Refining cell-based assay to detect MOG-IgG in patients with central nervous system inflammatory diseases.

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Highlights

- A full-length MOG transfected stable cell line and cell based assay was developed.
- In-house and Oxford CBA showed high concordance.
- IgG (H+L) and IgG1-Fc antibodies were comparable, and no IgM binding was observed.
- CBA-immunofluorescence assay score and CBA-flow cytometry yielded high correlation.
- No MS, AQP4-IgG positive NMOSD or healthy individuals were MOG-IgG seropositive.

Refining cell-based assay to detect MOG-IgG in patients with central nervous system inflammatory diseases.

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ABSTRACT

Background:

Given that the spectrum of myelin oligodendrocyte glycoprotein immunoglobulin G (MOG-IgG) associated disease is yet to be fully defined, development of sensitive and highly specific assays to identify MOG-IgG is crucial to precisely define the clinical phenotypes, disease courses and prognosis to describe the full spectrum of MOG-IgG associated diseases. Here, we aim to validate a new in-house live cell-based assay (CBA) for screening MOG-IgG in patients with central nervous system inflammatory diseases.

Methods:

We generated a full length MOG transfected HEK293 stable cell line using pIRES2-eGFP vector. Sera from 355 patients with central nervous system inflammatory diseases and 25 healthy individuals were evaluated for MOG-IgG seropositivity using in-house cell-based immunofluorescence assay (CBA-IF). The specificity of IgG (H+L) and IgG1-Fc secondary antibodies as well as IgM binding were determined by cell-based flow cytometry (CBA-FACS). The optimal cut-offs for determining seropositivity in CBA-FACS were calculated and the concordance of CBA-IF score and CBA-FACS was studied. The results of our CBA-IF were compared with the Oxford CBA-IF.

Results: 11.5% (41/355) of patients were seropositive for MOG-IgG and had clinical phenotypes that were within the known clinical spectrum of MOG-IgG associated diseases. No typical multiple sclerosis patients, aquaporin-4-IgG positive neuromyelitis optica spectrum disorder or healthy individuals were MOG-IgG seropositive. Using CBA-FACS, the anti-human IgG (H+L) was found to be comparable to IgG1-Fc antibody. No IgM binding was observed in all the samples tested. CBA-IF score and CBA-FACS yielded high correlation. The concordance of the NCC CBA-IF with the Oxford CBA-IF was 98%.

Conclusion: We have developed MOG-IgG CBAs that have different characteristics and benefits but with high specificity and concordance. The complementary use of two methods and follow-up study with larger cohort will increase the clinical usefulness of MOG-IgG CBAs.

Key words: MOG-IgG, cell-based assay, MOG-IgG assay, MOG-IgG associated diseases.

1. Introduction

Serostatus of autoantibodies is now a critical diagnostic criterion in a wide range of neurological diseases involving autoimmune conditions. Development of cell-based assays (CBAs) with native myelin oligodendrocyte glycoprotein (MOG) as the substrate identified MOG-IgG positive patients with various demyelinating phenotypes. Both pediatric and adults with acute disseminated encephalomyelitis (ADEM), seizures, encephalitis, aquaporin 4 (AQP4)-IgG negative neuromyelitis optica spectrum disorder (NMOSD), optic neuritis (ON), myelitis and brainstem encephalitis had MOG-IgG (Cobo-Calvo et al., 2017; de Mol et al., 2019; Kitley et al., 2014; Kitley et al., 2012; Probstel et al., 2011; Reindl et al., 2013; Rostasy et al., 2012). The MOG-IgG were not present in multiple sclerosis (MS) patients, hence, thought to distinguish non-MS central nervous system (CNS) demyelinating disorders from MS (Waters et al., 2015). Moreover, there are clinical, radiologic, pathologic and prognostic differences between MS and patients with MOG-IgG associated diseases. Recent studies have shown that persistent positivity of MOG-IgG is associated with clinical relapses despite immunotherapy whereas conversion to seronegativity resulted in no clinical relapses (Hyun et al., 2017; Jurynczyk et al., 2017; Lopez-Chiriboga et al., 2018). Furthermore, serologic findings and different experimental approaches suggested that MOG-IgG may be pathogenic (Dale et al., 2014; Ikeda et al., 2015; Marta et al., 2005; von Budingen et al., 2002). Nonetheless, the spectrum of MOG-IgG associated diseases is yet to be fully defined. Hence, development of a sensitive and highly specific assay to identify specific autoantibodies is crucial to precisely define the clinical phenotypes, disease courses and prognosis to describe the full spectrum of MOG-IgG associated diseases.

We have previously developed a sensitive and highly specific CBA to detect AQP4-IgG (Kim

et al., 2017). In this study, we have employed our previous method and built a new in-house CBA for screening MOG-IgG. The serostatus of MOG-IgG was evaluated in a large cohort of CNS inflammatory diseases at National Cancer Center (NCC) Korea.

2. Method

2.1 Patients

Sera from 355 patients diagnosed with CNS inflammatory diseases at the NCC and from 25 healthy individuals as control (HC) were included in this study. The demographics of 355 patients are summarized in table 2 (see Results 3.2). The serostatus of 355 patients were evaluated by in-house CBA. The cohort includes 333 patients who were selected randomly from patients who have CNS inflammatory diseases and are treated at the NCC and 22 patients who were previously confirmed as MOG-IgG positive by the live IgG1 Oxford CBA (Oxford-CBA) (Waters et al., 2015).

