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Method for quantitative detection of FAM19A4 by flow cytometry using latex beads as solid carrier

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FAM19A4 (family with sequence similarity 19 member A4; also TAFA4) is a classical secretory protein expressed mainly in the central nervous system and upregulated significantly in lipopolysaccharide (LPS)-stimulated monocytes and macrophages. It is a novel cytokine ligand of formyl peptide receptor 1 (FPR1), showing chemotactic activities on macrophages and promoting the phagocytosis capacity and the release of reactive oxygen species (ROS) by macrophages upon zymosan stimulation. Based on the same detection principle as ELISA, we developed a sandwich immunoassay for quantitative detection of FAM19A4 in biological fluids by flow cytometry, with latex beads as solid carrier. The method showed good performance in a wide range of 39–10,000 pg/mL and possessed excellent specificity, good precision, and favorable recovery in several different matrices. Native FAM19A4 secreted by phorblo 12-myristate 13-acetate (PMA) and LPS stimulated THP-1 cells could also be detected by this method. This method will be much helpful to FAM19A4 studies.
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FAM19A4 (family with sequence similarity 19 (chemokine (C–C motif)-like), member A4; also TAFA4) is a member of the FAM19/TAFA family of small secreted chemokine-like proteins, which were first discovered in Homo sapiens and Mus musculus using a novel database searching strategy (1). Like most of the other members of the FAM19/TAFA family, FAM19A4 contains 10 regularly spaced cysteine residues that follow the pattern Cx7CCx13CxCx14Cx11Cx4Cx5Cx10C, where C represents a conserved cysteine residue and x represents any noncysteine amino acid (1). Real-time PCR analysis indicates that FAM19A4 mRNA expression is restricted to the central nervous system (CNS), with the highest level in the thalamus (1). TAFAs may represent a novel class of neurokines that modulate immune responses in the CNS and act with other chemokines to optimize the recruitment and activity of immune cells in the CNS (1). TAFAs may also control axonal sprouting following brain injury (1). In addition, FAM19A4 has been reported to play a potent analgesic role in pain relief in mice models (2). It has also been well documented that *FAM19A4* methylation is highly correlated with cervical carcinomas, and methylation analysis of the *FAM19A4* gene in cervical samples is highly efficient in detecting cervical carcinomas in HPV-positive women (3–6).

Our recent study has shown by N-terminal sequencing that FAM19A4 is a classical secretory protein and comprises a 45 amino acids (aa) signal peptide in the 140 aa precursor and a 95 aa mature chain (7). Besides in the CNS, it is increasingly expressed in lipopolysaccharide (LPS)-stimulated monocytes and macrophages, especially in polarized M1, showing chemotactic activities on macrophages and promoting effects on the macrophage phagocytosis of zymosan and the release of reactive oxygen species (ROS) upon zymosan stimulation (7). We have further identified FAM19A4 as a novel cytokine ligand of formyl peptide receptor 1 (FPR1) (7), which is mainly expressed by myeloid cells mediating cell migration, phagocytosis and release of ROS (8), and even involved in antitumor immune response (9). These suggest that monocyte/macrophage-derived FAM19A4 may play a crucial role in the migration and activation of myeloid cells during pathogenic infections and antitumor immunity.

Therefore, the quantitative measurement of FAM19A4 in human serum, plasma and other biological fluids such as the cell culture supernatants may facilitate the further functional study of FAM19A4 and even the clinical diagnosis of some infectious diseases. The sandwich enzyme-linked immunoassay (ELISA) is by far the most commonly used method to determine the concentration of cytokines and other soluble proteins in biological samples. Generally, an antibody specific for the target protein is pre-coated onto a microplate for capturing the target protein in the samples; and another enzyme-linked antibody also specific for the target protein is added for detecting the bound protein. A substrate solution is used to develop a color reaction that is in proportion to the amount of the target protein captured by the first antibody. According to the same detection principle, bead-

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based immunoassays that can be monitored with flow cytometry platforms such as xMAP Technology (Luminex, Austin, TX, USA), FlowCytomix (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA), and the BD Cytometric Bead Array (CBA, BD Biosciences, Franklin Lakes, NJ, USA) have been developed using the so-called microspheres (beads) as the solid support for the sandwich immunoassay (10). These platforms have become more and more popular in the past years for their highly sensitive and multiplexed measurements of cytokines in small volumes, which provide a network perspective of cytokine profiles more valuable than single cytokine measurements (11,12).

To acquire a sensitive quantification method of FAM19A4 that has the potential for integrating with the multiplex cytokine analysis platforms, we have developed a flow cytometry-based sandwich immunoassay to quantify FAM19A4 in biological fluids using latex beads as the solid carrier that were pre-coated with the capturing antibody for FAM19A4.

