Journal of Autoimmunity 102 (2019) 38–49

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Myelin oligodendrocyte glycoprotein revisited—sensitive detection of MOG-specific T-cells in multiple sclerosis

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ARTICLE INFO

Keywords:
Autoimmunity
Multiple sclerosis
Myelin oligodendrocyte glycoprotein
Autoantigen
T-cell

ABSTRACT

Autoreactive $CD4^+$ T-cells are believed to be a main driver of multiple sclerosis (MS). Myelin oligodendrocyte glycoprotein (MOG) is considered an autoantigen, yet doubted in recent years. The reason is in part due to low frequency and titers of MOG autoantibodies and the challenge to detect MOG-specific T-cells. In this study we aimed to analyze T-cell reactivity and frequency utilizing a novel method for detection of antigen-specific T-cells with bead-bound MOG as stimulant.

Peripheral blood mononuclear cells (PBMCs) from natalizumab treated persons with MS (n = 52) and healthy controls (HCs) (n = 24) were analyzed by IFNγ/IL-22/IL-17A FluoroSpot. A higher number of IFNγ ($P = 0.001$), IL-22 ($P = 0.003$), IL-17A ($P < 0.0001$) as well as double and triple cytokine producing MOG-specific T-cells were detected in persons with MS compared to HCs. Of the patients, 46.2–59.6% displayed MOG-reactivity. Depletion of $CD4^+$ T-cells or monocytes or blocking HLA-DR completely eliminated the MOG specific response. Anti-MOG antibodies did not correlate with T-cell MOG-responses.

In conclusion, we present a sensitive method to detect circulating autoreactive $CD4^+$ T-cells producing IFNγ, IL-22 or IL-17A using MOG as a model antigen. Further, we demonstrate that MOG-specific T-cells are present in approximately half of persons with MS.

1. Introduction

Multiple sclerosis is an autoimmune disease of the central nervous system in which lymphocytes migrate through the blood brain barrier and cause inflammation, resulting in demyelination of axons and subsequent neurological damage [1]. As it is believed to be mainly $CD4^+$ T-cell driven, great efforts in the search for the target autoantigens have been made in the past decades [2]. Myelin-derived antigens, most importantly myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) have been implicated as autoantigens in MS, but the data are generally inconclusive and lack consistency. Although both T-cells and autoantibodies targeting these proteins have been found in some persons with MS, they have also been found in a fraction of healthy individuals [3,4]. Particularly, the role of MOG as a putative autoantigen has been clearly established in the mouse and rat model of MS, experimental autoimmune encephalomyelitis (EAE), where both MOG-autoantibodies and MOG-reactive T-cells are pathogenic [5,6]. However, more than other putative autoantigens, studies aiming to confirm the role of MOG in humans have not been as conclusive. MOG-reactive T-cells have been identified in some studies, while in others, they have been shown either to be present in both patients and healthy controls or absent in both [3,7–10]. Further, antibodies targeting MOG have in recent years been reported in other neuroinflammatory diseases like acute disseminated

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https://doi.org/10.1016/j.jaut.2019.04.013
Received 25 January 2019; Received in revised form 9 April 2019; Accepted 12 April 2019
Available online 01 May 2019
0896-8411/

encephalomyelitis and aquaporin-4 seronegative neuromyelitis optica but have rarely, or only in a smaller subset of patients been found in MS [11,12].

The interest in identifying autoantigens is not limited to MS but encompasses autoimmunity research in general as autoreactive T-cells are believed to play a key role in several autoimmune diseases. Besides their relevance from a basic disease-mechanism standpoint, means of identifying disease-eliciting rare circulating autoantigen specific T-cells pave the way for novel diagnostic and therapeutic strategies [13]. Antigen-specific immunotherapy has been suggested as the holy grail of treatments for autoimmune diseases as it has the potential to be highly effective while being free of the often severe side effects of current treatment strategies of non-specific immune-suppression [14]. In support of this notion, antigen-specific immunotherapy targeting myelin-antigens have been very effective in EAE. However, it has been difficult to translate such animal model results into treatment of humans [2,15–22]. One important reason for the lack of success is that the repertoire of autoantigens in MS is still not fully known and the required targets might not have been used in the clinical trials [2].

Several cellular analysis methods are available for detecting antigen-specific immune responses in clinical samples, e.g. ELISpot, proliferation assays and flow cytometry. Generally, challenges arise when the cells of interest are rare and currently available assays display a low signal to nonspecific cell noise [23]. Non-specific activation of many cell types may also be caused by the presence of minute amounts of contaminants such as endotoxins in the samples.

Here we aimed to develop a sensitive method able to detect autoreactive MOG-specific T-cells in MS patients, without a need of pure antigenic material.

2. Material and methods

2.1. Human subjects

Persons with MS (n = 57) were recruited at the neurology clinic at Karolinska University Hospital, Sweden. All patients were diagnosed with relapsing remitting MS according to the McDonald Criteria and were at the time of sampling on treatment with monthly injections of natalizumab. HLA-data from inclusion in previous studies at our institution was available for 31 out of 57 patients, 16 (52%) of whom were HLA-DRB1*15:01 positive. Healthy controls (HCs) were recruited and age and sex-matched (n = 27). Cohort demographics are presented in Table 1. Clinical data were collected retrospectively from electronic health records and the collection was blinded in regards to MOG-reactivity status. Another separate cohort consisting of patients (n = 16; 44% female; mean age ± SD 42.1 ± 5,8, range 32–53) and healthy controls (n = 9; 56% female; mean age ± SD 41.3 ± 12,6, range 24–58) was used for comparison of non-antigen beads and cell medium only.

Table 1
Cohort characteristics.

	Multiple sclerosis RRMS (n = 57)	Healthy controls (n = 27)
Age (years)	36.0 ± 9.8 (17-61)	34.5 ± 8.3 (20-48)
Sex (female)	40 (70.2%)	20 (74.1%)
EDSS	2.1 ± 1.4 (0.0–6.5)	–
Disease duration (years)	8.8 ± 5.4 (1-20)	–
Natalizumab treatment duration (years)	3.3 ± 1.9 (0.2–8.2)	–

EDSS: Expanded disability status scale. Numerical values presented as mean ± standard deviation with range in parenthesis.

2.2. Design of the MOG and ABD constructs

A full length *MOG* (UniProtKB - Q5SSB8) gene including flanking BsaI sites was designed, omitting the 29 aa signal peptide. The gene was subcloned into a modified pET28a expression vector (Merck-Millipore) containing an albumin binding domain (ABD) [24] in a one-step digestion-ligation reaction with BsaI and T4 DNA ligase as described [25]. The resulting fusion protein contained an N-terminal 6x histidine purification tag, ABD and MOG interspaced with a GGS linker. As a negative control protein, 6x histidine and ABD was produced (hereafter referred to as negative control (NC)) (Full sequences are available in the Supplementary Fig. 1).

2.3. Expression and purification of MOG and ABD

The vector containing the MOG and NC constructs were transformed into *E. coli* BL21-AI cells (Thermo Fisher Scientific). Cells were grown in 500 ml Super broth medium supplemented with 50 mg/L kanamycin, 0.6% Glycerol, 1 mM Mg2SO4, 0.2% arabinose, 0.2% lactose and 0.015% glucose for auto induction at 25 °C overnight. The cells were centrifuged at 9000 g, solubilized in 2.5 ml per gram pellet of lysis buffer containing 6 M Guanidinium-HCl, 10 mM Tris-HCl, 50 mM $NaHPO^4$, 100 mM NaCl and 20 mM β-mercaptoethanol, pH 8.0. Cells were frozen at −80 °C and thawed at 37 °C. The protein lysates were clarified at 23 500 g for 60 min and the supernatants stored at 8 °C until purification.

1.9 ml of protein solution was purified using a vial of His Mag Sepharose® Ni Beads (GE Healthcare), equilibrated in lysis buffer and incubated end-over-end for 2 h. The beads were washed two times with 1.5 ml, 6 M Urea, 500 mM NaCl, 0.05% Tween 20, 10 mM β-mercaptoethanol, 10 mM MES, pH 6.7, and one time with 1.5 ml non-salt wash buffer, 6 M Urea, 0.05% Tween 20, 10 mM β-mercaptoethanol, 10 mM MES, pH 6.7. The proteins were eluted with 3 × 300 µl elution buffer (6 M Urea, 0.05% Tween 20, 10 mM β-mercaptoethanol, 10 mM MES, pH 3.0). The eluted fractions were immediately adjusted to pH 5 by adding a pre-calibrated fraction of 0.82 M pH 6.0 MES buffer. The proteins were analyzed for purity by SDS-PAGE (Bolt 4–12%, Invitrogen) (Supplementary Fig. 1D) and the concentration was analyzed using NanoDrop (Thermo Fisher Scientific).

