Preparation and Characterization of Monoclonal Antibodies Against VSTM1

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VSTM1 (V-set and transmembrane domain containing 1) is a novel membrane molecule identified from immunogenomics, which has two main isoforms, VSTM1-v1 and VSTM1-v2. VSTM1-v1 is a type I transmembrane protein, and VSTM1-v2 is a classical secretory protein, lacking only the transmembrane domain compared with VSTM1-v1. This study was designed to generate VSTM1-specific monoclonal antibodies (MAbs) for further exploration of its expression and function. Mice were immunized with two recombinant prokaryotic proteins of GST-VSTM1-v2 and VSTM1-v2 without any tag. Hybridomas were generated by the fusion of the splenocytes to Sp2/0 myeloma cells. Three hybridoma cell lines (2C11, 6E11, and 7A8) stable in secreting anti-VSTM1 MAb were obtained and further characterized. All three MAbs were IgG2b isotype and effective in detecting the overexpressed VSTM1 in both Western blot and flow cytometry assays, while recognizing the endogenous VSTM1 in Western blot analysis only. These MAbs could be helpful in the basic study of VSTM1 and in revealing the interesting conformation difference between the overexpressed and endogenous proteins.

Introduction

CYTOKINES AND MEMBRANE MOLECULES MEDIATE the interaction of immune cells. They play essential roles in many physiological and pathological processes. More importantly, many cytokine and membrane molecule related products have been developed into biotechnological drugs.⁽¹⁻⁴⁾ Therefore, it will be of great value to identify and characterize novel potential cytokines and membrane molecules for both basic research and clinical application.

VSTM1 (V-set and transmembrane domain containing 1) is a potential leukocyte differentiation antigen gene selected by the strategy of immunogenomics, encoding two main splicing isoforms, VSTM1-v1 and VSTM1-v2. VSTM1-v1 contains 236 amino acids and is a type I membrane molecule, mainly expressed on human peripheral blood granulocytes and monocytes. There is an IgV-like domain in its extracellular region and two ITIM motifs in its cytoplasmic region. It may be a novel ITIM-bearing inhibitory immune receptor involved in the regulation of phagocytes.⁽⁵⁾ VSTM1-v2 contains 205 amino acids and has proven to be a classical secretory glycoprotein, lacking only the transmembrane domain compared with VSTM1-v1. Our studies show that recombinant VSTM1-v2 can promote the differentiation and activation of human Th17 cells.⁽⁶⁾

In order to further characterize the expression profile and function of VSTM1, generation of VSTM1 MAbs is highly desirable. Here we report the generation and characterization of three hybridoma clones specifically targeting the VSTM1 protein.

Materials and Methods

Cell lines

The HEK293T cells and the mouse myeloma cell line SP2/0 were respectively cultured in DMEM and RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD) at 37°C in a humidified atmosphere in the presence of 5% CO₂.

Recombinant proteins of VSTM1

Since VSTM1-v2 lacks only the transmembrane domain compared with VSTM1-v1 and is a classical secretory protein,⁽⁶⁾ it will be an ideal immunogen for production of anti-VSTM1 MAbs. Two recombinant prokaryotic proteins of VSTM1-v2, GST-VSTM1-v2, and His-VSTM1-v2 were expressed and purified as described previously.⁽⁷⁾ By cutting the GST tag from GST-VSTM1-v2 with thrombin, we obtained a protein of VSTM1-v2 without any tag. The cDNA encoding the extracellular domain of VSTM1-v1 was amplified by PCR with the primers 5'-GCTCTAGATACGAAGATGAGAAAAAGAATG-3' and 5'-GCTCTAGATCTGGTGTCTGTTTCA TTGAG-3' using pcDNA3.1-VSTM1-v1-myc-his plasmids as templates. This was then cloned in-frame into pYD11, a mammalian expression vector containing the Fc portion of human IgG1,⁽⁸⁾ to construct the plasmid expressing a fusion protein of extracellular region of VSTM1-v1 and Fc portion of human Ig (VSTM1-Fc). Then the plasmid was transfected into HEK293T cells with Vigofect (Vigorous Biotechnology,

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Beijing, China) according to the manufacturer's instructions. At 72 h after transfection, VSTM1-Fc protein was purified from the culture medium using protein G sepharose HP (GE Healthcare, Madison, WI).

