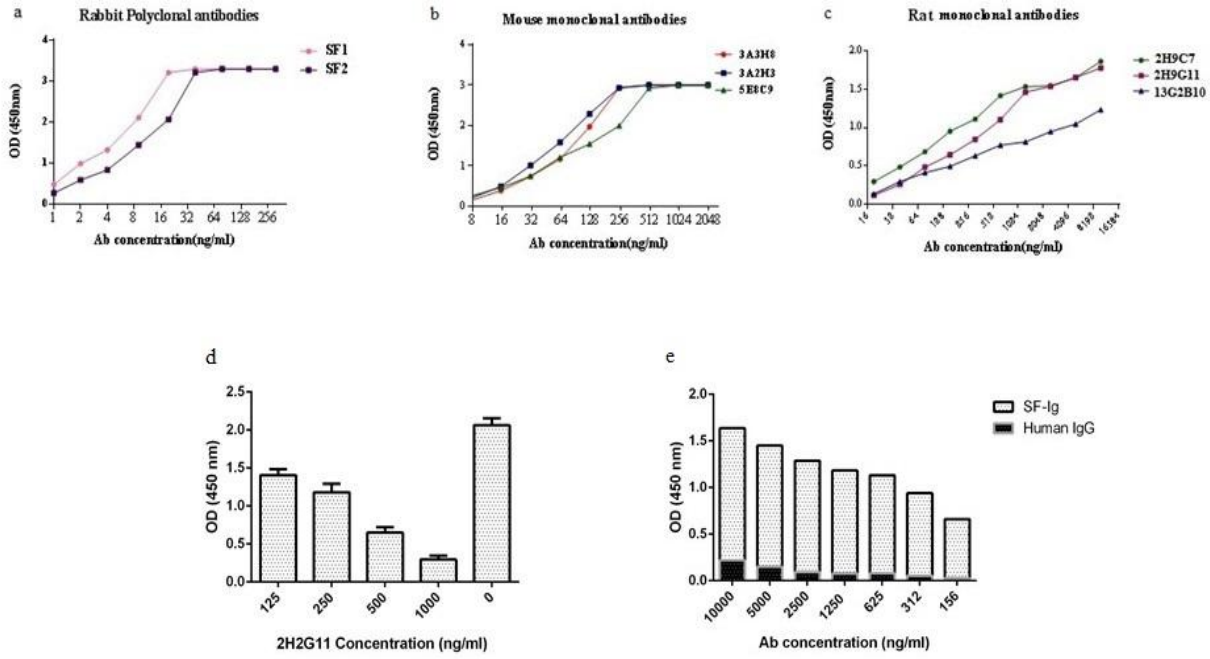

Figure 3. Reactivity assessment of purified anti-Siglec-F polyclonal and monoclonal antibodies by indirect ELISA. Purified rabbit polyclonal (a) and mouse (b) and rat (c) monoclonal anti-SF antibodies were serially titrated on ELSA plates coated with immunizing peptides (rabbit and mouse antibodies) or SF-Ig (rat antibodies). In order to determine whether or not the generated rat anti-SF clones (2H9C7 and 2H9G11) have different clonality, competitive ELISA was performed. The results showed that reactivity of 2H9C7 was inhibited by different concentrations of 2H9G11 indicating the same clonality of the aforesaid rat anti-SF monoclonal antibodies (d). Reactivity of generated rat monoclonal antibodies with human IgG as ELISA coating layer was also tested. None of the rat monoclonals reacted with human IgG indicating that generated rat mAbs were directed against SF but not Ig portion of the SF-Ig fusion protein. Stacked graph of 2H9C7 has been presented as representative data (e). SF, Siglec-F; SF-Ig, secreted Siglec-F composed of Ig domains of Siglec-F fused to the Fc region of human IgG; IgG,Immunoglobulin G; mAbs, monoclonal antibodies.

