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Regulatory T Cells Increase After rh-MOG Stimulation in Non-Relapsing but Decrease in Relapsing MOG Antibody-Associated Disease at Onset in Children

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Background: Myelin oligodendrocytes glycoprotein (MOG) antibody-associated disease (MOGAD) represent 25% of pediatric acquired demyelinating syndrome (ADS); 40% of them may relapse, mimicking multiple sclerosis (MS), a recurrent and neurodegenerative ADS, which is MOG-Abs negative.

Aims: To identify MOG antigenic immunological response differences between MOGAD, MS and control patients, and between relapsing *versus* non-relapsing subgroups of MOGAD.

Methods: Three groups of patients were selected: MOGAD (n=12 among which 5 relapsing (MOGR) and 7 non-relapsing (MOGNR)), MS (n=10) and control patients (n=7). Peripheral blood mononuclear cells (PBMC) collected at the time of the first demyelinating event were cultured for 48 h with recombinant human (rh)-MOG protein (10 µg/ml) for a specific stimulation or without stimulation as a negative control. The T cells immunophenotypes were analyzed by flow cytometry. CD4⁺ T cells, T helper (Th) cells including Th1, Th2, and Th17 were analyzed by intracellular staining of cytokines. Regulatory T cells (T_{regs}, Foxp3⁺), CD45RA⁻Foxp3⁺ T_{regs} and subpopulation naive T_{regs} (CD45RA⁺Foxp3^{int}), effector T_{regs} (CD45RA⁻Foxp3^{high}) and non-suppressive T_{regs} (CD45RA⁻Foxp3^{int}) proportions were determined.

Results: The mean onset age of each group, ranging from 9.9 to 13.8, and sex ratio, were similar between MOGR, MOGNR, MS and control patients as analyzed by one-way ANOVA and Chi-square test. When comparing unstimulated to rh-MOG stimulated T cells, a significant increase in the proportion of Th2 and Th17 cells was observed in

MOGAD. Increase of Th17 cells was significant in MOGNR (means: 0.63 ± 0.15 vs. 1.36 ± 0.43 ; Wilcoxon-test $p = 0.03$) but not in MOGR. $CD4^+$ T_{regs} were significantly increased in MOGNR (means: 3.51 ± 0.7 vs. 4.59 ± 1.33 ; Wilcoxon-test $p = 0.046$) while they decreased in MOGR. $CD45RA^+Foxp3^+$ T_{regs} were significantly decreased in MOGR (means: 2.37 ± 0.23 vs. 1.99 ± 0.17 ; paired t-test $p = 0.021$), but not in MOGNR. MOGR showed the highest ratio of effector T_{regs}/non suppressive-T_{regs}, which was significantly higher than in MOGNR.

Conclusions: Our findings suggest that $CD4^+$ Th2 and Th17 cells are involved in the pathophysiology of MOGAD in children. The opposite response of T_{regs} to rh-MOG in MOGNR, where $CD4^+$ T_{regs} increased, and in MOGR, where $CD45RA^+Foxp3^+$ T_{regs} decreased, suggests a probable loss of tolerance toward MOG autoantigen in MOGR which may explain relapses in this recurrent pediatric autoimmune disease.

Keywords: neuroinflammation, auto-immune diseases, acquired demyelinating syndromes (ADS), myelin oligodendrocyte glycoprotein, multiple sclerosis, regulatory T lymphocytes

INTRODUCTION

Pediatric acquired demyelinating syndromes (ADS) are rare immune-mediated acute demyelinating disorders of the central nervous system (CNS) with an incidence of 0.6 to 1.6 for 100,000 children per year in western countries (1–3). Myelin oligodendrocytes glycoprotein (MOG) antibodies (Abs) are found in about 25% of pediatric ADS (4) now referred to as MOG antibody-associated disease (MOGAD). MOG protein represents only 0.05% of myelin proteins and is expressed exclusively on the outer surface of the myelin sheath and the plasma membrane of oligodendrocytes. Its cell surface location makes it accessible to immune reactions (5) becoming a target of autoimmune responses that cause inflammation and CNS demyelination (6, 7). The course of MOGAD can be either non-relapsing (MOGNR) or relapsing (MOGR). Unlike MOGAD, multiple sclerosis (MS) patients do not have MOG-Abs (8–10). MS is an ADS characterized by the recurrence of demyelination episodes resulting in subsequent neurological damage. Both MOGAD and MS have T and B lymphocytes infiltration in the brain, but in MS, $CD8^+$ T cell and B cell infiltration is higher (11) than in MOGAD where $CD4^+$ T cell infiltration is predominant (12). There are evidence that autoreactive $CD4^+$ T-cells are involved in both MOGAD and MS pathogenesis, but further research is required to understand their role in the disease onset and evolution (13, 14).

In experimental autoimmune encephalomyelitis (EAE) in rodents, both cell-mediated and humoral immune responses are involved in brain inflammation and demyelination (15, 16), sustained by a T/B cell cooperation as studied using transgenic

mice with MOG-specific T and B cell receptors (17–21). *In vitro* experiments in humans have shown that patient sera containing MOG-Abs, activate the complement pathway (22–24), induce natural killer cells and antibody-dependent cell-mediated cytotoxicity (6), and can result in the disruption of the oligodendrocyte cytoskeleton (25, 26).

A non-human primate model of EAE in cynomolgus macaques was developed by sensitization with recombinant human (rh)-MOG, emulsified in incomplete Freund's adjuvant (IFA), which induced a disease similar to MOGAD in children (27). Interestingly, in this model, it was recently shown that the intradermal routing of MOG into resident dendritic cell asialoglycoprotein receptor (DC-ASGPR)⁺ cells using recombinant antibody DC-ASGPR fused to MOG prevented the breach of immune tolerance against MOG after rh-MOG/IFA sensitization. Phenotyping of blood lymphocytes indicated that only the control animals had an increase activation of $CD4^+$ T cells in the days preceding the onset of EAE. In contrast, animals treated with anti-DC-ASGPR-MOG had an increase in MOG-specific T_{regs} upon rh-MOG/IFA re-administration (28).

