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ORIGINAL ARTICLE

# Monocyte and Lymphocyte Activation and Regulation in Multiple Sclerosis Patients. Therapy Effects

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## Abstract

Analysis of gut barrier status, monocyte and lymphocyte activation and T regulatory (Treg) cells at diagnosis before and after therapy, in patients with multiple sclerosis (MS). Analysis of differential effects of interferon beta (IFN-β), glatiramer acetate (GA) and natalizumab. Thirty-five patients with untreated MS were included. Gut barrier status (serum concentrations of intestinal fatty acid binding protein), monocyte (serum levels of soluble CD14, soluble CD163 and interleukin 6) and T lymphocyte activation (CD4 + DR+ and CD8 + DR+) and Treg (CD4 + CD25highFoxP3+) cells were analyzed. Patients with clinical isolated syndrome and relapsing-remitting forms were treated with IFN-β or GA, and immune characteristics were reevaluated following up after 6 months. A sample of 56 stable RR MS patients, in treatment with IFN-β, GA or natalizumab, and 50 healthy individuals were included as controls. Gut barrier status was similar in MS patients and healthy controls. Untreated patients with relapsing-remitting and primary progressive patterns of MS showed increased serum levels of soluble CD14. At baseline, significant increases in activated T lymphocytes and Treg were detected in patients. A significant decrease of CD4 + DR+, CD8 + DR+, and Treg percentages after 6 months of therapy was observed. In previously treated patients, IFN-β, GA, or natalizumab therapies were associated with a comparable cell proportion of activated lymphocytes and Treg. MS patients have a baseline state characterized by monocyte and lymphocyte activation, not related with gut barrier lesion. An increase in Treg number, correlated with activated T CD8+ lymphocytes, was detected. Treatment with IFN-β, GA or natalizumab was associated with a comparable decrease in activated lymphocytes and Treg.

**Keywords** Multiple sclerosis · Gut barrier permeability · sCD14 · sCD163 · Interleukin 6 · Activated T lymphocytes · T regulatory cells

## Introduction

Histopathological studies of patients with multiple sclerosis (MS) have shown that the immune system has a pathogenic role (Hemmer et al. 2015). Acute demyelinating white matter lesions show myelin breakdown that is accompanied by infiltrates of dendritic cells, mononuclear phagocytes, T and B lymphocytes, and plasma cells (Henderson et al. 2009). In addition, alterations of innate and adaptive immunity are present in the peripheral blood of MS patients (Romme Christensen et al. 2013).

Recently, the influence of the gut-central nervous system (CNS) axis on MS progression has been suggested (Fleck et al. 2017). Chronic microbe translocation increases circulating microbial-associated molecular pattern (e.g., lipopolysaccharides, peptidoglycan, or flagellin) levels, which modifies

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the secretion profile of various Toll-like receptor (TLR)-expressing cells, including peripheral and tissue-resident immune cells, CNS-resident glial cells, and CNS neurons. This scenario results in chronic systemic inflammation and inflammation of the CNS (Crack and Bray 2007). A clear link between the gastro-intestinal microbiota composition and pathological outcomes in the CNS has been demonstrated in experimental models. Mice treated with antibiotics or maintained under germ-free conditions have a compromised gut microbiome. Under those circumstances, disease onset in spontaneous experimental autoimmune encephalomyelitis (EAE) mice was attenuated, and mice with induced EAE displayed a significant decrease in disease severity (Yadav et al. 2015). In humans, indirect evidence through the analysis of serum soluble CD14 (sCD14) levels has demonstrated that sCD14 levels are increased in MS (Lutterotti et al. 2006). The sCD14 is a soluble molecule derived from macrophages after interaction of either lipopolysaccharide from *Enterobacteriae* or cell lysis products with macrophage membrane TLR4 (Crack and Bray 2007).

A counterregulatory increase of T regulatory (Treg) cells is expected associated to immune activation state described in MS patients (Romme Christensen et al. 2013). Tregs are CD4 + CD25highCD127lowFoxP3+ (forkhead box P3) cells, which play a key role in maintaining self-tolerance; their dysfunction is well documented in multiple autoimmune diseases (Chi et al. 2007). Although significant differences in the number of circulating Treg cells in MS patients compared to healthy controls have not been frequently reported (Hellings et al. 2001; Michel et al. 2008), Treg cells from MS patients are reported to have lower suppressive capabilities (Noori-Zadeh et al. 2016), which could lead to increase of autoreactive T-cells and activation of autoantibody-producing B cells (Haas et al. 2005; van Mierlo et al. 2008). This suggests that deficits in Tregs may contribute to the pathogenesis of MS.

Attending to the different mechanisms of action of drugs used in MS, the effects of these drugs on immune parameters will be also different. Briefly, beta-interferon (IFN-β) decreases the expression of HLA class I molecules and T lymphocytes proliferation (Karp et al. 2000); glatiramer acetate (GA) blocks HLA class II molecules and increase the synthesis of immunosuppressive IL-10 (Lalive et al. 2011), and natalizumab prevents the entry of immune cells to the CNS (Stüve et al. 2008).

We hypothesize that monocyte and lymphocyte activation is present in peripheral blood of MS patients (maybe due to intestinal barrier permeability lesion), with an expected contrarregulatory increase of Treg cells. Immunomodulatory therapy with IFN-β, GA or natalizumab will revert, partially or totally, these immune modifications.