MATERIALS AND METHODS

Cells The HEK293T cells were kindly provided by T. Matsuda, Japan and cultured in complete Dulbecco's Modification of Eagle's Medium (DMEM, Gibico, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany). The HEK293T cells were transfected with the eukaryotic expression plasmid pcDB-FAM19A4 and the empty vector control pcDB (short for pcDNA 3.1 myc/his B, Invitrogen) using VigoFect (Vigorous, Beijing, China). The THP-1 (TIB-202) cells were purchased from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 (Gibico) with 10% inactivated FBS. The THP-1 cells were stimulated by pre-treated with phorblo 12-myristate 13-acetate (PMA, 20 ng/ml; MultiScience, Hangzhou, China) for 24 h and then stimulated with lipopolysaccharide (LPS, 100 ng/mL; Sigma–Aldrich, St. Louis, MO, USA) for another 48 h.

Recombinant proteins The eukaryotic recombinant FAM19A4-myc-his (NP_001005527) and LYG1-myc-his (NP_777558) proteins were expressed and purified by Crown Bioscience, Inc. (Beijing, China). The recombinant FAM19A1-myc-his and FAM19A5-myc-his proteins were kindly provided by Prof. Ying Wang, Peking University, Beijing, China. The protein concentrations were measured using a BCA protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard.

Anti-FAM19A4 antibodies The polyclonal antibody (pAb) against FAM19A4 was generated by immunizing rabbits with the FAM19A4-myc-his protein as described before (7,13). The antibody was purified by immunoaffinity chromatography. Briefly, the purified FAM19A4-myc-his protein and the synthesized myc-his polypeptide (EQKLISEEDLHHHHHH, chemically synthesized by BGI Genomics, Beijing, China) were coupling to CNBr activated sepharose 4B (GE Healthcare, Chicago, IL, USA) respectively according to the manufacture's instruction. The rabbit polyclonal antiserum was first absorbed by the myc-his peptide coupled gel to remove the antibodies against the myc-his tag. The flow-through serum was then incubated with the FAM19A4-myc-his protein conjugated gel at 4°C overnight. After thorough wash, the bound antibody was eluted using 0.1M glycine, pH 2.4 and neutralized immediately with 1 M Tris-Cl, pH 9.0. The specificity of the polyclonal antibody to recognize FAM19A4 was identified by Western blot.

The monoclonal antibodies (mAbs) against FAM19A4 were prepared by immunizing BALB/c mice with the FAM19A4-myc-his protein, which was commissioned to be completed by BGI Genomics (Beijing, China). After preliminary ELISA screening, 12 best hybridoma clones (marked as 1#–12#) were selected for expansion and their individual culture supernatants were further identified by Western blot and flow cytometry analyses. The selected clone was affinity-purified using protein G agarose and the concentration of purified IgG was determined by its absorbance at OD280 as described previously (14).

A commercial mAb against FAM19A4 (clone no. 480103) was purchased from R&D Systems (Minneapolis, MN, USA) and used as a positive control for the mAb screening.

Western blot assay The culture supernatants of HEK293T cells were collected and centrifuged (1500 rpm for 10 min at 4°C followed by 12,000 rpm for 10 min at 4°C) to eliminate the residual cells, debris and other insoluble particles. The culture supernatants of HEK293T cells and recombinant proteins were separated on 12.5%–15% SDS–PAGE and electrotransferred to nitrocellulose membranes (Hybond, Escondido, CA, USA) with standard procedures. After being blocked in 5% skim-milk in TBS-T (Tris-buffered saline containing 0.05% Tween 20) for 1 h at room temperature (RT), the membranes were incubated with the culture supernatants from individual hybridoma clones (1#–12#) or purified rabbit anti-FAM19A4 polyclonal antibodies (1 μg/mL in 5% skim-milk/TBS-T) at 4°C overnight. The HRP-conjugated goat anti-mouse or rabbit IgG antibodies (1: 5000 dilution, Cell Signaling Technology, Danvers, MA, USA) followed by enhanced chemiluminescence (ECL) Western blot detection reagents (Pierce, Rockford, IL, USA) were used to visualize the positive signals, which were then imaged on an ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA).

Preparation of capture beads Purified rabbit anti-FAM19A4 pAb was immobilized onto Aldehyde/Sulfate latex beads (Molecular Probes, Eugene, OR, USA) following the Passive Adsorption Protocol provided by the manufacture. Typically, about 45 μg of antibody was incubated with 50 μL beads in MES buffer (0.025 M, pH 6) at room temperature (RT) overnight. Any reactive sites not being covered on the microsphere surface were filled by blocking with 3% BSA in Phosphate-Buffered Saline (PBS). The antibody-coated capture beads were then kept at 4°C in 200 μL storage buffer (PBS, 0.1 M, pH 7.2, 0.1% BSA; 0.1% NaN3) until use.