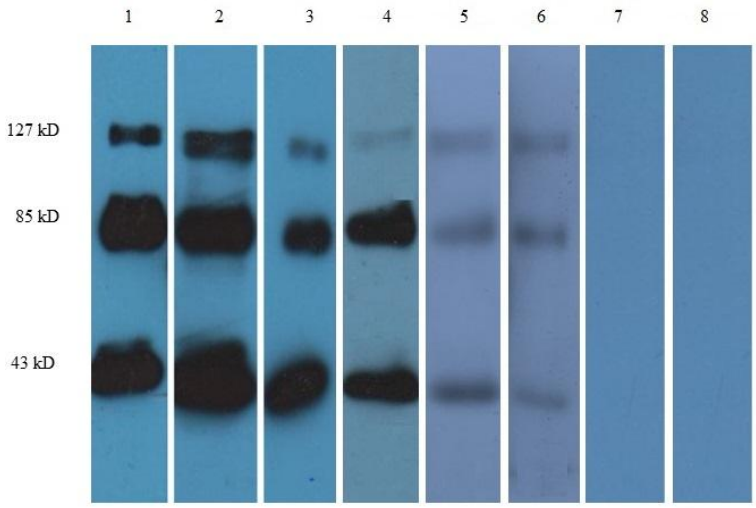

Figure 4. Western blot analysis of purified anti-SF polyclonal and monoclonal antibodies. SF-Ig recombinant protein was separated by SDS-PAGE under reducing condition, transferred to nitrocellulose membrane and probed with polyclonal and monoclonal anti-SF antibodies. Lane 1: HRP-conjugated anti-His6 tag antibody, Lane 2: Rabbit anti-SF1, Lane 3: Rabbit anti-SF2, Lane 4: Anti-SF1; clone 5E8C9, Lane 5 and 6: Anti-SF2; clones 3A3H8 and 3A2H3, respectively. Lane 7: Anti-SF; clone 2H9C7, Lane 8: Anti-SF; clone 2H9G11. SF, Siglec-F; HRP, Horseradish peroxidase.

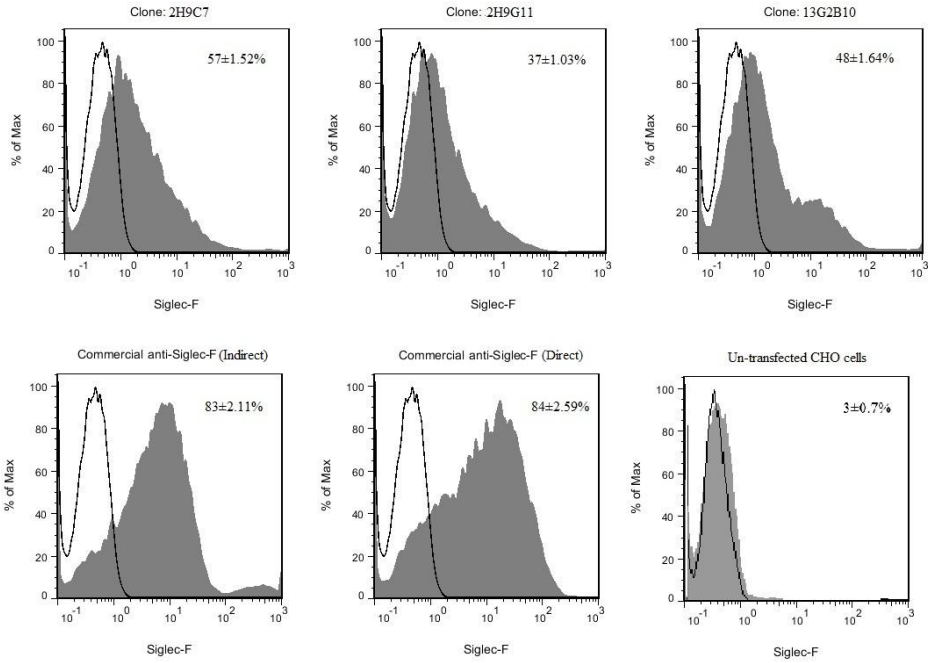

Figure 5. Flow cytometric analysis of rat anti-SF monoclonal antibodies. Reactivity of purified rat anti-SF monoclonal antibodies with native SF molecule were tested on CHO-SF cells using flow cytometry. No signal was observed when un-transfected CHO cells were tested with the generated antibodies (as a representative figure, reactivity of the 2H9C7 with un-transfected CHO cells has been shown in the lower right panel). Commercial anti-Siglec-F antibody was used as positive control by direct and indirect immunostaining methods. Grey and white and histograms show anti-SF antibodies and isotype controls, respectively.SF,Siglec-F;CHO, Chinese hamster ovary.

Flow Cytometric Analysis of mAbs

In order to determine whether or not anti-SF monoclonal antibodies are reactive with native surface SF, flow cytometric analysis was performed using CHO-SF cells expressing Siglec-F on their surface. Our results showed that rat monoclonals, 2H9C7, 2H9G11 and 13G2B10 recognized Siglec-F molecule in 57±1.52%, 37±1.03% and 48±1.64% of CHO-SF cells, respectively (Figure 5). Possible cross-reactivity of the rat anti-SF antibodies with irrelevant CHO cell surface antigens was ruled out by testing un-transfected CHO cells as negative cell control in all flow cytometry experiments and the results showed no signal in those cells (Figure 5). Contrary to Western blot experiment, mouse monoclonal anti-SF antibodies were not able to recognize native SF on the surface of CHO-SF cells. As positive control, commercial anti Siglec-F antibody was also tested for its reactivity in flow cytometry and the results showed that this antibody was reactive in 84±2.59% and 83±2.11% of CHO-SF cells when tested in direct and indirect immunostaining methods, respectively.