The specificity and sensitivity of three different secondary antibodies (anti-human IgG H+L, IgG1-Fc and IgM) were evaluated in 105/355 patients by flow cytometry. The specificity and sensitivity of secondary antibodies were also confirmed by CBA-IF in 10/355 patients. The concordance between the NCC CBA-IF (NCC-CBA) and Oxford-CBA-IF were evaluated in 125/355 patients (22 MOG-IgG seropositive patients included) (see Results). Sera from all participants were stored at -80℃ until analysis. The diagnosis of NMOSD and MS were based on the 2015 international Panel for NMO Diagnosis criteria and the 2010 McDonald criteria, respectively (Polman et al., 2011; Wingerchuk et al., 2015). The clinical, laboratory and radiological data of these patients were reviewed retrospectively. The Institutional Review Board of the NCC approved the present study and written informed consent was obtained

from all participants.

2.2 Generation of MOG-HEK293 cell

Full-length human MOG (FL-MOG) cDNA was isolated from FL-MOG/pIRES2-DsRed2 plasmid and cloned into the XhoI and SmaI sites of the pIRES2-EGFP vector (Clontech, Mountain View, CA, USA). All of the sequences were confirmed by automatic sequencing. The cloned FL-MOG plasmid or the empty vector (EV) plasmid was transfected into Human Embryonic Kidney 293 (HEK293) cells (ATCC, Manassas, VA, USA) using Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA). After 48 hours, the cells were split into the medium containing 2mg/mL G418 (Invitrogen). The G418-resistant cells were isolated after 2 weeks and then further cloned by limiting dilution. The individual stable transfectant clones were screened for green fluorescent protein (GFP) expression using flow cytometry. FL-MOG protein expression in GFP-positive cells was confirmed by Western blotting with anti-MOG antibody (Santacruz, Dallas, Texas, USA).

2.3 MOG-IgG Cell-based indirect immunofluorescence assay (CBA-IF)

CBA-IF was performed as previously described using MOG-HEK293 cell (Kim et al., 2017). Three different secondary antibodies were used :goat anti-human IgG (H+L) conjugated with Alexa Fluor-594 (Jackson Immunoresearch, PA, USA; diluted 1:2000 with PBS), goat anti-human IgG1 (FCγ Fragment specific) conjugated with Alexa Fluor-594 (Jackson Immunoresearch, PA, USA; diluted 1:750 with PBS) or goat anti-human IgM conjugated with Alexa Fluor-594 (Invitrogen, CA, USA; diluted 1:750 with PBS).

In order to exclude non-specific background staining, HEK293 cells stably transfected with

only empty vector (EV) was used as a control. Each experiment was performed in duplicate, and seropositivity was determined by two investigators who were blinded to the clinical and laboratory information of the studied patients, and to each other's results. Any disagreements regarding the results were resolved by performing the experiment again and getting a third opinion. The intensity of surface immunofluorescence was scored as table 1. The final score was the median of readings from 2 or 3 investigators and the IF scores ≥ 1 were considered positive and scores of 0.5 were considered borderline.

2.4 AQP4-IgG CBA-IF

The serostatus of AQP4-IgG in MOG seropositive patients were determined using in-house CBA at the NCC, as previously described (Kim et al., 2017).

2.5 Cell-based flow cytometry assay (CBA-FACS)

HEK293 cells were harvested and washed in staining buffer (2mM EDTA-1X PBS supplemented with 0.5% bovine serum albumin). 2 x 10^5 cells were incubated with patient serum (diluted 1:40 in staining buffer, 200 μL) for 30 minutes in 96 well U-bottom plate (Thermo Fisher Scientific, MA, USA) on ice. Cells were then washed two times with PBS and incubated with allophycocyanin-conjugated goat anti-human IgG (H+L) (Jackson Immunology, PA, USA; diluted 1:2000 in PBS) or phycoerythrin-conjugated mouse anti-human IgG1-Fc (SouthernBiotech, Birmingham, USA; diluted 1:1000 in PBS) or blue-violet 421-conjugated mouse anti-human IgM (BD Bioscience, CA, USA; diluted 1:50 in PBS) for 30 minutes on ice. The cells were washed, resuspended in 400 μL cold PBS, and before

acquisition, viability dye 7-AAD (BD Biosciences, CA, USA; 5 µl per sample) was added to the cells to exclude dead cells. A total of 10,000 cells were acquired on a FACSVerse and data were analyzed using Flow Jo software (TreeStar, Ashland, OR, USA). Binding was expressed as mean fluorescence intensity (MFI). MFI ratio was determined by MFI of MOG-transfected HEK293 cells divided by the MFI of EV-transfected HEK293 cells.

Receiver-operating characteristic (ROC) curve analysis was used to determine the utility of MFI ratio for determining MOG-IgG seropositivity, using CBA-IF as the reference standard. The optimal cut-offs with highest specificity and sensitivity were determined from ROC curve analysis.

The specificity was calculated as: [(Number of MOG-IgG negatives in the AQP4-IgG positive NMOSD, MS and other neurological diseases (OND) patients)/ Total number of AQP4-IgG positive NMOSD, MS and OND patients tested] x 100.

2.6 Statistical analysis

Concordance rate: (number of concordance samples / number of total samples) x 100.
Cohen's kappa statistic was used to evaluate the concordance between CBA-IF vs CBA-FACS and (NCC) CBA-IF vs (Oxford) CBA-IF using PASW 18.0.0 (IBM, New York, USA). A p value of < 0.05 was considered statistically significant.

The following statistical analysis was performed using Prism 6 (GraphPad, La Jolla, USA). Correlation of CBA-IF score and CBA-FACS was analyzed with Spearman's non-parametric correlation. A p value of < 0.05 was considered statistically significant.

3. Results

3.1 In-house MOG-IgG CBA-IF

To optimize an in-house CBA-IF, HEK293 cells stably transfected with FL-MOG were used, with successful transfection confirmed by western blot and flow cytometry (figure 1 A, B). The CBA-IF was performed as previously described (Kim et al., 2017), and fluorescence levels were measured by fluorescence microscopy.