2.4. Coupling of antigen to beads

The antigen was covalently linked using amine coupling to paramagnetic polystyrene beads pre-functionalized with carboxyl groups with a diameter of 1 µm (Dynabeads MyOne, Thermofisher). A magnetic rack (Magrack 6, GE) was used to separate beads from wash solution. The beads were washed twice in 25 mM MES buffer, pH 6, before adding 0.1 M N-hydroxysuccinimide (Sigma-Aldrich), and 0,4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich) in MES-buffer and left for 30 min in room temperature. They were washed twice with MES-buffer before addition of antigen, 1 mg/ml in MES buffer, and incubated at RT for 30min. Still reactive carboxylic acid groups were quenched by 50 mM Tris-buffer pH 7.4. The beads were subsequently washed five times in sterile PBS. The no-antigen beads were prepared using the same method, omitting antigen.

2.5. Endotoxin removal

Buffer was added, the beads were vortexed and then collected using a magnet while the supernatant was discarded. The procedure was repeated four times for each different wash buffer. Antigen beads were first washed in solutions with concentration of NaOH ranging between 0.1 and 2 M depending on the coupled protein's ability to stimulate PBMCs from HCs (n = 2) in an IFNγ/IL-22/IL-17A FluoroSpot assay. If needed, 1% v/v Triton X-100 was also used. Lastly, all beads were washed with sterile PBS. The wash-condition with optimal background

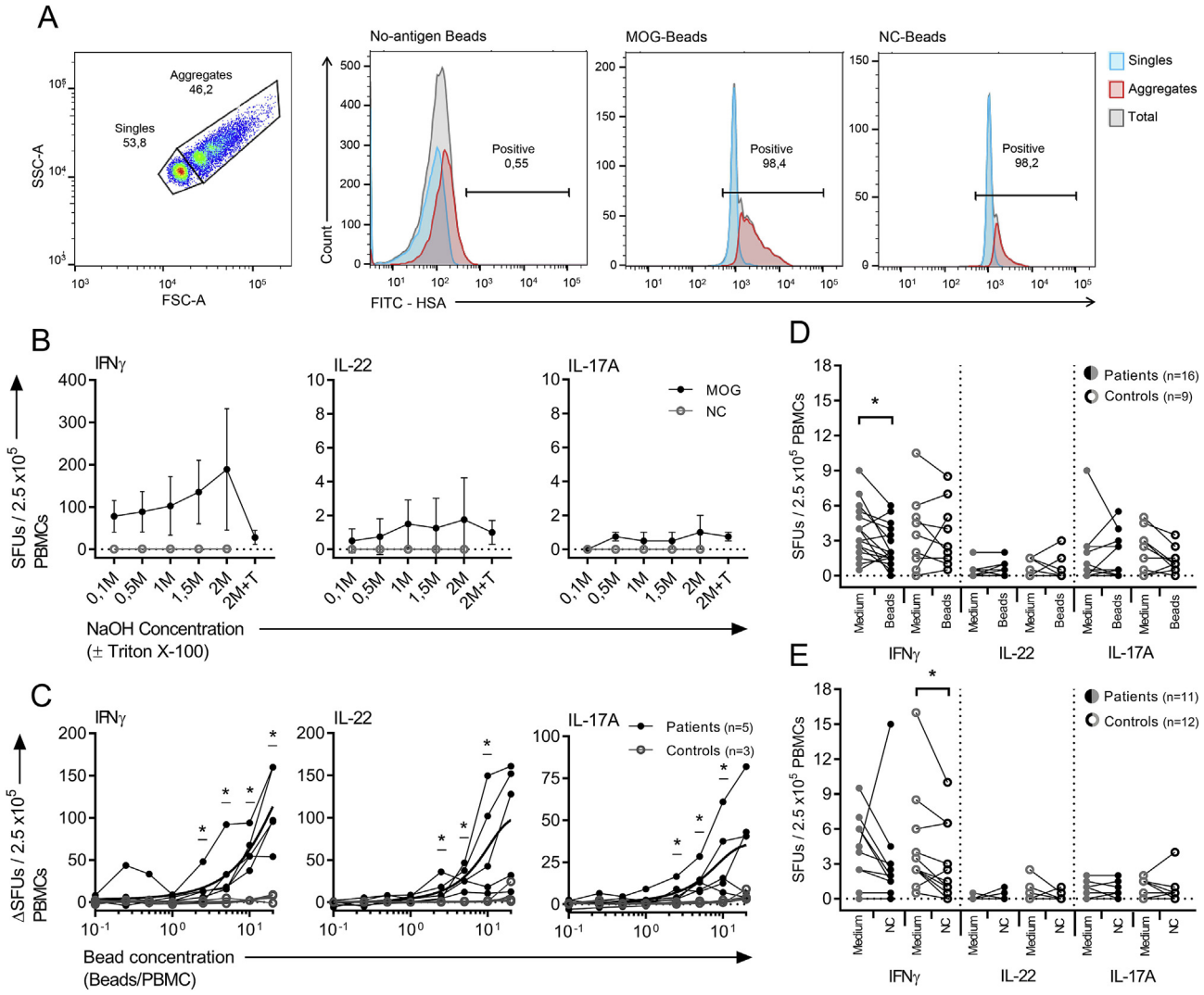

Fig. 1. Pre-study evaluation of the antigen beads. A. The albumin binding domain contained on both the MOG-beads and negative control beads were stained with FITC conjugated human serum albumin and analyzed by flow cytometry. Furthermost left plot shows representative forward and side-scatter gating for single and aggregate beads. Numbers denote the percentage of beads within the positive range (brackets). B. In order to determine which endotoxin removal wash to use, PBMCs from healthy controls (n = 2) were stimulated with MOG-beads washed with increasing concentration of sodium hydroxide and detergent in an IFNγ/IL-22/IL-17A FluoroSpot assay. MOG-stimulated in black, NC in grey. Circles and brackets represent mean and range. The concentration of sodium hydroxide in molarity (M) and addition of detergent (Triton X-100, 1% in PBS) (+T) is plotted on the X-axis. C. To evaluate the optimal bead concentration on patient and control response, PBMCs of MOG-reactive persons with MS (n = 5) and healthy controls (n = 3) were stimulated with increasing amount of MOG-beads. The thick line in represents the interpolated mean dose-response curve. To ensure that the response was antigen-dependent, PBMCs from patients (filled circles) and HCs (open circles) were stimulated with beads without antigen or beads with recombinant albumin binding domain (NC-beads) and compared to unstimulated PBMCs in an IFNγ/IL-22/IL-17A FluoroSpot assay. D. Comparison of no-antigen beads (black circles) and cell medium only (grey circles). E. Comparison of NC-beads (black circles) and cell medium only (grey circles) MOG: Myelin oligodendrocyte glycoprotein. NC: Negative control. HSA: Human Serum Albumin. P-values calculated with Mann-Whitney U test (C) or Wilcoxon Sign Rank test (D and E). *p < 0.05. SFUs: Spot forming units.

level was chosen for further experiments. For the NC beads 0.1 M NaOH and for the MOG-beads 2 M NaOH and 1% Triton X-100 was chosen.

2.6. LAL-assay

Endotoxin remaining after the bead-wash was measured in duplicates by a limulus amebocyte lysate (LAL) assay (Endpoint Endochrome Kit, R160K, Charles River Laboratories) according to the manufacturer's protocol. The beads were diluted to 10^9 beads/ml in endotoxin free water and incubated at 95 °C for 5 min prior to running the assay. Results outside the reference (0.018–0.6 EU/ml) were reported as above or below the closest standard point.

2.7. Flow cytometry assessment of bead-antigen coupling

To assess the coupling efficiency of the antigens to the beads, a flow cytometry protocol was devised, taking advantage of the ABD-tag. Five microliter FITC conjugated human serum albumin (FITC-HSA, Abcam) was incubated with 1–1.5 × 10^6 beads in 200 µl PBS for 15min. The beads were washed twice in PBS and resuspended in 150 µl PBS before analysis by flow cytometry (FACSVerse, BD biosciences, USA). Data-processing was performed in FlowJo™ 10 software (FlowJo LLC, USA). Percentage and homogeny of antigen coupled beads was evaluated based on the fluorescence in the single and aggregated bead gates. No-antigen beads were used as negative control to set the threshold for positivity.