DISCUSSION

Eosinophils are central for inflammatory processes associated with allergy and asthma. In such conditions, eosinophils accumulate in large numbers in skin and lung[16] leading to inflammatory processes. Inefficient mechanisms responsible for resolution of such inflammatory reactions may lead to persistent inflammatory responses. According to the some reports, apoptosis of eosinophils is delayed in asthma.[17] Therefore; antibodies with eosinophil specificity capable of induction of apoptosis in these cells could be viewed as one modality for immunotherapy of patients with asthma.

Siglec-F is a transmembrane molecule prominently expressed on mature circulating mouse eosinophils, and on some myeloid precursors in bone marrow.[18, 19] In fact, Siglec-specific antibodies and glycan-based probes of Siglecs are the primary tools for targeting Siglecs in vivo.[20] Zhang et al. observed enhanced apoptosis of mouse eosinophils by antibody cross-linking of Siglec-F in vitro.[21] It has been reported that targeting Siglec-F in vivo with specific antibody in IL-5-transgenic mice and in mice with experimental hypereosinophilic syndrome/chronic eosinophilic leukemia decreased eosinophil counts without affecting mast cell viability. In that way, administration of Siglec-F antibody significantly ameliorated airway inflammation and features of airway remodeling in a chronic mouse model of asthma by induction of apoptosis in resident eosinophils of lung and bone marrow.[22]

In this study, we produced a series of polyclonal and mAbs against Siglec-F using different immunogens and hosts and characterized their reactivity by different readout systems. In addition to the recombinant protein and CHO cells overexpressing Siglec-F and based on the predicted topology and structure of Siglec-F protein, two Siglec-F–specific peptides in extracellular region of the molecule were also designed and used for production of Abs. Except for rats immunized with cells overexpressing Siglec-F, other immunizations were successful leading to generation of monoclonal antibodies. Siglec-F-specific polyclonal and monoclonal antibodies exhibited excellent reactivity with immunizing peptides and recombinant protein in an almost similar manner. However, murine anti-peptide antibodies failed to react with recombinant Siglec-F protein indicating that the antibodies recognize only linear epitopes within molecule, a finding which were subsequently confirmed by Western blotting. In Western blot analyses, polyclonal antibodies and all three mAbs of mouse origin detected three bands of about 43, 127 and 85 kD. The recombinant protein used for immunization was a Siglec-F-Fc chimera protein with molecular mass of 72 kD. The higher MW of the protein observed in SDS-PAGE (85 kD) at reducing condition could be attributed to the post translational modification especially glycosilation, as Siglec-F is a glycoprotein containing several predicted N-linked and O-linked glycosilation sites. The band with lower MW probably reflects cleaved SF protein, but the nature of higher MW band is not clear for us. Nonetheless, as this band and also smaller one were sharply detected with anti-His6 tag antibody, they might be regarded as specific bands. Monoclonal antibodies of rat origin failed to detect Siglec-F recombinant protein in WB, although these monoclonals detected native surface Siglec-F in flow cytometry. These results indicate that in contrast to murine monoclonal antibodies, rat monoclonals produced in this study predominantly recognize conformational epitopes. One major reason behind this

finding may be the nature of immunizing antigen in rats which was recombinant protein. Regarding the highly glycosylation pattern of Siglec-F, recombinant protein produced in eukaryote hosts is more likely to retain conformational epitopes and thereby induce antibodies capable to react with native protein.[23] Although three rat clones against SF were produced, reactivity of 2H9C7 with SF-Ig was found to be inhibited by 2H9G11 indicating that these clones have the same clonality.

Although the peptides we selected for immunization of mice had no predicted N- or O-glycosylation sites, the presence of such sites in adjacent amino acids within protein sequence might be responsible for different conformation of the epitope within the native protein compared to the synthetic peptides. On the other hand, there is no data on the sequence of rat Siglec-F and it is probable that mouse Siglec-F phylogenically differ at least in some sequences with that of rat. In this context, one may expect that rats immunized with mouse Siglec-F produce more functional antibodies compared with mice immunized with the same molecule.

In conclusion, we introduced here a set of monoclonal antibodies against Siglec-F generated with different immunizing molecules in different hosts and concluded that antibodies produced with recombinant protein in rat are able to detect native molecule and suitable for flow cytometric applications, whereas those produced in mice with specific peptides recognize linear epitopes in such applications as Western blot. Monoclonal antibodies reported here are invaluable tools for studying linear and conformation epitopes of SF and tracing mouse eosinophils.

ACKNOWLEDGEMENTS

This study was funded by a grant from Tehran University of Medical Sciences (Grant No:16302).

