Development and Characterization of Domain-Specific Monoclonal Antibodies Produced Against Human SLAMF9

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SLAMF9 is a member of the signaling lymphocyte-activating molecule (SLAM) immunoreceptor family. The SLAM family receptors are expressed in a broad range of immune cells and play an important role in immunity. To date, SLAMF9 is the least studied member of this family. Its ligand, signaling properties, and cells on whose surface it is expressed are unknown. We generated hybridoma clones 6E11 and 7G5 secreting monoclonal antibodies specific to human SLAMF9. BALB/c mice were immunized with *Escherichia coli*-expressed purified SLAMF9 protein; splenocytes from these mice were fused with mouse myeloma cell line NS-1. Based on isotyping of the MAbs, clone 6E11 was referred to the IgG1 subclass, while 7G5 to IgG2b. The specificity of these MAbs was assessed by ELISA, immunoblotting, immunohistochemistry, and flow cytometry. According to the results of epitope analysis, clone 6E11 reacts with the C2-like domain, whereas 7G5 is specific to the V-like domain of the SLAMF9 molecule. The generated MAbs were demonstrated to be applicable in various immunochemical analyses. They may be useful tools in studies clarifying the expression and function of human SLAMF9.

Introduction

THE SIGNALING LYMPHOCYTE ACTIVATING MOLECULE (SLAM) is a group of immune cell-specific receptors that play an important role in immune regulation.^(1–5) The SLAM family, also known as the CD150 family, consists of nine structurally related leukocyte cell surface glycoproteins that belong to the immunoglobulin (Ig) superfamily. They possess an extracellular segment usually composed of an N-terminal V-Ig domain and a C-terminal C2-Ig domain, in addition to a transmembrane region and a cytoplasmic domain bearing multiple tyrosine-based motifs.⁽⁶⁾ SLAMF3 is exceptional in its structural organization. It has four Ig domains in its extracellular region (two V/C2-Ig sets).^(7,8)

Members of the SLAM family have a characteristic feature that makes them different from most receptors expressed in immune cells. They are homophilic receptors and can function as self-ligands. The only known exception to this is SLAMF4, which recognizes another immune cell receptor SLAMF2 as its ligand.^(8,9) When the SLAMF receptors interact with each other, the tyrosine-based switch motifs of the cytoplasmic domain are phosphorylated and recruit various adaptor proteins, such as the SLAM-associated proteins (SAP). Through its ability to transduce tyrosine phosphorylation signals, SLAMF receptors play an important role in regulation of immune cell interactions and adhesion required

for the normal development, homeostasis, and function of the immune system.^(7,10–13)

Most of the SLAMF immunoreceptors have been studied quite extensively. They were detected in a wide array of immune cells, the expression of SLAMF members being different in various cell types.^(13–16) To date, SLAMF9 (CD84-H1/SF2001/CD2F-10) is the least studied representative of the human SLAM family receptors. In contrast to the other SLAMF receptors, SLAMF9 and SLAMF8 have no signaling motifs in the short cytoplasmic tail. The SLAMF9 expression in several tissues and, more specifically, in some populations of immune cells was demonstrated by Northern blot and reverse transcription-PCR analyses only.^(17–20) There are as yet no data regarding SLAMF9 expression at the protein level; the ligand with which this receptor interacts is unknown too. To better analyze SLAMF9, its possible function and its unknown ligand, specific monoclonal antibodies are essential. Thus far, there have been no reports on SLAMF9-specific monoclonal antibodies, and SLAMF9 has not been included into the nomenclature of CD molecules originated from MAb reactivity to human antigens.

In this study, novel domain-specific anti-human SLAMF9 MAbs, 6E11 and 7G5, were generated and characterized by ELISA, immunocytochemistry, flow cytometry, and Western blotting. These two MAbs may be useful tools for further investigation of SLAMF9 expression and function.

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Materials and Methods

Cell cultures

NS-1 and P301 mouse myeloma cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Hyclone, Thermo Scientific, Cramlington, United Kingdom), 100 U/mL penicillin, and 100 µg/mL streptomycin. The human embryonic kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS (Hyclone), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cell lines were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Bacterial production of recombinant SLAMF9 proteins

SLAMF9 cDNA (Image 30915213) was purchased from Open Biosystems (Thermo Scientific, Surrey, UK). A cDNA fragment encoding two extracellular domains of SLAMF9 (32-235 amino acids) was PCR amplified and cloned either into pET21a vector (EMD Biosciences, San Diego, CA) using NdeI and HindIII sites or into pMal-2c vector (New England BioLabs, Bethesda, MD) using BamHI and HindIII sites. As a result, the former vector encoded SLAMF9 fused with the C-terminal 6xHis tag and the latter encoded SLAMF9 fused with the N-terminal maltose-binding protein (MBP). The His- and MBP-tagged SLAMF9 recombinant fusion proteins were expressed in *Escherichia coli* under isopropyl-β-D-1-thiogalactopyranoside induction. Then, His-SLAMF9 fusion protein was affinity-purified by use of Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purification of MBP-SLAMF9 fusion protein was performed by affinity chromatography using amylose resin column according to the manufacturer's instructions (New England BioLabs). Purified proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie Blue staining, pooled, quantified, and used for mouse immunization.