2.8. Isolation of peripheral blood mononuclear cells

Venous blood samples were drawn using EDTA vacutainer tubes (BD diagnostics). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque plus (GE healthcare) in SepMate^{tm} (Stemcell Technologies) system according to the manufacturer's protocol. Red blood cells were lysed using ACK-lysing buffer (Sigma-Aldrich) for 5 min. Viable cells were counted using an automated cell-counter (Scepter™ 2.0 Cell Counter, Merck-Millipore, USA or LUNA-II, Logos Biosystems, South Korea). The cells were transferred to freezing medium, 45% RPMI 1640 (Sigma-Aldrich), 45% heat inactivated FCS and 10% DMSO, at a concentration of 10–15 × 10^6 cells/ml, frozen at a rate of -1 °C/min in a CoolCell container (Biocision, USA) to −80 °C and transferred to −150 °C within 24–48 h. Median (IQR) number of PBMCs obtained were 2.17 (1.63–2.87) x10^6 per ml of blood from patients and 1.43 (1.19–1.66) x10^6 per ml of blood from controls (p = 0.0001) (Supplementary Fig. 2).

2.9. IFNγ/IL-22/IL-17A FluoroSpot assay

PBMCs were thawed in a water bath at 37 °C and subsequently washed twice in cRPMI (RPMI 1640 (R8758, Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum (FBS, F7524, Sigma-Aldrich), 2 mM L-Glutamine (G7513, Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (P4333, Sigma-Aldrich)). PBMCs, 250,000 cells in 200 μl cRPMI per well, were added to a pre-coated 96-well FluoroSpot plate (Human IFNγ/IL-22/IL-17A FluoroSpot kit, Mabtech, Sweden), together with stimuli and incubated for 44 h at 37 °C, 5% CO_2. As stimuli, ~3 × 10^6/well (~12 beads/cell) MOG-beads were used. Anti-CD3 Abs, per manufacturer's protocol, and NC-beads were used as positive and negative control respectively. Plates were developed according to the manufacturer's protocol. Mean spot forming units (SFUs) from duplicates were counted with an iSPOT Spectrum (AID, Germany) reader except for the cell depletion and HLA-blocking experiments which was counted on an IRIS reader (Mabtech). Camera setting and spot parameters were adapted individually for each filter for optimal spot detection and all settings were kept consistent during the study. For the comparison of responses to MOG, both patient and control samples were analyzed simultaneously in one FluoroSpot plate, at a proportion of 1:1 or 3:1. Five patients and three controls were excluded from analysis, either due to a high number of spots (> 90 for IFNγ or > 20 for IL-22 or IL-17A) in the negative control or a low number of spots (< 200 for IFNγ) in the positive control.

2.10. Cell depletion

PBMCs from MOG-reactive patients (n = 5) were thawed, washed, counted, resuspended in cold PBS supplemented with 2% FBS and divided into six 1 ml fractions containing 1.5–2.5 × 10^6 cells each (See Fig. 3A for graphical representation). To each fraction, 25 μl depletion beads anti CD4 (11145D), CD8 (11147D), CD14 (11149D) or CD19 Pan B (11143D) (Dynabeads, ThermoFisher) were added for cell depletion. For control purposes, one fraction was mock-depleted, using beads (Dynabeads M − 450 Epoxy, 14011, ThermoFisher) conjugated with mouse monoclonal IgG of irrelevant specificity and one fraction was left without beads. All fractions were incubated for 40 min at 4 °C with end-over-end mixing. The tubes were placed in a magnetic rack (MagRack 6, GE Healthcare) and the supernatant containing the non-depleted cells were put in fresh tubes and washed once. Cell depletion efficiency was analyzed from 30 μl cell suspension by flow cytometry. The fractions were resuspended in equal amounts of cRPMI and analyzed in parallel in a FluoroSpot plate as described in section 2.9.

Depletion efficiency was assessed by flow cytometry (LSRFortessa™, BD biosciences, USA) after staining the cells with αCD3-FITC, αCD4-APC, αCD8-PE-Cy7, αCD14-BrilliantViolet510 (300406 Clone UCTH1, 300514 Clone RPA-T4, 344712 Clone SK1, 301841 Clone M5E2, respectively, BioLegend), and αCD19-APC (561742, Clone HIB19, BD). Median (IQR) depletion efficiency was 99.6% (95.1–99.7%), 99.9% (99.0–100%), 91.8% (91.3–97.0%) and 99.7% (99.2–99.9%) for the $CD4^+$, $CD8^+$, $CD14^+$ and $CD19^+$ depletion respectively (Supplementary Fig. 3). Depletions with an efficiency of < 90% were excluded from analysis.

2.11. HLA-blocking

PBMCs from patients earlier shown to be MOG-reactive (n = 10) were tested in an IFNγ/IL-22/IL-17A FluoroSpot assay as described in section 2.9, either stimulated with MOG-beads only or with MOG-beads in the presence of 10μg/ml anti HLA-DR, anti HLA-DQ or anti HLA-DP monoclonal antibodies (ab136320 clone L243, ab23632 clone SPV-L3, ab20897 clone B7/21 respectively, Abcam).

2.12. Anti-MOG antibody assays

Anti-MOG antibodies were detected in patient serum samples (n = 29) using a cell-based assay essentially as previously described [12]. In short, HeLA cells were transfected with either human MOG fused with enhanced green fluorescent protein (EGFP) or EGFP only. The transfected cells were incubated with a 1:50 dilution of patient serum for 45 min at 4 °C. Afterwards the cells were incubated with a secondary antibody (biotin conjugated goat anti-human) for 30 min at 4 °C and subsequently stained with AlexaFluor 647-conjugated streptavidin for 30 min and analyzed with FACS. The readout was MFI ratio (MOG-EGFP transfected cells/MFI of EGFP-only transfected cells). The recombinant monoclonal anti-MOG human IgG1 Ab r8-18C5 [26] at different concentrations (0.5, 0.1, 0.05 μg/ml) was used as positive control. A cut-off value for positivity was determined as 3 SD above the mean MFI ratio of control samples (MFI = 2,27) tested in the same assay previously [12,27].

As antigen in the ELISAs, the recombinant extracellular part of MOG produced in HEK cells with an Avi-Tag was used [28]. MaxiSorp 96-well plates and Streptavidin-coated 96-well plates were coated with human-MOG (10μg/ml, overnight, 4 °C) or biotinylated human-MOG (3μg/ml, 2 h, room temperature). As a negative control, wells were coated with BSA (10μg/ml or 3μg/ml respectively). The plates were washed and subsequently blocked with 3% BSA or 3% BSA +5 μM d-biotin in PBS overnight at 4 °C. Patient sera (1:100 dilution in PBS 0.5% BSA) or anti-MOG mAb (clone r8-18C5 at 50, 10, 2 and 0.4 ng/ml) were added and incubated for 2 h at room temperature. The plates were washed and secondary antibody (HRP-conjugated anti-human IgG Ab diluted 1:5000 in 0.5% BSA in PBS (109036003, Jackson)) was added and incubated for 1 h at room temperature. Plates were developed with 100 μl/well of TMB-substrate for 5–20 min before reaction was stopped with H_2SO_4. OD was read at 450 nm, plate background at 540 nm. Anti-MOG OD was calculated as OD (MOG-wells) minus OD (BSA-wells) minus plate background.

2.13. Statistics

For statistical analysis Prism 7 (GraphPad, USA) was used and the level of significance was set to $P < 0.05$. Main FluoroSpot results were analyzed by two-tailed Mann-Whitney U test. Before analysis the mean number of spots in the negative control wells (NC-beads) were subtracted from the mean number of spots in the wells containing MOG-beads and presented as spot forming units above negative control (ΔSFU). Fischer's exact test was used to compare the proportion of positive patients and controls as well as the differences of ordinal data between of MOG-T-cell positive and MOG-T-cell negative patients. Differences in disease and treatment duration and age were analyzed by student's T-test and EDSS scores by Mann-Whitney U test. P-values were adjusted using Holm-Sidak correction for multiple comparisons. A two-tailed paired t-test with Holm-Sidak correction for multiple