These results prompted us to evaluate the response of $CD4^+$ T cells to rh-MOG stimulation *in vitro* from children with different forms of pediatric ADS. We compared the $CD4^+$ T cells immunological phenotypes of MOGAD patients to MS and control patients with non-inflammatory neurological diseases, and assessed the cells functional responses after stimulation with rh-MOG protein *in vitro*. We focused our analysis on the three main T helper (Th) cells corresponding to three lineages of $CD4^+$ lymphocytes triggered upon antigenic activation. These $CD4^+$ T cells are referred to as Th type-1 (Th1), Th type-2 (Th2) or Th type-17 (Th17) cells, according to their phenotype. Th1 cells produce interferon- γ (IFN- γ) and tumor necrosis factor-alpha (TNF- α) and are effective against intracellular bacteria and viruses, but are also involved in autoimmune diseases. Th2 cells secrete interleukin-4 (IL-4), -5, -10 and -13, which up-regulate antibody production through B cells activation. Th17 cells secrete IL-17 and TNF- α and are involved in tissue

Abbreviations: ADS, acquired demyelinating syndromes; ADEM, acute demyelinating encephalomyelitis; AQP, aquaporin; IL-6, interleukin-6; IL-4, interleukin-4; IL-17, interleukin-17; IFN- γ , interferon- γ ; NMOSD, neuromyelitis optica spectrum disorder; MOG, myelin oligodendrocyte glycoprotein; MOGAD, MOG antibody-associated disease; MOGNR, non-relapsing MOGAD; MOGR, relapsing MOGAD; MRI, magnetic resonance imaging; MS, multiple sclerosis; TCR, T-cell receptor.

inflammation, activation of neutrophils and in autoimmunity. We then studied regulatory T (T_{reg}) cells that secrete IL-10 and transforming growth factor-beta ($TGF-\beta$), which modulate Th cell activity and suppress some of their functions, inducing tolerance to antigens. We then subdivided the MOGAD group into non-relapsing MOGAD (MOGNR) and relapsing MOGAD (MOGR) to further evaluate relations between relapse and immunological response to rh-MOG.

MATERIALS AND METHODS

Patients and Controls

Twenty-two children ≤ 18 years old, from the French cohort KIDBIOSEP, followed for a first demyelinating episode in the national reference center for rare inflammatory brain and spinal diseases at Bicêtre Hospital, from January 2011 to May 2018, were included. ADS was defined as an acute neurological deficit lasting more than 24 h in the CNS, affecting the optic nerve, brain, cerebellum, brainstem and/or spinal cord associated with T2 lesions on magnetic resonance imaging (MRI). Relapse was defined as a new episode of CNS demyelination at least 1 month after the first episode or 3 months after the first episode if the first attack is an acute demyelinating encephalomyelitis (ADEM), and lasting for at least 24 h in the absence of fever or infection. The MS diagnosis was made according to the 2013 IPMSSG criteria and the revised 2010 MacDonald criteria. We classified our patients into 3 groups: MOGAD patients, including non-relapsing (MOGNR) and relapsing (MOGR) subgroups based on the progression of the disease after the blood sampling, ADS without MOG-Ab corresponding to MS patients and control patients (CTRL). Seven control patients were included from pediatric neurology department of Bicêtre Hospital for non-inflammatory neurological diseases, such as intracranial hypertension ($n=1$), psychosomatic syndromes ($n=2$), genetical peripheral neuropathies ($n=2$), psychomotor developmental delays ($n=1$) or stroke ($n=1$), for which blood samples were performed for diagnosis. Demographic data of included children are presented in **Table 1**.

Ethics

This study complied fully with French national and local ethics committee guidelines. The national cohort of first demyelinating episode “Kidbiosep 2004” (No. 910506) was authorized by the Commission Nationale de l’Informatique et des Libertés and the Comité de Protection des Personnes of Paris-Saclay University. An informed consent form was signed by parents of each included child.

Blood Samples

Blood samples were collected on heparinized tubes from all patients at their first demyelinating event, within the first 3 months and before starting immunosuppressive or immunoregulatory therapy. For all patients, PBMCs were isolated by Ficoll density gradient centrifugation. Briefly, heparinized blood was centrifuged at $700 \times g$ for 15 min and the top layer containing plasma was removed, transferred in cryovials and stored at -20°C . The remaining blood was diluted with an equal volume of isosmotic 0.9% wt/vol NaCl solution layered over 15 ml of the Ficoll-Paque PLUS (GE Healthcare). Gradients were centrifuged at $700 \times g$ for 30 min at room temperature. The PBMC interface was removed by pipetting and washed with 0.9% wt/vol NaCl solution by centrifugation at $700 \times g$ for 15 min. Non-viable cells were identified by staining with trypan blue and cell viability was calculated using the total cell count and the count of non-viable cells. Approximately 1 million PBMCs were transferred in cryovials in 1 ml 90% heat-inactivated fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO; Sigma) and immediately placed into a freezing box, placed overnight into an -80°C freezer. On the following day, the vials were placed at -150°C for storage.

Cell-Based Assay for Detection of Antibodies to Cell Surface MOG in Plasma

HEK293A cells transfected with full-length human MOG were used as antigenic substrate in combination with control cells as previously described (29). Briefly, these stable MOG cells were used to detect patient plasma Ig by flow cytometry. As a control,

TABLE 1 | Demographic data of included children.

