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ORIGINAL RESEARCH ARTICLE

# Administration of $CD4^+CD25^{high}CD127^-FoxP3^+$ Regulatory T Cells for Relapsing-Remitting Multiple Sclerosis: A Phase 1 Study

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## Abstract

**Background** Multiple sclerosis (MS) is an immune-mediated disease in which autoimmune T conventional ($T_{conv}$) cells break the blood–brain barrier and destroy neurons of the central nervous system. It is hypothesized that $CD4^+CD25^{high}CD127^-FoxP3^+$ T regulatory ($T_{reg}$) cells may inhibit this destruction through suppressive activity exerted on $T_{conv}$ cells.

**Methods** We present the results of a phase 1b/2a, open-label, two-arm clinical trial in 14 patients treated with autologous $T_{reg}$ cells for relapsing-remitting MS. The patients received either expanded ex vivo $T_{reg}$ cells intravenously (intravenous [IV] group, $n = 11$; dose $40 \times 10^6$ $T_{reg}$ cells/kg of body weight) or freshly isolated $T_{reg}$ cells intrathecally (intrathecal [IT] group, $n = 3$; dose $1.0 \times 10^6$ $T_{reg}$ cells). Importantly, patients were not treated with any other disease-modifying drugs for at least 6 months before the recruitment and during the follow-up.

**Results** No severe adverse events were observed. Self-assessed quality of life (EuroQol–5 Dimensions [EQ-5D] form) did not change and did not differ significantly between the groups. A total of 12 relapses were noted in five intravenously treated patients, who had from one to three attacks per year. Three out of ten participants who completed the trial in the IV group deteriorated more than 1 point on the Expanded Disability Status Scale (EDSS) during the follow-up. At the same time, no patients in the IT group experienced a relapse or such a deterioration in the EDSS. No significant differences were found in the Multiple Sclerosis Functional Composite (MSFC) scale in both the IV and IT groups. Magnetic resonance imaging (MRI) scans revealed a significantly lower change in the T2 lesion volume in the IT group compared to the IV group. The increase in the number of new T2 lesions during the follow-up was significant for the IV group only. There were no significant changes in the level of $T_{reg}$ cells or $T_{conv}$ cells in the peripheral blood throughout the follow-up or between the groups. Interestingly, $T_{reg}$ cells in all patients consisted of two different phenotypes: peripheral $T_{reg}$ cells Helios(−) (≈ 20%) and thymic $T_{reg}$ cells Helios(+) (≈ 80%). The analysis of the cytokine pattern revealed higher levels of transforming growth factor-α and proinflammatory factors MCP3, CXCL8, and IL-1RA in the IT group compared with the IV group.

**Conclusions** No serious adverse events were reported in the 14 patients with MS treated with $T_{reg}$ cells in this study. The results suggest that IT administration is more promising than IV administration. Because of the low number of patients recruited, the statistical results may be underpowered and further studies are necessary to reach conclusions on efficacy and safety.

**Trial registration** EudraCT: 2014-004320-22; registered 18 November 2014.

Kamil Chwojnicki, Dorota Iwaszkiewicz-Grześ and Anna Jankowska contributed equally to this study.

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Extended author information available on the last page of the article

**Key Points**

The safety results were good with intravenous and intrathecal routes of administration in the trial of T regulatory ($T_{reg}$) cells in the treatment of relapsing-remitting multiple sclerosis.

This trial was a proof of concept that intrathecal administration of $T_{reg}$ cells is more promising than intravenous administration in the treatment of relapsing-remitting multiple sclerosis

## 1 Introduction

The current knowledge on the pathogenesis of multiple sclerosis (MS) assumes that the disease is triggered by a dysregulation of immune effector–suppressor cell interactions. This ultimately leads to the formation of autoreactive adaptive immune cells (T conventional [$T_{conv}$] cells) that are capable of infiltrating and promoting damage within the central nervous system (CNS). However, progressive neurodegeneration after an autoimmune attack is highly dependent on chronic inflammation in the CNS. This self-sustaining process is kept going by both immune cells trafficking from the periphery via a damaged blood–brain barrier and, even more importantly, by tissue-resident cells [1]. These two phases of the disease are equally important for the progression of MS, as implied by the current clinical experience. For example, currently available approved immunomodulatory drugs target mainly the systemic inflammation and, therefore, they efficiently reduce the frequency and severity of relapses, but do not ultimately halt neurodegeneration dependent on the local environment of the CNS [1]. It seems that regaining a proper balance between effector and suppressor cells as well as quenching of the inflammation in the CNS should constitute the two parts of an efficient combined treatment of the disease. This implies the necessity to simultaneously administer several drugs affecting particular immune mechanisms or the administration of a single drug with multimodal activity in order to target both the peripheral immune system and the inflammation in the CNS. However, it seems that combined treatment with currently available drugs may cause severe adverse events [2].

For this reason, novel drugs with better safety and efficacy profiles targeting several mechanisms of MS, possibly as monotherapy, might be helpful. The population of $CD4^+CD25^{high}CD127^-FoxP3^+$ T regulatory ($T_{reg}$) cells is a good candidate for such a treatment. The cells occur physiologically in the immune system, in which they are responsible for immune tolerance. The function of $T_{reg}$ cells is recognized as crucial in the protection of the body against autoimmune reactions [3]. Importantly, a decreased function of T lymphocytes with a regulatory role has been implicated in MS [1, 4]. From the biological perspective, these are live cells with a myriad of different mechanisms through which they regulate and suppress exaggerated immune responses [5–7]. In addition, $T_{reg}$ cells are easily trafficked throughout the body and exert their action when attracted by inflammation [8]. From the clinical perspective, different populations of $T_{reg}$ cells are currently manufactured as cellular drugs and have been tested in dozens of clinical trials all over the world as a treatment for graft versus host disease (GvHD) after bone marrow transplantations, solid-organ allograft rejection, or autoimmune conditions [9].

In our center, $T_{reg}$ cells have been tested in clinical trials for over 10 years now [10]. Having had a positive experience with the treatment of GvHD and type 1 diabetes [11–15], we chose relapsing-remitting MS as the next therapeutic target. Taking into account that this kind of treatment has never been studied in this condition, we conducted a phase 1b/2a safety trial in order to evaluate safety (primary endpoint) and some measures of efficacy (secondary endpoints). Bearing in mind that immune cells that trigger the disease can traffic throughout the whole body, but the changes in MS affect mainly the highly insulated CNS, we designed the trial with two routes of administration of the cellular product. Administering the product intravenously, we could address the hypothesis that the systemic dysregulation between $T_{conv}$ cells and $T_{reg}$ cells might trigger the disease and relapses. From the safety point of view, this is a feasible low-risk procedure, as dictated by our experience from previous trials [14]. On the other hand, intravenous (IV) treatment requires a high number of $T_{reg}$ cells in order to counterbalance the systemic presence of a high number of autoimmune $T_{conv}$ cells. The intrathecal (IT) route of administration tested in the trial addressed the importance of local inflammation in the CNS. This approach has also been tested with good results in animal models [16]. It has been suggested that $T_{reg}$ cells given intrathecally bypass the blood–brain barrier and enter the CNS directly. It is also likely that they can operate in the meninges, where the compartmentalized inflammation, including pseudo-follicles, orchestrates the chronic CNS damage. An advantage of such an approach is that a significantly lower number of $T_{reg}$ cells is needed to cover the relatively low number of local proinflammatory cells present in the CNS. On the other hand, $T_{reg}$ cells are administered via a lumbar puncture, which requires an experienced physician and brings additional risks of complications associated with this procedure.

