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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).

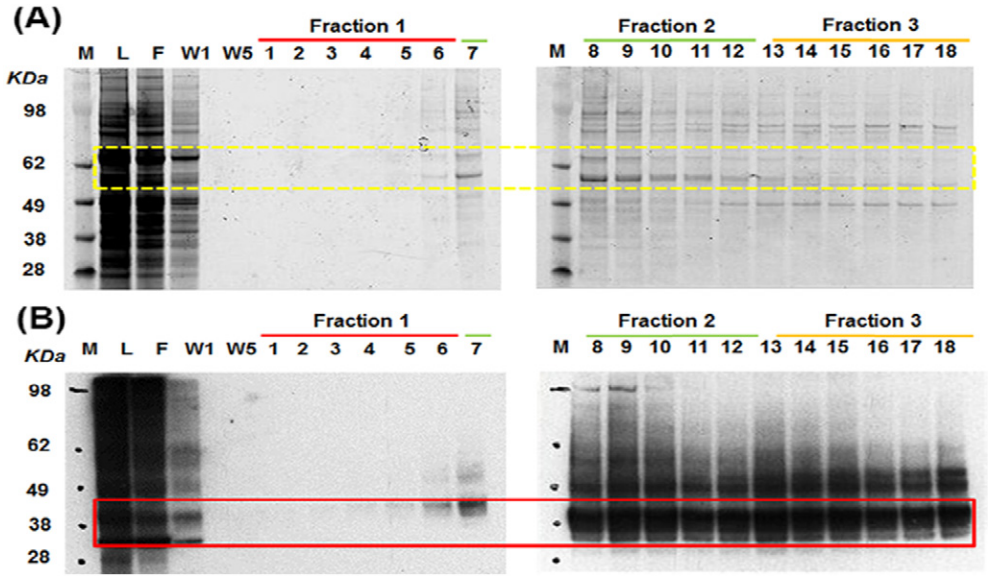

Fig. 1. Purification checkpoint: Coomassie staining and WB. The presence of LG-KIR4.1 protein was determined by running proteins on 4% to 12% bis-tris gel in reducing conditions; A: gels were stained with Coomassie to show the amount of proteins: in particular, progressive reduction of the ~62 KDa band (corresponding to higher-glycosylated KIR4.1) is shown in dotted-line box. B: Western blot was performed by mixing two different commercial anti-KIR4.1 antibodies: presence of LG-KIR4.1 (~38 KDa band) is shown in solid-line box. M = marker; L = total HEK293 lysate; F = purification flow through; 1–6: Fr1 aliquots; 7–12: Fr2 aliquots; 13–18: Fr3 aliquots.

Table 1
Data obtained in the entire set of working sessions.