Immunization and generation of hybridomas

AbMax Biotechnology (Beijing, China) was commissioned to complete the immunization and generation of hybridomas following their development and fast procedures.^(9–11) In brief, three BALB/c mice were immunized with 1:1 mixtures of purified GST-VSTM1-v2 and VSTM1-v2 without tag. Two weeks after immunization, blood sample was obtained from the tails of the immunized mice and tested for titers against VSTM1-v2 by ELISA. The mouse with the highest serum titer was selected for fusion, whose spleen was removed and splenocytes were fused with the mouse myeloma cell line SP2/0. Culture supernatant from individual hybridoma clones was screened by ELISA using VSTM1-v2 as coating antigen. From 700 monoclones screened, 627 positive clones (readings were two times more than the negative control) were obtained in the initial screening. Among them, 40 best clones were selected for further expansion and the repeated screening, in which the purified recombinant proteins of GST-VSTM1-v2, His-VSTM1-v2, VSTM1-v2, and VSTM1-Fc were coated separately onto the EIA plates (Corning, NY) for ELISA. A total of 18 clones that could recognize all of the four antigens was selected for further Western blot and flow cytometry analyses.

Preparation and purification of antibodies

To produce antibodies from different hybridoma clones, the clones were seeded in stationary bioreactors in DMEM (BRL-Gibco, Grand Island, NY) plus 10% low-IgG fetal bovine serum from HyClone (Logan, Utah). The bioreactor fluids were collected every 3 days, and IgG fractions were affinity-purified using protein G agarose columns (Upstate Biotechnology, Lake Placid, NY). The concentrations of purified IgG were determined by their absorbance at OD280.

Enzyme-linked immunoassay

Each well of 96-well high-binding EIA plates (Corning) was coated with 100 ng purified recombinant proteins of VSTM1 overnight at 4°C in PBS. After two washings with PBST (0.05% Tween-20 in PBS) and blocking with 5% skim-milk/PBS for 1 h at room temperature (RT), wells were incubated with tail bleeds (1:500 to 1:50,000 dilution) or culture supernatants (1:1) in 5% skim-milk/PBS for another hour at RT. After two more washings, wells were probed with HRP-conjugated goat-anti-mouse IgG Fc-specific secondary antibodies (1:2000) in 5% skim-milk/PBS for 1 h. After five washings with PBST, HRP substrate TMB solution was added and absorbencies were determined after 30 min at 450 nm with a microplate reader.

Antibody isotype determination

Immunoglobulin isotypes were determined using the mouse monoclonal antibody isotyping kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions.⁽¹²⁾

Peripheral blood granulocytes and monocyte isolation

Peripheral blood mononuclear cells (PBMC) were separated from granulocytes and red blood cells in buffy coats of healthy donors by Ficoll/Hypaque density-gradient centrifugation as previously described.⁽¹³⁾ Red blood cells were then lysed in a hypotonic buffer of 1/6×PBS at room temperature for 1.5 min and the reaction was stopped by adding an appropriate volume of 10×PBS to adjust the lysis buffer to be isotonic. Granulocytes were pelleted by centrifugation, and the lysis was repeated until there were very few red blood cells left. PBMC were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in Petri dishes for 2 h and the non-adherent cells were removed. After washing with PBS three times, monocytes were collected by scraping the adherent cells off the dishes.

Western blot analysis

Isolated granulocytes and HEK293T cells transfected with either pcDNA3.1-VSTM1-v1-myc-his or the empty vector (pcDNA3.1/Myc-His(-) B; pcDB for short, as a negative control) were lysed in 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% cocktail (Pierce, Rockford, IL), and 0.1 mmol/L ALLN. The protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. 100 µg of granulocyte total lysate and 1 µg of HEK293T cell lysate or 0.5 µg of purified recombinant proteins were applied to 12.5% SDS-PAGE in SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-ME, 25% glycerol, 0.02% Bromophenol blue). For native PAGE, 100 µg of granulocyte total lysate and 0.2 µg of purified VSTM1-Fc protein in 62.5 mM Tris/HCl (pH 6.8), containing 25% glycerol, and 0.2% Bromophenol Blue were analyzed using 4% stacking and 10% resolving gels with a Tris/glycine buffer (25 mM Tris, 192 mM glycine).⁽¹⁴⁾ Electrotransfer was performed with standard procedures using nitrocellulose membranes (Millipore, Temecula, CA). After being blocked in 5% skim-milk/TBST (0.1% Tween-20 in TBS) for 1 h at room temperature, the membranes were incubated with anti-VSTM1 MAbs (1 µg/mL) and mouse anti-myc MAb (1 µg/mL; MBL, Woburn, MA) as a control (positive for the overexpressed protein and negative for the endogenous protein) at 4°C overnight. The HRP-conjugated goat anti-mouse IgG antibody (1:2000 dilution, Cell Signaling Technology, Danvers, MA) followed by enhanced chemiluminescence (ECL) Western blot detection reagents (GE Healthcare, Madison, WI) was employed to visualize the positive signals after thorough washings. Beta-actin was detected with mouse anti-beta-actin MAb (1 µg/mL; MBL) as an internal standard to indicate the amount of input lysates and verify the integrity of these lysates.