In a previous study, concerns about the usage of anti-human IgG (H+L) secondary antibody to detect MOG-IgG were raised due to IgM cross-reactivity, particularly with MS patient samples (Waters et al., 2015). Secondary antibodies against IgG1-Fc or IgM were used in a CBA-IF with a limited number of samples (n=10; 1 AQP4-IgG negative NMOSD, 2 single or relapsing myelitis, 4 other demyelinating diseases including tumefactive demyelination or ADEM-like manifestation (ODD) and 3 healthy controls). We found no difference in the fluorescence between IgG (H+L) and IgG1-Fc, and no signal was seen when an IgM secondary antibody was used (figure 1 C-E). Green fluorescence in the cytosol was observed in MOG-transfected cells, and when patient serum that contained MOG-IgG was added, red fluorescence was observed surrounding the cell membranes by binding of MOG-IgG to FL-MOG (figure 1E). If the patient serum did not contain MOG-IgG, no red fluorescence was observed (figure 1C, D).

3.2 MOG-IgG serostatus in the NCC cohort

We evaluated the serostatus of 25 HC and 355 patients (65 with AQP4-IgG positive NMOSD, 37 with AQP4-IgG negative NMOSD, 111 with MS, 52 with single or relapsing myelitis (myelitis), 16 with single or relapsing optic neuritis (ON), 55 with ODD and 7 with OND) from a large cohort of CNS inflammatory diseases. The patient characteristics and MOG-IgG seropositivity of 355 patients are summarized in table 2.

Out of 355 patients tested in total, 41/355 (11.5%) were seropositive. 4/37 (10.8%) patients with AQP4-IgG negative NMOSD, 7/52 (13.5%) patients with single or relapsing myelitis, 6/16 (37.5%) patients with single or relapsing ON and 24/55 (43.6%) patients with ODD had MOG-IgG in their serum. None of the 183 patient samples with AQP4-IgG positive NMOSD, MS, OND and healthy controls were positive for MOG-IgG. All 41 patients positive for MOG-IgG had clinical phenotypes that were within the known clinical spectrum of MOG-IgG associated diseases.

The female to male ratio was 28:13 and the median age at disease onset was 28 years (range: 3-60 years). The clinical phenotype at onset included optic neuritis (37%), myelitis (29%), brain attacks (24%) and poly-regional involvements (10%). During the median disease duration of 78 months (range: 3-298 months), 10 patients were monophasic and 31 patients were relapsing: 26/41 (63%) patients experienced at least one brain attack, 23/41 (56%) patients experienced at least one optic neuritis attack, 20/41 (49%) patients experienced at least one myelitis attack. In 26 patients with brain attacks, 17 (65%) patients had ADEM-like lesions, 7 (27%) patients had fluffy brainstem lesions, and 2 (8%) patients had cortical lesions. Longitudinally extensive transverse myelitis was observed in 13 (65%) of 20 patients with myelitis, and 8 (35%) of 23 patients with optic neuritis showed bilateral involvement. At the last follow-up, simultaneous or successive poly-regional involvement was observed in 25/41 (61%) patients. In these patients, the most common clinical manifestation was combination of optic neuritis and brain attack (12/25, 48%), followed by combination of myelitis and brain attack (7/25, 28%), and optic neuritis and myelitis (3/25, 12%). Three (12%) patient showed optic neuritis, myelitis, and brain attack. The remaining 16 patients experienced isolated clinical syndromes such as isolated myelitis (n=7), optic neuritis (n=5), and brain attacks (n=4).

3.3 Determination of seropositivity by CBA-FACS

To further confirm the utility of IgG(H+L) with our FL-MOG stable cell line, sera from a total of 105/ 355 patients with CNS inflammatory diseases (65/105 patients were selected randomly and 40 MOG-IgG seropositive patients) and 25 HC were tested using CBA-FACS. Three different secondary antibodies, IgG (H+L), IgG1-Fc and IgM were used (figure 2A).

The serostatus of 105 patients found by CBA-IF were grouped into MOG-IgG seropositive and seronegative, and each patient's MFI ratio was determined by CBA-FACS. Using ROC curve analysis, the area under the curve for MFI ratio with IgG (H+L) was 1.00 and IgG1-Fc was 0.97 (figure 2B). We calculated the optimal cut-offs from the ROC curve to detect seropositivity of 105 patients. The optimal cut-offs for anti-human IgG (H+L) and IgG1-Fc secondary antibodies were 3.4 and 2.3, respectively.

The diagnosis at last follow-up of 105 patients and seropositivity of each patient group was determined (figure 3). All of patients with AQP4-IgG positive NMOSD, MS and ONDs were MOG-IgG negative when anti-human IgG (H+L) was used in the assay. On the other hand, anti-human IgG1-Fc secondary antibody detected MOG-IgG in 1 patient with AQP4-IgG seropositive NMOSD and 2 patients with MS (specificity = 95%). The MFI ratio of all 25 HC sera were below the cut-offs for both IgG (H+L) and IgG1-Fc antibodies. None of the samples bound to IgM antibody.

3.4 Correlation of CBA-IF score and CBA-FACS

The CBA-IF score was determined as described above and the correlation between CBA-IF

score and CBA-FACS results were also determined. The IF score of 40 MOG-IgG seropositive patients were: 10 patients had score 1, 13 had score 2, 10 had score 3 and 7 had score 4. The anti-human IgG (H+L) secondary antibody showed stronger positive correlation with CBA-IF score than IgG1-Fc secondary antibody (IgG (H+L): r=0.87, p <0.0001 vs IgG1-Fc: r=0.81, p <0.0001) (figure 4).