PURIFICATIONS [a]		TOTAL PROTEIN CONCENTRATION [b]				COOMASSIE BLUE STAINING [c]		WESTERN BLOT [d]		ELISA RESULTS [e]							ACCEPTED SESSIONS [f]
Working sessions		280 nm Absorbance				Protein pattern		Commercial Anti-KIR4.1 Ab reactivity		mAb 20F9 Anti-LG-KIR4.1			Assay Diluent + HRP Anti-Hu IgG		Assay Diluent + HRP Anti-Rat IgG		Criteria for rejection
N°	Data	W5	Fr1	Fr2	Fr3	Fr1	Fr3	Fr1	Fr3	Fr1 (OD)	Fr3 (OD)	Ratio %	Fr1 (OD)	Fr3 (OD)	Fr1 (OD)	Fr3 (OD)	
1	06/11/14	n.a.	0,01	0,06	0,01	Dirty	↓ 62KDa band	5	+++	1,733	2,965	71	n.a.	n.a.	n.a.	n.a.	Fr1, Ratio %
2	12/12/14	0,00	0,03	0,11	0,06	Dirty	↓ 62KDa band	4	+++	1,313	3,232	146	n.a.	n.a.	n.a.	n.a.	Fr1, Ratio %
3	17/12/14	0,00	0,01	0,11	0,06	Clean	↓ 62KDa band	6	+++	0,207	1,494	623	n.a.	n.a.	n.a.	n.a.	Ratio %
4	18/12/14	0,01	0,03	0,12	0,09	Clean	Dirty	6	+++	0,807	2,097	160	n.a.	n.a.	n.a.	n.a.	Fr1, Ratio %
5	21/01/15	0,02	0,07	0,29	0,14	Clean	Dirty	5	+++	0,3615	0,7265	1,1145	0,069	0,037	n.a.	n.a.	Fr1, Ratio %
6	03/02/15	0,00	0,02	0,21	0,04	Clean	↓ 62KDa band	No	+++	0,171	2,561	1397	0,027	0,029	n.a.	n.a.	Accepted
7	11/02/15	0,01	0,10	0,21	0,01	Clean	↓ 62KDa band	6	+++	0,635	3,881	512	0,069	0,816	n.a.	n.a.	Fr1, Ratio %
8	13/02/15	0,00	0,00	0,19	0,02	Clean	↓ 62KDa band	6	+++	0,103	0,457	344	0,037	0,098	n.a.	n.a.	Ratio %
9	19/02/15	0,00	0,34	0,08	0,03	Clean	dirty	5	+++	1,110	3,240	192	0,030	0,030	n.a.	n.a.	Fr1, Ratio %
10	10/03/15	0,00	0,05	0,09	0,06	Clean	↓ 62KDa band	4	++	0,440	2,725	519	0,025	0,025	n.a.	n.a.	Fr1, Ratio %
11	19/03/15	0,00	0,05	0,08	0,02	Clean	↓ 62KDa band	5	+++	1,030	3,607	250	0,258	0,037	n.a.	n.a.	Fr1, Ratio %
12	27/03/15	0,03	0,02	0,27	0,11	Clean	↓ 62KDa band	6	+++	0,175	2,790	1494	0,081	0,060	n.a.	n.a.	Accepted
13	02/04/15	0,00	0,01	0,12	0,07	Clean	↓ 62KDa band	No	+++	0,275	2,032	639	n.a.	0,088	n.a.	n.a.	Ratio %
14	29/04/15	0,00	0,00	0,12	0,03	Clean	↓ 62KDa band	6	+++	0,170	3,313	1854	0,033	0,024	n.a.	n.a.	Accepted
15	30/04/15	0,02	0,02	0,11	0,06	Clean	↓ 62KDa band	6	+++	0,022	1,727	7930	0,017	0,036	n.a.	n.a.	Accepted
16	19/05/15	0,00	0,02	0,10	0,03	Clean	↓ 62KDa band	6	+	0,301	3,333	1009	0,117	0,195	n.a.	n.a.	Ratio %
17	22/05/15	0,00	0,00	0,14	0,05	Clean	dirty	6	+	0,435	3,245	646	0,031	0,026	n.a.	n.a.	Fr1, Ratio %
18	18/08/15	0,00	0,01	0,07	0,09	Clean	↓ 62KDa band	No	+++	0,089	3,156	3466	0,012	0,019	0,029	0,079	Accepted
19	25/08/15	0,01	0,03	0,09	0,12	Dirty	—62Kda band	6	+++	0,383	2,709	608	0,048	0,020	0,031	0,050	Fr1, Ratio %
20	31/08/15	0,00	0,01	0,05	0,10	Clean	↓ 62KDa band	No	++	0,131	2,826	2057	0,020	0,078	0,040	0,075	Accepted
21	15/09/15	0,00	0,00	0,02	0,06	Clean	↓ 62KDa band	No	+++	0,073	2,575	3427	0,019	0,020	0,030	0,076	Accepted
1 NT	17/08/15	0,01	0,02	0,07	0,05	Clean	Weak bands	No	no	0,038	0,348	814	0,039	0,017	0,039	0,017	
2 NT	07/09/15	0,00	0,00	0,10	0,06	Dirty	Dirty	No	no	0,090	1,010	1028	0,044	0,038	0,044	0,038	
3 NT	17/09/15	0,00	0,02	0,06	0,09	Clean	Dirty	No	no	0,082	0,939	1055	0,012	0,028	0,012	0,028	

