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Research paper

Detection of potassium channel KIR4.1 antibodies in Multiple Sclerosis patients

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ABSTRACT

The presence of KIR4.1 antibodies has been proposed to be a characteristic of Multiple Sclerosis (MS). This could have a significant impact on disease management. However, the validation of the initial findings has failed till date. Conflicting results have been attributed to difficulties in isolating the lower-glycosylated (LG) KIR4.1 expressed in oligodendrocytes, the putative target antigen of autoantibodies.

The aim of this study is to verify the presence of KIR4.1 antibodies in MS patients, by independently replicating the originally-described procedure.

Assay procedure consisted of KIR4.1 expression in HEK293 cells, 3-step elution to isolate LG-KIR4.1 in elution fraction 3, and ELISA. Sera of 48 MS patients and 46 HCs were studied in 21 working sessions.

In a preliminary analysis, we observed different KIR4.1 antibody levels between MS patients and Healthy Controls (HCs). However, a high variability across working sessions was observed and the sensitivity of the assay was very low. Thus, stringent criteria were established in order to identify working sessions in which the pure LG-KIR4.1 was isolated. As per these criteria, we detected LG-KIR4.1 antibodies in 28% of MS patients and 5% of HCs.

Unlike previous findings, this study is in agreement with the original report. We propose further efforts be made towards the development of a uniform method to establish the detection of KIR4.1 antibodies in MS patients.

1. Introduction

The conflicting data regarding the presence of KIR4.1 antibodies in sera of MS patients (Srivastava et al., 2012; Watanabe et al., 2013; Kraus et al., 2014; Schirmer et al., 2014; Nerrant et al., 2014; Brickshawana et al., 2014; Brill et al., 2015; Chastre et al., 2016; Pröbstel et al., 2016; Higuchi et al., 2016) might be due to differences in the assay methodology (Hemmer, 2015; Gu, 2016). However, validation of the same could have tremendous impact on the management of MS. Therefore, we visited the laboratory that originally reported KIR4.1 antibodies (Srivastava et al., 2012) and replicated the described procedure. Notably, the expression and molecular form of KIR4.1 is highly regulated in a cell dependent fashion (Hibino et al., 2004). Therefore, it is important to build the assay on the protein that corresponds to lower-glycosylated (LG) KIR4.1 expressed in oligodendrocytes, the putative target of the immune response in MS (Hemmer, 2015). The primary aim of the present study is limited to provide the "Proof of Concept". We would like to highlight the importance of methodology in the correct purification and isolation of LG-KIR4.1, which is key for a successful ELISA. Additionally, we discuss critical issues related to the procedure, which may drastically affect the end results.

2. Materials and methods

2.1. Serum samples

Samples for this study were selected from the CRESM Bio-Bank. 48 sera of untreated Relapsing-Remitting MS patients and 46 sera of Healthy Controls (HCs), matched for age and sex, were studied.

All patients gave written informed consent for the use of their blood banked samples for this study (approvals n. 7777/2013 and 50/2016 by the Ethical Committee of AOU San Luigi Gonzaga).

Abbreviations: CRESM, Regional Referring Multiple Sclerosis Centre; NICO, Neuroscience Institute Cavalieri Ottolenghi; KIR4.1, inward-rectifying potassium channel 4.1; MS, multiple sclerosis; LG-KIR4.1, lower-glycosylated KIR4.1; HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay; HCs, healthy controls; OD, optical density; ROC, receiver operative characteristic; CNS, central nervous system; RR, relapsing remitting; DMEM, Dulbecco's modified eagle medium; FBS, foetal bovin serum; PBS, phosphate buffer saline; SDS, sodium dodecyl sulfate; WB, Western blot; LDS, lithium dodecyl sulfate; Fr1, 2, 3, fraction 1, 2, 3; PBST, PBS Tween; O.N., overnight; HRP, horseradish peroxidase; W5, wash 5; mAb, monoclonal antibody; TMB, 3,3′,5,5′-Tetramethylbenzidine; CV, coefficient of variation; SD, standard deviation; NT, non-transfected; IgG, immunoglobulin G.