Using a sample of MS patients before and after therapy, the objectives were: 1) Analysis of intestinal barrier lesion, detected by increased concentrations of intestinal fatty acid-binding protein (I-FABP). I-FABP is a cytosolic protein exclusively expressed by enterocytes and rapidly released into blood circulation upon cell damage (Piton and Capellier 2016). 2) Monocyte activation, measured by serum levels of soluble membrane receptors (sCD163 and sCD14), which are secreted after activation, and by the serum concentration of the proinflammatory interleukin (IL) IL-6. 3) Analysis of CD4+ and CD8+ T lymphocyte activation. 4) Evaluation of Treg cell counts. 5) Analysis of the immune parameters depending on MS pattern [clinical isolated syndrome (CIS), relapsing-remitting (RR), and primary progressive (PP) forms] and therapy.

## Patients and Methods

**Study Design** We conducted a prospective, observational study of consecutive cases of MS recruited from a cohort of patients who were followed-up in an outpatient clinic at the Puerta del Mar University Hospital, Cadiz, Spain.

According to the existence or not of a prior immunomodulatory treatment against MS, two groups of patients were selected: 1) Untreated MS patients. Thirty five adults with untreated (naïve) MS, diagnosed according the 2010 McDonald criteria (Polman et al. 2011), were recruited: 10 patients with CIS, 16 patients with RR form, and nine patients with PP form. 2) Previously treated MS patients. To analyze the persistence of immune changes, a sample of previously treated MS patients with the RR form of MS who demonstrated clinical and radiological stability at the time of inclusion was selected. Fifty healthy volunteers were recruited as controls from the hospital staff.

The exclusion criteria were as follows: (1) active infections (including human immunodeficiency virus, viral hepatitis or cytomegalovirus infections), other autoimmune diseases or neoplasms; (2) active drug use (cocaine, heroin, amphetamines) or significant alcohol ingestion (greater than 50 g/day); and (3) treatments that could have modified the serum levels of inflammation-related molecules or cells (e.g., pentoxifylline, immunoglobulin, or immunosuppressive drugs).

**Study Schedule** The study protocol included information about MS pattern, evolution time, number of previous clinical episodes, magnetic resonance imaging data, cerebrospinal fluid findings, visual and auditory evoked potentials, expanded disability status score (EDSS), and previous treatment, if any.

In untreated patients with CIS or RR clinical forms of MS, therapy with IFN-β or GA was initiated. No therapy was administered to patients with the PP pattern of disease. Follow up clinical revisions were scheduled at 1, 3, and 6 months after

treatment start. Immune parameters were analyzed at baseline and after 6 months of treatment.

Current therapy was continued for those RR MS patients who were already undergoing treatment (IFN-β, GA, or natalizumab). Immune parameters were analyzed at baseline.

Doses of drugs were the following: IFN-β1a, 22 or 44 mcg, sc, three times a week; IFN-β1b, 250 mg/48 h, sc; GA 20 mg/24 h, sc; natalizumab, 300 mg/28 days, iv.

**Laboratory Methods** After centrifugation of blood samples, plasma was stored at −80 °C until the time of analysis. Plasma concentrations of I-FABP, sCD14, sCD163, and IL-6 were measured with specific sandwich enzyme-linked immunosorbent assays (R&D systems, Minneapolis, USA).

A separate, fresh aliquot of peripheral blood was used to analyze lymphocyte populations. Activation of CD4+ and CD8+ T-cells were determined by the membrane expression of HLA-DR (CD4 + DR+ and CD8 + DR+). Treg count was determined after lysis and upon reaction with a mix of anti-CD4, anti-CD25, anti-CD127, and anti-FoxP3 antibodies. The anti-FoxP3 expression was performed using intracellular staining after fixation and permeabilization using Stain Buffer (FBS) and Human FoxP3 Buffer Set (Becton Dickinson, San Jose, CA, USA). Stained cells were washed, acquired, and analyzed in a FACSCalibur cytometer, using FACS Diva software (Becton Dickinson).

**Statistical Analysis** Patients with diverse patterns of MS (CIS, RR and PP) were included to analyze differences among patients with different evolutive patterns. Analysis of immune parameters was performed before and after therapy to examine differences attributable to treatment. Finally, we evaluate the differential effect of the diverse therapies used in them (IFN-β, GA, or natalizumab) on previously treated MS patients with RR pattern, in a clinical and radiological situation of absence of activity.

Data were expressed as absolute values (percentages) or as a sample median [25–75 interquartile range (IQR)]. Categorical variables were compared using the chi-square test or Fisher's exact test. Quantitative variables from independent groups were compared using the Mann-Whitney U test. The Spearman's correlation test was used to analyze the association between quantitative variables. A paired analysis of variables was performed using the Wilcoxon's rank test. A two-tailed $p$ value of <0.05 was considered statistically significant. Statistical analyses were performed using the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA).

**Ethical Aspects** This study was performed according to the Helsinki Declaration. The project was approved by the hospital ethical research committee. Written informed consent was obtained from each participant.

## Results

Clinical characteristics of the patients and controls are shown in Table 1.