[a]Twenty-one working sessions were performed on HEK293 cells transfected with KIR4.1 DNA (1−21). Three working sessions were performed on HEK293 cells non transfected with KIR4.1 DNA (NT 1–3).
[b]Total protein concentration in wash 5 (W5), and in each elution fraction (Fr1, Fr2, and Fr3) was determined by Nanodrop (Biorad) at 280 nm absorbance.
[c]The purified protein pattern on Coomassie gel (Fig. 1C) was outlined, describing Fr1 and Fr3 quality. Fr1 (*on the left*) should be free of any proteins: Fr1 quality is therefore described as "clean" or "dirty". Fr3 quality (*on the right*) is also described, indicating in addition the trend of 62 KDa band which correspond to the higher-glycosylated KIR4.1 ("↓" = reduction of band intensity, "—" = constant band intensity).
[d]Presence of LG-KIR4.1 was checked by Western Blot using commercial anti-KIR4.1 antibodies (Fig. 1C). *On the left*, reactivity in Fr1 (which should be free of LG-KIR4.1) is described ("*no*" = absence of the specific reactivity; a "*number*" refers to the first aliquot of Fr1 showing LG-KIR4.1 specific band). *On the right*, intensity of LG-KIR4.1 specific band in Fr3 was reported.
[e]Positive Control (mAb 20F9) and Negative Control (Assay diluent stained with HRP anti-human IgG or anti-rat IgG secondary antibody) reactivities in ELISA test against Fr1 and Fr3 were reported as OD values and Ratio % [(Fr3 − Fr1) / Fr1 ∗ 100].
[f]Acceptance criteria causing rejection of each working session were reported ("Fr1" and "Ratio %" refers to acceptance criteria, Fig. 3). *Grey rows* show accepted working sessions, in which pure LG-KIR4.1 was isolated.
n.a. = not available.

Mann-Whitney test was used to compare the distributions of HC and MS samples reactivity anti LG-KIR4.1. Fisher's exact test was used to analyze contingency tables. p values < 0.05 were considered statistically significant. The optimal cut-off for KIR4.1-Abs was determined by means of the receiver operative characteristic (ROC) curve.

3. Results

3.1. LG-KIR4.1 antibodies detection: preliminary analysis

A total of 21 working sessions were performed from KIR4.1-transfected HEK293 cells. 3 working sessions were performed from non-transfected (NT) HEK293 cells, as a negative control. The entire data obtained from each working session have been summarized in Table 1.

In these 21 sessions, a total of 94 serum samples (48 MS and 46 HC) were tested. Each purification session had mAb 20F9 as the positive control. The mAb 20F9 reactivity was higher in Fr3 than in Fr1 ($p < 0.0001$, Mann-Whitney test; Fig. 2A). 805 total determinations were performed, 511 for MS samples (median 13 tests, range 1–21 tests), and 294 for HCs (median 5 tests, range 1–21 tests). Median OD values for each patient were calculated.

The serum levels of LG-KIR 4.1 antibodies were found to be significantly higher in MS patients as compared to HCs ($p = 0.0150$, Mann-Whitney test). Although this result agrees with the initial findings, the cut-off identified by the ROC analysis allowed the detection of LG-KIR4.1 antibodies only in 4/48 (8%) MS patients and 2/46 (4%) HCs (Fig. 2B). This result is similar to the findings of independent studies before us that failed to replicate this method (Watanabe et al., 2013; Nerrant et al., 2014; Brickshawana et al., 2014; Brill et al., 2015; Chastre et al., 2016; Pröbstel et al., 2016; Higuchi et al., 2016). Key