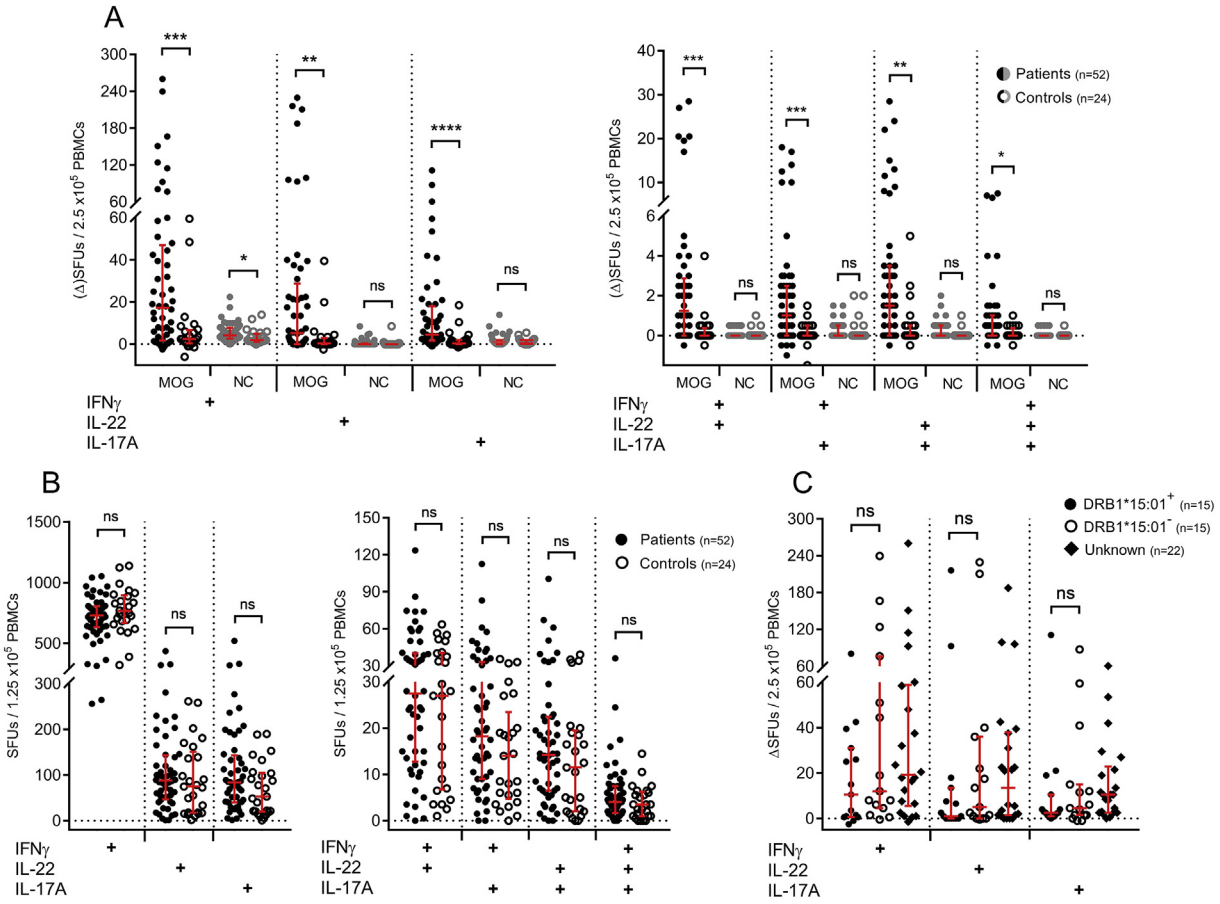

Fig. 2. MOG-beads activate PBMCs from persons with MS to a higher degree than PBMCs from healthy controls. PBMCs from persons with MS (filled circles, n = 52) and age- and sex-matched healthy controls (open circles, n = 24) were stimulated for 44 h with recombinant antigen coupled to 1 μm paramagnetic beads in an IFNγ/IL-22/IL-17A FluoroSpot assay. A. Single and multiple cytokine FluoroSpot results when stimulated with MOG (black circles) or the negative control (NC) (grey circles). MOG-results are plotted as ΔSFUs, NC-results as SFUs. B. FluoroSpot results when stimulated with the polyclonal T-cell activator anti-CD3 Ab. C. FluoroSpot results of persons with MS when stimulated with MOG, stratified based on HLA-DRB1*15:01 status. Dots represents the response of one individual. Staples denote median and IQR. P-values calculated with Mann-Whitney U test. ns $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. SFUs: Spot forming units. ΔSFUs: Number of spot forming units above the negative control. MOG: Myelin oligodendrocyte glycoprotein. NC: Negative control. PBMCs: Peripheral Blood Mononuclear cells.

comparisons (four per readout) was used to compare the proportional FluoroSpot results ($\Delta SFU^{depleted}/\Delta SFU^{PBMCs}$) after cell depletion to that of the mock-depletion. The effects of different HLA-blockers were tested with a two-tailed Wilcoxon signed rank test and adjusted P-values were calculated with the Holm-Sidak correction for multiple comparisons (three per readout). Spearman's test was used to test correlation between FluoroSpot SFUs and autoantibody MFI. Wilcoxon signed rank test was used for comparison of the response towards NC-beads, no antigen-beads and media.

2.14. Study approval

Patients and controls gave their written informed consent to participate in the study. The study was conducted in accordance to the Declaration of Helsinki principles and approved by the regional ethical board, Regionala Etikprövningsnämnden in Stockholm (application no. 2009/2107-3112 and no. 2015/1161-31/4).

3. Results

3.1. Evaluation of antigen-coupled beads

Before use as stimuli in the study, the antigens were covalently coupled to paramagnetic polystyrene beads with a diameter of 1 μm, a size chosen after testing different beads in a pilot experiment (data not shown). In order to ensure a comparable and even coupling of the proteins, the beads were labeled with fluorescent human serum albumin (HSA) and examined by flow cytometry. The beads were sorted into singles and aggregates (Fig. 1A). Approximately half of the events (MOG-beads: 47.7%, NC-beads: 66.2% no-antigen beads: 53.8%) were detected as single beads while the remaining were aggregated. Regardless of single or aggregated, the amount of antigen on the beads was high and homogenous for both the MOG- and negative control (NC)-beads. More than 98% stained positive compared to 0.55% of the no-antigen beads (Fig. 1A).

As both MOG and Albumin binding domain (ABD) protein, used for the MOG- and NC-beads respectively, were produced in *E. coli*, they may have been contaminated with a considerable amount of endotoxin. After bead coupling of antigens, stringent removal of the endotoxins was performed using sodium hydroxide and detergents. To choose an appropriate wash for optimal signal to noise ratio in follow-up assays, different wash-conditions were evaluated by stimulating PBMCs from healthy controls (n = 2) in an INFγ/IL-22/IL-17A FluoroSpot assay (Fig. 1B). For the subsequent experiments, the chosen wash conditions were 2 M NaOH, 1% Triton X-100 and PBS for MOG and 0.1 M NaOH and PBS for the NC beads. Post-wash, the amount of remaining endotoxin was measured using a limulus amebocyte lysate-assay (Table 2). The NC-beads contained 3.5 times more endotoxin than the