### Serum Concentrations of I-FABP, sCD163, sCD14 and IL-6

At baseline, healthy controls and untreated MS patients with CIS, RR, or PP forms of the disease showed similar I-FABP concentrations (Mann-Whitney U test).

Patients with RR and PP forms showed significant increased serum sCD14 levels compared with healthy controls. No significant differences in serum levels of sCD163 were observed among the different groups of MS patients and healthy controls. Only patients with PP pattern showed a significant increase in IL-6 detected (Table 2).

No significant differences in the serum concentration of I-FABP, sCD14, or sCD163 were observed among the different groups of MS patients. Serum levels of IL-6 were significantly elevated in patients with PP MS, compared to those with the CIS and RR forms (Table 2).

Serum IL-6 concentration was correlated with the MS evolution time of MS ($r = 0.332$, $p = 0.039$). No significant correlation was detected between macrophage markers and either lesions in magnetic resonance imaging at T1 or T2, EDSS, or with the number of clinical events during evolution (Spearman's correlation test).

### Activated CD4+ and CD8+ T Lymphocytes and Tregs in Patients and Healthy Controls

No significant differences were observed in the monocyte or lymphocyte cell number between healthy controls and MS patients (Table 2). Thus, in the following paragraphs, values of lymphocyte populations will be expressed as absolute percentages.

Untreated MS patients showed a significant increase on CD4+ T cell percentages, whereas no differences were observed in the proportions of CD8+ T-cells comparing to healthy controls.

The percentages of CD4 + DR+ and CD8 + DR+ and Treg were significantly increased in MS patients, compared to healthy controls. No significant difference in the CD4 + DR+, CD8 + DR+ or Treg percentages was observed between the different groups of MS patients (Table 2).

A significant correlation was observed between the percentage of Treg and the proportion of CD8 + DR+ cells ($r = 0.398$, $p < 0.001$), but not with CD4 + DR+ cells ($r = 0.173$,

**Table 1** Age, sex and multiple sclerosis-related characteristics of patients' and healthy controls

| | Healthy controls (n = 50) | Previously untreated (naïve) MS patients ($n = 35$) | | | Previously treated MS patients ($n = 56$) |
|---|---|---|---|---|---|
| | | CIS (n = 10) | RR (n = 16) | PP (n = 9) | |
| Age (years) | 40 (30–53) | 35 (28–42) | 33 (27–39) | 53 (46–65) | 40 (34–45) |
| Sex male (n,%) | 22 (44) | 4 (40) | 7 (44) | 5 (56) | 16 (29) |
| Evolution time (months) | | 0 (0–1) | 12 (6–14) | 168 (84–204) | 96 (36–144) |
| Number of episodes | | 1 (1–1) | 2 (2–3) | | 3 (2–5) |
| Expanded disability status score | | 1.0 (0.0–1.3) | 0.0 (0.0–2.0) | 6.5 (6.0–7.0) | 1.0 (0.0–2.9) |

*MS* multiple sclerosis; *CIS* isolated clinical and radiological syndrome; *RR* relapsing-remitting form; *PP* primary progressive form; *SP* secondary progressive form

$p = 0.096$). No significant correlation was detected between lymphocyte parameters and either lesions in magnetic resonance imaging at T1 or T2, EDSS, evolution time with MS, or number of clinical events during evolution (Spearman's correlation test).

## Evolution of Monocyte and Lymphocyte Parameters after Treatment in Untreated Patients

Those patients with CIS and RR, untreated at the beginning of the study, initiated therapy with IFN-β or GA.

Patients with CIS received IFN-β ($n = 8$) or GA ($n = 2$). They did not have any new clinical events. After 6 months of treatment they showed similar serum concentrations of I-FABP [4 (3–5) vs 4 (3–5) ng/ml, $p = 1.000$], sCD163 [571 (409–673) vs 493 (409–673) ng/mL, $p = 0.508$], sCD14 [2663 (1592–3195) vs 2343 (1586–3532) ng/mL, $p = 0.878$] and IL-6 [1 (0–1) vs 1 (0–1) pg/mL, p = 1.000], as well as CD4+, CD8, and Treg levels, compared to their values measured at the beginning of the study (Wilcoxon's rank test). Interestingly, a significant decrease in CD4 + DR+ and CD8 + DR+ percentages were detected at 6 months after therapy, compared to values obtained before therapy (Fig. 1a).

Patients with RR clinical pattern received IFN-β ($n = 11$) or GA ($n = 5$) after inclusion in the study. No clinical relapses were detected in the following 6 months after initiating the assigned therapy. Similar serum concentrations of I-FABP [4 (3–5) vs 4 (3–5) ng/mL, p = 1.000], sCD163 [710 (468–786)

