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Multiplex array analysis of circulating cytokines and chemokines in natalizumab-treated patients with multiple sclerosis

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ABSTRACT

Natalizumab greatly reduces inflammatory relapses in multiple sclerosis (MS) by blocking the integrin-mediated leukocyte traffic to the brain, but less is known about its effects on the systemic immunity. We measured 48 cytokines/chemokines in sera from 19 natalizumab-treated MS patients. Serum concentrations of both anti-(IL-10, IL1ra) and pro-inflammatory (IL7, IL16) molecules decreased after 21-month treatment, without associations to unbalanced Th2/Th1cytokine ratios, clinical responses, and blood/urine replication of polyomavirus JC (JCPyV). No patient developed the JCPyV-related progressive multifocal leukoencephalopathy (PML), the major risk factor of natalizumab therapy. Our data suggest that natalizumab has marginal impact on the systemic immunity.

1. Introduction

The transmigration of autoreactive-activated T cells from peripheral blood into the central nervous system (CNS) is a crucial step in the initiation and maintenance of brain inflammatory reaction in multiple sclerosis (MS) (Steinman et al., 2002). The α4β1-integrin (VLA-4), expressed on the leukocytes' surface and interacting with the vascular cell adhesion molecule 1 (VCAM-1), is critically involved in this process because it mediates both the adhesion and migration of lymphocytes across the blood-brain barrier (BBB) (Kumar et al., 2005; Kummer and Ginsberg, 2006; Libbey and Fujinami, 2010; Polman et al., 2006; Rose et al., 2002; Sawcer et al., 2011).

Natalizumab is a monoclonal antibody directed against the α4 chain of the VLA-4 and α4β7 integrins and is used as a monotherapy for treating relapsing-remitting MS (RRMS)·(Stuve and Bennett, 2007). Natalizumab substantially reduces the relapse rate and the worsening symptoms, but its use is associated the development of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease that occurs due to the lytic replication of the Polyomavirus JC (JCPyV) (Polman et al., 2006).

Chemokines affect the trafficking of leukocytes to the sites of inflammation and have a crucial role in establishing a balance between subpopulations of T helper (Th) cells (Baggiolini, 1998). Th2-related cytokines are associated with inflammatory reduction and improvement of MS symptoms. In contrast, Th1-related cytokines, which are known as pro-inflammatory proteins are increased in brain, cerebrospinal fluid (CSF) and/or blood of MS patients, especially during acute exacerbations (Imitola et al., 2005; Mellergard et al., 2010; Miller et al., 2004; Sharief and Hentges, 1991).

The effects of the cytokine/chemokine levels have been occasionally analyzed, with contrasting results (Khademi et al., 2009; Kivisakk et al., 2009; Mellergard et al., 2010; Ramos-Cejudo et al., 2011). To gain increased insight into the immunomodulating effect of natalizumab systemically, we used a multiplex array to measure the levels of 48 cytokines/chemokines in sera from MS patients treated with natalizumab from 21 months.

2. Materials and methods

2.1. Patients and samples collection

After obtaining signed, informed consent based on the local ethics committee guidelines, 19 patients with RRMS, treated with natalizumab, were enrolled at the "Fondazione Istituto Neurologico C.

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Table 1
Demographic, clinical and radiological characteristics in 19 relapsing remitting multiple sclerosis (RRMS) patients receiving Natalizumab.

Demographic, clinical, and radiological characteristics in 19 RRMS patients receiving Natalizumab during 21 months of treatment	
Sex (F/M)	16/3
Age at recruitment (mean ± SD)	34.2 ± 9.8
Disease duration at baseline (mean years ± SD)	10.2 ± 6.2
EDSS at baseline (mean ± SD) (range)	1.3 ± 1.5 (0.0–6.5)
EDSS after 12 months of treatment (mean ± SD) (range)	1.4 ± 1.6 (0.0–6.5)
EDSS after 21 months of treatment (mean ± SD) (range)	1.7 ± 1.7 (0.0–7.0)
Relapsing/non-relapsing patients	5/15
Patients with/without new MRI lesions at the end of treatment	4/15

EDSS = Expanded Disability Status Scale; MRI = Magnetic Resonance Imaging; SD = standard deviation.

Mondino" (Pavia, Italy). Natalizumab was administered intravenously to RRMS patients once every 4 weeks at a dose of 300 mg. Blood, serum and urine samples were collected before the first infusion (T0) and subsequently after 12 (T12) and 21 (T21) months of treatment. At the corresponding time point, Kurtzke's Expanded Disability Status Scale (EDSS) was scored, and Magnetic Resonance Imaging (MRI) scans were performed (Castellazzi et al., 2015).