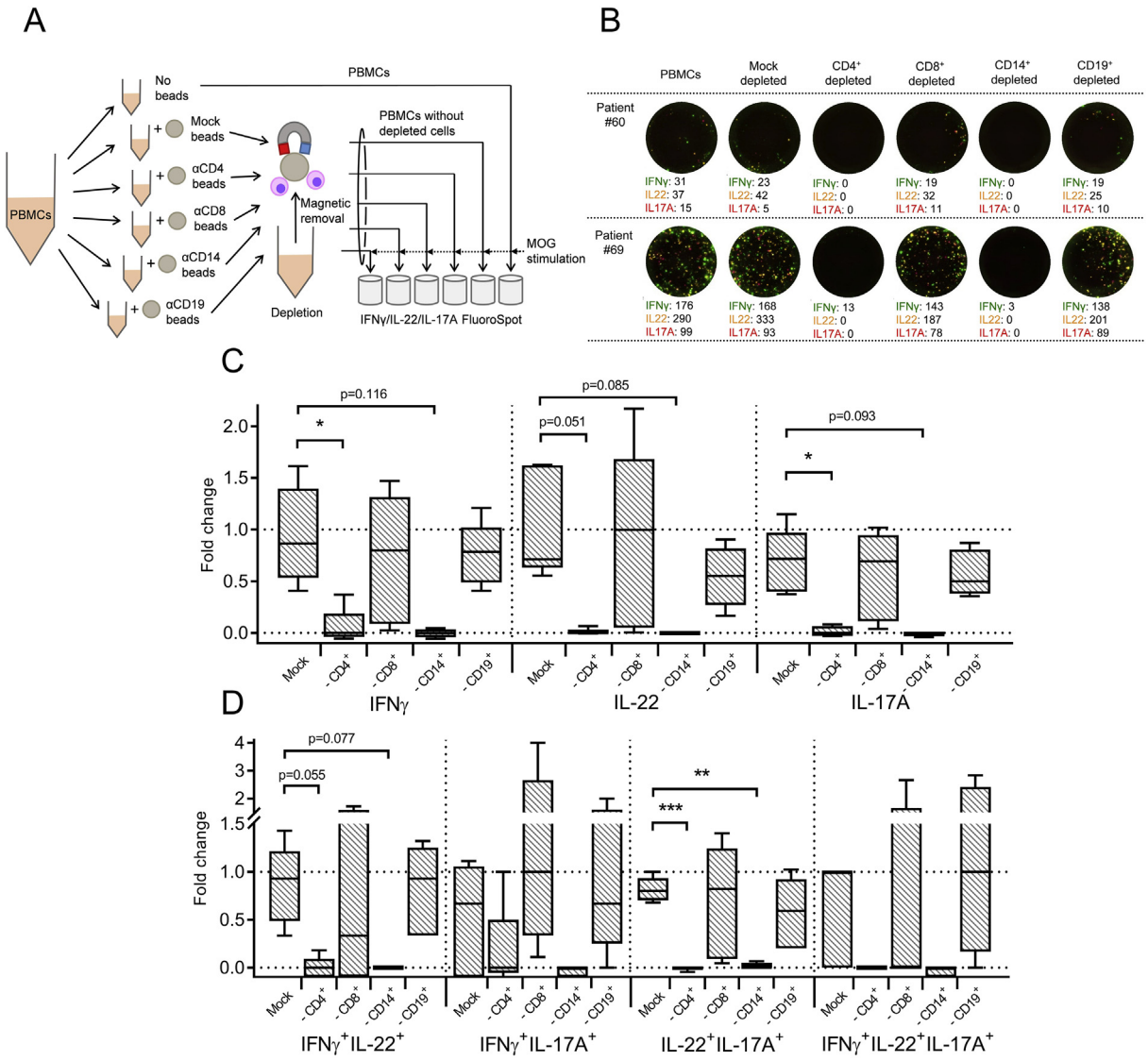

Fig. 3. MOG-reactivity is $CD4^+$ T-cell and $CD14^+$ cell dependent. PBMCs from MOG-reactive MS patients (n = 5) were depleted of either $CD4^+$, $CD8^+$, $CD14^+$ (n = 4) or $CD19^+$ cells or underwent mock-depletion before stimulation for 44 h with MOG in an IFNγ/IL-22/IL-17A FluoroSpot assay. A. Schematic overview of the experiment. B. Representative FluoroSpot pictures from two patients after MOG stimulation with spot forming unit (SFU) counts. C. Single cytokine responses to MOG after depletion of different cell subsets. D. Multiple cytokine responses to MOG after depletion of different cell subsets. Fold change calculated as $\Delta SFUs^{Depleted}/\Delta SFUs^{PBMC}$. Box represents median and IQR. Whiskers denote range. P-values calculated by comparing mock-depletion with cell depletion using a paired t-test with Holm-Sidak correction for multiple comparisons. P-values that were < 0.05 before correction but not after are written as exact post-correction values. *p < 0.05, **p < 0.01, ***p < 0.001.

Table 2
Endotoxin contamination of the antigen-beads.

	LPS EU/10^9 beads	Resulting LPS in FluoroSpot assay EU/ml
MOG-beads	0.025	0.00031
NC-beads	0.088	0.0011
FBS	–	> 0.06

LPS: Lipopolysaccharide MOG: Myelin oligodendrocyte glycoprotein. NC: Negative control. FBS: Fetal bovine Serum.

MOG-beads. However, when adjusted for the amount used in the FluoroSpot assay, both beads contained < 2% of the endotoxin level that was found in the fetal bovine serum used in the cell-medium in following assays.

The effect of bead concentration in the FluoroSpot-assay was tested in five MOG-reactive patients and three representative non MOG-reactive healthy controls (Fig. 1C). The concentration of MOG-beads was titrated from 0.1 to 20 beads per PBMC. T-cell reactivity was recognized at 2.5 beads/cell and increased with number of beads, following a sigmoidal dose-response curve. An optimal separation of patients and controls was determined at ~10 beads per PBMC.

To ensure that the beads did not induce cell activation intrinsically, PBMCs from persons with MS (n = 16) and HCs (n = 9) were stimulated with no-antigen beads or no stimuli (cell medium only) (Fig. 1D). Similarly, to explore whether the ABD tag contained in the MOG-construct could illicit a significant, potentially confounding, response in our assay, a comparison between NC-beads, i.e. ABD-beads, and no stimuli was made with patients (n = 11) and HCs (n = 12) (Fig. 1E). Neither the beads without antigen nor with ABD activated PBMCs to a higher degree than no stimuli. On the contrary, patients displayed a significantly lower IFNγ response towards the non-antigen beads (p = 0.02) and HCs a significantly lower response towards the NC-beads compared to medium (p = 0.0156). However, the difference in absolute number of spots was very low. Altogether, this shows that the

antigen-bead induced cell activation in this context is antigen-specific.

3.2. Increased frequencies of MOG-specific autoreactive cells in persons with MS

To investigate autoreactivity against MOG, PBMCs from 52 MS patients and 24 age -and sex matched healthy controls (HCs) were stimulated with bead-bound MOG and evaluated for cytokine production in an INFγ/IL-22/IL-17A FluoroSpot assay (Fig. 2). There was a significantly higher response of persons with MS compared to HCs with median (IQR) spot forming units above negative control (ΔSFUs)/ 2.5×10^5 PBMCs for IFNγ: 17.25 (1.75–47.1) vs 2.5 (0.5–6.75) (P = 0.001), IL-22: 5.25 (0–28.9) vs 0.5 (0–3.0) (P = 0.003) and IL-17A: 4.5 (1.6–18.0) vs 0.5 (0–2.0) (P < 0.0001). There was also a difference in reactivity in response to the negative control-beads (NC-beads) for IFNγ: Patients ΔSFUs 4.25 (2.6–7.9) vs Controls ΔSFUs 2 (1.1–5.1) (P = 0.016), while no difference in reactivity was recorded for IL-17A or IL-22 (Fig. 2A). Additionally, for patients the amounts of SFUs correlated between the different cytokines (p < 0.0001 for all combinations) when stimulated with MOG (Supplementary Fig. 4). Interestingly, IL-22 and IL-17A were more closely correlated than either were with IFNγ, with an r^2 of 0.82 as compared to 0.41 and 0.37 respectively.

With the FluoroSpot assay, simultaneous detection of cells producing several cytokines is possible [29]. While rarer than single cytokine producing cells, a similar comparatively higher response for persons with MS was observed for all cytokine combinations; $IFN\gamma^+IL\text{-}22^+$: 1.25 (0–2.875) vs 0 (0–0.375) (P = 0.0001), $IFN\gamma^+IL\text{-}17A^+$: 1 (0–2.5) vs 0 (0–0.5) (P = 0.0004), $IL\text{-}22^+IL17^+$: 1.5 (0–3.5) vs 0 (0–0.5) (P < 0.0033) and triple cytokine producing cells: 0 (0-1) vs 0 (0–0.375) (P = 0.0290) (Fig. 2A). The median responses to the NC-beads were 0 in both patients and controls for all combinations and no individual had > 2 SFUs, demonstrating a very low background noise level.

To ensure that the responses to MOG were not due to an over-representation of cytokine producing T-cells in general, a comparison between the responses to the polyclonal T-cell activator anti-CD3 Ab was made. There was a large inter-individual variation, but no difference between patients and controls was observed for any cytokine or combination thereof (Fig. 2B). Additionally, there was no correlation between the absolute number of PBMCs in the blood and MOG-specific cells for any cytokine or cytokine combination (p > 0.05 for all cases) (data not shown).

Further, we explored whether the MS-risk related HLA variant HLA-DRB1*15:01 correlated to the MOG-response. HLA typing from inclusion in previous studies was available in our database for 30 out of the 52 analyzed patients, 15 (50%) of which were HLA-DRB1*15:01 positive (hetero- or homozygote). There was no difference in MOG-response between HLA DRB1*15:01 positive and negative patients (Fig. 2C).

These data strongly suggest that there is an increased frequency of MOG-specific autoreactive cells producing pro-inflammatory cytokines in persons with MS compared to in healthy controls.

3.3. Sensitivity and specificity of MOG autoreactivity

In order to compare MOG reactive frequencies in patients and HCs we first defined the cut-off for a positive response as a ΔSFU above three standard deviations of the background (NC-beads) (Supplementary Table 1). As the background differed slightly, the cut-off was set separately for patients and HCs. Out of the single cytokines, IFNγ was the most sensitive analyte with 28 of 52 patients and 3 of 24 controls (p = 0.0009) showing a positive response while IL-17A was the most disease specific with a response in 24 of 52 patients and 2 of 24 (p = 0.0014) controls. Out of all readouts, cells producing both IFNγ and IL-17A were the most disease specific, with 24 of 52 positive patients and 0 of 24 positive controls (p < 0.0001). Based on a mean + 3SD cutoff of NC-response as a threshold for detection, the method was able to detect frequencies of cytokine producing antigen specific cells of > 1 in 13,000 PBMCs for IFNγ, > 1 in 40,000 for IL-22 and > 1 in 25,000 for IL-17A. For double-positive cells the detection threshold was as low as > 1 in 125,000 for all combinations.