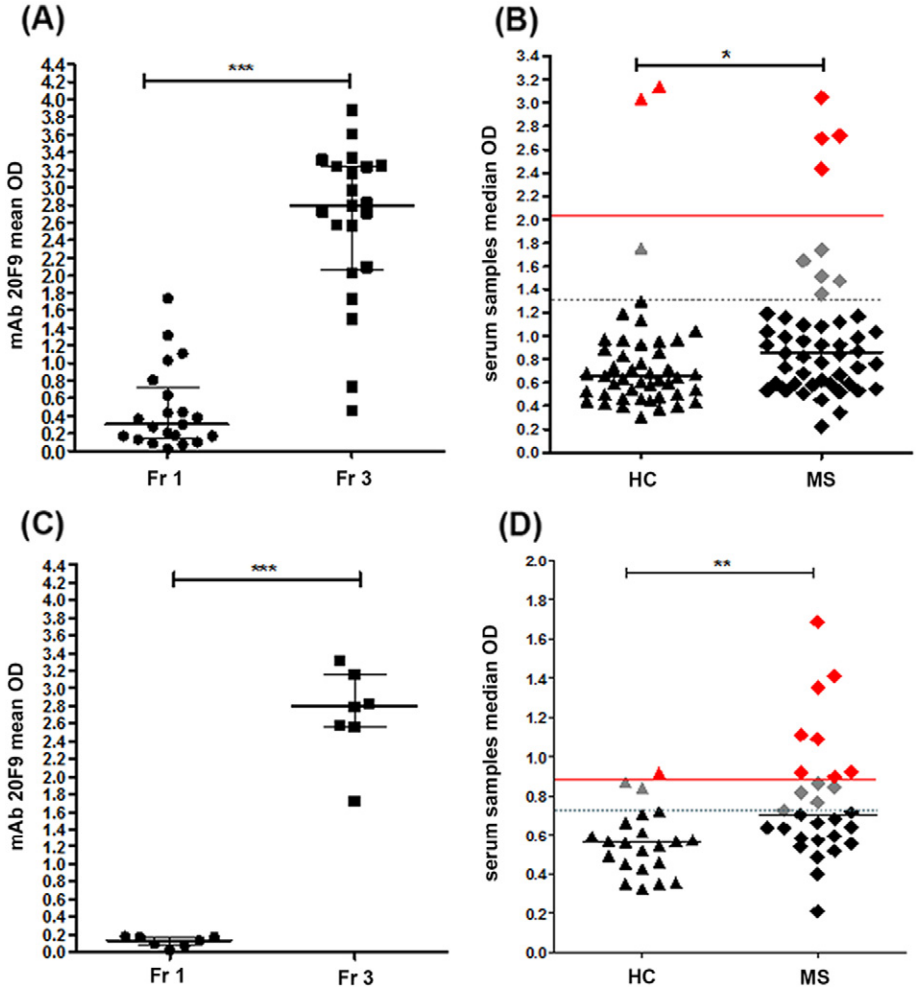

Fig. 2. Detection of LG-KIR4.1 antibodies: preliminary and final analysis. LG-KIR4.1 antibodies detection was evaluated by analyzing ELISA reactivity of 20F9 mAb antibody along with serum samples from HC and MS patients, in the whole set of 21 sessions and in the 7 accepted sessions. mAb 20F9 was used in the assay as positive control to show the enrichment of LG-KIR4.1 in Fr3 related to Fr1. 20F9 mAb was tested in duplicate in each session: each dot on the graph (Panels A and C) represents the mean OD value obtained in each session; median OD and interquartile range are shown. Each serum sample was tested in duplicate in different working sessions: each dot on the graph (Panels B and D) represents the median OD value obtained for each sample. A and B: first analysis was performed on the entire set of 21 working sessions. ELISA results of 20F9 mAb reactivity are shown (A): median OD was 0.301 for Fr1 and 2.790 for Fr3 ($p < 0.0001$, Mann-Whitney test), showing the enrichment of LG-KIR4.1 in Fr3 as compared to Fr1, despite a high variability in OD values. Panel B shows the results of ELISA obtained from the whole cohort of 48 MS patients and 46 healthy controls: median OD was 0.8595 in the MS group and 0.6520 in the control group ($p = 0.0150$, Mann-Whitney test). ELISA cut off was defined by ROC Analysis using a threshold of 2.095 (*upper solid line*) corresponding to 96% of specificity, and 8% sensitivity; LG-KIR4.1 antibodies were found in 2/46 (4%) HCs and 4/48 (8%) MS patients. A "grey zone" of positivity was also defined (*dotted line*) by selecting a lower cut-off at 1.328 OD corresponding to 93% specificity, and 19% sensitivity. C and D: LG-KIR4.1 antibodies detection was further performed on samples from the 7/21 working sessions fulfilling the acceptance criteria. In