REFERENCES

1. Varki A, Angata T. Siglecs--the major subfamily of I-type lectins. Glycobiology 2006; 16(1):1R-27R.
2. Macauley MS, Crocker PR, Paulson JC .Siglec-mediated regulation of immune cell function in disease. Nat Rev Immunol 2014; 14(10):653-66.
3. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. Nat Rev Immunol 2007; **7**(4):255-66.
4. Robertson SA, Mau VJ, Hudson SN, Tremellen KP. Cytokine-leukocyte networks and the establishment of pregnancy. Am J Reprod Immunol 1997; 37(6):438-42.
5. Daëron M, Jaeger S, Du Pasquier L, Vivier E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 2008; 224:11-43.
6. Hartnell A, Steel J, Turley H, Jones M, Jackson DG, Crocker PR. Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. Blood 2001; 97(1):288-96.
7. Avril T, Floyd H, Lopez F, Vivier E, Crocker PR. The membrane-proximal immunoreceptor tyrosine-based inhibitory motif is critical for the inhibitory signaling mediated by Siglecs-7 and -9, CD33-related Siglecs expressed on human monocytes and NK cells. J Immunol 2004; 173(11):6841-9.
8. Crocker PR, Varki A. Siglecs in the immune system. Immunology 2001; 103(2):137-45.
9. Tateno H, Crocker PR, Paulson JC. Mouse Siglec-F and human Siglec-8 are functionally convergent paralogs that are selectively expressed on eosinophils and recognize 6'-sulfo-sialyl Lewis X as a preferred glycan ligand. Glycobiology 2005; 15(11):1125-35.
10. Song DJ, Cho JY, Lee SY, Miller M, Rosenthal P, Soroosh P, et al. Anti-Siglec-F antibody reduces allergen-induced eosinophilic inflammation and airway remodeling. J Immunol 2009; 183(8):5333-41.
11. Possa SS, Leick EA, Prado CM, Martins MA, Tibério IF. Eosinophilic inflammation in allergic asthma. Front Pharmacol 2013; 4:46.
12. Farid SSh, Mirshafiey A, Razavi A. Siglec-8 and Siglec-F, the new therapeutic targets in asthma. Immunopharmacol Immunotoxicol 2012; 34(5):721-6.
13. Bousquet j, Chiron R, et al. Biologics in asthma: difficulties and drawbacks. Expert Opin Biol Ther 2008; Dec;8(12):1921-8.
14. Ghods R, Ghahremani MH, Darzi M, Mahmoudi AR, Yeganeh O, Bayat AA, et al. Immunohistochemical characterization of novel murine monoclonal antibodies against human placenta-specific 1. Biotechnol Appl Biochem 2014; 61(3):363-9.
15. Shojaeian S, Allameh A, Zarnani AH, Chamankhah M, Ghods R, Bayat AA, et al. Production and characterization of monoclonal antibodies against the extracellular domain of CA 125. Immunol Invest 2010; 39(2):114-31.
16. Simon D, Wardlaw A, Rothenberg ME. Rothenberg.

Organ-specific eosinophilic disorders of the skin, lung and gastrointestinal tract. J Allergy Clin Immunol 2010; 126(1):3-13.

17. Crocker PR, Varki A. Siglecs, sialic acids and innate immunity. Trends Immunol 2001; 22(6):337-42.
18. Zhang JQ1, Biedermann B, Nitschke L, Crocker PR. The murine inhibitory receptor mSiglec-E is expressed broadly on cells of the innate immune system whereas mSiglec-F is restricted to eosinophils. Eur J Immunol 2004; 34(4):1175-84.
19. Pope SM, Fulkerson PC, Blanchard C, Akei HS, Nikolaidis NM, Zimmermann N, et al. Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation. J Biol Chem 2005; 280(14):13952-61.
20. O'Reilly MK, Paulson JC. Paulson. Siglecs as targets for therapy in immune cell mediated disease. Trends Pharmacol Sci 2009; 30(5):240-8.
21. Wang X, Mitra N, Secundino I, Banda K, Cruz P, Padler-Karavani V, et al. Specific inactivation of two immunomodulatory SIGLEC genes during human evolution. Proc Natl Acad Sci U S A 2012; 109(25):9935-40.
22. Cho JY, Song DJ, Pham A, Rosenthal P, Miller M, Dayan S, et al. Chronic OVA allergen challenged Siglec-F deficient mice have increased mucus, remodeling, and epithelial Siglec-F ligands which are up-regulated by IL-4 and IL-13. Respir Res 2010; 11:154.
23. Zhang M1, Angata T, Cho JY, Miller M, Broide DH, Varki A. Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. Blood 2007; 109(10):4280-7.