In order to test the method's functionality as a possible diagnostic test, a second method, receiver operating characteristic (ROC) analysis, was used to find the cutoff which optimally separated patients from controls (Supplementary Table 1 and Supplementary Fig. 5). For the most sensitive cytokine, IFNγ, this analysis yielded a sensitivity (95% CI) of 53.9% (39.5–67.8%) and specificity of 91.7% (73.0–99.0%). Again, $IFN\gamma^+IL\text{-}17A^+$ generated the most specific result with a sensitivity of 42.3% (28.7–56.8%) and specificity of 100% (85.8–100%). In summary, using bead-bound MOG as stimuli and an appropriate negative control for deciding a threshold for positivity, MOG-reactivity was found in approximately half of persons with MS.

3.4. MOG-specific auto-reactivity is $CD4^+$ T-cell mediated

To further explore the MOG-reactivity in persons with MS, we depleted either $CD4^+$, $CD8^+$, $CD14^+$ or $CD19^+$ cells from PBMCs of persons with MS (n = 5, n = 4 for $CD14^+$ depletion) before performing the IFNγ/IL-22/IL-17A FluoroSpot analysis (Fig. 3A). Depleting $CD4^+$ T-cells removed essentially the whole MOG-response for all cytokines, with a mean (95% CI) reduction of 94.1% (72.3–100%), 98.8% (95.6–100%) and 98.6% (92.4–100%) for IFNγ, IL-22 and IL-17A respectively (Fig. 3C). Compared to mock-depletion the reduction was significant for IFNγ (p = 0.0448) and IL-17A (p = 0.0269), but not for IL-22 (p = 0.051). A similar reduction was seen for all multiple cytokine combinations, however only significant when compared to mock-depletion for $IL\text{-}22^+IL\text{-}17A^+$ (p = 0.0004) (Fig. 3D).

$CD14^+$ depletion likewise extinguished essentially the whole MOG-response, with a mean reduction of > 98% for all cytokines and cytokine combinations, however only statistically significant compared to mock-depletion for $IL\text{-}22^+IL\text{-}17A^+$ (p = 0.0024). In contrast to the $CD4^+$ T-cell and $CD14^+$ depletion, neither $CD8^+$ T-cell nor $CD19^+$ cells affected the MOG induced activation for any cytokine readout. This suggests that the MOG-response is mediated by MOG-specific $CD4^+$ T-cells and that the method requires the presence of phagocytizing cells for antigen presentation.

3.5. HLA-restriction of MOG-autoreactivity and correlation with antibody responses

In order to investigate whether the MOG-specific T-cell responses are restricted in regards to MHC class II subclasses, PBMCs from MOG-reactive patients were stimulated with MOG-beads in a FluoroSpot assay in the presence of anti-HLA-DR, -DQ or -DP (n = 9, n = 7 for anti-HLA-DP) blocking antibodies (Fig. 4A). Anti-HLA-DR antibodies extinguished the response for all cytokines (p = 0.0117 for IFNγ, p = 0.0232 for IL-22, p = 0.0117 for IL-17A). Contrastingly, neither anti HLA-DQ nor -DP antibodies decreased the MOG-specific response significantly, rather -DP antibodies increased the IFNγ response (p = 0.031). In all, this suggests that MOG-epitopes have to be presented via HLA-DR to be recognized by autoreactive T-cells.

We also investigated the prevalence of MOG-specific autoantibodies in our patient cohort, using a cell-based assay for detection of antibodies in serum (Fig. 4B). Of the 29 patients tested, 16 (55%) were positive for MOG-specific T-cells in the FluoroSpot assay but only one were positive for MOG-autoantibodies, demonstrating that MOG-specific B-cell responses do not correlate with T-cell reactivity to MOG (p > 0.05 for all comparisons) and are rarer than T-cell responses in MS (p < 0.0001). Additionally, two different ELISA-tests for MOG-antibodies were performed, showing similar non-correlation and low MOG-Ab levels (Supplementary Fig. 6).

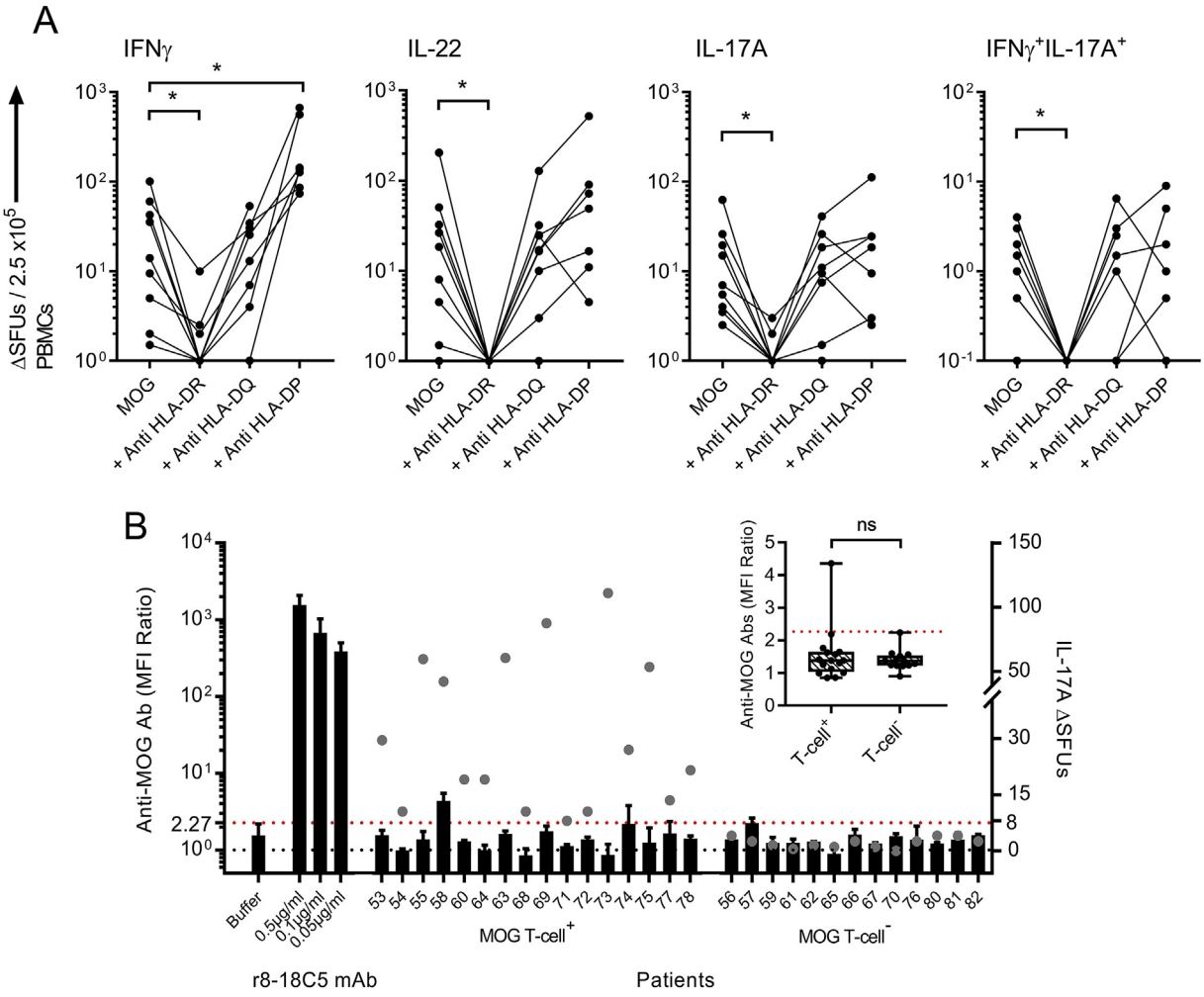

Fig. 4. MOG-recognition of T-cells is HLA-DR restricted and does not correlate with antibody response. A. FluoroSpot results after stimulation of PBMCs from MOG-reactive patients (n = 9) with MOG alone or MOG in the presence of HLA-DR, -DQ or -DP (n = 7) blocking antibodies. Each line represents one individual. Adjusted P-values were calculated using Wilcoxon signed rank test with Holm-Sidak correction for multiple comparisons. *p < 0.05. ΔSFU-values below zero are shown as the lowest Y-axis value. B. Presence of MOG specific antibodies detected with a cell-based assay testing patient sera (n = 29, 16 of which had MOG responses in the FluoroSpot assay). Bars and staples denote mean and SD of two separate tests of anti-MOG antibodies (plotted on left axis). Grey circles represent IL-17A FluoroSpot responses (right axis). Red dotted line denotes threshold for positivity on both Y-axes (2.27 for MFI, 8 for ΔSFUs). Comparison of MOG-antibody-MFI between MOG-T-cell positive and negative patient in inlayed graph. P-values calculated using Mann-Whitney U test. ns p > 0.05. MFI ratio calculated as MFI of MOG-EGFR cells/ MFI of EGFR-only cells. MFI: Mean fluorescent intensity. EGFR: Enhanced green fluorescent protein. mAb: Monoclonal antibody.