2.2. Cytokines/chemokines analysis

Quantification of 48-cytokines/chemokines was performed on the serum samples at T0, T12 and T21 using magnetic bead-based multiplex immunoassays (Bio-Plex®) (BIO-RAD Laboratories, Milano, Italy), following the manufacturer's instructions. This procedure uses Luminex Xmap technology with magnetic multi-analyte profiling beads. Briefly, a standard curve was created via dilution of premixed standards to 50,000 pg/mL, followed by series dilution to 8 concentrations. 50 µL of serum (diluted 1:4), and 50 µL of standard curve samples were added to a 96-well filter plate containing anti-cytokine antibody-conjugated beads. After incubation for 30 min at room temperature, followed by washing plate with Bio-Plex Wash Buffer, 25 µL of the antibody-biotin reporter was added and incubated for 30 min shaking at 1100 rpm, and then 50 µL of Streptavidin-PE was added to each well. After incubation of 10 min 125 µL of Bio-Plex assay Buffer was added to plate for reading. The concentration of cytokines was determined by using the Bio-Plex 100 Analyzer (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. A digital processor managed data output, and the Bio-Plex Manager® software presented the data as median fluorescence intensity (MFI) and concentration (pg/mL) (BIO-RAD Laboratories, Milano, Italy). These assays have a limit of detection of 1–20 pg/mL, depending on the protein target.

Th2/Th1ratio was defined based on the ratio of IL-4, IL-5, IL-6 or IL-10 Th2 related cytokines and pro-inflammatory IFN-ɣ or TNF-α cytokines Th1-related cytokines.

2.3. DNA extraction and JCPyV detection

DNA was isolated from 0.2 mL of blood samples using the QIAampDNA Mini Kit (Qiagen, USA) and 0.15 mL of urine samples with Nucleospin RNA Virus Kit (MachereyNagels, Germany) following the manufacturer's instructions. Quantitative real time PCR (Q-PCR) assay was performed to quantify JCPyV genome using a Taqman chemistry with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA), with specific primers targeting the Large T antigen viral region, following a protocol described previously (Campello et al., 2011; Delbue et al., 2005). Data were expressed as copies/µg of DNA isolated from blood and as copies/mL of urine samples.

2.4. Anti-JCPyV IgG antibodies in serum samples

Serum concentrations of anti-JCPyV IgG were measured using a home-made ELISA previously described, using a GST-JCPyV VP1 protein as the capture antigen (Elia et al., 2016).

2.5. Statistical analysis

The values of the cytokines were analyzed under log transformation considered the skewedness of the distributions. Linear mixed models were used to analyze the change in time of the cytokines with a random intercept for each patient. The same approach was used to compare the values in time of the cytokines of patients with and without JCPyV viruria with an interaction with time of the virus presence. The false discovery rate was used to adjust the *p*-values, considering the multiple testing problem due to the analysis of 48 cytokines. The same analysis strategy was used to evaluate the change over time of the 8 Th2/Th1 ratios. Differences were considered significant at $p < 0.05$ (CI 95%).

3. Results

3.1. Patient characteristics

A total of 19 RRMS patients treated with natalizumab for 21 months was included in the study. Demographic and clinical characteristics are described in Table 1.

3.2. Cytokine/chemokine profile and natalizumab treatment

A panel of 48 soluble immune proteins was measured in the sera for the evaluation of their expression profile in response to natalizumab treatment at T0, T12 and T21. The concentration of the molecules measured as pg/mL, their role and the degree of statistical significance are reported in Table 2. Some of these proteins, including IL-2, IL-15, RANTES, IL-1a, IL-12p40, β-NGF, TNF-β, GM-CSF, MCP-1 and IFN-α, showed a concentration below the detection limit; therefore, they were not considered in the analysis of results. The concentration of seven molecules was significantly increased (Eotaxin) or decreased (IL-1ra, IL-7, IL-10, PDGFbb, IL-16 and HGF) during this time (Table 2 and Fig.1).

Th2/Th1 ratios did not exhibit any differences in the three analyzed time points, except for the IL-10/IFN-ɣ and IL-10/TNF-α ratios (Fig. 2).

3.3. JCPyV DNA detection, seropositivity and natalizumab treatment

JCPyV DNA was not found in any of the blood samples (Data not shown). No significant changes were observed in the urine JCPyV viral load or the levels of serum JCPyV antibodies (Fig. 3).

3.4. Cytokine/chemokine profile and JCPyV detection

The cytokine profiles of patients with JCPyV viruria were compared to those of patients without JCPyV viruria. No significant differences were observed for the considered cytokines (Data not shown).