Importantly, the patients recruited for the trial did not receive any disease-modifying drugs for MS at least 6

months before recruitment and throughout the follow-up. Together with other inclusion criteria, this gave us the ability to monitor the homogenous group of MS patients. As a result, the majority of the observed effects during the follow-up could be attributed exclusively to the administered $T_{reg}$ cells and not to other forms of treatment, with the exception of therapy for relapses. On the other hand, this made it very difficult to find and recruit patients and, therefore, we were not able to enroll as many patients as planned in the clinical protocol of the trial. For this reason, the statistical results may be underpowered, and further studies are necessary for final conclusions. Still, the acquired data allowed us to present this proof-of-concept report.

## 2 Methods

### 2.1 Protocol and Treatment

The study recruited patients with the relapsing-remitting form of MS, diagnosed according to the McDonalds criteria or revised McDonald criteria [17, 18], and these patients were randomized to IV or IT administration of $T_{reg}$ cells. Other inclusion criteria were as follows: at least one relapse during the last year or at least two relapses in the preceding 2 years; up to 4 points on the Expanded Disability Status Scale (EDSS); the ability to provide written informed consent; and appropriate venous access for blood drawing. The most important exclusion criterion was any immunosuppressive therapy administered up to 6 months before the administration of the $T_{reg}$ cell preparation. The only exception was glucocorticoids, which could be administered as a treatment for relapses only. Importantly, patients had full access to the standard of care before and at the time of the study. Other exclusion criteria included the following: other autoimmune diseases; diagnosed immunodeficiencies; presence or history of active infections, including hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), tuberculosis, and systemic fungal infections; any history of malignancy; diagnosed cytopenia; elevated thrombotic activity or history of past thrombosis; hospitalization for cardiovascular events in the last 2 years before inclusion; increased intracranial pressure defined as papilledema; any retinopathy; arterial hypertension; presence or history of macroalbuminuria; excessive anxiety of the patient related to the procedures; any medical condition that, in the opinion of the investigator, may interfere with safe participation in the trial; known active alcohol or substance abuse; positive pregnancy test (for female participants); unwillingness to use effective contraceptive measures during the study and for 4 months after discontinuation, when appropriate; and intent to procreate during the study or within 4 months after discontinuation, when appropriate (for male participants).

The follow-up started at administration of $T_{reg}$ cells (day 0) and lasted 12 months, with visits at +14 days, +3 months, +6 months, +9 months, and +12 months post-administration. The endpoints measured included the number and intensity of the therapy side effects, the number of annual relapses, worsening on the EDSS scale by at least 1 point, changes in the Multiple Sclerosis Functional Composite (MSFC) scale, changes in magnetic resonance imaging (MRI) scans according to the Magnetic Resonance Imaging in MS (MAGNIMS) 2015 consensus, changes in quality-of-life questionnaire [EuroQol–5 Dimensions [EQ-5D] form] score, peripheral blood lymphocyte immunophenotype, and serum cytokines levels. The design of the study followed the "Guideline on Clinical Trials in Small Populations" [19], and received opinions and approval from the appropriate ethics committee.

### 2.2 Manufacture and Administration of $T_{reg}$ Cells

The manufacture of $T_{reg}$ cells was performed under Good Manufacturing Practice (GMP) conditions, similarly to our previous trials [11–14].

The cells were isolated from patients' venous peripheral blood (450 mL) with a high efficiency particulate air filter (HEPA) enclosed fluorescence-activated cell sorting (FACS) sorter (Influx, BD Biosciences, USA) using exchangeable sterile sample lines to the following phenotype: $CD3^+CD4^+CD25^{high}CD127^-lin^-doublet^-$. The sort itself was based on the staining and gating of the cells with GMP-grade monoclonal antibodies from Miltenyi Biotec, Germany (fluorochrome/class/clone): anti-CD4 (VioBlue/IgG1/M-T466), anti-CD25 (phycoerythrin [PE]/IgG1/3G10), and anti-CD127 (allophycocyanin [APC]/IgG1/MB15-18C9). Average post-sort $T_{reg}$ cell purity was ≈ 98% (range 97–100%). The phenotype and impurities were additionally confirmed from post-sort samples of $T_{reg}$ cells using monoclonal antibodies from BD Biosciences, Poland (fluorochrome/class/clone): anti-CD3 (PacificBlue/IgG1/UCHT1), anti-CD4 (V-500/IgG1/RPA-T4), anti-CD8 (peridinin-chlorophyll-protein [PerCP]/IgG1/SK1), anti-CD19 (PerCP/IgG1/4G7), CD14 (PerCP/IgG2b/MφP9), anti-CD16 (PerCP-Cy5.5/IgG1/3G8), anti-CD25 (PE/IgG1/M-A251), and anti-CD127 (APC/IgG1/hIL-7R-M21).

For IV administration, the expansion of $T_{reg}$ cells was performed using clinical grade anti-CD3/anti-CD28 beads (Miltenyi Biotec), interleukin (IL)-2 (aldesleukin, Novartis), and inactivated autologous serum for up to 14 days (median [minimum–maximum] = 11 [10–14]). The medium (X-Vivo20, Lonza) was supplemented with 10% serum and 1000 UI/mL of IL-2 throughout the entire expansion. The beads were added to the cells in a 1:1 ratio at the beginning

of expansion and then during passages on days +7, +8, and +9 to restore the 1:1 ratio. The culture was washed out from the beads and left in 10% serum and a low level of IL-2 (100 UI/mL) for the last 24–48 h of the culture. The sentinel culture with autologous CD4+ $T_{conv}$ cells was performed in 10% serum and a low level of IL-2 (100 UI/mL) as a source of T responders for functional tests. Quality control for the cultures was performed on day +7 and on the release of the product. The interferon (IFN)γ suppression assay was performed as previously described [15]. Briefly, a sample of $T_{reg}$ cells from the expansion cultures (washed out from the beads and left resting for at least 24 h) was co-cultured with autologous sentinel $T_{conv}$ cells in a 1:1 ratio. The controls consisted of the cultures of $T_{conv}$ cells or $T_{reg}$ cells only, either stimulated or not stimulated to produce IFNγ. Immediately prior to the assay, $T_{conv}$ cells were stained with cell tracer CFSE (CFDA kit Thermo, USA) in order to distinguish them from $T_{reg}$ cells, and therefore, it was possible to give separately the proportions of IFNγ-positive $T_{reg}$ cells and $T_{conv}$ cells at the end of the assay. The stimulation of the cultures and staining were performed with an intracellular staining kit (BD Biosciences, Poland) according to the manufacturer's description. The cultures were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate, 500 ng/mL of ionomycin (Sigma, Poland), and 2 µL/mL of cytokine leakage inhibitor GolgiPlug (BD Biosciences, Poland) for 5 h. Then, the cells were stained with anti-IFNγ antibodies. The positive readout of the assay was the suppression of IFNγ production by $T_{conv}$ cells co-cultured with $T_{reg}$ cells by at least 25% (median [minimum–maximum] = 69% [52–95]), when compared to the production of IFNγ in the cultures with $T_{conv}$ cells only. The production of IFNγ by $T_{reg}$ cells never exceeded 2% of the cells. Microbial safety was confirmed through negative results of microbiology cultures of supernatants from expansion media (BD Bactec system, BD Biosciences, Europe), negative endotoxin tests from supernatants of expansion media (Endosafe – PTS Endotoxin Cartridge/Cartridge reader, Charles River, USA), negative Gram staining of the supernatants from expansion media (Gram Stain Kit, BD Biosciences, Europe), and the absence of genetic material of HBV, HCV, HIV-1, and HIV-2 in the product (Cobas MPX, Roche, Europe). Patients were followed for any adverse symptoms related to the possible contamination of the product until all microbial post-release results were confirmed negative. The ready-to-use preparation of $T_{reg}$ cells had to be administered within 2 h of the release from tissue establishment. The final dose was $40 \times 10^6$ $T_{reg}$ cells per kg of the body weight. Upon release, the preparation was washed out completely, suspended in 250 mL of 0.9% NaCl for injection (Polfa, Warsaw), and then administered in a slow IV infusion to the patient.