	MOGR n=5	MOGNR n=7	MS n=10	Controls n=7	p value
Female (n (%))	5 (100)	2 (29)	4 (40)	2 (29)	0.051
Age at onset (mean, years \pm SD)	9.9 \pm 2.4	10.2 \pm 5.2	13.8 \pm 2.2	11 \pm 3.2	0.114
Presentation (n (%))					
Optic neuritis	2 (40)	1 (14)	3 (30)		0.594
Transverse myelitis	2 (40)	4 (57)	0		0.025
ADEM	0	2 (29)	0		0.48
Rhombencephalitis	1 (20)	1 (14)	2 (20)		0.9
Long tract involvement	0	0	6 (60)		0.007
Others	0	0	1 (10)		0.454
Follow-up time (mean, years \pm SD)	4.9 \pm 2.2	1.9 \pm 2.2	2.3 \pm 2	0.5 \pm 0.5	0.005

Data are expressed as mean \pm standard deviation (SD) unless otherwise stated. MOGR: relapsing MOGAD, MOGNR: non-relapsing MOGAD. MS: multiple sclerosis patients. Controls: other non-inflammatory neurological disorder patients. For age at onset and follow-up time, one-way ANOVA p values are given. For gender and presentation, the Chi-square p value are given, except for ADEM, Rhombencephalitis and Others for which only Fisher’s exact test could be used.

non-transfected HEK293 cells were used for each sample. Cells were harvested using PBS containing 0.2 mM EDTA. Cells were rinsed in 2% FCS/PBS, blocked with 10% FCS/PBS. About 150,000 cells were incubated with patient plasma at a 1:2 dilution for 1 h at 4°C. Cells were then washed three times with 2% FCS/PBS and incubated with fluorescein isothiocyanate (FITC) conjugated anti-human immunoglobulin anti-IgG H + L Fab'2 secondary antibody (Kallestad FITC conjugate, Bio-Rad, Marnes-la-Coquettes, France) for 15 min at 4°C. Cells were washed with 2% FCS/PBS, fixed in 2% formaldehyde-PBS and re-suspended in 300 μ l PBS before analysis. A total of 50,000 events per sample were recorded on a FACS Canto II instrument. Data analysis was performed using Flow Jo software (Ashland, OR, USA) and Excel. Binding was expressed as mean fluorescence intensity (MFI). Levels of specific antibody binding in transfected cells were expressed as Δ MFI. Δ MFI was determined by the subtraction of MFI obtained with HEK293 control cells from the MFI obtained with HEK293MOG⁺ cells. A Δ MFI greater than mean + 6 standard deviations (SDs) of values of the control patients' samples was considered positive. Each experiment was performed at least twice. Positive plasmas were further tested at dilutions of 1:10 to 1:640 by serial dilution with a threshold of 1:160 to define MOG-Ab positivity.

T Lymphocytes Activation and Flow Cytometry

PBMCs were thawed by warming cryovials rapidly in a 37°C water bath for approximately 1 min until the ice disappeared. The cell suspension was transferred to a 15 ml centrifuge tube and slowly mixed with 7 ml of warmed culture medium. After centrifugation (400 \times g for 10 min), the supernatant solution was removed, and cell pellets suspended in 1 ml fresh culture medium. PBMCs (2 \times 10⁵ cells/well) were cultured for 44 h (37°C, 5% CO₂) in 200 μ l DMEM supplemented with 10% FCS and 1% penicillin/streptomycin, with or without 10 μ g/ml rh-MOG, or with 5 μ g/ml phytohemagglutinin (PHA, Sigma-Aldrich) as a positive stimulation control as described (28, 30). After 44 h of incubation time, Golgi plug (1 μ l/ml, BD Biosciences) and Golgi stop (0.67 μ l/ml, BD Biosciences) were added to the media in each well and the cultures incubated for another 4 h. Cells were washed and stained to detect antigen-specific CD4⁺ T cell subsets, as previously described (28), using commercial mAbs according to the manufacturer's guidelines: anti-CD3-BV768 (SP34-2, BD), anti-CD4-BV605 (L200, BD), anti-CD45RA (HI100 PE Cy7, BD), and anti-FOXP3-APC (236A/E7, BD). Intracellular staining for Foxp3 required permeabilization buffer and the Foxp3 buffer kit (BD) was used following the manufacturer's instructions. Intracellular staining also included anti-IFN- γ (clone B27, V450, BD), anti-IL-4 (clone 8D4-8, AF488, BD) and anti-IL-17 (clone N49-653, AF700, BD). Cells were analyzed by flow cytometry with BD-LSR Fortessa (BD Biosciences) using BD FACSDiva software, with at least 100,000 events collected. FlowJo software was used for analysis.

Statistical Analysis

We performed statistical analyses of cellular immunophenotyping using GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA). Data are presented as mean \pm standard error of

the mean (SEM). For all data sets which could be accurately modeled by a Gaussian normal distribution, an unpaired t-test was used for analysis of differences between groups; otherwise, the Mann-Whitney U-test was used. Within each group, paired comparison of non-stimulated *versus* rh-MOG stimulated was performed for normal distribution using the paired t-test; otherwise, the non-parametric Wilcoxon signed rank test was used. Statistical significance was assigned to values of $p < 0.05$, and the symbols used were $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). For demographic data, one-way ANOVA test, Chi-square test and Fisher's exact test were used.

RESULTS

Demographic Characteristics of Patients

Demographic data are summarized in **Table 1**. Twenty-two children were included in this study. The mean onset age of each group were similar as analyzed by one-way ANOVA, but that of MS was significantly higher than that of MOGR (unpaired t-test $p = 0.009$). The gender proportion of each group was similar as analyzed by Chi-square test, but MOGR occurred only in female in our cohort (5/5, 100%), a proportion significantly higher than that in MOGMR (Fisher's exact test $p = 0.027$) and in MS (Fisher's exact test $p = 0.044$).

