Journal of Neuroimmunology 303 (2017) 75–80

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Lymphocyte subsets as biomarkers of therapeutic response in Fingolimod treated Relapsing Multiple Sclerosis patients

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ARTICLE INFO

Article history:
Received 2 November 2016
Received in revised form 19 December 2016
Accepted 19 December 2016

Keywords:
Multiple Sclerosis biomarkers
Immune system re-modulation
Fingolimod
Lymphocytes
Lymphocyte subsets

ABSTRACT

We investigated, lymphocyte count (LC) and lymphocyte subpopulations (LS) in a real life setting of Fingolimod (FTY) treated Relapsing MS (RMS) patients.
Peripheral blood counts with LS, relapses and MRI scans were recorded in a cohort of 119 FTY patients, during one year of treatment. Simple and multivariate logistic regression models, were performed. ROC analysis identified cut-off values of LS predicting a higher risk of relapses and of Gd+ lesions.
We demonstrated a FTY-induced re-modulation of the immune system, suggesting that LS in RMS FTY treated patients can predict the clinical response to the drug.

1. Introduction

Multiple Sclerosis (MS) is an extremely heterogeneous pathology, during its first phases, rate and severity of relapses, pattern of disease progression, radiological appearance, and response to the disease modifying treatments (DMTs). In the last 20 years a considerable number of treatment options have been approved for Relapsing-MS (RMS), and the more recent therapeutic strategies aim to target the immune system, influencing lymphocyte activation, migration towards the Brain Blood Barrier (BBB) and the recruitment of different cellular populations (Sellebjerg and Sorensen, 2015). Fingolimod (FTY) acts by preventing the migration of lymphocytes from peripheral lymphoid organs to the Central Nervous System (CNS) with the reduction of the lymphocyte count in the peripheral blood (PB) (Brinkmann et al., 2002). CD4+ T lymphocytes are the most affected cells by FTY treatment and are reduced both in the PB as well as in the Cerebral Spinal Fluid (CSF) of the treated patients (Mehling et al., 2010). Also CD8+ T lymphocytes are reduced in the PB of the patients treated with FTY, but not to the same extent as CD4+ (Kowarik et al., 2011). The effect of FTY on Natural Killer cells (NK) is less clear; these cells seem to be relatively unaffected during the treatment (Vaessen et al., 2006) or slightly reduced in the PB (Johnson et al., 2011). Other studies, however, report an increase in NK cells in both the PB and the CSF of MS patients treated with FTY, compared to untreated patients (Kowarik et al., 2011). Furthermore, B lymphocytes in the PB are reduced during FTY treatment (Kowarik et al., 2011; Vaessen et al., 2006), while the count of B lymphocytes in the CSF and the intrathecal synthesis of immunoglobulin (Ig) G are not influenced by the treatment (Kowarik et al., 2011; Nakamura et al., 2014). For this reason, the monitoring of the "immune-phenotype" of FTY treated patients can be useful in clinical practice.

The objective of this study was to evaluate, the temporal profile of white blood cells (WBC), lymphocyte count (LC) and lymphocyte subpopulations (LS) in a cohort of FTY-treated RMS patients, during the first year of treatment. Moreover, we correlated clinical variables (disease duration, number of relapses, score at the Expanded Disability Status Scale – EDSS) and Magnetic Resonance Imaging (MRI) variables (presence/absence of Gadolinium enhancing - Gd+ - and new T2 lesions) with LC and LS variation to identify potential biomarkers of treatment response.

2. Material and methods

2.1. Patients

We included a cohort of 119 consecutive RMS patients according to McDonald's criteria (McDonald et al., 2001) who underwent FTY treatment at the Centre for Multiple Sclerosis of the University of Bari between 2012 and 2014. The entire cohort was monitored for the first six months of treatment, and 84 patients were monitored for twelve

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http://dx.doi.org/10.1016/j.jneuroim.2016.12.012
0165-5728/

months. The study-protocol was approved by the Local Ethics Committee. All patients signed a written informed consent before the study.

At the beginning of the treatment, demographic and clinical data were collected from all patients (age, gender, disease duration, number of relapses, previous DMTs) and were assessed for neurological disability using the EDSS, a clinical tool used to evaluate the level of functioning of MS patients, providing a total score on a scale from 0 to 10 (Kurtzke, 1983). Patients were re-evaluated every six months; an additional neurological assessment was performed in case of relapses (Polman et al., 2011).

MRI scans at month 6 and at month 12 were examined to assess the Gd + lesions and T2 hyperintense lesions.

PB samples were collected at baseline and at the sixth and twelfth month of treatment (T0, T6 and T12, respectively); total WBC, LC and LS (CD3+, CD4+, CD8+, CD16/56+, CD19+) were performed using the quality reference of the laboratory at the Centre for Multiple Sclerosis of the University of Bari.