For patients treated intrathecally, 1 million ($1 \times 10^6$) of freshly isolated $T_{reg}$ cells (without expansion) was examined according to the release criteria described above and then suspended in 10 mL of 0.9% NaCl. Afterwards, it was administered in a slow injection during L4/L5 or L5/S1 lumbar puncture through a puncture needle. There was a 6-h bed regimen post-injection.

The predefined block randomization was used to assign the patients to a particular interventional arm. Both IV and IT doses received opinions and approval from the ethics committee (Fig. 1S, see the electronic supplementary material).

### 2.3 Clinical Assessment

Apart from routine physical/neurological examinations at the site visits, patients were also assessed according to the EDSS and MSFC scales by certified neurologists [20–22] to monitor disease progression and according to the EuroQol–5 Dimensions (EQ-5D) questionnaire to monitor quality of life [23]. The following laboratory tests were performed (only significantly abnormal values are shown): complete blood count, metabolic, kidney, and liver panels, C-reactive protein levels, and urinalysis. For safety reasons, the clinical team was not blinded to the administration route used to treat particular patients.

### 2.4 MRI Assessment

MRI of the brain was performed according to the MAGNIMS 2015 standard protocol (3D T1-weighted, 3D T2-fluid-attenuated inversion recovery [FLAIR], 3D T2-weighted, and post–single-dose gadolinium-enhanced T1-weighted imaging, all with a nongapped section thickness of ≤ 3 mm, and a diffusion-weighted MRI [DWI] sequence [≤ 5-mm section thickness, 1,5 Tesla Magnetom Aera, Siemens, Germany]) [24, 25]. MRI scans were performed during visits at +3 months, +6 months, and +12 months post-administration. The assessment of lesions and their progression was made using BrainMagix software (Brussels, Belgium) and Philips IntelliSpace Portal 10; the total number of plaques and number contrast-enhanced plaques were counted by two observers blinded to the administration route used to treat the patients.

### 2.5 Immune Responses

Immune phenotyping was performed using ten-color panels to follow $CD3^+CD4^+CD25^{high}CD127^-FoxP3^+$ $T_{reg}$ cells and $CD3^+CD4^+CD25^{low/-}CD127^+FoxP3^-$ $T_{conv}$ cells in the peripheral blood. In both populations, the expression of antigens important for the functioning of these subsets was followed. We specifically determined the percentage of naïve/memory subsets based on the following phenotypes: naïve ($T_n$) ($CD62L^+CD45RA^+$), central memory

($T_{cm}$) ($CD62L^+CD45RA^-$), and effector memory ($T_{em}$) ($CD62L^-CD45RA^-$). $CD3^+CD4^+CD25^{high}CD127^-FoxP3^+$ $T_{reg}$ cells were further divided based on the expression of transcription factor Helios into peripheral [$pT_{reg}$ Helios(−)] and thymic [$tT_{reg}$ Helios(+)] subsets [26] (Fig. 2S, see the electronic supplementary material).

The following anti-human monoclonal antibodies purchased from BD Biosciences, Poland, were used in this procedure (fluorochrome/class/clone): anti-CD3 (PacificBlue/IgG1/UCHT1 or V500-C/IgG1/clone SK7), anti-CD4 (PerCP or AlexaFluor700/IgG1/RPA-T4), anti-CD25 (PE or BV786/IgG1/M-A251), anti-CD127 (FITC or BUV737/IgG1/hIL-7R-M21), anti-CD45RA (PE-Cy7/IgG1/L48), anti-CD73 (BUV737/IgG1/AD2), anti-CD279 (BV605/IgG1/EH12.1), anti-CD137 (BV650/IgG1/4B4-1), anti-CD134 (BV711/IgG1/ACT35), anti-CD152 (BV786/IgG1/BNI3), anti-CD18 (FITC/IgG1/L130), anti-CD184 (PE-CF594/IgG1/12G-5), anti-CD194 (BV605/IgG1/1G1), anti-CD39 (BV650/IgG1/TU66 or BUV737/IgG1/TU66), and anti-CD103 (BUV395/IgG1/Ber-ACT8). Anti-CD62L (APC-Cy7/IgG1/3B5) was supplied by Invitrogen, USA; the FoxP3 staining kit and anti-Helios (eFluor450/IgG1/22F6), by eBioscience/Thermo Fisher, USA; and anti-CCR8 (PerCP/IgG1/91704) and anti-CCR10 (PE/IgG1/314305) by R&D/Biotechne, UK.

Serum levels of 38 cytokines (IFNα2, IFNγ, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, sCD40L, IL-17, IL-2, IL-1RA, IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-9, tumor necrosis factor [TNF]α, TNFβ, epidermal growth factor [EGF], fibroblast growth factor [FGF]2, transforming growth factor [TGF]α, granulocyte colony-stimulating factor [G-CSF], granulocyte–macrophage colony-stimulating factor [GM-CSF], vascular endothelial growth factor [VEGF],

Fms-related tyrosine kinase 3 ligand [FLT3L], IL-7, eotaxin, CX3CL1, CXCL1, MCP3, CCL22, IL-8, interferon gamma-induced protein 10 [IP-10], MCP1, MIP1α, and MIP1β) were measured with the Bead-based Multiplex Assay on a Luminex analyzer (Merck, USA). All assays were performed according to the manufacturers' instructions.

The same cytometer and protocols were used throughout the study. The cytometer and Luminex analyzer underwent routine internal quality checks and periodical operational and process qualifications conducted by the manufacturers.

### 2.6 Statistical Analysis

Data were computed with the software Statistica 12.0 (StatSoft, Poland). Cluster analysis was performed with ClustVis software (https://biit.cs.ut.ee/clustvis/#mathematics). The analysis was carried out with nonparametric tests. $p \leq 0.05$ was considered statistically significant.

## 3 Results

### 3.1 Population

Fourteen MS patients (18–55 years old) were recruited into the two groups treated with $T_{reg}$ cells either intravenously (IV group, $n = 11$) or intrathecally (IT group, $n = 3$) (Table 1 and Fig. 1S [see the electronic supplementary material]). One patient from the IV group dropped out of the trial due to pregnancy during the follow-up.