**Table 2** Monocyte-derived characteristics, CD4+ and CD8+ activated T cells and T regulatory cells in untreated multiple sclerosis patients' and healthy controls

| | Healthy controls (n = 50) | Previously untreated (naïve) MS patients ($n = 44$) | | |
|---|---|---|---|---|
| | | CIS (n = 10) | RR (n = 16) | PP (n = 9) |
| Leukocytes/mm3 | 5845 (4868–7125) | 7160 (6015–8195) | 6925 (5658–7978) | 6930 (5725–8340) |
| Monocytes/mm3 | 615 (395–715) | 550 (475–763) | 495 (393–665) | 490 (360–605) |
| I-FABP (ng/ml) | 4 (3–5) | 4 (3–5) | 4 (3–5) | 3 (2–4) |
| sCD163 (ng/ml) | 644 (432–774) | 493 (409–673) | 565 (516–699) | 732 (529–881) |
| sCD14 (ng/ml) | 2213 (1251–2593) | 2343 (1586–3532) | 3343 (2414–4302) ** | 3709 (2421–4900) ** |
| IL-6 (pg/ml) | 1 (0–3) | 1 (0–1) § | 2 (0–4) § | 4 (0–8) ** |
| Lymphocytes/mm3 | 2005 (1725–2655) | 1980 (1645–2863) | 2285 (1878–2778) | 2440 (1395–2590) |
| CD4+ T cells (percentage of CD3+ T cells) | 42 (38–45) | 47 (40–53) * | 47 (42–53) * | 52 (42–56) * |
| CD4 + DR+ T cells (percentage of CD4+ T cells) | 13 (9–18) | 51 (41–53) *** | 47 (42–53) *** | 46 (42–50) *** |
| CD8+ T cells (percentage of CD3+ T cells) | 23 (20–25) | 23 (20–28) | 27 (19–30) | 25 (16–30) |
| CD8 + DR+ T cells (percentage of CD8+ T cells) | 18 (15–24) | 47 (43–54) *** | 55 (31–62) *** | 53 (44–59) *** |
| CD4 + CD25highFoxP3+ (percentage of CD4+ T cells) | 17 (15–20) | 31 (14–48) * | 41 (10–57) ** | 33 (25–44) *** |

Comparison of MS patients' with healthy controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Comparison of PP MS patients' with CIS or RR MS patients: § $p < 0.05$

Quantitative variables from independent groups were compared using the Mann-Whitney U test

*MS* multiple sclerosis; *CIS* isolated clinical and radiological syndrome; *RR* relapsing-remitting form; *PP* primary progressive form; *SP* secondary progressive form. *I-FABP* intestinal fatty acid-binding protein. *IL-6* interleukin 6

vs 565 (516–699) ng/mL, $p = 0.535$], sCD14 [2837 (1636–4608) vs 3343 (2414–4302) ng/mL, $p = 0.301$] and IL-6 [1 (0–2) vs 2 (0–4) pg/mL, $p = 0.655$], as well as CD4+, and CD8+ percentages were detected after 6 months of undergoing therapy, compared with values obtained at the beginning of the study (Wilcoxon's rank test). A significant decrease of CD4 + DR+, CD8 + DR+, and Treg percentages after 6 months of therapy was observed (Fig. 1b).

To evaluate the persistence of immune changes, a sample of 56 MS patients with RR clinical pattern, previously treated for a median of 28 (15–50) months, who were clinically stable (they did not show any clinical event in the last 6 months), was analyzed. These patients were receiving IFN-β [$n = 26$, time on treatment 42 (30–53) months], GA [$n = 21$, time on treatment 38 (26–50) months] or natalizumab [$n = 9$, time on treatment 13 (7–19) months]. No significant differences were found in immune characteristics compared with those observed in previously untreated RR patients after 6 months of therapy (Table 3).