2.2. Flow cytometry

Whole PB samples (5 ml) of MS patients were collected in ethylene diamine tetra acetic acid (EDTA) (8.55 mg/tube) for immunophenotyping by flow cytometry from MS patients. PB samples (150 μl) were incubated with monoclonal antibodies, at room temperature (RT) for 15 min in the dark. After incubation, red cells were lysed with fluorescence activated cell sorter (FACS) lysing solution (Becton-Dickinson, San Diego, CA, USA) for 10 min at RT, in the dark. Cells were washed with 1 ml of FACS buffer (phosphate-buffered saline, 0.2% fetal bovine serum, 0.02% sodium azide), centrifuged twice for 5 min at 500*g*, and then resuspended in 200 μl of FACS buffer and analyzed by flow cytometry. To characterize lymphocyte subpopulations $CD3^+$ (T-cells), $CD3^+$ $CD4^+$ (T helper cells), $CD3^+$ $CD8^+$ (cytotoxic T-cells), $CD19^+$ (B-cells), $CD16/56^+$ (Natural Killer-cells), we used the monoclonal antibodies from the Becton-Dickinson multitest kit (San Diego, CA, USA).

3. Calculation

The analysis of the quantitative variation of WBC, LC and LS, considered both in terms of absolute value and of the "delta" (the difference between the absolute values of LC and LS between two consecutive time-points), was performed. We compared two different periods of treatment to the baseline (T0): T6 (sixth month) and T12 (twelfth month). Variations of the lymphocytes during the observation period were analyzed using a student *t*-test.

To describe the demographic, clinical, and radiological characteristics, we used proportions or statistical means. For the comparison between continuous variables we used independent sampled *t*-test, and for the comparison between categorical variables, we used $\chi 2$ test. We compared clinical and radiological data of the cohort with LC and LS using a non parametric test (Spearman test). Then we performed a correlation analysis using simple and multiple linear and logistic regressions. All analyses were adjusted for age and gender. A 2-sided $p < 0.05$ was considered significant.

We used the receiver-operating characteristic (ROC) curve to assess the predictive accuracy of each potential biomarker of clinical and/or radiological activities at T6 and T12; we only considered the ROC curves with Area under the curve (AUC) ≥ 0.7. The optimal cut-off value was set according to Youden's index, which depended on the maximized value of sensitivity plus specificity minus 1. All analyses were performed using the SPSS version 19.0 (SPSS Inc., Chicago, Illinois).

4. Results

We included in our cohort 84 female patients and 35 male patients. The mean age at FTY was 38.3 ± 8.9 years. The mean disease duration at FTY beginning was 12.5 ± 7.3 years. All patients, except one "treatment naive" patient, had previously been treated with DMTs: 71 with Interferon beta (IFN β), 34 with Glatiramer Acetate (GA), and 13 with Natalizumab (NTZ). The mean wash-out period pre-FTY treatment was 76.5 ± 207.7 days for IFNβ, 89.7 ± 168.4 days for GA and 171.9 ± 100.4 for NTZ (Table 1).

4.1. Temporal profile of WBC and LC

WBC and LC values at the beginning of FTY, at the sixth and twelfth months of treatment, are reported in Fig. 1A.

At T0 we observed a direct correlation between the LC and the duration of the wash out period from the previous treatment ($r = 0.18$; $p = 0.02$). The LC at baseline did not correlate with any other clinical-demographical variable and no statistically significant difference was found in the mean baseline number of lymphocytes stratifying the patients according to their previous DMTs.

Considering the T0-T6 interval we observed a statistically significant reduction of WBC ($p < 0.01$) and a 71.9% reduction in LC ($p = 0.01$), while from T6 to T12 we observed a statistically significant increase of 13.1% in the number of LC ($p = 0.01$) (Fig. 1A).

4.2. Lymphocyte subsets temporal profile

The variations of the mean values of each LS at the three subsequent follow-ups, are reported in Fig. 1B.

Considering the T0–T6 interval, we observed a statistically significant reduction of 78.03% in CD3+ ($p = 0.001$), of 89.1% in CD4+ ($p = 0.002$), of 65.01% in CD8+ ($p = 0.001$) and of 89.4% in CD19+ ($p < 0.0001$), while the reduction of CD56+/CD16+ did not achieve statistical significance. Considering the T6–T12 interval, CD3+ ($p < 0.05$), CD8+ ($p = 0.02$) and CD56+/CD16+ lymphocytes ($p = 0.02$) significantly increased by14.1%, 19.5% and of 10.4% respectively.

During the twelve months observation period, the LS which mostly showed a reduction in comparison to the baseline were the CD4+ and the CD19+ lymphocytes.

4.3. Correlation between LS variation and disease activity

We evaluated the correlation between LS and outcomes of disease activity during the first and second semester of treatment.

Table 1
Baseline characteristics of the 119 FTY treated patients.

Sex (F/M)	84/35
Age at disease onset (years)	25.8 ± 8.7
Age at FTY start (years)	38.3 ± 8.9
Last treatment pre-FTY	
• IFNβ/GA	105 (71/34)
• NTZ	13
• Naive	1
Duration of pre FTY (years)	
• IFNβ	2.5 ± 2.6
• GA	2.7 ± 2.5
• NTZ	2.2 ± 3.3
Duration of the pre-FTY wash-out period (days)	
• IFNβ	76.5 ± 207.7
• GA	89.7 ± 168.4
• NTZ	171.9 ± 100
Mean disease duration at FTY beginning (years)	12.5 ± 7.3

FTY = Fingolimod, IFNβ = Interferon-β, GA = Glatiramer Acetate, NTZ = Natalizumab. Values expressed as mean (±SD) and median (range).