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2.2. Assay procedure

The procedure described by Hemmer's group (Srivastava et al., 2012; Srivastava et al., 2014) includes three phases: KIR4.1 expression in HEK293 cells, 3-step elution to isolate LG-KIR4.1 in elution fraction 3 (Fr3), and ELISA. Elution and ELISA must be performed only on fresh material on the very next day and are considered a single working session. Here, each working session was also monitored through different "checkpoints" in order to evaluate quality of purification and ELISA performance.

A monoclonal antibody targeting the extracellular domain of KIR4.1 (mAb 20F9, produced and provided by B. Hemmer), which binds specifically to LG-KIR4.1, was used in each working session as positive control (Srivastava et al., 2014) to evaluate the enrichment of LG-KIR4.1 in Fr3.

2.2.1. KIR4.1 expression in HEK293 cells

pcDNA 3.1(+)/KIR4.1 was produced and provided by the laboratory of Bernhard Hemmer. KIR 4.1 DNA was replicated in DH5 alpha cells, following standard procedures. Plasmid DNA was extracted using Xtra Maxi Plus kit (Macherey-Nagel).

pcDNA of KIR4.1 was transfected in HEK293 cells grown in DMEM 10% FBS (Life Technologies) containing penicillin and streptomycin, according to standard procedures. HEK293 cells were transiently transfected with pcDNA 3.1(+)/KIR4.1, using Lipofectamine 2000 transfection reagent (Life Technologies), according to the manufacturer's instructions. At 3 hour post-transfection medium was supplemented with 3 ml of DMEM 20% FBS and plates were incubated 24 h at 37 °C, before scraping. Transfected HEK293 cells were pelleted, washed with Phosphate Buffer Saline (PBS) and dry pellets were stored at −80 °C until usage.

2.2.2. LG-KIR4.1 purification

300 million HEK293 cells transfected with KIR4.1 DNA were lysed for 2 h at 25 °C on an orbital shaker in 15 ml of lysis buffer [50 mM phosphate buffer, 550 mM Potassium chloride, 10 mM imidazole, 2.0% Maltoside, Benzoase nuclease (Sigma) and protease inhibitor cocktail (Sigma); pH 7.4]. Crude lysates were centrifuged at 20,000 rpm for 1 h at 10 °C. The supernatant (cleared lysate) was kept for the purification process.

Purification of LG-KIR4.1 was performed using a 3-step elution procedure (Srivastava et al., 2012, 2014). His-Pur cobalt resin (Thermo Scientific) was equilibrated at room temperature (optimal condition at 25 °C) and packed with 5–6 ml of cobalt resin; the storage buffer was drained from resin by gravity flow (final volume 3 ml of beads in the column). Later, the purification column was equilibrated with 15 ml of equilibration/wash buffer (50 mM phosphate buffer, 550 mM potassium chloride, 10 mM imidazole, 0.01% Maltoside). Cleared lysate was added onto the resin, incubated with it for 15 min, and then, the flow-through was collected in a tube. The resin was washed 5 times with 5 resin bed-volume (15 ml) of equilibration/wash buffer, collecting each wash fraction in a separate centrifuge tube (W1–5). Finally, KIR4.1 was eluted from the resin in a 3 step-procedure adding respectively one resin bed-volume (3 ml) of 50 mM imidazole (Fraction 1 - Fr1), 100 mM imidazole (Fraction 2 - Fr2), and 150 mM imidazole (Fraction 3 - Fr3) Elution Buffer (50 mM phosphate buffer, 150 mM potassium chloride, 50/100/150 mM imidazole; pH 7.4). The 3 different elution fractions were initially collected in 6 aliquots for each elution buffer used.

Purification checkpoint: since the crucial point for pure antigen isolation is the 3-step elution purification method (Hemmer, 2015; Srivastava et al., 2014), the procedure needs to be checked by evaluating eluted proteins both qualitatively and quantitatively. The quality of antigen purification was verified by running single elution fraction aliquots on denaturant SDS gels (4–12% bis-tris gel, Life Technologies), and performing both a Coomassie stain and a Western Blot (WB). 25 μl of each elution aliquot, HEK293 cleared lysate, flow-through, and wash fractions were mixed with the correct amount of sample reducing agent (Life Technologies) and LDS Sample Buffer (Life Technologies), for a total volume of 50 μl, and heated at 90 °C for 10 min. 20 μl of protein reaction mixture was loaded and separated on two SDS gels, one for Coomassie staining, and one for WB (Fig. 1).