3.5 High concordance of (NCC) CBA-IF with (Oxford) CBA-IF

In order to further validate our assay, the serostatus of 125 patients (36 with AQP4-IgG positive NMOSD, 13 with AQP4-IgG negative NMOSD, 27 with MS, 21 with single or relapsing myelitis, 6 with single or relapsing ON and 22 with ODD) were compared with live IgG1 (Oxford) CBA-IF. The anti-human IgG (H+L) secondary antibody was used for (NCC) CBA-IF. Two methods had consistent results for 122/125 (98%) samples (κ = 0.919, p < 0.0001) where both methods found 21/125 patients as MOG-IgG positive and 101/125 patients as MOG-IgG negative. 3/125 (2%) patients were only positive by one assay, 2 by (NCC) CBA-IF and 1 by (Oxford) CBA-IF (table 4). Two patients who were only MOG-IgG positive by (NCC) CBA-IF were AQP4-IgG negative NMOSD patients. One other sample (patient 5) was strong positive for AQP4-IgG and borderline positive for MOG-IgG by (Oxford) CBA-IF, while positive for AQP4-IgG and negative for MOG-IgG by (NCC) CBA-IF. We have found positive binding not only in MOG-transfected cells and AQP4-transfected cells but also in EV-transfected cells by (NCC) CBA-IF and CBA-FACS (Supplementary figure 1A-D). The MOG-IgG positive binding was observed with both anti-human IgG (H+L) and IgG1-Fc secondary antibodies. The MOG-IgG MFI ratios were below the cut-offs with all three secondary antibodies (anti-human IgG (H+L), IgG1-Fc and IgM) (Supplementary figure

1B). The optimal cut-off for AQP4-IgG has not been established yet, so AQP4-IgG seropositivity cannot be determined by CBA-FACS. However, the level of AQP4-IgG was higher than MOG-IgG (MFI ratio MOG-IgG: 1.1 vs AQP4-IgG: 2.9). Notably, this patient had typical combination of optic neuritis and longitudinally extensive transverse myelitis with poor recovery.

4. Discussion

As with any other autoantibody detection, the methodology of antibody detection immensely impacts the clinical usefulness of the antibody assay. Here, we have developed a stable HEK293 cell line transfected with FL-MOG using a similar approach to our previous study (Kim et al., 2017). With this assay, we evaluated the sera from 355 patients with CNS inflammatory diseases and found seropositivity in 11.5% (41/355) of patients with diverse clinical phenotypes and courses, but within the known clinical spectrum of MOG antibody associated diseases. Neither typical MS patients nor HC showed seropositivity. No patients were double positive for MOG and AQP4 antibodies. CBA-IF was validated through comparison with live IgG1 (Oxford) CBA-IF using identical sera. Using CBA-FACS, we have confirmed that the specificity of IgG (H+L) secondary antibody is comparable to IgG1-Fc secondary antibody when used with FL-MOG transfected stable cell line, while MOG-specific IgM were absent. Moreover, performing CBA-FACS with anti-human IgG (H+L) secondary antibody yielded high concordance with CBA-IF score.

The most compelling finding about this study is maintenance of high specificity with the use of anti-human IgG (H+L) and our FL-MOG transfected stable cell line. This finding is in line with a recent study which reported the sensitivity and specificity were similar between anti-

human IgG (H+L) and IgG1 in MOG transfected stable cell line (Tea et al., 2019). Waters et al. found low specificity with the use of FL-MOG (α-1 isoform) and anti-human IgG (H+L) secondary antibody, partially due to cross-reactivity of the secondary IgG antibody with IgM antibody. They found that the specificity can be enhanced significantly with the use of IgG1-specific antibody (Waters et al., 2015). Despite concerns about using FL-MOG and IgG (H+L) antibody, the concordance of (NCC) CBA-IF and (Oxford) CBA-IF was 98% using same sera. There were three samples that did not correlate between the two centres. Two samples, only positive by (NCC) CBA-IF were from AQP4-seronegative NMOSD patients and had no IgM binding while the final sample, which was only positive by the (Oxford) CBA-IF for AQP4 and low positive for MOG, had a classical NMOSD clinical phenotype. This patient had typical combination of optic neuritis and longitudinally extensive transverse myelitis with poor recovery.

We speculate the difference in the expression of MOG between transiently and stably transfected cells could be the most plausible explanation for the discrepancy. This may also account for how (NCC) CBA-IF is able to maintain high specificity at serum dilution 1:20 while most other groups use 1:160 or higher to maintain specificity. Indeed, other groups maintained high specificity at low serum dilution (e.g. < 1:50) when FL-MOG transfected stable cell line and anti-human IgG secondary antibody was used (Dale et al., 2014; Ramanathan et al., 2014; Zhou et al., 2006).

In CBA, the MOG antigen is presented to the autoantibody repertoire in its native state where the tertiary structure of folded MOG is not altered. This allows autoantibodies to bind to MOG as it is expressed *in vivo*, hence positive antibodies identified using this methodology is more likely to have disease relevance. The production of recombinant proteins with proper

folding and post-translational modifications traditionally were limited to stably transfected cells, however, recent advances in transient transfection has changed this. The stable cell lines and the transiently transfected cells lines have antagonistic benefits and researchers can choose whichever method of transfection they prefer. The transient transfection is more labor intensive as the transfection must be carried out repeatedly, hence using stable cell lines, which is more flexible once made, would be beneficial for long-term use where CBA needs to be performed on a large scale and daily basis. In contrast, the stable transfection is a longer process hence, the ability to use transient expression when appropriate would be beneficial.