Immunofluorescence staining and flow cytometry analysis

Isolated granulocytes and monocytes, as well as HEK293T cells transiently transfected with either pcDNA3.1-VSTM1-v1-myc-his or pcDB empty vector, were blocked in 100 µL FACS buffer (PBS containing 2% FCS) at RT for 30 min. 1×10^6 cells were then incubated on ice for 1 h with anti-VSTM1 MAbs (2 µg/sample in 100 µL FACS buffer) or rabbit anti-VSTM1 polyclonal antibody⁽⁷⁾ as a positive control. After

FIG. 1. Western blot analysis of the purified recombinant VSTM1 proteins using VSTM1 MAbs. Purified His-VSTM1-v2 and VSTM1-Fc proteins were recognized by 2C11, 6E11, and 7A8 MAbs. Normal mouse IgG (mIgG) was used as a negative control.

FIG. 2. Reactivity of VSTM1 MAbs with overexpressed VSTM1. (A) HEK293T cells transfected with the empty vector (pcDB, lane 1) or pcDNA3.1-VSTM1-v1-myc-his (lane 2) were lysed and analyzed by Western blot. Anti-myc MAb was used as a positive control. All three VSTM1 MAbs detected a ~45 kDa and ~55 kDa protein on Western blot in VSTM1-v1 transfected but not pcDB transfected HEK293T cells. (B) HEK293T cells transfected with VSTM1-v1 or the empty vector (pcDB) were stained with VSTM1 MAbs or normal mouse IgG2b (mIgG2b) control and subsequent FITC-conjugated anti-mouse IgG, and then analyzed by flow cytometry.

FIG. 3. Reactivity of VSTM1 MAb with endogenous VSTM1. (A) Isolated granulocytes (lane 3) were analyzed by Western blot, and lysates of HEK293T cells transfected with the empty vector (pcDB, lane 1) or pcDNA3.1-VSTM1-v1-myc-his (lane 2) were electrophoresed and detected at the same time as controls. Anti-myc MAb was used as a negative control in the detection of endogenous VSTM1. Beta-actin was detected as an internal standard to indicate the amount of loaded proteins and verify the integrity of these lysates. ~40 kDa and a weaker ~37 kDa specific bands were detected in granulocytes by 2C11 and 7A8. (B) Isolated granulocytes and monocytes were stained with VSTM1 MAb or normal mouse IgG2b (mlgG2b), and subsequent FITC-conjugated anti-mouse IgG, and then analyzed by flow cytometry. Rabbit anti-VSTM1 pAb was used as a positive control for the detection of VSTM1. CD11b and CD14 were stained as markers for granulocytes and monocytes respectively. (C) Granulocytes were stained as in flow cytometry assay and observed under a fluorescence microscope after being spun onto glass slides. (D) Western blot analysis was done with granulocytes (lane 1) and HEK293T cell-expressed VSTM1-Fc protein (lane 2) under native conditions. The MAb recognized non-denaturing VSTM1-Fc protein well, but only a nonspecific band could be detected in granulocytes.

FIG. 3. (Continued).

being washed in PBS, cells were stained with FITC-conjugated anti-mouse (or rabbit) IgG (Biolegend, San Diego, CA; diluted 1:100 in FACS buffer) in the dark at 4°C for 30 min, followed by washings in PBS. Granulocytes and monocytes were also stained with PE-conjugated anti-human CD11b and anti-human CD14 (BD Pharmingen, San Diego, CA), respectively, to verify the purity of the cells. Cells were then analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ).