Transient expression of recombinant SLAMF9 proteins in 293T cells

For transfection experiments, the full-length cDNA sequence for SLAMF9 was cloned into the pCI-neo expression vector (Promega, Madison, WI). A portion of SLAMF9 cDNA encoding V (16-131 aa), C2 (132-222aa), or VC2 (16-222aa) domains was cloned into the pAP-Tag5 (GenHunter, Nashville, TN) using HindIII and BspEI sites in-frame with mouse Ig kappa leader on the N-end and thermostable alkaline phosphatase (AP) on the C-end. The constructs were confirmed by sequencing; 293T cells were cultured in 60 mm dishes at 70–80% confluence. The pAPTag5-V-hSLAMF9, pAPTag5-C2-hSLAMF9, pAPTag5-VC2-hSLAMF9, and pCI-neo-hSLAMF9 plasmids were transfected separately into 293T cells using FuGENE HD (Roche, Mannheim, Germany) following the manufacturer's instructions. The plasmid concentration for transfection was 4 µg/plate. After 48 h, the cells were harvested and analyzed by Western blotting and flow cytometry. In this study, we designated 293T cells transfected with the recombinant pAPTag5-V-hSLAMF9 plasmid as V-SLAMF9-293T cells, those with the pAPTag5-C2-hSLAMF9 plasmid as C2-SLAMF9-293T cells, and

those with the pAPTag5-VC2-hSLAMF9 plasmid as VC2-SLAMF9-293T cells.

Monoclonal antibody production

BALB/c female mice (6–8 weeks old) were immunized by intraperitoneal injection with 20 µg of the purified recombinant His-fused human SLAMF9 protein in Freund's complete adjuvant (Sigma-Aldrich). After two booster injections, they received 20 µg of SLAMF9 each emulsified in incomplete Freund's adjuvant (Sigma-Aldrich) at 2-week intervals; the sera were collected and antibody titer was examined by the enzyme-linked immunosorbent assay (ELISA). Three days after mice were given a final boost, the splenocytes from an immunized mouse were fused with myeloma cells at a 3:1 ratio in the presence of 50% polyethylene glycol 1500 (Merck, Darmstadt, Germany). Fused cells were distributed to 96-well tissue culture plates, and hybrids were selected using HAT (hypoxanthine, aminopterin, thymidine) medium. The hybridoma supernatants were screened using ELISA with the purified recombinant MBP-SLAMF9 as antigen. Cells from the wells with positive signals were cloned by limiting dilution. Positive hybridoma clones were expanded, and antibodies were purified by 50% ammonium sulfate precipitation followed by DEAE-cellulose chromatography (DE-52, Whatman, Maidstone, UK). Purity of MAbs was confirmed by SDS-PAGE. The isotypes of monoclonal antibodies were determined using mouse monoclonal antibody isotyping reagents (Sigma-Aldrich) following the manufacturer's instructions.

MAb biotinylation

The purified antibodies were biotin-conjugated as described previously⁽²¹⁾; MAbs (5 mg in 1 mL of 0.1 M NaHCO₃, 0.15 M NaCl [pH 8.4]) were mixed with solution of N-hydroxysuccinimidobiotin ether (250 µg) in dimethylsulfoxide (50 µL), incubated for 2 h at room temperature, and dialyzed against 0.1 M NaHCO₃ and 0.15 M NaCl (pH 8.4).

ELISA screening

The purified recombinant MBP-fused SLAMF9 protein and the purified recombinant MBP protein as negative control (both at 20 µg/mL) were adsorbed to the surface of 96-well microtiter plates in 0.1 M NaHCO₃ by overnight incubation at 4°C. After blocking with 5% rabbit sera, 100 µL of hybridoma supernatants were added to the wells and incubated for 1 h at room temperature. The plates were then washed five times with 0.1% Triton X-100 /0.1 M NaHCO₃, and 100 µL of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins (Sigma-Aldrich, diluted 1:1000) were added to each well. The plates were incubated for 1 h at room temperature and then washed with 0.1% Triton X-100 /0.1 M NaHCO₃ as described above. O-phenylenediamine-H₂O₂ was used as substrate in the peroxidase reaction. Absorbance at 492 nm was measured using a microplate reader (EIA Reader 2550, Bio-Rad Laboratories, Tokyo, Japan).