In this study, we have employed our previous method and generated a stable cell line that co-express MOG and GFP without any interference in their expression, using pIRES2-eGFP vector (Kim et al., 2017). Although both CBA-IF and CBA-FACS are both based on the expression of MOG in HEK293 cells, they have different advantages. When CBA-FACS was used, the analysis method and generation of optimal cut-off is critical to determine the serostatus. The MFI can be used to semi-quantify MOG antibodies without the need to perform serial dilutions in every experiment. Most studies used difference in MFI to determine seropositivity (Brilot et al., 2009; Dale et al., 2014; Selter et al., 2010; Waters et al., 2015). On the other hand, some studies used MFI ratio method (Lalive et al., 2011; McLaughlin et al., 2009; Probstel et al., 2011). This could be because experimental conditions, such as antibody dilution, degradation of tandem dyes and laser fluctuations, immensely affect the basal fluorescence intensity. As the level of background differs greatly between sera, there is a risk of evaluating samples with high background as negative using MFI differences as the MFI difference would be small. We used MFI ratio and optimal cut-off was determined through ROC analysis. Another advantage of CBA-FACS is that data can be saved and reanalyzed in the future and allows analysis of larger number of samples at one time. Most

importantly, flow cytometry based analysis can produce objective quantification results since scoring by visual inspection through fluorescence microscopy can be subjective. Nonetheless, the CBA-IF is widely used, and its clinical utility has already been well established. In the current study, CBA-FACS showed high correlation with CBA-IF score despite inclusion of 23/105 patients with low MOG-IgG seropositivity (IF score 1 or 2). Hence, both CBA-IF and CBA-FACS can be applied on a daily basis, whilst both methods have different characteristics and benefits.

Nonetheless, these results must be interpreted with caution and several limitations should be considered. Firstly, there was a selection bias. While 333 patients were selected randomly, 22 patients with known MOG-IgG serostatus was included. Hence, seropositivity of 11.5% does not represent MOG-IgG seroprevalence of our cohort. Secondly, the sample size was inconsistent across different experiments. Lastly, although we did not detect MOG-specific IgM antibodies in our samples, we cannot convincingly conclude that there was no IgM cross-reactivity with IgG (H+L) antibody as we did not have any MOG-IgM positive sera in our cohort to confirm this. Possible explanations may be that our patients never had IgM antibodies in their sera in the first place. Previous reports found that MOG-specific IgM antibodies are more frequently found in pediatric samples (Pedreno et al., 2019). From our total samples tested for IgM antibodies, we had only 8 pediatric samples (< 18 years). Also, since we have tested only a proportion of our entire cohort, MOG-specific IgM antibodies may have not been detected yet. Further study with larger number of samples is necessary to firmly conclude high disease specificity with no IgM cross reactivity and high concordance of both CBAs.

5. Conclusions

In conclusion, using our stable cell line, in-house CBA-IF was validated through comparison with the Oxford live MOG-IgG1 CBA. We have developed a FACS based CBA that has equivalent metrics to the CBA-IF. Prospective studies involving long-term follow-up of a larger unselected cohort with CNS inflammatory diseases are warranted to further support the clinical utility of the assay.

Declarations

- Ethics approval and consent to participate

The Institutional Review Board of NCC approved the present study and written informed consent was obtained from all participants.

- Consent for publication

Not applicable

- Availability of data and material

All data generated or analysed during this study are included in this published article.

- Competing interests

Kim YS, Hyun JW, Woodhall MR, Oh YM, Lee JE, Jung JY, Kim SY, Lee MY, Kim SH, Kim W and Choi K has nothing to declare. Irani SR and Waters P are co-applicants and receive royalties on patent application WO/2010/046716 entitled 'Neurological Autoimmune Disorders'. The patent has been licensed to Euroimmun AG for the development of assays for LGI1 and other VGKC-complex antibodies. SRI and PW are coinventors on ‘ADiagnostic Strategy to improve specificity of CASPR2 antibody detection.’ Kim HJ received a grant from the National Research Foundation of Korea;

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- Author Contributions

Kim YS had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Kim YS, Choi K and Kim HJ. Acquisition of data: Kim YS, Oh YM, and Lee JE and Woodhall, MR. Analysis and interpretation of data: All authors. Drafting of the manuscript: Kim YS and Kim HJ. Critical revision of the manuscript for intellectual content: All authors. Statistical analysis: Kim YS. Study supervision: Choi K and Kim HJ.

- Competing interests

Kim YS, Hyun JW, Woodhall MR, Oh YM, Lee JE, Jung JY, Kim SY, Lee MY, Kim SH, Kim W and Choi K has nothing to declare. Irani SR and Waters P are co-applicants and receive royalties on patent application WO/2010/046716 entitled 'Neurological Autoimmune Disorders'. The patent has been licensed to Euroimmun AG for the development of assays for LGI1 and other VGKC-complex antibodies. SRI and PW are coinventors on 'ADiagnostic Strategy to improve specificity of CASPR2 antibody detection.' Kim HJ received a grant from the National Research Foundation of Korea; received research support Genzyme, Merck Serono, Teva-Handok, and UCB; received consultancy/speaker fees from Celltrion, Eisai, HanAll BioPharma, MedImmune, Merck Serono, Novartis, Sanofi Genzyme, Teva-Handok, and UCB; serves on a steering committee for MedImmune/VielaBio; is a co-editor for the Multiple Sclerosis Journal – Experimental, Translational, and Clinical, and an associated editor for the Journal of Clinical Neurology.