Panel C, 20F9 mAb reactivity is shown: median OD was 0.131 for Fr1 and 2.790 for Fr3 ($p = 0.0006$, Mann-Whitney test), showing the enrichment of LG-KIR4.1 in Fr3 related to Fr1, and less variability if compared to the previous analysis. In Panel D, ELISA results from serum samples of 29 MSs and 22 HCs are shown: median OD was 0.7060 in the MS group and 0.5640 in the control group ($p = 0.0042$, Mann-Whitney test). The cut off for ELISA test was defined by using ROC analysis considering a threshold of 0.8855 OD (*upper solid line*), 1/22 HC (5%) and 8/29 MS patients (28%) resulted positive for LG-KIR4.1antibodies, corresponding to a specificity of 95% and a sensitivity of 28%. A "grey zone" of positivity was also defined (*dotted line*) by selecting a lower cut-off at 0.7250 OD, corresponding to 86% specificity, and 45% sensitivity.

observations from the current study include high variability across working sessions in 1) mAb 20F9 OD values both in Fr3 and Fr1, 2) total protein concentrations, 3) protein patterns shown by Coomassie staining and 4) anti-KIR4.1 antibodies reactivity as shown by WB and ELISA (Table 1 and Fig. 2A). We believe that this variability may contribute to the failure of replication of the initial findings.

3.2. Establishment of acceptance criteria

Isolation of pure LG-KIR4.1 represents the critical point for a successful ELISA test (Hemmer, 2015).

The high variability observed in purification procedure and the low sensitivity obtained from the preliminary analysis of 21 working sessions could be due to an imperfect purification of the antigen. Therefore, we established two main acceptance criteria (Fig. 3):

1. *Purification criteria* to identify working sessions in which LG-KIR4.1 was correctly purified and enriched in Fr3. As a consequence, in these sessions, Fr1 was devoid of LG-KIR4.1.