Immunoperoxidase and immunofluorescence staining

For immunocytochemical analysis, HEK293T cells were suspended in phosphate buffered saline (PBS) containing 20% FBS and smeared over slides. After drying, cells were

fixed in cold acetone-methanol (1:1) for 10 min, followed by incubation in 3% H₂O₂, 0.1% NaN₃/PBS for 30 min at room temperature to block endogenous peroxidase activity. The preparations were then blocked with PBS containing 20% FBS for 30 min, followed by incubation with supernatants of hybridoma clones at 4°C overnight. The preparations were washed in PBS and incubated for 1 h at room temperature with HRP-conjugated rabbit anti-mouse immunoglobulins (Sigma-Aldrich). The HRP complex was visualized by staining with a substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

In immunofluorescence analysis, the cells were stained according to the above protocol for immunoperoxidase staining, eliminating the blocking of the endogenous peroxidase activity. The cells were incubated with biotinylated anti-SLAMF9 MAbs (2 µg/mL) as primary antibodies followed by incubation with streptavidin-labeled Texas Red (1:1500; Molecular Probes, Eugene, OR).

Images of the cells were obtained using an Axioskop 2 plus equipped with an AxioCam MRc digital camera and Axiovision Release 4.7 software (Zeiss, Oberkochen, Germany).

Flow cytometry

HEK293T cells transfected with pCI-neo-hSLAMF9 full-length cDNA and HEK293T cells transfected with pCI-neo as control were used for flow cytometric analysis. Cells (1×10^6) were washed two times with FACS buffer (PBS containing 2% FBS and 0.06% NaN₃) and incubated with biotinylated MAbs 6E11 and 7G5 for 30 min at 4°C. Thereafter, cells were washed two times with FACS buffer and incubated with streptavidin-PE (Invitrogen/Molecular Probes). After washing, stained cells were analyzed using a FACSCanto II (Becton Dickinson, San Jose, CA); 7-AAD (Calbiochem, La Jolla, CA) was applied to exclude dead cells.

Western blot analysis

Transfected HEK293T cells were lysed for 5 min in a loading SDS buffer at 100°C and subjected to reducing 12% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes Hybond-C (Amersham Biosciences, Buckinghamshire, UK) for Western blotting. Immunodetection was performed with the SNAP i.d. Protein Detection System (Millipore, Billerica, MA) using the hybridoma supernatants as the primary antibodies and HRP-conjugated rabbit anti-mouse antibodies (1:1000 dilution, Sigma) as the secondary antibodies.

The SLAMF9-specific signals were visualized by using ECL Select Western blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and subsequent exposure to Amersham Hyperfilm ECL (GE Healthcare).

Results and Discussion

Generation and characterization of novel hybridoma clones producing anti-human SLAMF9 MAbs

To produce human SLAMF9-specific monoclonal antibodies, we expressed the His-fused human SLAMF9 fragment corresponding to its extracellular region (amino acids 32-235) in *E. coli*. The purified recombinant His-SLAMF9 was used to immunize BALB/c mice, and hybridoma cells were generated by fusing the immune spleen with NS1 and

P301 mouse myeloma cells. Four fusions were performed to obtain SLAMF9-positive hybridomas. The fusions yielded about 550 hybrid colonies. The antibodies produced by the hybridomas were screened by indirect ELISA with the recombinant MBP-SLAMF9 as antigen. One anti-SLAMF9 antibody producing hybridoma clone was obtained from the third fusion with P301 myeloma cells, two from the fourth fusion with NS-1 cells. The MAbs of these clones specifically reacted with the recombinant MBP-hSLAMF9, but did not cross-react with MBP-protein and *E. coli* proteins.

The positive clones (designated as 6G2, 6E11, and 7G5) were subjected to two rounds of cloning by limiting dilution. To determine antibody titers, the hybridoma supernatants were harvested from hybridoma cultures containing equal numbers of 5×10^5 viable cells/mL and tested by ELISA. ELISA assay showed that two anti-SLAMF9 MAbs 6E11 and 7G5 strongly reacted with *E. coli*-expressed human SLAMF9 protein, the ELISA titers for these MAbs being 1:64000 and 1:8000, respectively (Fig. 1). The titer of 6G2 was 1:64 in the supernatant. The control supernatant from NS-1 myeloma cell culture was negative in the ELISA analysis (Fig. 1).