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CRediT author statement

Yeseul Kim: Conceptualization, methodology, validation, formal analysis, investigation, writing, visualization. **Jae-Won Hyun:** Validation, formal analysis, resources, writing. **Mark R Woodhall:** investigation, formal analysis. **Yu-Mi Oh:** methodology, resources. **Ji-Eun Lee:** methodology, resources. **Ji Yun Jung:** validation, resources. **So Yeon Kim:** validation, investigation. **Min Young Lee:** resources. **Su-Hyun Kim:** resources, formal analysis, validation. **Woojun Kim:** resources. **Sarosh R Irani:** resources, funding acquisition. **Patrick Waters:** resources, methodology, validation, supervision, funding acquisition. **Kyungho Choi:** resources, methodology, supervision, writing. **Ho Jin Kim:** conceptualization, methodology, validation, formal analysis, resources, writing, supervision, project administration, funding acquisition.

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Figure legend

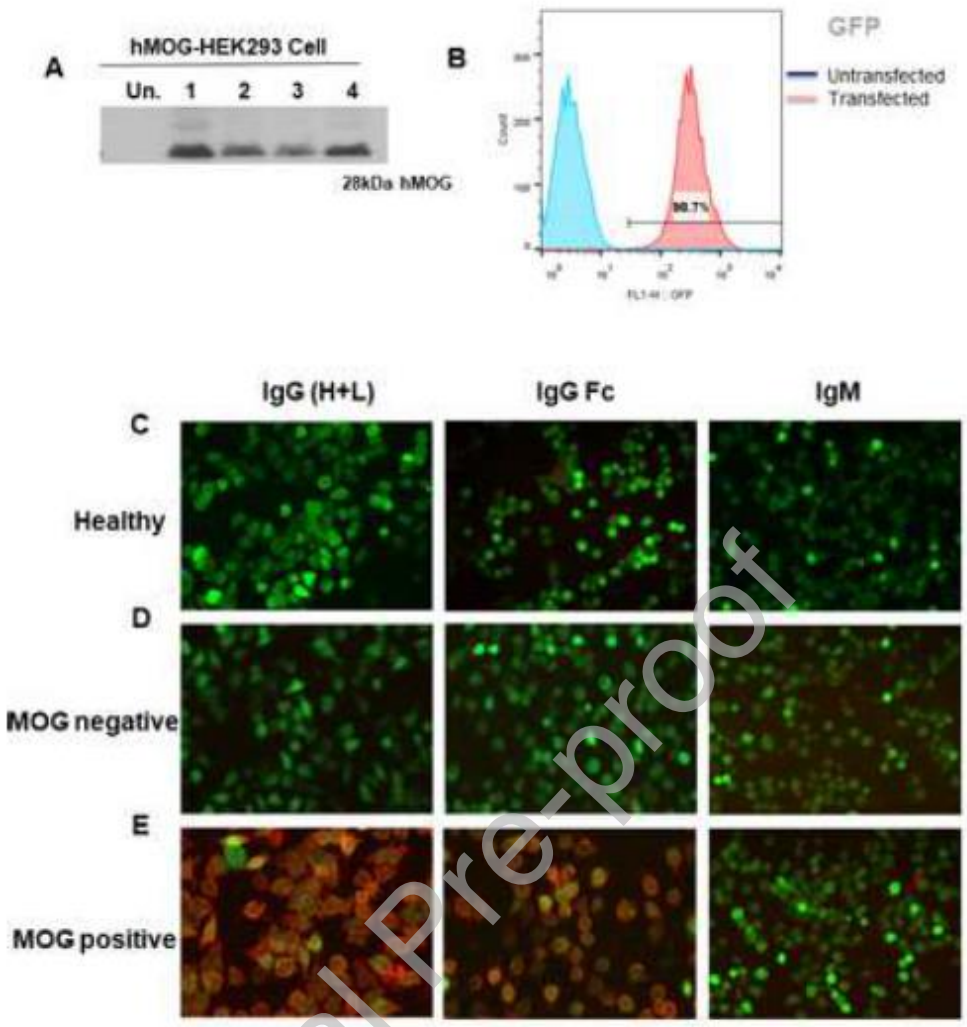

FIGURE 1. Development of in-house CBA-IF for MOG-IgG using HEK 293 cells stably transfected with MOG-IRES2-eGFP vector. A) MOG protein expression in the established cell lines analyzed by Western blot with anti-MOG antibody. Un, lysates of untrasfected cells; 1-4, lysates of the individual transfectant cell lines. B) GFP reporter expression in a representative cell line analyzed by flow cytometry. (C-E) CBA-IF was performed. Cells were stained with Alexa-594 for MOG-IgG after incubation with patient serum, then fluorescence was visually analyzed using fluorescence microscopy. MOG-IgG negative result when (C) healthy serum was used and (D) MOG-IgG negative patient serum was used. (E) MOG-IgG positive result. Serum IgG-binding to MOG is detected in HEK 293 cells expressing MOG (in red).