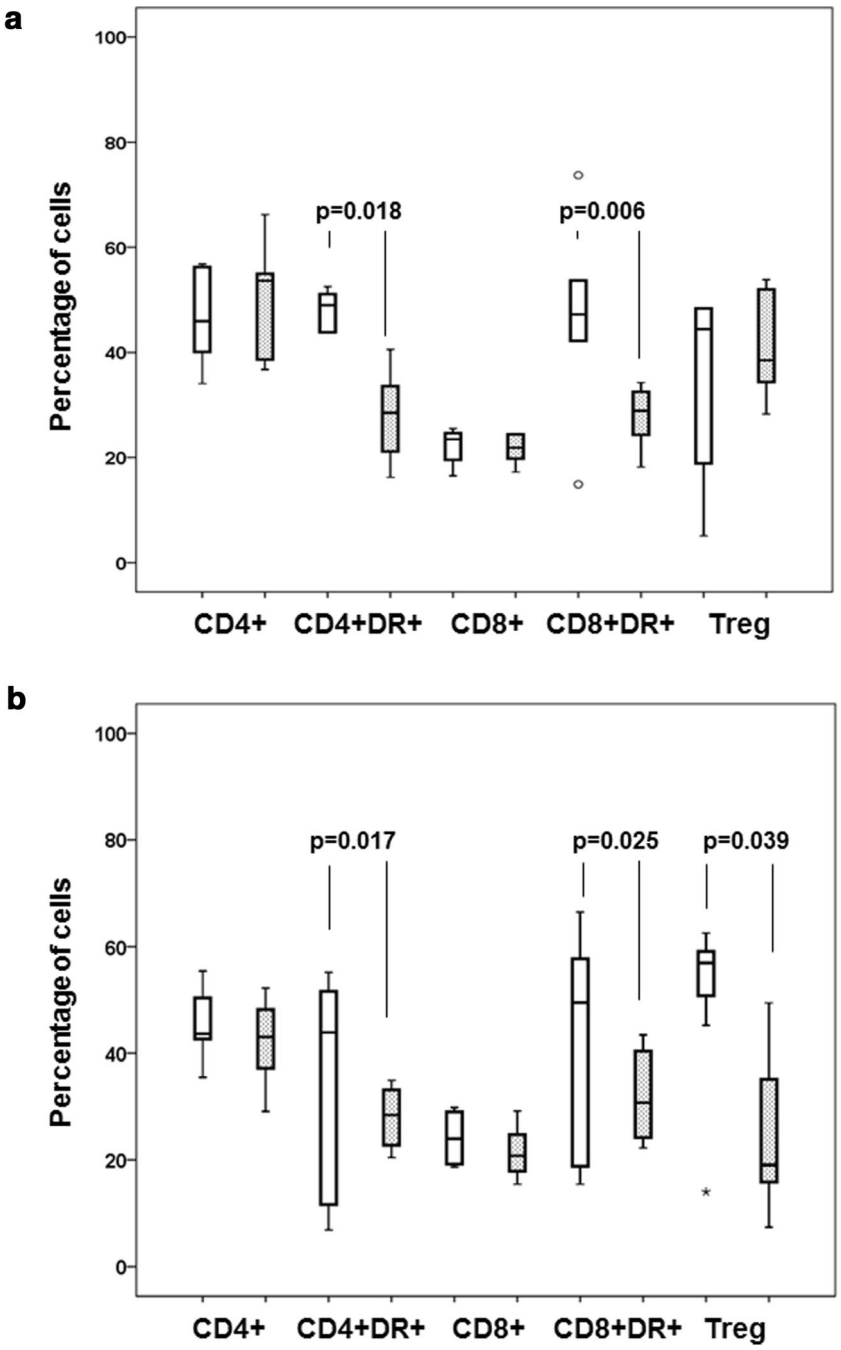


**Fig. 1** Percentages of lymphocyte populations in patients with multiple sclerosis, clinical isolated syndrome ($n = 10$) (Panel **a**) and relapsing-remitting form ($n = 16$) (Panel **b**), detected at baseline (white box) and after 6 months of therapy (grey box). Data are provided as follows: CD4+, percentage of lymphocytes (CD3+ T cells); CD4 + DR+, percentage of CD4+ T lymphocytes; CD8+, percentage of lymphocytes (CD3+ T cells); CD8 + DR+, percentage of CD8+ T lymphocytes; and Treg (CD4 + CD25highCD127lowFoxP3+), percentage of CD4+ T lymphocytes. Data are shown as median (25–75 interquartile range), with error bars. Paired analysis of variables was performed using the Wilcoxon's rank test

### Monocyte and Lymphocyte Parameters in Previously Treated and Stable MS Patients

Finally, to analyze the possible alterations in immune marker expression as a result of different mechanisms of action of immunomodulatory drugs, we compared the values of these immune markers as a function of the MS treatment (IFN-β, GA, or natalizumab) in the sample of 56 previously treated MS patients. An increased sCD14 serum concentration was detected in patients when compared with healthy controls. No significant differences in I-FABP concentration, sCD14, sCD163 or IL-6 were detected in the groups of patients treated with IFN-β, GA, or natalizumab (Fig. 2). Percentages of CD4 + DR+, CD8 + DR+ and Treg cells were significantly increased in patients when compared with healthy controls. No significant difference was detected in lymphocyte parameters when the groups of patients treated with IFN-β, GA, or natalizumab were compared (Fig. 3).

## Discussion

The results of this work in MS patients' demonstrated continuous immune activation associated with a concurrent increase in Treg, and these results are only partially modified by MS treatment.

These changes could be a result of increase permeability of the intestinal barrier. In response to the stress frequently detected in MS patients, the hypothalamic-pituitary-adrenal releases glucocorticoids or catecholamines, which can alter microbiota composition and gut permeability (Bailey et al. 2011). In our study, patients with MS showed similar serum levels of I-FABP to those observed in healthy controls. Serum concentrations of I-FABP are a very sensitive marker of increased intestinal permeability in relation to intestinal barrier lesion (Piton and Capellier 2016). Consequently, our findings don't support the hypothesis that altered gut permeability is a factor of immune activation in MS.

Three markers were selected to measure monocyte activation. sCD163 and sCD14 are expressed on the monocyte membrane. The CD163 antigen participates as a receptor for hemoglobin-haptoglobin complexes, in monocyte-endothelium interactions and as macrophage receptor for bacteria (Fabriek et al. 2009). CD14 receptor recognizes bacterial lipopolysaccharide of *Enterobacteriae*, as well as cell lysis products (Bas et al. 2004). Increased shedding of these receptors occurs due to infectious/inflammatory stimuli, and they may be valuable diagnostic parameters for monitoring macrophage activation under inflammatory conditions (Ríos-Toro et al. 2017). After interacting with inflammatory stimuli, macrophages secrete proinflammatory cytokines, such as IL-6, and contribute to the systemic inflammatory response (Bas et al. 2004; Crack and Bray 2007; Fabriek et al. 2009).