### 3.2 The Characteristics of $T_{reg}$ Cell Preparation on Release

The final product on release kept FoxP3 expression above 90% (median [minimum–maximum] = 91% [90–97]) and CD62L expression above 80% (median [minimum–maximum] = 87% [81–95]). Passed IFNγ suppression assay and microbiological tests were negative.

### 3.3 Safety

No serious adverse events were reported throughout the trial. Moderate adverse events were noted in patients treated with $T_{reg}$ cells intravenously. The most common adverse events were relapses and the presence of new or enlarging T2 lesions in the CNS. Interestingly, no adverse events were noted in patients administered with $T_{reg}$ cells intrathecally (Table 2).

**Table 1** Epidemiological characteristics of the patients

| Trait | Intravenous administration [$n = 10$] | Intrathecal administration [$n = 3$] |
|---|---|---|
| Age (years), median (min–max) | 28 (19–51) | 34 (26–45) |
| Sex (M/F), *n* | 6/5 | 3/0 |
| Age at diagnosis (years), median (min–max) | 25 (18–39) | 31 (25–44) |
| Disease duration at recruitment to the study (years), median (min–max) | 5 (0.3–13) | 1.5 (1–3) |

*F* female, *M* male, *max* maximum, *min* minimum

**Table 2** Adverse events in the trial

| Adverse event | Number of patients/events/% of patients | Severity |
|---|---|---|
| **$T_{reg}$ cells administered intravenously ($n = 10$)** | | |
| Relapse of MS | 5/12/50% (3 patients experienced 3 relapses, 1 patient experienced 2 relapses, and 1 patient experienced 1 relapse) | Moderate |
| New or enlarging T2 lesions in the CNS on MRI | 5/5/50% | Moderate |
| Progression of visual impairment | 1/1/8% | Moderate |
| Liver injury (increased AST and ALT without clinical symptoms, unknown etiology) | 1/1/8% | Moderate |
| **$T_{reg}$ cells administered intrathecally ($n = 10$)** | | |
| No adverse events reported | | |

*ALT* alanine transaminase, *AST* aspartate transaminase, *CNS* central nervous system, *MRI* magnetic resonance imaging, *MS* multiple sclerosis, $T_{reg}$ T regulatory

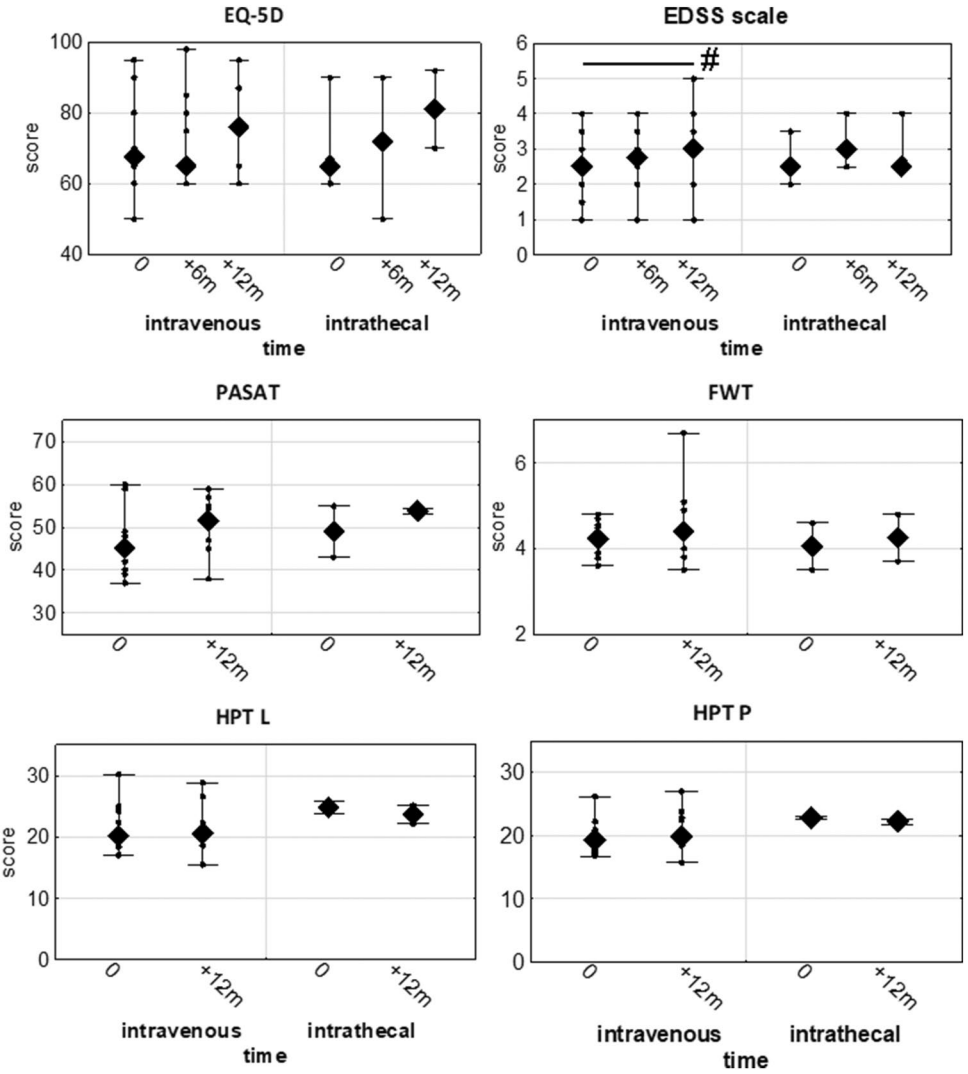


**Fig. 1** Clinical outcomes in the study. The patients underwent protocol-planned neurological examinations throughout the trial. Quality of life was assessed with the EuroQol–5 Dimensions (EQ-5D) questionnaire, and physical/neurological status was monitored with the Expanded Disability Status Scale (EDSS) and the components of the Multiple Sclerosis Functional Composite (MSFC) scale, such as Timed 25-Foot Walk (FWT), Dominant (9-HPT P) and Non-dominant (9-HPT L) Nine-Hole Peg Test (9-HPT), and the Paced Auditory Serial Addition Test (PASAT). The scores are presented throughout the follow-up separately for the patients administered intravenously and intrathecally as medians (minimum–maximum), and dots represent raw data. The significant changes over time in particular groups are marked with a line and hash (#)