To further characterize the MAbs, we transfected HEK293T cells with the pCI-neo-hSLAMF9 construct containing the full-length cDNA of human SLAMF9. Transiently transfected HEK293T cells expressing human SLAMF9 were subjected to immunoperoxidase staining (Fig. 2A). The results revealed that MAbs 6E11, 7G5, and 6G2 specifically stained the SLAMF9 transfectants, but did not react with the control HEK293T cells transfected with pCI-neo vector alone. However, 6G2 stained weaker SLAMF9-transfected cell smears in immunocytochemistry; this was consistent with the ELISA titration results.

Based on the results of titration and immunocytochemical analyses, we chose clones 6E11 and 7G5 for further study. Purification of monoclonal antibodies from the hybridoma culture supernatants was performed by 40% ammonium sulfate precipitation followed by DEAE chromatography. The purified MAbs were characterized with respect to isotypes. They were IgG1 for 6E11 and IgG2b for 7G5. Next, the

FIG. 1. Titration analysis of hybridoma supernatants. MAbs titers were determined by ELISA. 6G2, 6E11, and 7G5 hybridoma supernatants were serially diluted and added in MBP-SLAMF9-coated 96-well ELISA plates. Supernatant from NS-1 cell culture served as negative control.

FIG. 2. Immunocytochemical analysis of HEK293T cells expressing human SLAMF9. HEK-293T cells were transfected with pCI-neo-hSLAMF9 plasmid (293T-SLAMF9) or pCI-neo vector alone (control 293T). **(A)** Transfected and control cells were stained with 6E11, 7G5, and 6G2 MAb supernatants as primary antibodies. Peroxidase-conjugated rabbit anti-mouse immunoglobulins were used to detect anti-SLAMF9 MAb. **(B)** Immunofluorescence staining of transfected and control cells using biotinylated 6E11 and 7G5 MAb followed by incubation of streptavidin-labeled Texas Red. Texas Red images (left) and phase contrast images (right) are shown for each MAb. Imaging was performed using an Axioskop 2 Plus microscope ($\times 400$).

purified MAb were biotinylated and used for staining of the SLAMF9-transfected cells for immunofluorescence microscopy (Fig. 2B). The microscopic images show that the MAb 6E11 and 7G5 can specifically detect SLAMF9 in the transfected cells by immunofluorescence and immunoperoxidase staining (Fig. 2).

To further characterize the produced MAb, we examined whether or not these MAb can recognize human SLAMF9 expressed on the cell surface of transfected cells. The pCI-neo-

hSLAMF9-HEK293T and control pCI-neo-HEK293T cells were stained with biotinylated anti-SLAMF9 MAb 6E11 and 7G5, followed by PE-conjugated streptavidin, and analyzed by FACS. Flow cytometry results showed that MAb reacted with the membrane SLAMF9 on pCI-neo-hSLAMF9-HEK293T cells. It should be noted that labeling with MAb 6E11 displayed high fluorescence intensity on SLAMF9-transfected cells compared with the control cells transfected with empty vector pCI-neo (Fig. 3A). The extent of fluorescence signal

FIG. 3. Flow cytometry analysis of binding of the MAb 6E11 and 7G5 to transfected 293T cells expressing human SLAMF9. HEK293T cells transfected with human SLAMF9 cDNA express SLAMF9 on their surface (open histograms), whereas the empty vector-transfected control cells (pCI-neo-HEK293T) do not (shaded histograms). Cells were stained with the biotin-labeled MAb 6E11 **(A)** and 7G5 **(B)** followed with PE-conjugated streptavidin.

FIG. 4. Western blot analysis of transiently transfected 293T cells expressing human SLAMF9. Immunoblot analysis of whole 293T cell lysates was performed using the MAbs 7G5 (**A**) and 6E11 (**B**) followed by HRP-conjugated rabbit anti-mouse immunoglobulins and visualized using an ECL detection system. Lanes 1, 8, 293T cells expressing the full-length SLAMF9; lanes 2, 5, 293T cells expressing AP fusion protein containing the V and C2 SLAMF9-domains; lanes 3, 6, 293T cells expressing AP fusion protein containing the V SLAMF9-domain; lanes 4, 7, 293T cells expressing AP fusion protein containing the C2 SLAMF9-domain; lane M, pre-stained protein markers. Molecular weight markers are shown on the right. Positions of the proteins of interest are indicated by arrows.

from clone 7G5 was weaker than from clone 6E11 (Fig. 3B). The MAb 7G5 poorly recognized SLAMF9 on the living transfected cells in flow cytometry, but it efficiently detected SLAMF9 in the fixed cells in immunofluorescence microscopy. These results may be explained as follows: the epitope recognized by 7G5 became more available for binding when the SLAMF9 molecule was denatured (e.g., through fixation for immunoperoxidase staining).