Fluorescence microscope observation

Granulocytes were stained as in flow cytometry assay. After they were spun onto glass slides, the cells were observed under an Olympus BX51 fluorescence microscope equipped with an Olympus DP72 camera (Olympus Optical, Tokyo, Japan).

Results

Generation of MAbs of human VSTM1

After preliminary screenings using purified recombinant VSTM1 protein as antigen in the indirect ELISA, we obtained three hybridoma clones—2C11, 6E11, and 7A8. The isotypes of the MAbs were determined, all of which were IgG2b.

Detection of overexpressed VSTM1 with VSTM1 MAbs

The MAbs from all three hybridoma clones were able to react specifically with the purified recombinant His-VSTM1-v2 and VSTM1-Fc proteins (Fig. 1) and overexpressed VSTM1-v1-myc-his protein in the lysate of HEK293T cells on Western blot (Fig. 2A), indicating that the antibodies can recognize the extracellular domain of VSTM1-v1. Therefore, we subsequently detected the overexpressed VSTM1-v1 on the surface of HEK293T cells in flow cytometry analysis and found that all of the three MAbs recognized it well (Fig. 2B).

Reactivity of VSTM1 MAbs with endogenous VSTM1

The ability of MAbs to detect endogenous VSTM1 was tested by Western blot and flow cytometry analyses with human peripheral blood granulocytes and monocytes. As shown in Figure 3A, 2C11 and 7A8 specifically reacted with endogenous VSTM1 proteins in granulocytes in the Western blot, whereas 6E11 did not, or its affinity was not high enough. However, these MAbs failed to detect native VSTM1 on granulocytes and monocytes in flow cytometry analysis, while the rabbit polyclonal antibodies reacting with VSTM1 showed a strong positive signal (Fig. 3B). Observation under the fluorescence microscope also confirmed the loss of staining on granulocytes assessed by flow cytometry (Fig. 3C). To further verify the failure of the binding of these MAbs to native VSTM1, we used Western blots with granulocytes under native conditions. HEK293T cell-expressed VSTM1-Fc protein was used as a positive control. As shown in Figure 3D, MAbs recognized non-denaturing VSTM1-Fc protein well, but only a nonspecific band could be detected in granulocytes.

Discussion

VSTM1 encodes two main splicing isoforms, VSTM1-v1 and VSTM1-v2. VSTM1-v1, also known as SIRL-1 (signal inhibitory receptor on leukocytes-1), is mainly expressed on human peripheral blood granulocytes and monocytes. It contains an extracellular IgV-like domain and two ITIM mo-

tifs in its cytoplasmic tail, which can recruit SHP-1 and SHP-2 and is capable of inhibiting Fc ϵ R I-mediated signaling. It may be a novel ITIM-bearing inhibitory immune receptor involved in the regulation of phagocytes.⁽⁵⁾ VSTM1-v2, lacking only the transmembrane helix, is a soluble protein⁽⁶⁾ and thus provides an ideal antigen for production of the antibodies against VSTM1.

In the present study, we expressed and purified several recombinant proteins of VSTM1-v2 with different tags or no tag and used them respectively in immunization and screening procedures to improve the specificity of the antibodies. Finally, we obtained three strains of MAbs, 2C11, 6E11, and 7A8, and characterized them with Western blot and flow cytometry analyses. The clones do well in recognizing overexpressed VSTM1 in Western blot and flow cytometry analyses, while they detect the endogenous VSTM1 in the Western blot analysis only. The different molecular sizes between the overexpressed and endogenous VSTM1 detected in Western blot may be due to the different degree of modifications and the myc-His tag in the overexpressed protein.

Since the function of VSTM1 remains largely unknown, the specific monoclonal antibodies developed in this study would serve as useful tools in the further exploration of its biological functions. However, perhaps due to the differences in glycosylation and conformation between the prokaryotic and eukaryotic proteins, as well as between the overexpressed and the native forms, none of the three MAbs could recognize the native VSTM1 in flow cytometry analysis, which is essential for the expressional study of a membrane molecule. Therefore, we will improve our procedure of immunization and screening to get monoclonal antibodies that can recognize or even cross-link the native form of the target protein. On the other hand, these three MAbs may contribute to revealing the differences of the modification and/or the conformation between endogenous and overexpressed VSTM1, which will help us to optimize the antigens for immunization and screening.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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