Previous studies in MS patients have shown contradictory results about serum sCD163 concentration (Fabriek et al. 2007; Stilund et al. 2014). This could be a consequence of the heterogeneous population of analyzed MS patients, due to inclusion of several forms of MS, both treated and not treated (Fabriek et al. 2007), and the different times of evolution (Stilund et al. 2014). Also, in previous studies, serum sCD14 levels were increased in MS and did not correlate (Brettschneider et al. 2002), or correlated inversely (Lutterotti et al. 2006), with

**Table 3** Monocyte-derived characteristics, CD4+ and CD8+ activated T cells and T regulatory cells in multiple sclerosis patients' with relapsing-remiting forms, both untreated and previously treated at inclusion

| | MS patients with RR form, untreated at baseline, after 6 months of therapy (n = 16) | MS patients with RR form, undergoing therapy at inclusion (n = 56) | p |
|---|---|---|---|
| Leukocytes/mm3 | 6108 (5008–7520) | 6730 (5155–8675) | 0.887 |
| Monocytes/mm3 | 622 (412–688) | 555 (453–700) | 0.241 |
| I-FABP (ng/ml) | 4 (3–5) | 4 (3–5) | 0.543 |
| sCD163 (ng/ml) | 710 (468–786) | 682 (500–804) | 0.856 |
| sCD14 (ng/ml) | 2837 (1636–4608) | 2642 (1712–3923) | 0.856 |
| IL-6 (pg/ml) | 1 (0–2) | 1 (0–2) | 1.000 |
| Lymphocytes/mm3 | 2022 (1980–2534) | 2335 (1778–3208) | 0.876 |
| CD4+ T cells (percentage of CD3+ T cells) | 46 (40–52) | 48 (39–57) | 0.354 |
| CD4 + DR+ T cells (percentage of CD4+ T cells) | 29 (25–34) | 30 (22–34) | 0.986 |
| CD8+ T cells (percentage of CD3+ T cells) | 21 (18–27) | 23 (18–29) | 0.401 |
| CD8 + DR+ T cells (percentage of CD8+ T cells) | 37 (26–43) | 35 (28–39) | 0.506 |
| CD4 + CD25highFoxP3+ (percentage of CD4+ T cells) | 29 (16–36) | 26 (18–35) | 0.692 |

Quantitative variables from independent groups were compared using the Mann-Whitney U test

*MS* multiple sclerosis; *RR* relapsing-remitting form. *I-FABP* intestinal fatty acid-binding protein. *IL-6* interleukin 6

disease activity in RR MS patients. Finally, normal or minimally elevated IL-6 values have been detected in RR MS patients (Chen et al. 2012; Matsushita et al. 2013). Data about PP are lacking.

At the beginning of our study, patients with RR and PP patterns showed increased concentrations of sCD14, but not of sCD163. The normal concentration of sCD163, whose functions include macrophage receptor for bacteria (Fabriek et al. 2009), is compatible with the normal gut barrier permeability previously indicated and the consequent absence of bacterial translocation. In contrast, sCD14 also recognizes cell lysis products (including heat shock proteins) (Litvack and Palaniyar 2010) and it is possible to speculate that apoptosis-associated chronic activation provides such elements to activate monocytes and shed sCD14 to the plasma.

Serum levels of IL-6 were normal in all groups of patients analyzed, except in PP MS patients. In those patients, serum levels were correlated with the evolution time of the disease, indicating the existence of slight and persistent chronic monocyte activation, increased IL-6 levels being detected only after a long period of time had passed, such as that observed in MS patients with PP pattern.

An increase in the activation state of T lymphocytes has been previously demonstrated both in CIS, untreated RR, and PP forms (Jones et al. 2017), and our results are in accordance with that. Tregs, characterized as CD4 + CD25highCD127lowFoxP3+, play a key role in counteracting immune activation. Previous data on peripheral blood Tregs are controversial showing a decrease (Huan et al. 2005) or normal values (Venken et al. 2006; Noori-Zadeh et al. 2016) in MS patients, compared to healthy controls. Furthermore, it has been reported that Tregs in MS patients have lower immune suppressive capabilities (Haas et al. 2005; Venken et al. 2006), a finding that has been questioned when defining Tregs with different markers (Michel et al. 2008). Our data, like those provided by Dalla Libera et al. (2011), show increased Treg cell number in patients with MS, without differences between those groups with different clinical pattern. Considering Treg cells as a homeostatic mechanism aimed at controlling excessive immune activation