Standard preparation The recombinant FAM19A4-myc-his protein was firstly diluted to 10 ng/mL with Assay Diluent Buffer (Pierce, Rockford, IL, USA) and a double dilution series of 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.312 ng/mL, 0.156 ng/mL, 0.078 ng/mL, and 0.039 ng/mL, was produced by two-fold serial dilutions. The Assay Diluent Buffer serves as the zero standard (0 ng/mL).

Sandwich immunoassay of FAM19A4 by flow cytometry After thorough mixture by vortex, the appropriate volume of capture beads (1 μL beads/test) were aspirated and 10-fold diluted in PBS. 10 μL of the diluted beads were then incubated with 100 μL of each standard or sample on a rotary mixer at 4°C overnight. The liquid was then removed by centrifugation at 6000 rpm for 10 min and the culture supernatants from individual hybridoma clones (1#–12#, 100 μL/test) or the purified detection mAb of FAM19A4 (diluted in 2%FBS/PBS, 50 μL/test) was added to each test, mixed, and incubated at RT for 1 h. The detection antibody was removed by another centrifugation and the beads were washed once with PBS. APC Goat anti-mouse IgG (minimal x-reactivity) Antibody (Biolegend, Santiago, CA, USA; diluted 1:100 in 2%FBS/PBS) was used to stain the beads in the dark at RT for 30 min. After washed in PBS, the beads were acquired on a BD FACSVerse flow cytometer using BD FACSuite software (BD, Franklin Lakes, NJ, USA). The FSC and SSC voltages were adjusted on logarithmic scales until the singlet bead population fell between 10^4 and 10^5 in the FSC channel, which was gated in P1 (Fig. 1A). 2000 beads within P1 were collected for each standard and sample.

Calculation of results The APC-A Geo Mean Fluorescence Intensity (GMFI) of gate P1 was counted for each standard, control and sample and subtracted that of the zero standard. A standard curve was created by plotting the net APC-A GMFI for each standard against the corresponding concentration and a best fit curve through the points was determined by regression analysis using CurveExpert 1.4 (Daniel G. Hyams, https://www.curveexpert.net/). Then the concentrations of samples could be read from the standard curve.

Determination of specificity, recovery and precision The specificity of this assay for detection FAM19A4 was evaluated by determining the cross-reactivity for FAM19A1 (NP_001239145) and FAM19A5 (NP_001076436), two analogues of FAM19A4 also belonging to the TAFA family and sharing 74% and 68% similarities with FAM19A4 respectively. 10 ng/mL and 1 ng/mL of the two proteins in Assay Diluent Buffer were measured in the assay.

The recovery of the target protein was evaluated by spiking different matrices with certain level (1 ng/mL) of recombinant FAM19A4. The percent recovery is defined as 100 × [(concentration detected − endogenous concentration)/concentration added] (15). The matrices tested here included cell culture media RPMI1640 and DMEM supplemented with 10% FBS, FBS that might represent the serum to some extent, and sera from 6 apparently healthy volunteers. Protocols performed in the present study were approved by the Ethics Committee of Peking University Health Science Center (Beijing, China). Written informed consent was obtained from all healthy donors.

The intra-assay precision (precision within an assay) was assessed by testing 3 samples with low (0.156 ng/mL), middle (1.25 ng/mL) and high (10 ng/mL) level FAM19A4 9 times within an assay. The inter-assay precision (precision between assays) was assessed by testing 3 samples with low (0.156 ng/mL), middle (1.25 ng/mL) and high (10 ng/mL) level FAM19A4 in 9 separate assays. Coefficients of variation (CV) were calculated.

Reverse-transcription PCR Total RNA was extracted from the THP-1 cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed according to standard protocols using the 5 × All-In-One RT MasterMix (Applied Biological Materials, Richmond, BC, Canada). PCR was performed using the primers FAM19A4F: 5′-GCGGCCGCCACCATGAGGTCCCCAAGGATGAGAGTCT-3′ and FAM19A4R: 5′-GGTACCGCCCGCGTTACCTTCGTAGTTTTGACT-3′. Thermal cycling consisted of initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; and a final extension at 72°C for 7 min. GAPDH was used as an internal control and amplified for 20 cycles using the primers 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

Enzyme-linked immunoassay (ELISA) Each well of 96-well high-binding EIA plates (Corning) was coated with rabbit anti-FAM19A4 pAb (1 μg/mL, 2 μg/mL, 5 μg/mL, and 10 μg/mL diluted in Coating Buffer (Pierce, Rockford, IL, USA),

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