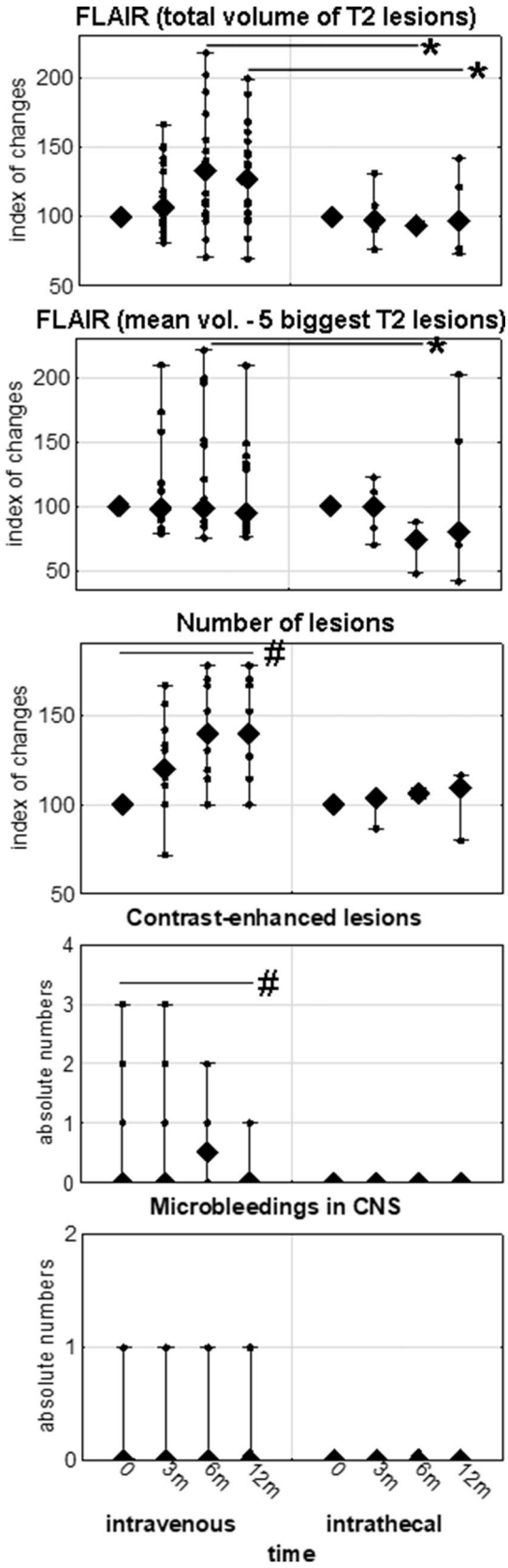


**Fig. 2** The progression of disease in the central nervous system using magnetic resonance imaging (MRI). The patients underwent protocol-planned MRI examinations throughout the trial. The most important changes are presented as the index of change of the T2 lesion volume on fluid-attenuated inversion recovery (FLAIR) sequence, of the volume of the five biggest T2 lesions on FLAIR sequence, and of the number of plaques. The index of changes on *y* axes was calculated from the individual values of the variables from day 0 (immediately before administration of regulatory T [$T_{reg}$] cells), which were treated as '100,' and the changes in the following examinations were calculated proportionally. The changes in the values of contrast-enhanced lesions and the number of microbleeds are presented as absolute numbers. The indexes and the absolute values are presented throughout the follow-up separately for the patients administered intravenously and intrathecally as medians (minimum–maximum), and dots represent raw data. The between-group differences are linked with a line and marked with an asterisk (*), and the changes over time in particular groups are marked with a line and hash (#)

The analysis of patients' quality of life revealed no deterioration in the self-assessment using the EQ-5D form. The results were similar in both groups throughout the follow-up (all tests $p > 0.05$, Fig. 1).

### 3.4 Clinical Assessment

The clinical status of patients assessed using the EDSS scale did not differ between the groups throughout the study (Kruskal-Wallis analysis of variance [ANOVA]: day 0: $H = 0.18$, $p = 0.66$; +6 months: $H = 0.36$, $p = 0.54$; +12 months: $H = 0.029$, $p = 0.86$). Longitudinal analysis performed for each of the groups separately revealed a significant increase in EDSS score in the IV group only (Friedmann ANOVA: IV group: $\chi^2 = 7.32$, $p = 0.02$; IT group: $\chi^2 = 4.71$, $p = 0.09$) (Fig. 1). One-year deterioration on the EDSS scale within the IT group and within the IV group was from 0 to 0.3 and from 0 to 1, respectively. In the IV group, three out of ten participants (30%) had a deterioration higher than 1 point on the EDSS scale. No such deterioration was seen in those treated intrathecally (0%). A total of 12 relapses were noted in five patients treated intravenously (50%), with the frequency of 1–3 episodes per year during the follow-up. At the same time, no relapses were observed in the IT group (0%).

The clinical status of patients assessed using the MSFC scale did not change in any group and did not differ between the groups in any of the scale components throughout the study (all tests $p > 0.05$, Fig. 1).

### 3.5 MRI Assessment

When compared to the IV group results, the analysis of MRI scans revealed lower disease activity in the IT group (Fig. 2).

The FLAIR sequence revealed that the total volume of T2 lesions in the CNS throughout the follow-up increased in the IV group, while it did not change in the IT group (Friedman's ANOVA: IV group: $\chi^2 = 12.79$, $p = 0.005$; IT group:

$\chi^2 = 4.5$, $p = 0.21$). The difference between the groups was significant at 6- and 12-month follow-up (Kruskal-Wallis ANOVA: 3 months: $H = 1.65$, $p = 0.19$; 6 months: $H = 6.14$, $p = 0.013$; 12 months: $H = 5.33$, $p = 0.047$). The difference was also seen when the volume of the five biggest T2 lesions (Kruskal-Wallis ANOVA: 3 months: $H = 0.01$, $p = 0.91$; 6 months: $H = 7.77$, $p = 0.005$; 12 months: $H = 2.34$, $p = 0.067$) and the number of new plaques (Kruskal-Wallis ANOVA: 3 months: $H = 3.76$, $p = 0.15$; 6 months: $H = 5.10$, $p = 0.076$; 12 months: $H = 4.61$, $p = 0.091$) were compared between the groups. Interestingly, it was the increasing number of plaques in the IV group that was responsible for the increase in the total volume of the plaques during the follow-up (Friedman's ANOVA for the number of plaques: IV group: $\chi^2 = 20.77$, $p = 0.0001$; IT group: $\chi^2 = 5.5$, $p = 0.13$), rather than the changes of the existing biggest plaques (Friedman's ANOVA for mean volume from the five biggest plaques: IV group: $\chi^2 = 3.66$, $p = 0.30$; IT group: $\chi^2 = 3.90$, $p = 0.27$). In addition, contrast-enhanced T1 lesions in the IV group decreased significantly at the end of the trial. This was not the case for intrathecally treated patients, as these lesions were not seen in this group throughout the follow-up (Friedman's ANOVA: IV group: $\chi^2 = 11.41$, $p = 0.009$; IT group: all numbers 0). Neither the volume of the main CNS structures nor the volume of T1 hypointensiveness differed between the groups (Fig. 3S, see the electronic supplementary material).

## 3.6 Immune Response

### 3.6.1 $T_{reg}$ Subsets

There were no significant changes in the level of FoxP3$^+$ $T_{reg}$ cells and $T_{conv}$ cells throughout the follow-up or between the groups (all tests $p > 0.05$, Fig. 3a). However, $T_{reg}$ cells differed from $T_{conv}$ cells in several measured subsets in all patients, regardless of the route of administration of $T_{reg}$ cells. When all patients were taken into account together, in the phenotype comparison of $T_{reg}$ cells and $T_{conv}$ cells, $T_{reg}$ cells contained mostly the $T_{cm}$ phenotype (50% or more), while $T_{conv}$ cells contained mostly the $T_n$ phenotype (50% or more) (Fig. 3b and Table 1S [see the electronic supplementary material]). We also found that $T_{reg}$ cells expressed several receptors, such as chemokine receptors CCR10, CXCR4, CCR4, integrin CD103, ectonucleotidase CD39, and two costimulatory molecules (CTLA-4 and 4-1BB), which were almost undetectable in $T_{conv}$ cells (Fig. 4Sa and b and Table 1S, see the electronic supplementary material). The difference between $T_{reg}$ cells and $T_{conv}$ cells in the expression of these receptors was confirmed with cluster analysis (Fig. 3c).