Rh-MOG Induced Th2 and Th17 Lymphocytes in MOGMR

The percentage of CD4⁺ Th2 cells (CD3⁺CD4⁺IL-4⁺) was significantly increased after rh-MOG stimulation in the MOGAD group (means: 1.09 \pm 0.42 vs. 2.15 \pm 0.98; Wilcoxon-test $p = 0.035$) while no change was detected in the MS group (means: 1.58 \pm 0.38 vs. 1.43 \pm 0.26; Wilcoxon-test $p = 0.677$) and in the control group (means: 0.92 \pm 0.19 vs. 0.86 \pm 0.25; Wilcoxon-test $p = 0.687$) (**Figure 1A**). The ratio of rh-MOG-stimulated-Th2 cells to unstimulated-ones was significantly higher in the MOGAD group than in the control group (means: 1.83 \pm 0.64 vs. 0.91 \pm 0.8; Mann-Whitney U-test $p = 0.036$) (**Figure 1D**). When comparing MOGMR and MOGR, the percentage of Th2 cell was increased upon rh-MOG stimulation in MOGMR (means: 1.28 \pm 0.67 vs. 3.07 \pm 1.62; Wilcoxon-test $p = 0.078$) but not in MOGR (means: 0.82 \pm 0.44 vs. 0.86 \pm 0.41; Wilcoxon-test $p = 0.437$) (**Figure 1G**). The ratio of rh-MOG-stimulated-Th2 cells to unstimulated-ones was higher in MOGMR than in MOGR without reaching significance (**Figure 1J**).

The percentage of CD4⁺ Th17 lymphocytes (CD3⁺CD4⁺IL-17⁺), was also significantly increased in the MOGAD group when comparing unstimulated to rh-MOG stimulated PBMC (means: 0.53 \pm 0.1 vs. 1.02 \pm 0.28; Wilcoxon-test $p = 0.013$), but not in MS (means: 0.34 \pm 0.07 vs. 0.35 \pm 0.06; Wilcoxon-test $p = 0.537$) nor in the control groups (means: 0.55 \pm 0.16 vs. 0.72 \pm 0.25; Wilcoxon-test $p = 0.437$) (**Figure 1B**). No significant differences were observed when considering the ratio of rh-MOG-stimulated-Th17 cells to unstimulated-ones among the three groups (means: 1.95 \pm 0.44; 1.47 \pm 0.28 and 1.6 \pm 0.45 in MOGAD, MS and control, respectively) (**Figure 1E**). When separating MOGAD into MOGMR and MOGR, it appeared that

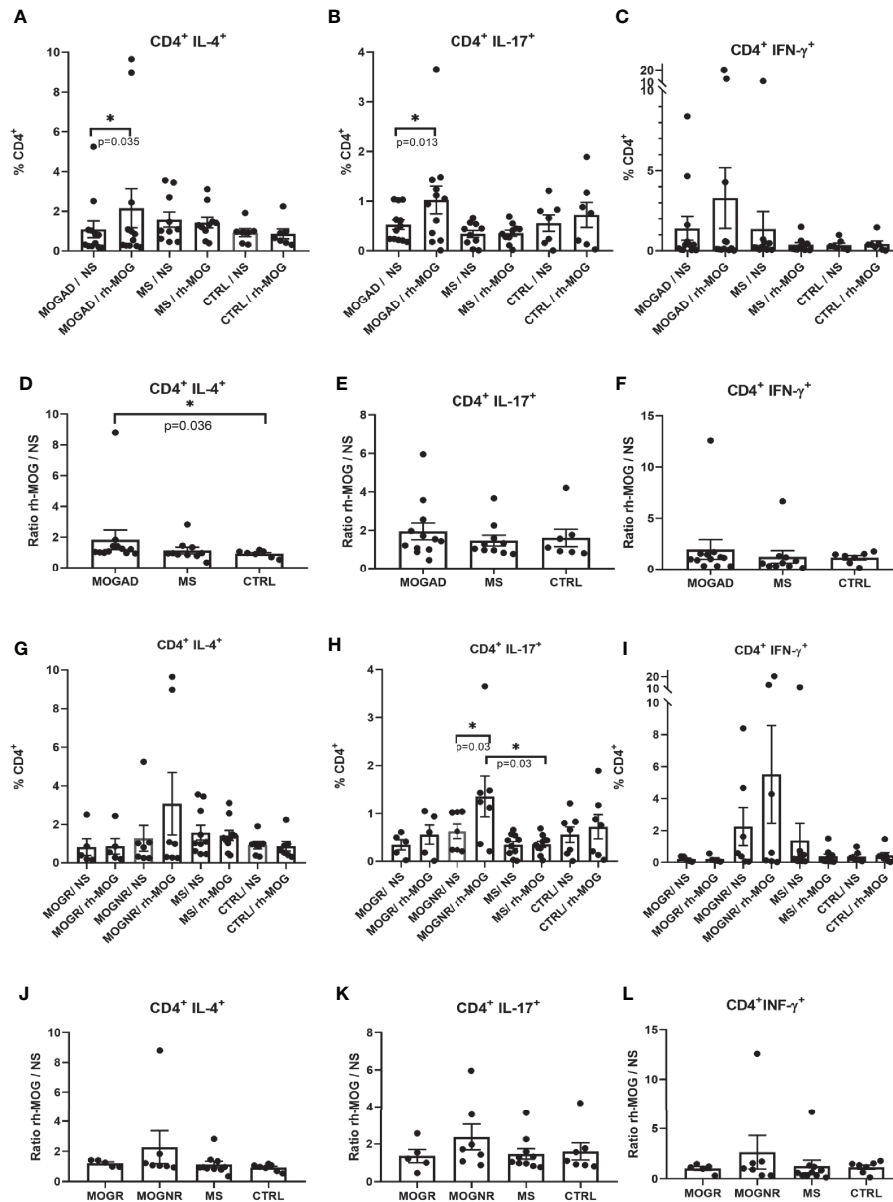


FIGURE 1 | Th2 and Th17 is increased upon rh-MOG stimulation in MOGAD. The percentages of Th2 (A, G), Th17 (B, H) and Th1 (C, I) cells in the CD4⁺ gate is shown in MOGAD (A–C), or in relapsing ones (MOGR) and non-relapsing ones (MOGNR) (G–I), multiple sclerosis (MS), and controls patients (CTRL), either not stimulated (NS) or stimulated by rh-MOG. Ratio of Th2 (D, J), Th17 (E, K) and Th1 (F, L) stimulated by rh-MOG versus NS are given. Mean and SEM values are indicated as well as *p* values when significant (Wilcoxon for NS vs. MOG and Mann-Whitney for groups comparison). **p* < 0.05.

the Th17 percentages increased significantly upon rh-MOG stimulation in MOGNR (means: 0.63 ± 0.15 vs. 1.36 ± 0.43 ; Wilcoxon-test *p* = 0.03) as well as in MOGR but without reaching significance (means: 0.34 ± 0.1 vs. 0.56 ± 0.2 ; Wilcoxon-test *p* = 0.25) (Figure 1H). The ratio of rh-MOG-stimulated-Th17 cells to unstimulated-ones was higher in MOGNR than in MOGR without reaching significance (Figure 1K).