3.6. MOG specific T-cells are present regardless of clinical status

To test whether MOG-specific T-cell status was correlated with any clinical parameters, patients were divided into MOG-reactive or MOG-non reactive based on their IL-17A response as it was the most disease specific single cytokine. The two groups were compared based on age, sex, EDSS at time of sampling, disease duration, natalizumab treatment duration, presence of spinal lesions and presence of optic neuritis at any time (Table 3). There were no clear clinical differences between the two groups. A weak trend was implied for natalizumab treatment, were the MOG-responders trended to a shorter treatment duration, however not significant after adjusting for the multiple comparisons made. Interestingly, MOG-specific T-cells were present in both recently diagnosed patients and persisted over time as it was identified in one patient 20

Table 3
Characteristics of MOG-reactive and non MOG-reactive patients.

	IL-17A MOG-reactive (n = 24)	Non IL-17A MOG-reactive (n = 26)	P-values (adjusted)
Age (years)	38.3 ± 8.7 (27-61)	36.4 ± 10.2 (17-56)	0.86
Sex (female)	62.5%	84.6%	0.5
EDSS	2.1 ± 1.6 (0.0–6.5)	2.2 ± 1.2 (0.0–5.5)	0.86
Disease duration (years)	7.8 ± 5.3 (1-20)	10.1 ± 5.2 (1-19)	0.5
Natalizumab treatment duration (years)	2.8 ± 1.8 (0.2–8)	3.8 ± 2 (1.2–8.2)	0.3 (0.05 unadjusted)
Optic Neuritis	41.6%	45.8%	0.99
Spinal Lesions	95.8%	80.7%	0.57

EDSS: Expanded disability status scale. Numerical values presented as mean ± standard deviation with range in parenthesis. Nominal data presented as percentage of group.

years after diagnosis. Similarly, despite the natalizumab treatment blocking T-cells from entering the CNS, MOG-specific T-cells were detected in patients after up to 8 years of ongoing treatment.

4. Discussion

Here we have developed a novel, simple and sensitive method for detection of rare autoreactive T-cells. To show proof-of-concept we chose MOG as model antigen. MOG was covalently linked to paramagnetic polystyrene beads and used to activate and identify single MOG-specific T-cells in persons with MS. The role of MOG as an autoantigen in MS has been debated but here we provide further conclusive evidence of its association with the disease. Some previous studies have identified MOG-reactivity in patients [7,8,30–32] and our findings corroborate with these results, 46.2–59.6%, depending on cytokine readout, of patients showing a significant response towards MOG. Although the number of MOG-reactive cells producing IL-22 and IL-17A were generally slightly lower than IFNγ producing cells, a similar frequency of positive patients was seen for the three cytokines. Also in line with previous studies, we could detect MOG-reactivity in a fraction of the healthy controls strengthening the hypothesis that while MOG-reactive cells are associated with MS, they are neither essential nor exclusive to disease and do not cause it independently of other factors.

A direct link between MS and MOG-specific cells is difficult to prove in humans. The strongest evidence stems from the experiments in the animal model EAE, where immunization with MOG or MOG-peptides generates an MS-like neuroinflammation in several different species (mice, rats and non-human primates) [5,33]. Additionally, adoptive transfer of MOG-specific T-cells, both of the Th1 and Th17 type, to naïve recipients can induce EAE [6,34,35]. In MS this causal link is not established, or even possible to prove, as the presence of MOG-specific T-cells may constitute an epiphenomenon. However, Johnson et al. [36] could demonstrate a correlation between MOG-responses and location of lesions, indicating that they may play a role in MS pathogenesis as well. We could not prove clinical differences between patients with or without MOG-specific T-cells in this study. This comparison is however hampered by the natalizumab treatment, as it essentially inactivates the disease and the patients very rarely experience relapses or disease activity. Moreover, MOG constitutes one out of several anticipated CNS-autoantigens in MS, and a broader mapping of autoantigen-reactivity is likely needed for strong clinical correlations. Interestingly, the MOG reactivity was detected in blood from persons with MS that had been on the treatment for years, consistent with a hypothesis that a disease driving autoreactivity persists in the circulation despite the chronic block of cells entering the CNS. This is compatible with the observation that MS CNS disease activity reappears shortly after stopping natalizumab treatment [60].

Using a cell depletion approach, we show that the IFNγ, IL-22 and IL-17A secretion in response to MOG is due to activation of antigen-specific $CD4^+$ T-cells. While the cytokines used in this study were mainly chosen to be able to detect proinflammatory $CD4^+$ MOG-reactive T-cells, $CD8^+$ T-cells can also respond with IFNγ secretion in response to activation. In addition, bead-bound antigens have been shown to enhance cross-presentation via MHC class I compared to soluble antigen suggesting that our assay is able to detect antigen-specific $CD8^+$ T-cells [37,38]. Yet, while autoantigen specific $CD8^+$ T-cells have been implied to play a part in MS pathogenesis, we could not detect any such MOG-specific cells in this study [39,40]. Still, this does not allow us to exclude their existence as it could be due to methodological bias.

The method of bead-bound antigens used in our assay was completely dependent on the presence of $CD14^+$ cells, i.e. monocytes, for antigen processing and presentation and subsequent $CD4^+$ T-cell activation. Additionally, we show that the recognition of MOG by $CD4^+$ T-cells is HLA-DR restricted. This result is consistent with the HLA-DRB locus as the largest known genetic association.

There has been an increasing interest in the role of B-cells in MS, in part due to the effectiveness of B-cell depleting therapies. The focus is mainly on the ability of B-cells to present antigens rather than their production of autoantibodies. Supporting this is the observation that while anti-CD20 therapy reduces B-cell counts in cerebrospinal fluid, antibody-levels are not affected regardless of clinical improvement [41,42]. We found a low prevalence of anti-MOG-Abs in our study, with only one (out of 29) patients being positive, which is in line with previous studies showing a prevalence of ~5% [11]. We also found no correlation between T-cell responses and anti-MOG-Abs. These observations strengthens the hypothesis that the role of B-cells in MS-pathology is mainly antibody-independent, and rather refers to their interplay with and activation of autoreactive T-cells [43]. In our study we found no support for the B-cells' role as antigen presenting cells in MS, as depletion of $CD19^+$ B-cells did not affect the MOG induced cytokine responses, meaning that they were not able to act as antigen presenting cells and to activate $CD4^+$ T-cells in our assay. Our assay design may however be biased against using B-cells as antigen presenting cells, as our bead-bound antigens are dependent on phagocytosis, something B-cells generally do not excel at. Thus, translating this observation to an *in vivo* situation requires further studies.

IFNγ is commonly analyzed in autoantigen related studies as it constitutes a main pro-inflammatory Th1 cytokine. However, an increasing amount of evidence suggest that Th17 cells play a vital role in autoimmune diseases [44,45]. In MS, several studies have implied that Th17 cells might be an even more important driver of disease than Th1 cells [46–48]. Our results confirm the presence of MOG-specific $CD4^+$ T-cells that produce IL-17A and IL-22, demonstrating the presence of cells of the Th17 phenotype. Even though our data do not allow conclusions regarding the causative role of these cells in MS, they raise the interest for performing further studies regarding their role in the disease. We also identified double and triple cytokine secreting MOG-specific cells at a frequency of approximately one tenth of the single cytokine-producing ones. Similar proportions of double positive $IL\text{-}17A^+IL\text{-}22^+$ cells among Th17 lymphocytes have been reported previously, and they have been shown to be able to migrate through the blood-brain barrier, co-express granzyme B and induce neuronal death [48,49]. Similar findings have also been obtained for other autoimmune diseases, as both $IFN\gamma^+IL\text{-}17A^+$ and $IFN\gamma^+IL\text{-}22^+IL\text{-}17A^+$ $CD4^+$ T-cells have been shown to be enriched in inflammatory lesions in inflammatory bowel disease and to be resistant to glucocorticoids, indicating their importance in treatment resistant autoimmunity [50,51]. Interestingly, MOG-reactive $IFN\gamma^+IL\text{-}17A^+$ cells were particularly disease-specific in our study. Studies further investigating the role of these cells in MS are required.