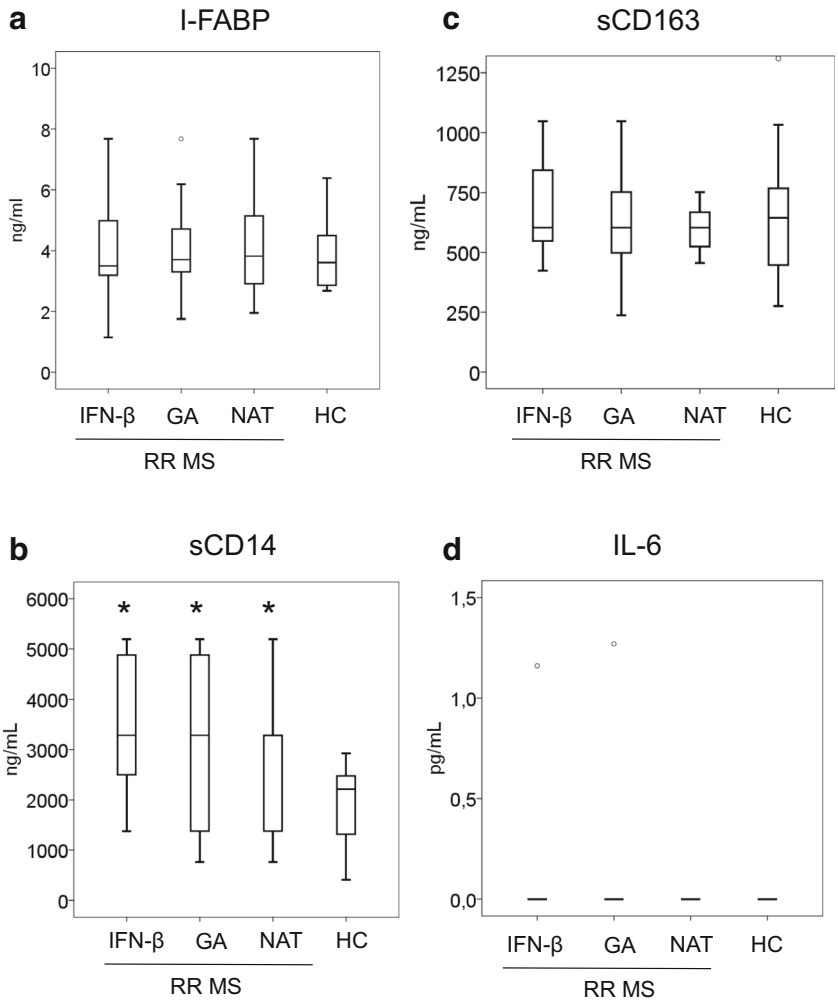


**Fig. 2** Serum levels of intestinal fatty acid binding protein (I-FABP) (Panel **a**), soluble CD14 (sCD14) (Panel **b**), soluble CD163 (sCD163) (Panel **c**) and interleukin 6 (IL-6) (panel **d**) in healthy controls (HC) ($n = 50$) and in patients with multiple sclerosis, relapsing-remitting form, previously treated with interferon-β (IFN-β) ($n = 26$), glatiramer acetate (GA) ($n = 21$) or natalizumab (NAT) ($n = 9$). Data are provided as follow: I-FABP, sCD14 and sCD163, ng/ml; IL-6, pg/ml. Significant differences were detected in the serum concentration of sCD14 between MS patients and healthy controls (*, $p < 0.001$ in each case). No significant difference was detected in the proportions of any lymphocyte population between MS patients undergoing different therapies ($p > 0.05$ in each case). Data are shown as median (25–75 interquartile range), with error bars. Quantitative variables from independent groups were compared using the Mann-Whitney U test

(Josefowicz et al. 2012), its percentage correlate with CD8+ activated cell proportions, a finding previously not communicated in patients with MS. Their decrease after therapy, in association with a decrease in the activated CD4+ and CD8+ lymphocytes, supports their function as immune activation control.

Evolution of the patients reinforces previous findings. In CIS and RR patterns of MS, serum monocyte activation markers were similar at baseline and after 6 months of therapy. Increased serum levels of sCD14 continued to be detected, suggesting a continuous and unmodified, apoptosis-driven chronic monocyte activation. In contrast, the percentage of activated CD4+ and T CD8+ cells, as well as the counteractive Treg proportions, significantly decrease in naïve RR MS patients after 6 months of therapy. Interestingly, values detected after 6 months of treatment in naïve patients were similar to those observed in the sample of 56 patients with a history of therapy (median 28 months), suggesting that a set point is attained after a short period of treatment.

Differential effects of drugs used to treat MS were analyzed in the sample of 56 previously treated patients. IFN-β decreases the expression of HLA class I molecules and the proliferation of T lymphocytes (Karp et al. 2000) and GA blocks HLA class II molecules and contributes to decreases in immune activation via secretion of immunosuppressive IL-10 (Lalive et al. 2011). Natalizumab blocks the adhesion molecule VLA-4, preventing the entry of immune cells to the CNS (Stüve et al. 2008). Data regarding the effects of IFN-β, GA, and natalizumab in our work suggested that the aforementioned mechanisms were not responsible for the detected findings, but a nonspecific effect of these drugs on lymphocyte activation or regulation. In fact, the concentrations of monocyte markers and the percentages of CD4 + DR+, CD8 + DR+, and Tregs were similar in the three groups of treated RR MS patients. The main pathogenic theory for MS indicates that antigen-specific immune activation occurs in the periphery and then is transferred to the unaffected CNS (Hemmer et al. 2015; Romme Christensen et al. 2013). Because natalizumab does not change the percentage of activated T-cells (Putzki et al. 2010), our results suggest an alternative mechanism to explain the decrease of peripheral blood-derived activated T-lymphocytes and Tregs. We hypothesize that IFN-β, GA, or natalizumab decreased the occurrence of immune-mediated lesion in the CNS. Thereby, the number of cell death-derived antigens and consequent activation of