In addition, around 20% of $T_{reg}$ cells in all patients did not express the transcription factor Helios, suggesting a peripheral origin for these cells (Fig. 3a). Having that in mind, we performed a deeper analysis, dividing $T_{reg}$ cells into thymic FoxP3$^+$Helios(+) $tT_{reg}$ cells and peripheral FoxP3$^+$Helios(−) $pT_{reg}$ cells. When compared, $tT_{reg}$ cells contained a higher percentage of CCR10$^+$ cells, CD103$^+$ cells, CD73$^+$ cells, and CD39$^+$ cells, while $pT_{reg}$ cells contained a higher percentage of CTLA-4$^+$ cells (Fig. 4Sc and Table 1S, see the electronic supplementary material). The cluster analysis confirmed that the higher expression of CCR10, CD103, and CD39 and lower expression of CTLA-4 receptors differs $tT_{reg}$ cells from $pT_{reg}$ cells (Fig. 3d).

### 3.6.2 Cytokines

The study also included an array of 38 different cytokines measured in the sera of patients. When compared to the intravenously treated patients, those treated intrathecally revealed higher levels of some factors associated with inflammation, such as MCP3, IL-1RA, and IL-8. Interestingly, the level of brain trophic factor TGFα was also higher in the IT group than in the IV group (Table 2S, Fig. 5S, see the electronic supplementary material). The levels of MCP3 and IL-1RA positioned in the same cluster differing the IT group from IV group (Fig. 4). The levels of other measured cytokines did not differ between the trial groups or within each group throughout the follow-up.

# 4 Discussion

The current trial confirms that both IV administration and IT administration of $T_{reg}$ cells are safe forms of treatment in MS patients. The results are also a proof of concept that IT administration of a $T_{reg}$ cell preparation is a promising way forward in the development of this therapy in MS, but this conclusion is limited by a small number of intrathecally treated participants in our trial. The analysis of $T_{reg}$ cell phenotypes revealed a surprisingly high percentage of peripheral Helios(−) $T_{reg}$ cells in the total $T_{reg}$ cell pool in all MS patients.

Immune intervention in MS is the treatment of choice in this condition. There are around 100 genes associated with the functioning of the immune system in this disease [27]. Although neurodegeneration is the major problem and it is ultimately responsible for the symptoms of MS, the erroneously functioning immune system is the primary cause of the disease. Importantly, the presence of autosensitized $T_{conv}$ cells does not provide the full explanation for the disease onset, as such cells have been also detected in healthy individuals [28]. It is a dysregulation between $T_{reg}$ cells and $T_{conv}$ cells that ultimately unleashes clinically relevant autoimmunity in MS [29]. There are clear animal models in which depletion of $T_{reg}$ cells can trigger experimental