Gels stained with Coomassie staining (Colloidal blue staining kit, Life Technologies) were used to check protein pattern in each fraction; in particular, this strategy allowed us to verify that contamination from higher-glycosylated KIR4.1 (~62 KDa), dominant in recombinant expression (Srivastava et al., 2014), decreased from Fr1 to Fr3 (Fig. 1A). WB was performed to check the absence of LG-KIR4.1 (~38 KDa) in Fr1, and its presence in Fr3 (Fig. 1B). Proteins were transferred to a nitrocellulose membrane and blocked with non-fat dry milk (Biorad, 5% in PBS Tween, PBST) for 30 min; membranes were incubated overnight (O.N.) with a mixture of two rabbit anti-human KIR4.1 antibodies (Alomone Laboratories and Millipore, 1:1000) in a blocking solution. Membranes were washed with PBST, and then incubated with horseradish-peroxidase (HRP)-conjugated-anti-rabbit secondary antibody (Dianova, 1:20,000) in blocking solution, for 1 h. After 5 washes, the HRP-conjugated antibody was visualized by using the Super Signal West Femto Reagent (Thermo Scientific), following the manufacturer's instructions. Proteins from each aliquot were then mixed obtaining total Fr1, Fr2 and Fr3. Total protein concentration was determined using Nanodrop (Biorad) at 280 nm absorbance of wash 5 (W5), Fr1, Fr2, and Fr3, to check the absence of detectable proteins in W5 and Fr1, and their presence in Fr3.

2.2.3. ELISA

ELISA was performed coating 5 μg/ml of freshly purified proteins from Fr1, Fr2 and Fr3 (diluted in PBS to a final volume of 100 μl/well) on different Nunc Immobilizer amino plates (Thermo Scientific). Here, we do not expect the presence of any antigen in Fr1, whereas Fr2 may contain partial traces of both higher-glycosylated and lower-glycosylated KIR4.1. However, the purest LG-KIR4.1 antigen containing fraction is expected to be the Fr3.

mAb 20F9 was tested as positive control whereas assay diluent buffer was used as negative control. Serum samples from both HCs and MS patients along with controls were tested in duplicate in each ELISA plate.

Plates were incubated O.N. at 4 °C on an orbital shaker. After 3 washes in PBST, plates were blocked with 200 μl of ultra-blocking buffer BUF033 (Biorad) for 1 h at 25 °C on the orbital shaker. After washing (3 times with PBST), plates were incubated with 100 μl of serum samples (1:100) or mAb 20F9 (1:1000) diluted in assay diluent buffer BUF037B (Biorad) for 3 h at 25 °C on the orbital shaker.

After incubation, plates were washed 5 times with PBST before probing with the proper secondary antibody (HRP anti-human IgG diluted 1:10,000, Sigma; HRP anti-rat diluted 1:5000, Dianova) in 100 μl of assay diluent buffer. Plates were incubated for 1 h at 25 °C on the orbital shaker, and washed 5 times with PBST. Finally, immunoreaction was developed using 100 μl of 3,3′,5,5′-Tetramethylbenzidine (TMB, BioFX) solution (equilibrated at 25 °C) for 22–25 min. Reaction was stopped with 50 μl of 2 N H2SO4 (BioFX).

OD values were read at 450 nm and 620 nm (reference wavelength) on a microplate reader (Thermo).

ELISA checkpoint: the technical quality of each ELISA test was checked by evaluating the reactivity of negative and positive controls: the assay diluent buffer OD in each plate (Fr1, Fr2 and Fr3) should ideally approach the baseline, while the mAb 20F9 should show a strong reactivity mostly in Fr3.

2.3. Statistical analysis

Statistical analyses were performed using Graph Pad Software version 5.0 (GraphPad).