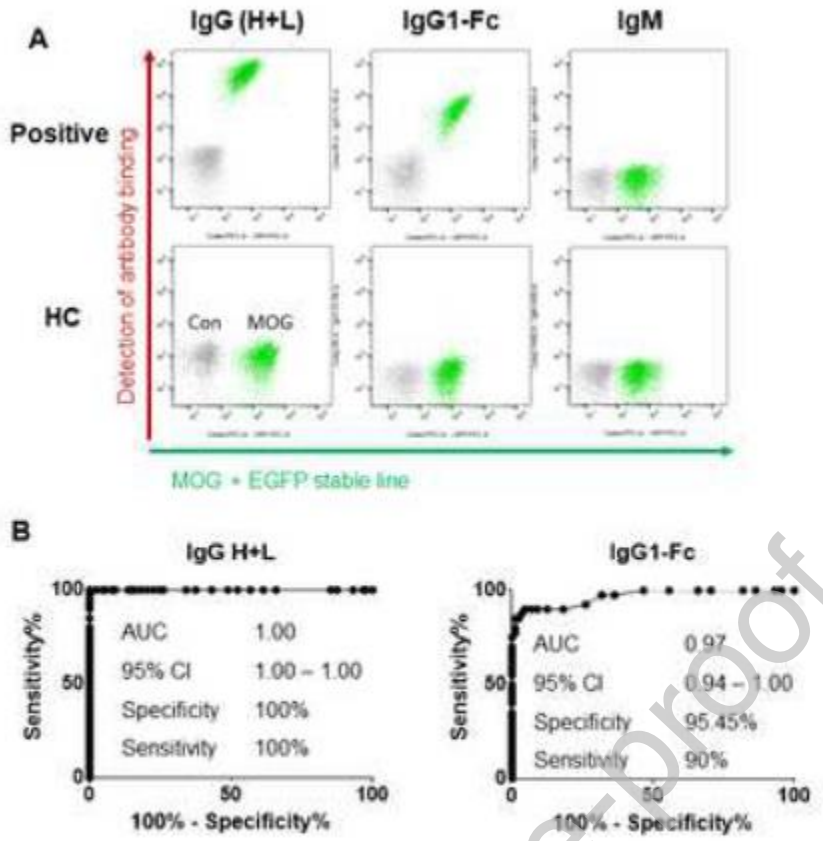

FIGURE 2. MFI ratio for determination of seropositivity by CBA-FACS (in colour).

(A) Representative dot plots of patient serum and HC serum binding to MOG transfected HEK cells**.** CBA-FACS was performed using both MOG-transfected HEK293 cells (MOG, green) and EV-transfected HEK293 cells (con, grey). Overlay of dot plots showing MOG-transfected cells as GFP+ cells and EV-transfected cells as GFP- cells. Positive control sera causes a specific shift in the MOG-transfected cells compared to EV-transfected cells when anti-human IgG (H+L) or anti-human IgG1-Fc secondary antibodies are used, but not when anti-human IgM secondary antibodies are used. Healthy control sera does not bind MOG using any of the secondary antibodies. (B) The 105 sera from a large cohort of CNS inflammatory diseases and 25 HC sera were tested using CBA-FACS. The receiver-operating characteristic (ROC) curve analysis was used to determine the specificity and

sensitivity of two different secondary antibodies. Using anti-human IgG (H+L) secondary antibody and MFI ratio method resulted in 100% specificity and sensitivity.

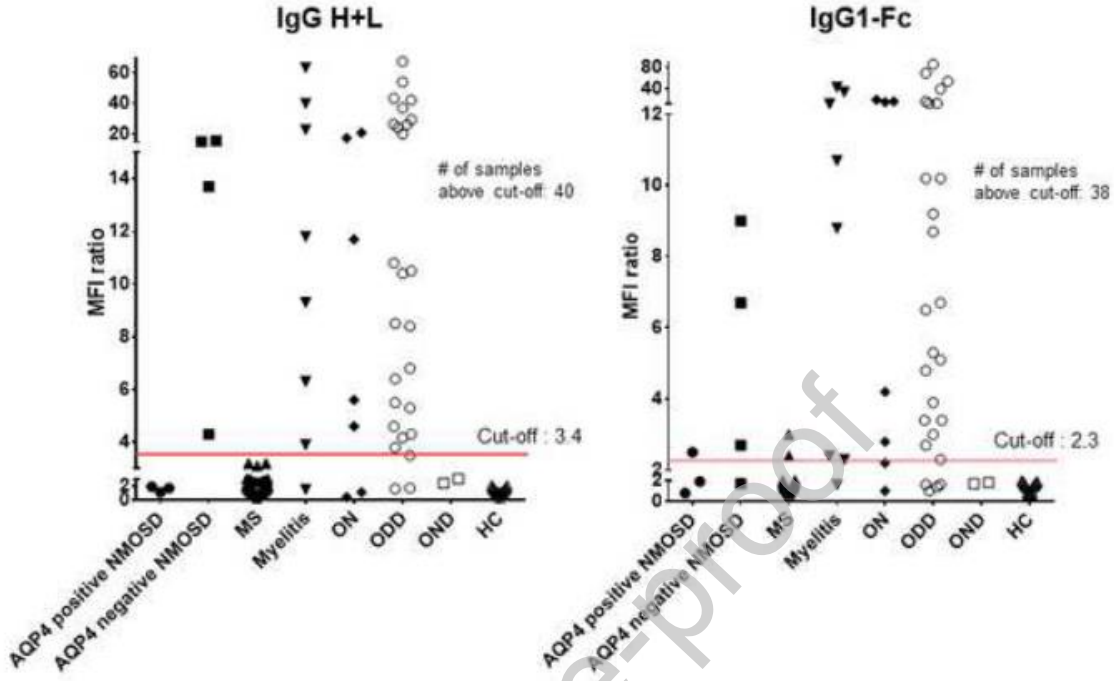

FIGURE 3. Evaluation of seropositivity of each patient group.

MFI ratio and seropositivity of 105 patients grouped according to their diagnosis at last follow-up. 40 samples were above cut-off for IgG (H+L) and 38 samples were above cut-off for IgG1-Fc.