The bead-induced cytokine response seems to be antigen-specific as neither the negative control protein, nor beads without antigen induced a higher response than cell-medium only. Rather, medium only gave a marginally, higher IFNγ response when compared to no-antigen beads for patients or when compared to NC-beads for controls. The relevance of this difference is uncertain, as the absolute difference in number of cells counted were low. It does however indicate that the response seen for MOG is antigen dependent, and not due to intrinsic properties of the beads or *E. coli* produced proteins in general. A higher IFNγ response was found in PBMCs of patients compared to controls towards the negative control. This could be explained by e.g. a higher number of pre-activated circulating cells in patients, autoproliferation [43] or that PBMCs of patients could be more responsive towards bead or protein endotoxin contaminants. A last possibility is that there is a true antigen-specific response towards the ABD-protein on the NC-beads, which for an undetermined reason differs between patients and controls. In any of these cases, it would not influence the results regarding MOG as the endotoxin contamination was lower for the MOG-beads and any other ABD-directed response or pre-activation have been counteracted by subtracting the individual negative control response before making the

analysis.

One of the reasons that the role of MOG in MS is debated is that previous studies using recombinant MOG to stimulate T-cells have often been unsuccessful. Van der Aa et al. performed a study similar to ours, where PBMCs were stimulated with recombinant MOG in an IFNγ ELISpot assay [10]. While the response of MS patients closely resembled that of our study, they saw a similar high response in the control group and no statistical significant difference was detected, indicating that a bead-based method could be better in distinguishing patients from controls in this context, possibly due to a higher signal-to-noise ratio. Hellings et al. also performed an ELISpot-based study, but observed a lower absolute number of IFNγ-producing cells, with no difference between patients and controls [9]. Accordingly, Lindert et al. did not detect a difference in a cell-proliferation assay between MS patients and HCs when stimulating PBMCs with recombinant MOG [52]. The antigen-specificity of the IFNγ response in some previous studies can be questioned. It is challenging to remove bacterial contaminants such as LPS from proteins and while LPS can increase the strength of an antigen specific response, it also induces IFNγ secretion without any other stimuli in PBMCs [53,54]. It is possible that LPS-induced IFNγ secretion obscures antigen-specific responses, reducing the ability to detect true antigen-specific responses. For example, during method development we could see a high IFNγ response of healthy controls when using too mild washes of our antigen beads (Fig. 1B). Interestingly, IL-22 and IL-17A were not affected to the same extent, indicating that they are not as sensitive to endotoxin contamination. This effect could be a reason why both Rosbo et al. and Sun et al., by using whole MOG isolated from human brain myelin rather than recombinantly produced protein, could detect MOG-reactivity in persons with MS [8,31].

One aspect, apart from the antigen processing, that separates our study from previous ones is the fact that the patients in our cohort were undergoing natalizumab treatment. As natalizumab blocks the very late antigen 4 dependent cell migration it is likely that there is an accumulation of autoantigen specific T-cells in the circulation of natalizumab-treated patients which in turn would increase their numbers in our assay, even if there are studies indicating this effect is not major [55]. As expected, we could see higher numbers of PBMCs in the natalizumab treated patients compared to controls (Supplementary Figure 2), a phenomenon that has been demonstrated before [56]. This difference should not influence the FluoroSpot assay where the total amount of cells is the same. Additionally, there was no significant difference in spot-number between patients and controls for any cytokine in response to the polyclonal activator anti-CD3, which shows that even if the absolute number of lymphocytes in circulation differs during natalizumab-treatment, the proportion of the different cell-types in the FluoroSpot assay was not significantly affected. However, natalizumab has also been shown to have an impact on the cytokine expression of immune cells [57]. For example, circulating IFNγ and IFNγ expression of PBMCs is increased during treatment [57–59]. Trends of increased production of IL-17A in response to autoantigens have also been demonstrated [55]. These changes of cytokine expression could carry over and influence our assay, by further increasing the cytokine response of the patients. Altogether, one could argue that natalizumab treatment may constitute a powerful tool that facilitates the search for new candidate autoantigens which could increase the sensitivity of similar assays.

The reasoning behind using bead-bound antigens comes from the field of vaccination, where micro- and nanoparticles have been used as adjuvants due to their ability to effectively induce antigen specific Th1 response *in vivo* [61,62]. There are several advantages with the bead-based method. One main strength is making it possible to use *E. coli* produced recombinant full-length antigens available for testing without the background noise obscuring the antigen-specific responses. For example, we were able to reduce the endotoxin content to levels < 2% of the upper limits of the cell-culture grade fetal calf serum used in our cell stimulation assays. Further, solubility does not become an issue when using bead-bound antigens.

The low endotoxin content allows for the use of PBMCs without the need of preparing specialized antigen presenting cells or target cells of interest, e.g. $CD4^+$ T-cells to reduce background noise. Rather, it relies on the phagocytosis of the antigen-beads, utilizing the combination of antigen-presenting cells present in the PBMC population, i.e. mainly monocytes. It has been shown that this mode of antigen delivery increases surface expression of MHC class II and co-stimulatory molecules on dendritic cells (DCs), which enhances the antigen presentation and T-cell activation [38]. Although DCs efficiently process and present particulate antigens, efficient antigen presentation can also be done by monocytes, which eliminates the need of specialized DCs in subsequent assays [37,63]. The size of the beads seemed to matter in our pilot experiments, with peak efficacy at a bead diameter of 1 μm, replicating previous studies showing micro-spheres are able to facilitate phagocytosis well [37,63–65].

Another strategy to solve some of the problems of high background noise or solubility is the usage of synthetic peptides. This approach is however generally only applicable when testing for a restricted number of possible T-cell epitopes as the amount of patient material needed otherwise becomes limiting. The phagocytosis and intracellular proteolytic degradation of full-length antigens rather than direct MHC-binding by synthetic peptides might also have other advantages. One can argue that antigen processing and generation of MHC presented peptides through this pathway will lead to presentation of peptides mimicking an *in vivo* setting, not always being the case when using synthetic peptides in T-cell activation assays [66]. Even though the central binding core aa-sequence may be identical, other factors such as the composition of peptide flanking residues and side chain modification can affect the TCR recognition. Utilizing the natural cell antigen processing machinery could potentially increase the sensitivity of follow-up assays [67].

4.1. Conclusions

In summary, this study demonstrates the presence of HLA-DR restricted MOG-specific $CD4^+$ T-cells, producing IFNγ, IL-22 and IL-17A in approximately half of the persons with MS investigated, strengthening the hypothesis that MOG is an important autoantigen in MS. Further, we demonstrate the functionality of a novel method for detecting very rare antigen-specific T-cells with high sensitivity and throughput.

Author contributions

MB, HG, TO and GG conceived the study. HG, GG, TO, MK and LB supervised the study. MB, SR and CCQ performed the FluoroSpot experiments. MB and SR performed the depletion and HLA-blocking assays. ON, AK and EH designed, produced and processed the antigens. CCQ performed the quality control of the antigen-beads. CM and SW performed the antibody assays. LB collected the clinical data. TO, MK, LB, EM contributed to study design, sample collection and discussions on data interpretation. MB prepared the manuscript with input from ON, AK, SR, CCQ, GG and HG. All authors reviewed the final manuscript.

Conflicts of interest

HG is the co-founder of the company TCER AB, which holds patents and pending patents regarding bead-based antigen processing. MB and HG are inventors of said patents. CCQ, AK and ON hold positions at TCER AB. TO has received unrestricted MS research grants and/or lecture/advisory board honoraria from Biogen, Novartis, Genzyme, Merck and Roche, of which non is applicable to this study.

Funding sources

This study was supported by grants from CBD solutions, Neuroförbundet, Swedish Research Council (2017-00777), Swedish Brain Foundation, Margareta af Ugglas Foundation, Stratneuro and the DFG (SFB TR128). None of the funding sources had a role in the study design, collection, analysis or interpretation of the data.

Acknowledgements

We want to thank the staff at MabTech for help with setting up and reading the FluoroSpot assays, the staff at the Neurology Clinic at Karolinska University Hospital, in particular Gunn Jönsson for recruitment of study participants and blood-sampling, Brinda Acharjee for PBMC preparation, Marta Nazzari for technical support, Sara Enoksson and Theresa Neimert Andersson for help with early assay optimization, Torbjörn Gräslund at KTH Royal Institute of Technology, for help and advice during method development.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.04.013.

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