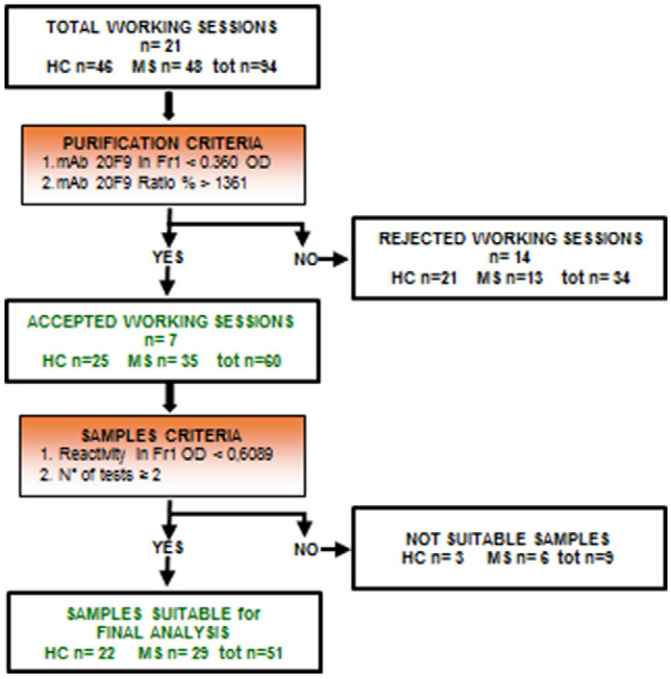

Fig. 3. Acceptance. Twenty-one purification sessions were performed on HEK293 cells transfected with KIR4.1 DNA (Total 46 HC and 48 MS samples were tested). Two main categories of acceptance criteria were established. "Purification criteria" were defined based on the ELISA reactivity of mAb 20F9, to identify working sessions in which pure LG-KIR4.1 was enriched in Fr3 compared to Fr1. "*Purification criterion 1*": mAb 20F9 reactivity in Fr1 < 0.360 OD. This reference value is the equivalent of 1 standard deviation (SD) above the mean OD for mAb 20F9 in Fr1, obtained in the working sessions where the Fr1 was free of any proteins, as evaluated from total protein concentration and WB reactivity (working session no. 8, 14, 17, 21, Table 1). "*Purification criterion 2*": mAb 20F9 Ratio % [(Fr3 − Fr1) / Fr1 ∗ 100] > 1361. The Ratio % expresses the enrichment of LG-KIR4.1 in Fr3 compared to Fr1. This reference value is the equivalent of 3 SD above the mean rRatio % for mAb 20F9 obtained in the three working sessions performed from non-transfected HEK293 (working session no. 1NT, 2NT, 3NT, Table 1). 7 out of 21 purification sessions were accepted (in these accepted purification sessions, 25 HC samples and 35 MS samples were tested). "Samples criteria" were established to identify samples showing a non-specific reactivity in Fr1. These criteria were applied on samples tested in the 7 accepted working sessions fulfilling the "purification criteria". "*Samples criterion 1*": serum reactivity in Fr1 < 0.6044 OD. This reference value is the equivalent of 1 SD above the mean OD for HCs (n = 25) in Fr1, obtained in accepted working sessions. "*Samples criterion 2*": no. of tests for each serum sample ≥ 2. Only serum samples tested in duplicate in at least 2 different accepted working sessions were accepted. Nine samples (6 MS and 3 HCs) were excluded from the final analysis, because they did not fulfill "*samples criteria*". 22 HC samples and 29 MS samples were suitable for the final analysis.

These criteria were met by 7/21 working sessions, in which 60/94 samples (35 MS and 25 HC) were tested.

2. *Samples Criteria* to identify and exclude samples with non-specific reactivity. These criteria were applied on samples tested in the 7 accepted working sessions fulfilling the "purification criteria". Hence, any possible non-specific serum reactivity in Fr1 of these samples could be due to antibodies recognizing other incorrectly eluted proteins.

51/60 samples (29 MSs and 22 HCs) fulfilled the above criteria, and were considered suitable for the final analysis.

3.3. LG-KIR4.1 antibodies detection: final analysis

The application of the acceptance criteria allowed us to analyze total 248 ELISA determinations, 155 from MS samples (median 5 tests, range 3–7 tests), and 93 from HCs (median 4 tests, range 2–7 tests). Median OD values for each patient were determined.

MAb 20F9 reactivity was found to be higher in Fr3 than in Fr1, reiterating the preliminary findings ($p = 0.0006$, Mann-Whitney test). However, the variability was far lesser as compared to the preliminary analysis. This may suggest a superior isolation of LG-KIR4.1 in all of the included working sessions (Fig. 2C).

A significant difference was found in the serum levels of LG-KIR 4.1 antibodies in MS patients as compared to HCs, confirming our preliminary observations ($p = 0.0042$, Mann-Whitney test). From the ROC analysis, an OD value of 0.8855 was selected as an appropriate cut off with 95% specificity and 28% sensitivity. LG-KIR4.1 antibodies were detected in 1/22 HCs (5%) and 8/29 MS patients (28%) (Fig. 2D).

4. Discussion

Antibodies against the inward rectifying potassium channel KIR4.1 have been identified in the sera of almost 50% of MS patients (Srivastava et al., 2012). These findings may be of impact for MS patients from a diagnostic, prognostic and, possibly, therapeutic point of view.

However, validation of these original findings has failed so far (Watanabe et al., 2013; Nerrant et al., 2014; Brickshawana et al., 2014; Brill et al., 2015; Chastre et al., 2016; Pröbstel et al., 2016; Higuchi et al., 2016). Such conflicting data could be attributed to methodological factors (Gu, 2016). A crucial component for a successful ELISA is the correct isolation of LG-KIR4.1 (Hemmer, 2015).