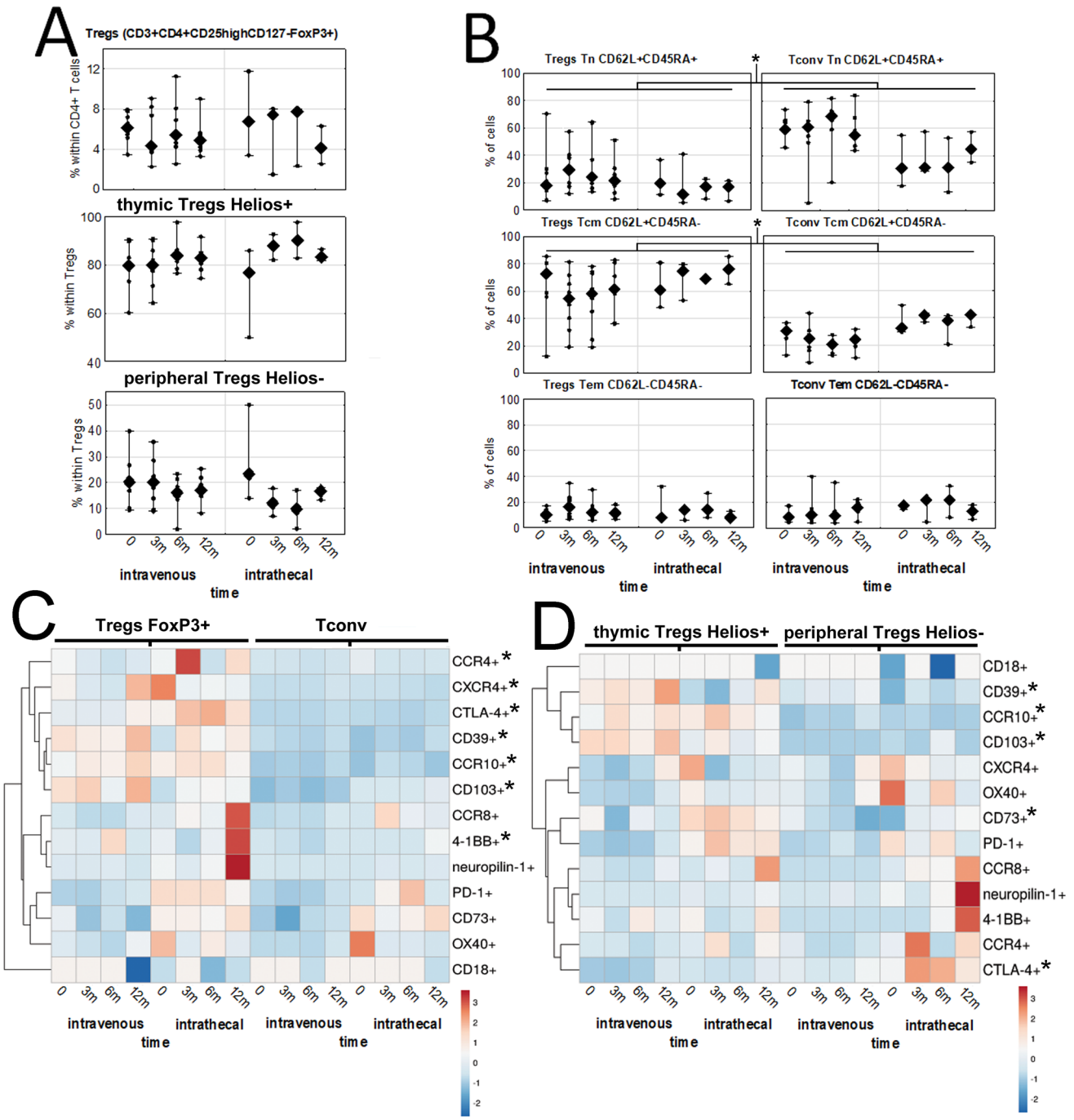


**Fig. 3** The levels of T regulatory ($T_{reg}$) and T conventional ($T_{conv}$) cells throughout the study. The levels of all $T_{reg}$ cells ($CD3^+CD4^+CD25^{high}CD127^-FoxP3^+$), thymic $T_{reg}$ ($tT_{reg}$) cells ($CD3^+CD4^+CD25^{high}CD127^-FoxP3^+Helios^+$), and peripheral $T_{reg}$ ($pT_{reg}$) cells ($CD3^+CD4^+CD25^{high}CD127^-FoxP3^+Helios^-$) are shown throughout the study in charts (**a**). The levels of naïve ($T_n$) ($CD62L^+CD45RA^+$), central memory ($T_{cm}$) ($CD62L^+CD45RA^-$), and effector memory ($T_{em}$) ($CD62L^-CD45RA^-$) phenotypes in all $T_{reg}$ cells ($CD3^+CD4^+CD25^{high}CD127^-FoxP3^+$) and $T_{conv}$ cells ($CD3^+CD4^+CD25^{low/-}CD127^+FoxP3^-$) throughout the study are shown in charts (**b**). The percentages of cells expressing the following markers are presented as the heatmaps: CCR10, CXCR4, CCR4, CD103, CCR8, CD18, CD39, CD73, CTLA-4, PD-1, 4-1BB, and, OX40. The tree of clusters was ordered to find the markers with contrasting expression between $T_{reg}$ cells and $T_{conv}$ cells (heatmap **c**; detailed levels also in Fig. 4S, see the electronic supplementary material). A similar clustering analysis was performed to compare and contrast $tT_{reg}$ cells and $pT_{reg}$ cells (heatmap **d**). In charts **a** and **b**, the percentages of cells are presented at administration of the $T_{reg}$ cell preparation (day 0), +3, +6, and +12 months post-administration separately for the intravenous group and intrathecal group. The results are presented as medians (minimum–maximum), and dots represent raw data. The asterisks (*) throughout the figure mark significant differences

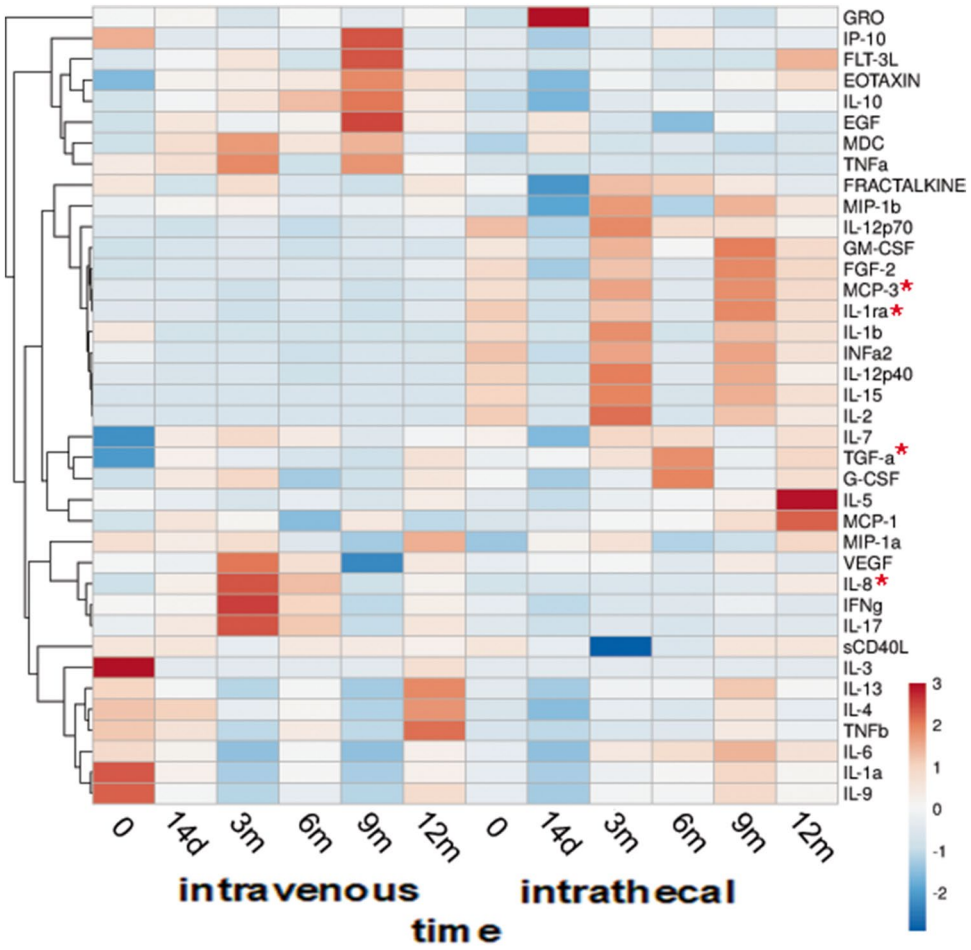


**Fig. 4** The levels of serum cytokines throughout the study. The levels of cytokines in the serum of the patients treated with intravenous or intrathecal injection of T regulatory ($T_{reg}$) cells are presented in the heatmap. The tree of clusters was ordered to find the cytokines with contrasting levels between these two groups of patients (detailed levels also in Fig. 5S, see the electronic supplementary material). The levels of cytokines are presented at administration of the $T_{reg}$ cells preparation (day 0), +14 days, +3, +6, +9, and +12 months post-administration separately for each trial group. The asterisks (*) mark the cytokines whose levels were significantly different between these two groups of patients

autoimmune encephalomyelitis (EAE), and adoptive transfer of these cells could protect from it [30]. In MS patients, when compared to healthy participants, decreased activity of $T_{reg}$ cells, rather than a difference in the number of cells, has been found. Interestingly, the impairment of suppressive function was reported in relapsing-remitting MS rather than in secondary progressive MS [31, 32]. Accumulation of $T_{reg}$ cells in cerebrospinal fluid and not in the blood of MS patients at remission has been found, which suggests the real site of the 'battlefield' [33]. Altogether, this evidence clearly points at $T_{reg}$ cells as being a tool to treat the disease, which we have tried to verify in the current trial.

Being aware of the immune dysregulation that triggers the disease, we administered a large number of $T_{reg}$ cells intravenously. It is the approach that we have already tested in other conditions, and these studies did not reveal any major safety issues [9]. The safety of $T_{reg}$ cells was also suggested by the results of the current trial. Nevertheless, the efficacy of such an approach appeared to be not exceptional. The first reason for that might be an intrinsic defect of $T_{reg}$ cells in MS patients reported by many studies [4]. However, it is rather not the case here, as the quality control of the preparation performed at release of the product proved the cells had a good suppressive profile. In addition, the number of the cells administered intravenously was relatively high, which should compensate for functional impairments on a single-cell level. Still, this group of patients suffered relapses and progression of the disease suggested objectively in MRI scans. In fact, this might be expected as we worked with real patients and not the animal model, which differ in the phase of MS. In animals, the interventions usually occur at the very beginning—often as a kind of prophylaxis—of the imminent pathological process which may result in EAE. In humans, the clinical onset occurs at the stage when immune processes are already heavily dysregulated and treatment is administered in the late stage of the disease. Given that, inhibition of local inflammation in the CNS rather than suppression of systemic processes should be followed as a therapeutic option. The second reason that IV therapy may have limited efficacy is the blood–brain barrier, which may limit bioavailability of the cells in the CNS. The eradication of local inflammation requires $T_{reg}$ cells, as suggested by their accumulation in the cerebrospinal fluid in MS patients at remission [33]. On the other hand, although the barrier is damaged in MS, we cannot exclude that proinflammatory cells traffic through more efficiently than $T_{reg}$ cells at relapse.