Th1 cells (CD3⁺CD4⁺IFN- γ ⁺) increased upon rh-MOG stimulation in PBMC from MOGAD patients (means: 1.4 ± 0.74 vs. 3.3 ± 1.89 ; Wilcoxon-test *p* = 0.747) while it decreased in

MS patients without reaching significance (means: 1.37 ± 1.08 vs. 0.37 ± 0.14 ; Wilcoxon-test *p* = 0.232) (Figure 1C). The ratio of rh-MOG-stimulated-Th1 cells to unstimulated-ones was higher in MOGAD than in MS (means: 1.97 ± 0.97 vs. 1.23 ± 0.61 ; Mann-Whitney U-test *p* = 0.159) (Figure 1F). When MOGAD patients were subdivided in MOGNR and MOGR, we observed that, upon rh-MOG stimulation, the proportion of Th1 cells in MOGNR was increased by 2.4-fold, although not reaching a statistical significance (means: 2.25 ± 1.19 vs. 5.51 ± 3.06 ; Wilcoxon-test *p* = 0.812), whereas it was unchanged in MOGR

(means: 0.2 ± 0.07 vs. 0.19 ± 0.09 ; Wilcoxon-test $p = 0.875$) (Figure 1I). Accordingly, the ratio of rh-MOG-stimulated-Th1 cells to unstimulated-ones tended to be higher in MOGNR than in MOGR (means: 1.03 ± 0.19 vs. 2.64 ± 1.67 ; Mann-Whitney U-test $p = 0.755$) without reaching significance (Figure 1L).

CD4⁺Foxp3⁺ T_{regs} Increased Upon rh-MOG Stimulation in MOGNR

The percentage of regulatory T lymphocytes T_{regs} (CD3⁺CD4⁺Foxp3⁺), was not different when comparing MOGAD, MS and control groups. No significant change in the T_{reg} percentages was observed following the rh-MOG stimulation in MOGAD (means: 3.42 ± 0.41 vs. 3.91 ± 0.8 ; Wilcoxon-test $p = 0.505$), in MS (means: 3.92 ± 0.49 vs. 3.78 ± 0.41 ; Wilcoxon-test $p = 0.492$) and in control patients (means: 3.73 ± 0.74 vs. 3.69 ± 0.72 ; Wilcoxon-test $p = 0.687$) (Figure 2A). Accordingly, the ratio of rh-MOG-stimulated-T_{reg} cells to unstimulated-ones was not significantly different among MOGAD, MS, and control groups (Figure 2B). Interestingly, when MOGAD patients were separated in two subgroups, there was a significant increase in T_{regs} after rh-MOG stimulation in MOGNR (means: 3.51 ± 0.7 vs. 4.59 ± 1.33 ; Wilcoxon-test $p = 0.046$), but not in MOGR (means: 3.3 ± 0.29 vs. 2.95 ± 0.36 ; Wilcoxon-test $p = 0.375$) (Figure 2C). When considering the ratio of rh-MOG-stimulated-T_{reg} cells to unstimulated-ones, it was significantly higher in MOGNR than that in MOGR patients (means: 1.23 ± 0.09 vs. 0.89 ± 0.07 ; unpaired t-test $p = 0.022$) and that in MS (mean: 1 ± 0.05 ; unpaired t-test $p = 0.045$) and in control patients (mean: 0.95 ± 0.03 ; unpaired t-test $p = 0.026$) (Figure 2D).

CD45RA⁻Foxp3⁺ T_{regs} Decreased Upon rh-MOG Stimulation in MOGR

We next evaluated the percentage of Foxp3⁺ T_{regs} among CD45RA⁻ cells to evaluate the balance between CD4⁺Foxp3⁺ T_{regs} and effector/memory CD45RA⁻Foxp3⁺ T_{reg} cells. This latter cell percentage did not differ after rh-MOG stimulation in MOGAD (means: 1.91 ± 0.2 vs. 1.83 ± 0.26 ; Wilcoxon-test $p = 0.294$), MS (means: 2.52 ± 0.33 vs. 2.51 ± 0.28 ; Wilcoxon-test $p = 0.865$) and control groups (Figure 2E). The same result was observed when expressed as a ratio (ratio of Foxp3⁺ T_{reg} cells among CD45RA⁻ cells in rh-MOG-stimulated *versus* unstimulated conditions) (Figure 2F). When subdividing MOGAD into MOGNR and MOGR, it appeared that the percentage of Foxp3⁺ cells in CD45RA⁻ fraction was significantly decreased in the MOGR group after rh-MOG stimulation (means: 2.37 ± 0.23 vs. 1.99 ± 0.17 ; paired t-test $p = 0.021$) but not in the MOGNR group (Figure 2G). Interestingly, the ratio of rh-MOG-stimulated-CD45RA⁻Foxp3⁺ T_{reg} cells to unstimulated-ones was significantly lower in MOGR as compared to MS (means: 0.84 ± 0.03 vs. 1.04 ± 0.05 ; unpaired t-test $p = 0.035$), and as compared to MOGNR (mean: 1.1 ± 0.08 ; unpaired t-test $p = 0.037$). These results indicate a decrease in Foxp3⁺ cells among CD45RA⁻ cells in MOGR after stimulation by rh-MOG (Figure 2H).