Identification of SLAMF9 domains that are recognized by MAbs 6E11 and 7G5

To identify the SLAMF9 domains targeted by produced MAbs, a series of constructs expressing truncated forms of SLAMF9 protein fused with alkaline phosphatase (AP) were developed and subcloned into eukaryotic expression vectors. The resulting transiently transfected V-SLAMF9-293T, C2-SLAMF9-293T, and VC2-SLAMF9-293T cells were used to analyze the domain binding specificity of anti-human SLAMF9 MAbs by Western blotting. Also, the pCI-neo-hSLAMF9-293T cells, expressing the full-length SLAMF9, were used for Western blot analysis of MAbs.

As shown in Figure 4, 6E11 and 7G5 MAbs reacted with the whole protein lysates of pCI-neo-hSLAMF9-293T cells and VC2-SLAMF9-293T cells. These SLAMF9 transfectants expressed V and C2 domains of the SLAMF9 molecule. The 6E11 and 7G5 MAbs detected protein bands at approximately 38 kDa and 82 kDa in lysates from transfected pCI-neo-hSLAMF9-293T cells and VC2-SLAMF9-293T cells, respectively, which were consistent with predicted values for the full-length of human SLAMF9 and for the V-C2-SLAMF9-AP fusion protein.

Immunoblotting also reveals that the MAb 7G5 recognizes a 69 kDa band in lysates of V-SLAMF9-293T cells and does not react with C2-SLAMF9-293T cell lysates (Fig. 4A). In contrast, the 6E11 reacts with a 72 kDa band in C2-SLAMF9-293T and does not show reactivity against V-SLAMF9-293T cells (Fig. 4B). Thus, the two MAbs recognize different epitopes: MAb 6E11 recognizes an epitope within the C2 domain of the SLAMF9 molecule, while MAb 7G5 resides within the V domain.

SLAMF9 is a member of the SLAM immunoreceptor family that plays an important role in innate and adaptive immunity.^(1-3,13) Like other members of the SLAM family, SLAMF9 is composed of two extracellular domains (an N-

terminal V and a membrane-proximal C2), the transmembrane segment, and a short cytoplasmic tail. The cytoplasmic tail of SLAMF9 does not contain signaling tyrosine-based motifs. This is a structural feature distinguishing it from the other SLAMF receptors.⁽¹⁷⁻²⁰⁾ To date, human SLAMF9 is the least characterized member of SLAM family; its function is still unknown. There are reports regarding the mRNA expression pattern of SLAMF9. According to the reports, the expression of SLAMF9 was mainly restricted to hematopoietic tissues; however the details of the mRNA expression pattern of SLAMF9 in various populations of immune cells and various tissues are controversial.⁽¹⁷⁻²⁰⁾ The protein expression pattern of SLAMF9 has not yet been studied. It is timely and appropriate to generate new MAbs against the human SLAMF9 with the aim of using them as effective tools for clarifying the SLAMF9 protein expression.

Here, to generate MAbs against SLAMF9, we expressed human SLAMF9 both in bacteria and mammalian cells. In immunization and primary screening we used bacterially expressed recombinant proteins, i.e., His-tagged SLAMF9 fusion protein served as immunogen and MBP-tagged SLAMF9 protein served as coating detection antigen for ELISA. The eukaryotic expression human SLAMF9 protein was used for analysis of the specificity of MAbs. We succeeded in raising and screening anti-SLAMF9 MAbs by combining the advantages of prokaryotic and eukaryotic expression systems. As a result of four fusions, three new monoclonal antibodies against human SLAMF9 were derived. Based on ELISA titration and immunocytochemical analyses, clones 6E11 and 7G5 were selected for further study. Although we produced MAbs against SLAMF9 using bacterially expressed recombinant protein, the resulting MAbs 6E11 and 7G5 were able to detect SLAMF9 expressed in eukaryotic transfected cells. These MAbs work with the eukaryotic-expressed SLAMF9 in both immunohistochemistry and Western blotting; in addition, the anti-SLAMF9 MAb 6E11 is also suitable for flow cytometry analysis. In summary, we established new domain-specific monoclonal antibodies against human SLAMF9 that can be applied in various immunochemical analyses and can be effective tools in further analysis of human SLAMF9 expression and function.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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