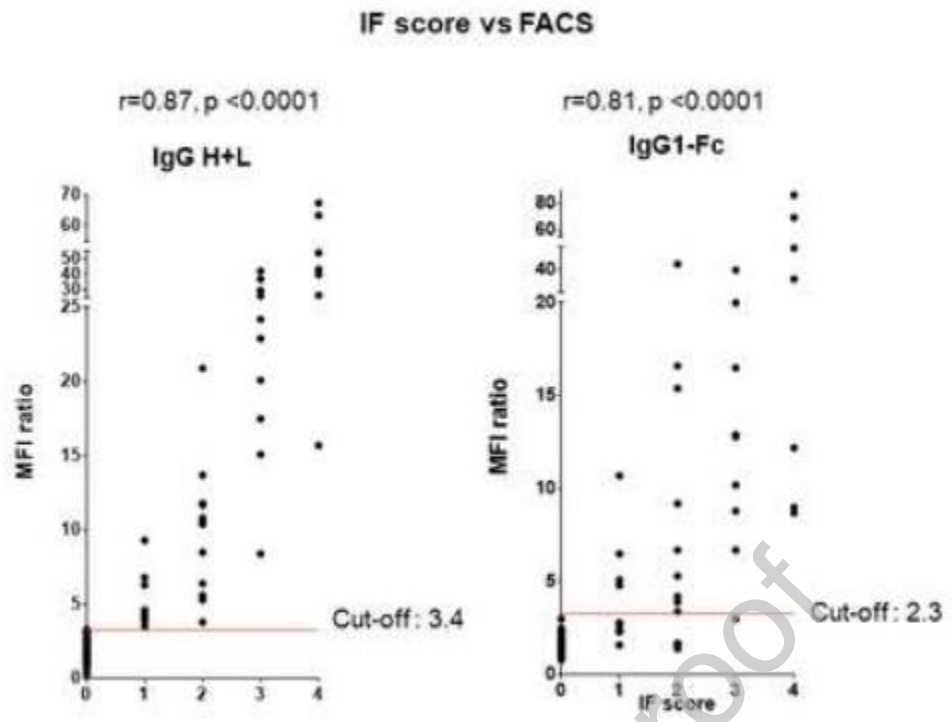

FIGURE 4. Correlation of CBA-FACS with CBA-IF score. Anti-human IgG (H+L) secondary antibody was used for both CBA-IF and CBA-FACS using identical sera. The optimal cut-offs were determined from ROC curve. For CBA-FACS, MFI ratio was used to determine seropositivity. The correlation of CBA-IF score and MFI ratio was determined.

Supplementary figure 1. MOG-IgG detection in serum from patient 5. Positive binding was observed in both MOG-HEK293 cells and HEK293 cells transfected with empty vector, when IgG (H+L) and IgG1-Fc was used in both A) CBA-IF and B) CBA-FACS. No IgM binding was observed. Positive binding was observed in both AQP4-HEK293 cells and HEK3293 cells transfected with empty vector by both C) CBA-IF and D) CBA-FACS.

Tables

Table 1. Description of CBA-IF score.

IF Score	Description
0	No binding around the cell membrane or staining that does not surround the cell membrane in a ring shape
0.5	Weak binding around the cell membrane but not surrounding the entire membrane of ~50% of visible cells
1	Clear but weak binding surrounding the entire membrane in a ring shape in > 50% of visible cells
2	Clear and moderate brightness surrounding the entire membrane in >75% of visible cells
3	Clear and bright binding similar to the brightness of positive control in >90% of visible cells
4	Very bright binding stronger than positive control in >90% visible cells

Table 2. Demographics and seropositivity of 355 patients with CNS inflammatory diseases at NCC.

Diagnosis at last follow-up	Number of patients	Males:Females	Median age at sampling (years,	Median disease duration at sampling	Number of MOG-IgG positive patients

			range)	(months, range)	
AQP4 positive NMOSD	65	11:54	37 (13-67)	43 (14-73)	0/65 (0%)
AQP4 negative NMOSD	37	9:28	41 (12-72)	29 (0-351)	4/37 (10.8%)
Multiple sclerosis	111	34:77	32 (16-65)	24 (0-286)	0/111 (0%)
Single or relapsing myelitis	52	32:20	40 (14-74)	18 (0-196)	7/52 (13.5%)
Single or relapsing optic neuritis	16	6:10	34 (15-63)	2 (0-87)	6/16 (37.5%)
Other demyelinating diseases including tumefactive demyelination or ADEM-like manifestation	55	23:32	34 (7-61)	7 (0-325)	24/55 (43.6%)
Other neurological diseases	19	11:8	49 (21-68)	6 (0-212)	0/19 (0%)

Abbreviations: AQP4 = aquaporin 4; NMOSD = neuromyelitis optica spectrum disorder; ADEM = acute disseminated encephalomyelitis.

Table 3. Demographics and clinical features of 41 patients with MOG-IgG.

Female-to-male ratio	28:13
Median age at sampling (years, range	31 (7-60)
Median disease duration at sampling (months, range)	8 (0-153)
Clinical phenotype	
Patients with at least one brain attack	26/41 (63%)
ADEM-like lesions	17/26 (65%)
Fluffy brainstem lesions	7/26 (27%)
Cortical lesions	2/26 (8%)
Isolated clinical syndrome	16/41 (39%)
M	7/16 (44%)
O	5/16 (31%)
B	4/16 (25%)
Poly-regional involvement	25/41 (61%)
O+B	12/25 (48%)
M+B	7/25 (28%)
O+M	3/25 (12%)
O+B+M	3/25 (12%)

Abbreviations: AQP4-IgG = aquaporin 4 immunoglobulin G; ADEM = acute disseminated

encephalomyelitis; M = myelitis; O = optic neuritis; B = brain.

Table 4. The concordance rate of NCC-CBA and Oxford-CBA

		NCC-CBA			κ = 0.919
		Positive	Negative	SUM	*p* <0.0001
Oxford-CBA	Positive	21	1	22	
	Negative	2	101	103	
	SUM	23	102	125	

Abbreviations: CBA = cell-based assay.