T_{regs} Subpopulation Differences Between MOGR and MOGNR

Distinct T_{reg} subsets have been identified based on their phenotypic and functional properties. On the basis of the expression of CD45RA and Foxp3, one can distinguish 3 important Foxp3-expressing

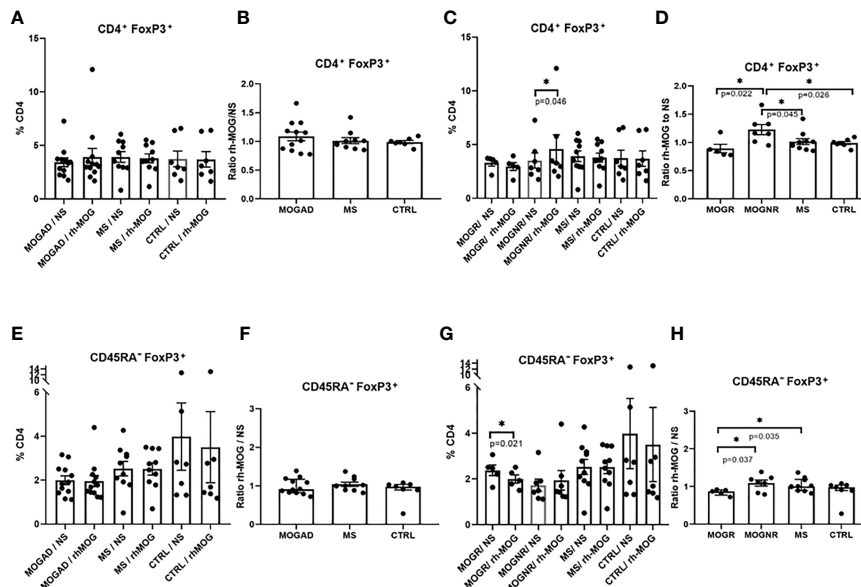


FIGURE 2 | T_{regs} are increased in MOGNR unlike in MOGR. The percentage of T_{regs} (Foxp3⁺) in CD4⁺ gate (A, C) and in CD45RA⁻ (E, G) are shown in each group of patients, either not stimulated (NS) or stimulated by rh-MOG. Ratio of CD4⁺Foxp3⁺ (B, D) and CD45RA⁻Foxp3⁺ (F, H) stimulated by rh-MOG *versus* NS are given. Mean and SEM values are indicated as well as p values when significant [Wilcoxon for NS vs. MOG, Mann-Whitney for groups comparison for all figures except paired t-test for (F) and unpaired t-test for (B, D-F, H)]. * $p < 0.05$.

subsets: naive T_{reg} cells (nT_{regs} : $CD4^+CD45RA^+Foxp3^{int}$), effector T_{reg} cells (eT_{regs} : $CD4^+CD45RA^-Foxp3^{high}$) which are both suppressive, and non-suppressive $Foxp3^+$ cells (non- T_{regs} : $CD4^+CD45RA^-Foxp3^{int}$). The majority of nT_{regs} are thought to recently originate from the thymus, that may subsequently convert into eT_{regs} (31). This strategy of analysis allows better identification of the $Foxp3$ expressing cells exhibiting suppressive properties (31). To investigate whether differences in these T_{reg} subsets existed among our groups of patients, we quantified these populations (nT_{regs} , eT_{regs} and non- T_{regs} among $Foxp3^+$ cells) as well as T effector/memory cells among $Foxp3^-$ cells ($T_{eff/mem}$: $CD4^+CD45RA^-Foxp3^-$). The gating strategy used is presented in **Figure 3A**.