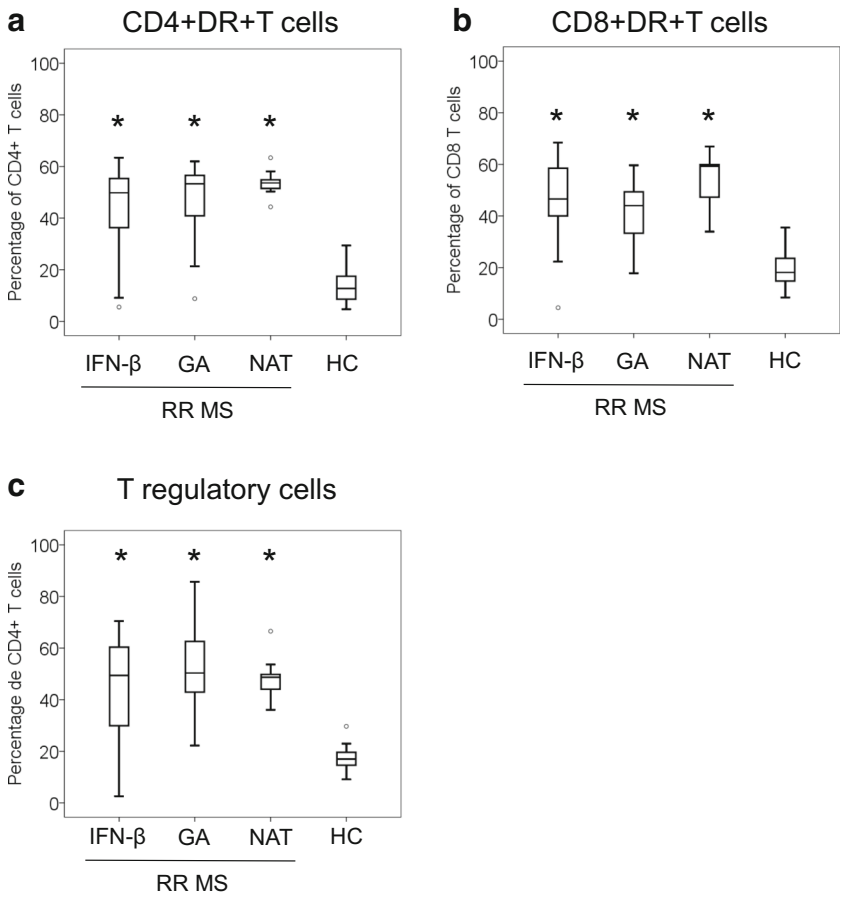


**Fig. 3** Percentages of lymphocyte populations in healthy controls (HC) (n = 50) and in patients with multiple sclerosis, relapsing-remitting form, previously treated with interferon-β (IFN-β) (n = 26), glatiramer acetate (GA) (n = 21) or natalizumab (NAT) (n = 9). Data are provided as follows: CD4 + DR+, percentage of CD4+ T lymphocytes (Panel **a**); CD8 + DR+, percentage of CD8+ T lymphocytes (Panel **b**); and Treg (CD4 + CD25highCD127lowFoxP3+), percentage of CD4+ T lymphocytes (Panel **c**). Significant differences were detected in the percentage of CD4 + DR+, CD8 + DR+, and Treg populations between MS patients and healthy controls (*, $p < 0{,}001$ in each case). No significant difference was detected in the proportions of any lymphocyte population between MS patients undergoing different therapies ($p > 0{,}05$ in each case). Data are shown as median (25–75 interquartile range), with error bars. Quantitative variables from independent groups were compared using the Mann-Whitney U test

microglial cells decrease, although it did not normalize. The secondary adaptive immune response in the periphery, due to drainage of these antigens into deep cervical lymph and peripheral blood [this different pathway from the CNS has been demonstrated (Prodinger et al. 2011)], will be also lower, justifying the diminution of peripheral blood activated T-cells.

**Conclusions** 1) Our study has demonstrated that the existence of monocyte and lymphocyte activation in MS patients is not related with a gut barrier lesion. An increase in Treg number, which correlated with CD8 + DR+ cells, was detected. 2) In previously untreated patients with CIS or RR pattern, immunomodulatory therapy induces a significant decrease of lymphocyte activation and Treg, but does not modify the monocyte activation state. 3) In previously treated and stable RR clinical pattern, treatment with IFN-β, GA, or natalizumab was associated with a comparable decrease in activated lymphocytes and concomitantly of Tregs. Because IFN-β, GA, or natalizumab have different mechanisms of action, these results suggest that all these drugs decrease the occurrence of immune-mediated lesion in the CNS with a secondary reflection in the periphery.

**Contributions Made by each of the Authors to the Article** Conceived and designed the experiments: María Carmen González-Oria, Mercedes Márquez-Coello, José A Girón-González.

Clinical follow-up of patients`: María Carmen González-Oria, Joaquín Argente-Alcaraz, Miguel Moya-Molina.

Contributed reagents/materials/analysis tools: Mercedes Márquez-Coello; José Antonio Girón-González.

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Wrote the draft: José Antonio Girón-Ortega, José Antonio Girón-González.

All authors contributed to conception of the study, and critical revision of the manuscript, and saw and approved the final version.

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## Compliance with Ethical Standards

**Ethics Approval** The project was approved by the hospital ethical research committee. Written informed consent was obtained from each participant.

**Consent for Publication** Not applicable.

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