After visiting the laboratory that first reported on KIR4.1 antibodies, we independently set up the whole procedure. Results emerging from a preliminary analysis of the samples tested in all working sessions showed that LG-KIR4.1 antibody levels were significantly different between MS patients and HCs. However, similar to the results obtained by other groups, the sensitivity of the test was observed to be very poor (Watanabe et al., 2013; Nerrant et al., 2014; Brickshawana et al., 2014; Brill et al., 2015; Chastre et al., 2016; Pröbstel et al., 2016; Higuchi et al., 2016). This was probably due to a high variability in eluted proteins and mAb 20F9 ELISA reactivity (Table 1; Fig. 2A and B). This led us to adopt a systematic approach for data analysis.

In order to verify the purity of isolated LG-KIR4.1, two main categories of acceptance criteria were defined, namely, Purification Criteria and Samples Criteria (Fig. 3). This systematic approach allowed us to highlight some critical issues related to the whole procedure, and to carry out a thorough analysis ensuring reliable results.

Due to these rigorous criteria, only 7/21 working sessions and 51/94 serum samples were found acceptable for final analysis. In these samples as well, ELISA test showed higher levels of LG-KIR4.1 antibodies in MS patients compared to HCs ($p = 0.0042$). As per the OD value (cut-off, 0.8855) by ROC analysis (95% specificity and 28% sensitivity), 1/22 HCs (5%) and 8/29 MS patients (28%) were found to be positive for LG-KIR4.1 antibodies (Fig. 2D).

In addition, our experience highlights the importance of using mAb20F9 as positive control to monitor the successful purification of LG-KIR4.1. Failure in replicating the original data by other groups could be caused by a lack of monitoring of these steps (Watanabe et al., 2013; Nerrant et al., 2014; Brickshawana et al., 2014; Brill et al., 2015; Chastre et al., 2016; Pröbstel et al., 2016; Higuchi et al., 2016) and a lack of a specific positive control (Watanabe et al., 2013; Nerrant et al., 2014; Brickshawana et al., 2014; Brill et al., 2015; Higuchi et al., 2016).

5. Conclusions

Our data show that a systematic approach based on the establishment of acceptance criteria is able to detect LG-KIR4.1 antibodies in a significant subset of MS patients. To date, this is the first study that confirms the original findings by Hemmer's group, although, with a lower sensitivity. However, the method described here for LG-KIR4.1antibodies quantification is very complex. Given the importance and the strong impact that KIR4.1 antibodies could have in the management of MS, further studies are warranted to develop an assay allowing easier and reliable quantification of the antibodies in serum samples.

Conflicts of interest

Fabiana Marnetto received speaker honoraria from Biogen Idec, Merck Serono and Euroimmun, and received research support from Italian Multiple Sclerosis Society.

Paola Valentino received speaker honoraria from Biogen Idec, Merck Serono and Euroimmun.

Marzia Caldano received speaker honoraria from Biogen Idec, Merck Serono and Teva.

Antonio Bertolotto served on the scientific advisory boards of Almirall, Bayer, Biogen Idec, and Genzyme, and received speaker honoraria from Biogen Idec, Genzyme, Novartis, Sanofi-Aventis and Teva. His institution has received grant support from Bayer, Biogen Idec, Merck, Novartis, Teva, the Italian Multiple Sclerosis Society, Fondazione Ricerca Biomedica ONLUS, and San Luigi ONLUS; is on the editorial board of *Multiple Sclerosis International*, *Progress in Neuroscience*, *Dataset Papers in Neuroscience*, *Journal of Multiple Sclerosis*, *Neurology and Therapy*, and *Multiple Sclerosis and Demyelinating Disorders*; and received research support from Regione Piemonte, Italian Multiple Sclerosis Society, Associazione Ricerca Biomedica ONLUS, and San Luigi ONLUS.

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