As a result, disruption of effector–suppressor balance triggers the flare of the disease [34].

Together, these are the arguments for the administration of $T_{reg}$ cells directly into the CNS. Interestingly, IT administration of $T_{reg}$ cells in our trial was not associated with a significant number of adverse events. In addition, in patients treated in this way, we did not record significant deterioration of their clinical status, which was confirmed by the lack of progression in MRI scans. When compared to the intravenously treated patients, the disease progression was slower in intrathecally treated patients. If true, this confirms that the local inflammation is the most important target in the treatment of this disease in the clinic. Currently, MS is mainly diagnosed at the moment of clinical onset. Hence, earlier systemic phases of the pathogenesis are negligible from the treatment point of view, as self-sustaining neuroinflammation in the CNS has already developed. For obvious reasons, we could not follow the proportions of the cells and soluble factors in the CNS affected by the local inflammation, but we could follow them in the peripheral blood of the patients. Interestingly, despite having a better clinical profile, the group treated intrathecally was characterized by higher levels of proinflammatory factors than patients treated intravenously. It confirms that the systemic changes may not correspond to the severity of local changes.

Although we screened the patients with a significant number of laboratory immune markers, we did not find anything unique. The analysis of the immune phenotype revealed two interesting features of $CD4^+$ T cells in the MS patients. First, $T_{reg}$ cells expressed mainly the central memory phenotype, while $T_{conv}$ cells remained naïve. It is usually the other way round, as $T_{conv}$ cells are the primary fighters against infections or accelerators of autoimmunity, which quickly turn them towards memory compartments, while $T_{reg}$ cells require the naïve phenotype for the superior performance of the immune system. The high percentage of memory $T_{reg}$ cells suggests that this compartment is actively involved in immune responses in MS patients. This may also explain the impaired suppressive function of these cells after the clinical onset of MS [31], as the memory phenotype, notably reduced expression of the CD45RA receptor, has been reported as a marker of instability and inferior function of $T_{reg}$ cells [35]. A second finding, somehow linked to the first one, was that $T_{reg}$ cells in all patients contained a surprisingly high percentage of peripheral Helios(−) $T_{reg}$ cells ($pT_{reg}$). Our experience from other chronic conditions, such as kidney allotransplantation or GVHD, but also from healthy adults, tells us that $pT_{reg}$ cells rarely consist of more than 10% of $FoxP3^+T_{reg}$ cells. $pT_{reg}$ cells are extrathymic suppressors that arise in response to a challenge with antigen via infectious tolerance when $tT_{reg}$ cells are around [36]. It is possible that $tT_{reg}$ cells excessively involved in immune responses not only 'erode' from the naïve to memory phenotype, but also 'infect' other $CD4^+$ T cells, shifting them towards $pT_{reg}$, which in turn accumulate with the time. Nevertheless, it is mainly the population of $tT_{reg}$ cells that protects from autoimmunity [37], so decaying $tT_{reg}$ cells unleash pathological responses of $T_{conv}$ cells sensitized against antigens from the CNS.

In this work, we tried to build some concepts of MS and the role of $T_{reg}$ cells in the treatment of this disease, but the obvious flaws of the trial execution, such as no control group, males only in the IT group, longer (insignificantly) duration of the disease in the IV group, and low number of patients recruited to the trial, require careful conclusions. The statistics given in the results are helpful, but the differences found may not survive with bigger numbers. Nevertheless, the strong point of these conclusions is the fact that the patients were not treated with any other MS drugs. For this reason, the effects observed in the trial are specific and may be attributed almost entirely to the treatment with $T_{reg}$ cells. It is very difficult to find such patients nowadays as there are many different MS therapies offered and usually even skeptical patients accept with time some form of treatment with one of the approved drugs. For that reason, we did not include an untreated control group, and we had to reduce the number of patients recruited as well as the time of the follow-up. Nevertheless, in an effort to reference our results to the natural course of the disease or results of routine treatment, we went through the results of completed placebo-controlled trials. In our search of the literature, per-year percentage of relapses in placebo-treated groups varied between 41% and 76%, the annual progression rate was between 17% and 46%, and significant progression in 1 year of follow-up in MRI was noted in at least 52% of patients, with an increase of new lesions of around 50%. At the same time, the patients on monotherapy (we chose IFNβ, glatiramer, and dimethyl fumarate for analysis) experienced an up to twofold reduction in per-year percentage of relapses (rate between 25% and 44% of patients), the annual progression rate was between 11% and 25%, and an increase of MRI changes of at least 10% was noted in around 29% of treated patients [38–43].

## 5 Conclusions

The primary endpoint of the study, which was safety, has been achieved, as no serious adverse events happened with either of the tested routes of $T_{reg}$ cell administration. This would allow a move to the next stage of development. Our results also suggest that IT administration should be of special interest in future trials. Nevertheless, these conclusions need to be confirmed in a larger, phase 2 trial, which is currently under preparation in our centre. The findings on the content of the $T_{reg}$ cell compartment require further

separate studies, which might allow us to get a deeper insight into the pathogenesis of MS and possibly provide an answer regarding which $T_{reg}$ cell phenotype is the best to administer in the treatment of this disease.

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## Declarations

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**Conflict of interest** PT and KC are co-inventors of patents related to the presented content. PT is a stakeholder and board member of POLTREG venture. Medical University of Gdańsk received payment for the license to the presented content. AJ, AJC, AK, BK, DIG, ES, JK, JS, KK, MG, MGrz, MK, MZ, PG, PŁ, and WN have no conflicts of interest relevant to the contents of this article.

**Ethics approval** The study was conducted according to the Declaration of Helsinki principles. The protocol has been registered in the EudraCT database under the number 2014-004320-22 and received approval from the Institutional Review Board of the Medical University of Gdańsk (no. NKBBN/414/2012 and NKBBN/414-163/2017).

**Consent to participate** Written informed consent was received from all the participants at recruitment, before any medical procedure was commenced.

**Consent for publication** All the patients signed written consent that the data obtained in the study could be used for scientific publications, provided the data are anonymized.

**Availability of data and material** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

**Author contributions** KC was the principal investigator and contributed to the study design, protocol writing, data collection, analysis, interpretation, and writing and reviewing the report. DIG was a head of the R&D team and contributed to the study design, cell preparation, data collection, analysis, and writing and reviewing the report. AJ contributed to the study design, protocol writing, MRI data collection, analysis, interpretation, and writing and reviewing the report. MZ contributed to lab assays, data collection, and interpretation. PŁ contributed to patient care, data collection, and interpretation. MG contributed to the cell preparation, data collection, analysis, and reviewing the report. MGrz contributed to MRI data collection, analysis, and interpretation. KK contributed to patient care, data collection, and interpretation. AK contributed to MRI data collection, analysis, and interpretation. PG contributed to patient care, data collection, and interpretation. AJC contributed to clinical assessment, and data collection. JS and JK contributed to the cell preparation, data collection, and analysis. MK contributed to patient care, data collection, and interpretation. BK, WN, and ES contributed to study design, data collection, and interpretation and reviewed the report. PT is the guarantor of the study, and supervised the whole process and contributed to the study design, protocol writing, cell preparation, data collection, analysis, interpretation, and writing and reviewing the report.

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