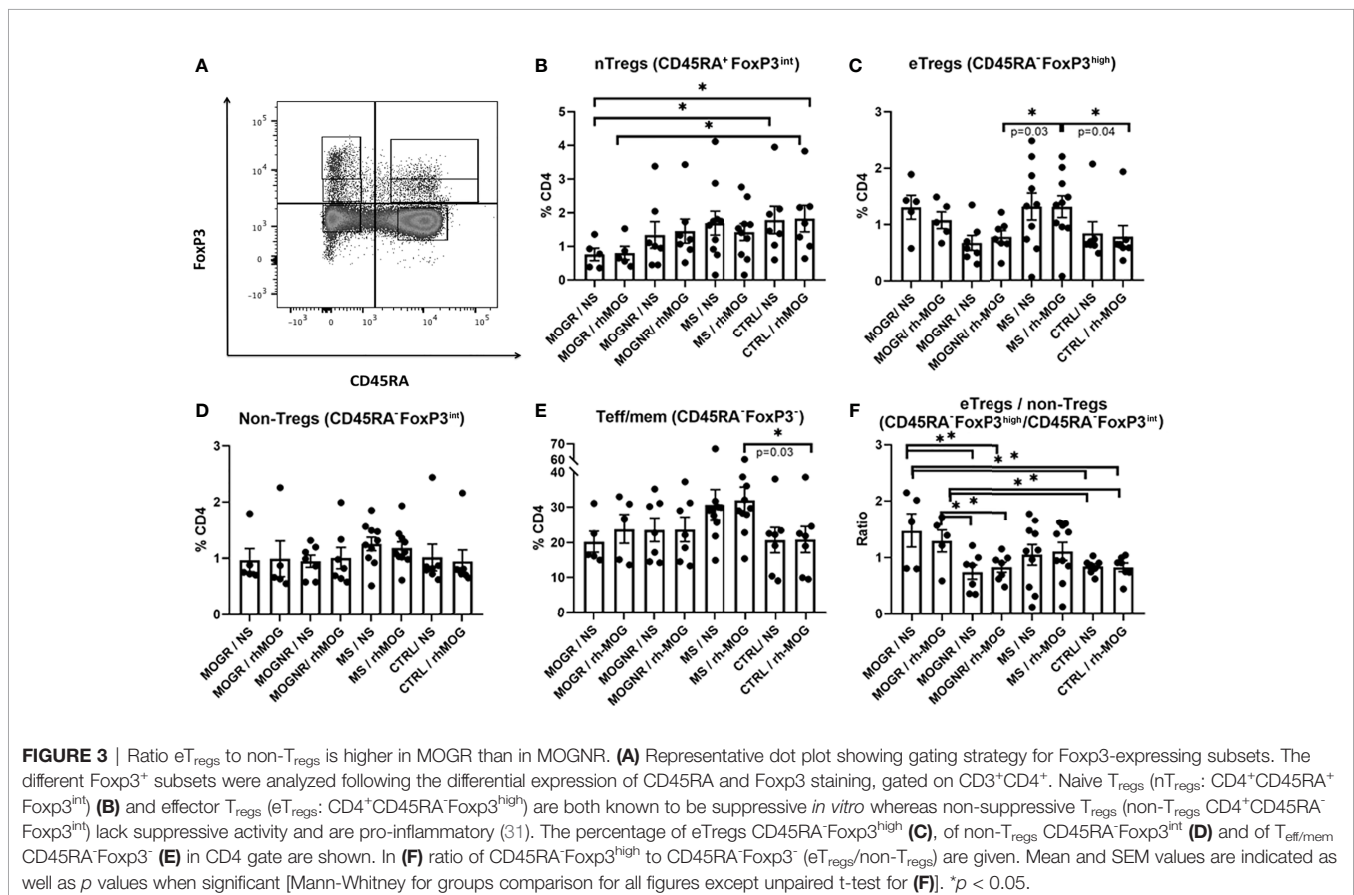
We observed that nT_{regs} tended to be lower in MOGR than in any other group of patients, and significantly lower than in control patients (**Figure 3B**). No change in the percentage of eT_{regs} was observed between MOG-stimulated and unstimulated conditions in MOGR, MOGR, MS and control groups. However, when comparing the proportions of eT_{regs} upon rh-MOG stimulation, MS exhibited a higher proportion of eT_{regs} than MOGR and control (**Figure 3C**). This was specific to the eT_{regs} fraction since no difference in the percentages of non- T_{regs} $Foxp3^+$ cells among MOGR, MOGR, MS and control, in MOG-stimulated or unstimulated conditions was observed (**Figure 3D**). As a control, we also determined the proportion of $T_{eff/mem}$ $Foxp3^-$ cells ($CD4^+CD45RA^-Foxp3^-$). This fraction was not affected by rh-MOG stimulation as no difference was

observed between stimulated and unstimulated conditions. We observed that MS exhibited higher proportion of $T_{eff/mem}$ $Foxp3^-$ cells compared to the control group (**Figure 3E**). Lastly, we evaluated the ratio of eT_{regs} $Foxp3^+$ cells/non- T_{regs} $Foxp3^+$ cells to evaluate the balance between suppressive and effector cells within the activated fraction. The impact of rh-MOG stimulation on this ratio was not significant in all group considered. However, the MOGR showed a significantly higher eT_{regs} /non- T_{regs} ratio than MOGR and control, in both non-stimulated and rh-MOG stimulated conditions (**Figure 3F**).

DISCUSSION

One main finding of this study is that there are differences in the $CD4^+$ T cells immunological phenotypes of ADS clinical subsets, with a significant increase in $CD4^+$ Th2 and Th17 cells following stimulation with rh-MOG in MOGAD children at onset of demyelinating events but not in MS and control patients. Within MOGAD patients, a significant increase of Th17 induced by rh-MOG stimulation was observed in patients without relapse (MOGR). Importantly, $CD4^+Foxp3^+$ T_{regs} were significantly increased in response to rh-MOG in MOGR, while $CD45RA^-Foxp3^+$ T_{regs} decreased upon rh-MOG stimulation in MOGR patients.

Our results point at a major role of MOG-specific $CD4^+$ T cells in MOGAD pathogenesis. The absence of significant changes in MS cells upon rh-MOG stimulation suggests that $CD4^+$



lymphocytes do not respond to MOG antigen in MS, reminiscent of the absence of MOG-Abs in this pediatric ADS (9, 10). Several groups also showed no difference in number of circulating MOG-autoreactive T-cells in MS compared to healthy controls (32). However, in a recent study, a novel and highly sensitive method for detection of antigen-specific T-cells using bead-bound MOG as stimulant allowed to detect circulating autoreactive CD4⁺ T-cells producing IFN- γ , IL-22 or IL-17A in 46–59% of adult MS patients. The patients included in this study were adults with MS under natalizumab, which blocks the very late antigen 4 (VLA-4) dependent cell migration across the blood-brain barrier into CNS, and could result in an accumulation of MOG specific T-cells in the circulation of treated patients, that may have increase their numbers in this assay (33) whereas our patients, at onset of the disease, had not been treated yet.

The rates of MOG-specific Th1 lymphocytes are low in our ADS patients as compared to that found in other studies (34). This may be explained by the fact that in this latter study PMA-ionomycin is used to stimulate the cells, whereas we compared rh-MOG stimulated cells to unstimulated ones.

Involvement of Th2 inflammation in autoimmunity including ADS is increasingly found as a component of these diseases (35). In some EAE models, a harmful Th2 response upon exposure to MOG autoantigen was observed beside the classical Th1/Th17 responses in mice (36) and in marmosets (37). These studies suggest that a Th2-type immune response plays a role in the development of EAE, giving further importance to the increase in Th2 cells observed in our MOGAD patients. Recently, in neuromyelitis optica spectrum disorder (NMOSD), an autoimmune demyelinating disorder characterized by auto-Abs targeting the astrocytic aquaporin-4 (AQP4) water channel in the serum of patients, it was found that a therapeutic strategy promoting a shift from Th1/Th17 to Th2 responses is potentially deleterious in NMOSD (35). In other autoimmune diseases with autoantibodies such as systemic lupus erythematosus (SLE), beside the well-established role of Th1/Th17, a Th2 environment and increased basophils are associated with lupus nephritis in human (38).

In rodent EAE, it has also been reported that IL-17-deficient mice have a less severe disease than wild-type mice (39), and that IL-17 worsened EAE in mice (40). In EAE in marmosets, it was found that treatment with an anti-IL-17A antibody induced a moderate delay of clinical scores, without abrogating EAE development (41). Th17 have often been associated with demyelinating diseases in children and adults, such as MS (42, 43) and MOGAD (44). Our results support this finding and suggest that Th17 may be more particularly involved in MOGAD patients where it is significantly increased as compared to MOGR patients.

In MOGAD patients, beside the increase of MOG-specific Th2 and Th17 responses, we observed a significant increase in CD4⁺Foxp3⁺ T_{reg} cells. On the contrary, in MOGR patients, effector/memory CD45RA⁺Foxp3⁺ T_{regs} significantly decreased upon rh-MOG stimulation. Th17 is known to induce autoimmune tissue injury, whereas T_{regs} inhibit autoimmunity and tissue injury. Disruption of the Th17/T_{reg} balance is thought to be involved in the development of various autoimmune disorders and chronic inflammation (45, 46). When we assessed

specifically T_{regs}, the ratio of CD4⁺Foxp3⁺ T cell percentage upon stimulation *versus* unstimulated conditions was higher in MOGAD compared to MOGR, MS and control. Focusing on the percentage of Foxp3⁺ cells among CD45RA⁺ cells showed the relative contribution of Foxp3⁺ cells among effector/memory cells. Interestingly, we observed a reduced percentage of Foxp3⁺ cells among CD45RA⁺ cells upon rh-MOG-stimulation in MOGR but not MOGAD. Because Foxp3 expressing cells may include cells with different suppressive activity and notably a non-suppressive fraction, we further studied the Foxp3⁺ fraction. In MOGR, nT_{regs} tended to be lower than in any other group of patients. On the contrary, eT_{regs} were higher in MOGR than in MOGAD and control groups. Although the eT_{regs} fraction decreased upon stimulation by rh-MOG in MOGR, the percentages in stimulated conditions in MOGR remained higher than in MOGAD and control group. Foxp3 is essential for differentiation and suppressive function of T_{regs}. In human, conventional non T_{reg} T-cells have been shown to transiently express Foxp3 upon activation (47). Additionally, emerging evidence suggests that Foxp3 expression is not always stable in T_{regs} and can be lost. Several studies on T_{reg} in other autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis, suggest a loss of Foxp3 expression and the generation of pathogenic Th17 cells (48), in association with Foxp3 instability in T_{regs} in these diseases. Different mechanisms would be involved in controlling the stability and expression of Foxp3. Demethylation of an evolutionarily conserved element within the Foxp3 genomic locus, conserved noncoding sequences 2 (CNS2), was described as an important feature regarding its expression stability (49, 50). Recently, downregulation of Foxp3 mRNA expression was described in PBMC of NMOSD patients (51). The decrease, significant for CD45RA⁺Foxp3⁺ T_{regs} and trend for eT_{regs}, we found in MOGR upon rh-MOG stimulation could also be explained by apoptosis of T_{regs}. In healthy condition, T_{regs} are resistant to apoptosis induced by T-cell receptor (TCR) (52), including T_{regs} expressing self-reactive TCR (53). However, in autoimmune thyroiditis, it was recently suggested that apoptosis of T_{regs} through self-reactive TCR activation can drive autoimmunity (54). In a study based on PBMC stimulation *in vitro*, examination of AQP4-specific T-cells revealed a significantly reduced frequency of T_{regs} in NMOSD patients in response to rhAQP4, in comparison to healthy controls (55). Decrease of Foxp3⁺ T_{reg} cells could influence the multiphasic evolution of MOGAD and be linked to a poor control of inflammation. In our study, it is tempting to speculate that MOGAD patients, which have higher Th17 in response to rh-MOG than MOGR at onset, can control autoimmunity since their T_{regs} are also increased upon rh-MOG stimulation. On the contrary, MOGR patients, in which T_{regs} and particularly CD45RA⁺Foxp3⁺ T_{regs} are decreased in response to rh-MOG, may have intermittent loss of tolerance toward this autoantigen and therefore enter into a relapsing form of MOGAD.

In primates treated with rhMOG/IFA, an EAE is induced having immune-inflammatory characteristics closer to MOGAD than to MS (27). Subsequent treatment of these primates with anti-DC-ASGPR-MOG increased their number of MOG-specific CD4⁺CD25⁺FOXP3⁺CD39⁺ T_{regs} as compared to controls. This increase likely precluded EAE seen in the controls (28) and

reminded the increase in T_{regs} upon rh-MOG stimulation found in MOGMR patients.

One major limitation of our study is the small sample size of patients including MOGAD, but this is inherent to the fact that ADS are rare diseases. The timing of the samples was not always similar in all patients and there might be temporal modifications of immune cells, which could also have biased our work. The absence of healthy control is also a limitation.

CONCLUSIONS

In conclusion, an increase of Th2 and Th17 after stimulation by rh-MOG was observed in ADS children with MOGAD, particularly in MOGMR. T_{regs} have differences in their subset pattern and behave differentially in MOGR and MOGMR. $CD4^+Foxp3^+$ T cell are increased upon rh-MOG stimulation specifically in MOGMR while in MOGR $CD45RA^+Foxp3^+$ T_{regs} decreased upon rh-MOG stimulation. This may reflect instability or apoptosis of T_{regs} induced by rh-MOG that may subsequently contribute to a probable loss of tolerance toward MOG autoantigen in MOGR which may explain relapses in this recurrent pediatric autoimmune disease. These results suggest that T_{regs} are targets to develop new therapeutic strategies of MOGAD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Commission Nationale de l'Informatique et des Libertés. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PH, CS, RL and KD conceived and designed the experiments. PH, AC, LG, CL and PC performed the experiments. PH, AC, LG, CL, PC and SH-B-A analyzed and interpreted the data. PH, CB, XM and KD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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