

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.

Keywords:

JC Virus, cytokines, natalizumab

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 $\alpha 4\beta 1$ -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 $\alpha 4$ chain of the VLA-4 and $\alpha 4\beta 7$ 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, O'Connor, 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, Edstrom, 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. 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 minutes at room temperature, followed by washing plate with Bio-Plex Wash Buffer, 25 ul of the antibody-biotin reporter was added and incubated for 30 minutes shaking at 1,100 rpm, and then 50 ul of Streptavidin-PE was added to each well. After incubation of 10 minutes 125 ul 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 1-20pg/mL, depending on the protein target.**

Th2/Th1 ratio was defined based on the ratio of IL-4, IL-5, IL-6 or IL-10 Th2 related cytokines and pro-inflammatory IFN- γ 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/ μ g 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 viraemia 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- γ 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 (Figure 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).

4. Discussion

The beneficial effects of natalizumab in MS patients are associated with the reduction of the inflammatory status in the CNS (Jain et al. , 2010, Yednock et al. , 1992).

The marked decline of the inflammation intrathecally indicates a decline in the CSF levels of cytokines and chemokines, at both the mRNA and protein levels after one year of treatment (Khademi, Bornsen, 2009, Mellergard, Edstrom, 2010).

How natalizumab affects the systemic status of inflammation is instead less understood. Khademi and colleagues observed increases in systemic pro-inflammatory cytokine expression (mRNA levels) after 6 and 12 months of treatment. The high levels of pro-inflammatory cytokines in the blood were associated with the lymphocytosis and fatigue, occurring in association with natalizumab treatment (Khademi, Bornsen, 2009, Khademi et al. , 2008). High numbers of activated T cells expressing pro-inflammatory cytokines in blood have also been observed (Kivisakk, Healy, 2009).

Opposite results were reported by Mellegard and colleagues (Mellergard, Edstrom, 2010), who showed a marked decline in circulating plasma GM-CSF, TNF α , IL6 and IL 10 (proteins) after 12 months of treatment. In addition, it has been reported that 20 months of treatment with the antibody did not modify globally the cytokine profile in the blood, although an increase in some pro-inflammatory and anti-inflammatory cytokines has been observed after starting treatment (IFN- γ , IL-4, IL-10, IL-5 and IL-13) and after long-term treatment (IL-1 β , IL-2, IL17,IL-5 and IL-13) with natalizumab. In addition, in the same study, the treatment with the antibody did not modify regulatory T cell function. These findings suggested different mechanisms of action of early versus prolonged exposure to Natalizumab on the target immune cells. (Ramos-Cejudo, Oreja-Guevara, 2011) .

Our study adds to this debate, given that the sera cytokine/chemokine profiles were defined in 19 patients with clinically defined RRMS before the beginning of natalizumab treatment and after 12 and 21 months. It was not possible to conduct the same analysis on healthy donor subjects in order to obtain data on biological variation and reference range values.

Most of the 48 studied proteins did not exhibit any significant variations during the course of the treatment, except for IL7, IL16, PDGFbb, HGF, IL1ra, and IL10, which decreased over 21 months. The first three listed proteins are pro-inflammatory, produced by Th1 cells, whereas the last three proteins act as anti-inflammatory mediators, secreted by Th2 cells. Despite these variations, the Th2/Th1 ratio was not subjected to any changes at the three analyzed time points, except for the IL-

10/IFN- γ and IL-10/TNF- α ratios. Thus, the beneficial effect of natalizumab cannot be associated with a shift in favor of a Th2 anti-inflammatory profile. These data had no clinical counterparts, as they did not correlate with the clinical findings.

As for single cytokines, a significant decrease in IL7, which is a non-redundant cytokine for T-cell development and survival, has been observed. Several reports have indicated that the IL-7 levels increased in MS and experimental allergic encephalomyelitis (EAE) (Chou et al. , 1999). IL-7 enhances proliferation and IFN- γ secretion by myelin-activated T cells cultured from both normal controls and MS patients. Consistent with our results, IFN- β 1a treatment led to significantly reduced serum IL-7 levels, whereas natalizumab treatment mitigated the serum IL-7 levels (Lundstrom et al. , 2014). Analogously to IL-7, IL-16 is a chemoattractant for CD4⁺ T lymphocytes, monocytes and eosinophils and is mainly produced by T lymphocytes (Cruikshank et al. , 2000); further, CD4⁺ T-cell infiltration in the CNS correlates with increased IL-16 levels in brain lesions in EAE (Skundric, 2015). The IL-16 concentration in sera from MS patients was increased compared with healthy controls, and the IL-16 serum levels were significantly reduced after two years of IFN- β 1a treatment (Nischwitz et al. , 2014). **In accordance with these previous findings**, in our study, 21 months of natalizumab treatment demonstrated the same ability to induce the reduction in IL-16 concentration.

Recent studies have described the anti-inflammatory action of CCL11 (Eotaxin) in a model of experimental autoimmune encephalitis, where the increased concentration of this chemokine was associated with milder disease phenotype, reduced antigenic specific response and a preferential activation of the Th2 immune response (Adzemovic et al. , 2012, Tejera-Alhambra et al. , 2015). Consistent with this finding, in our study a significant increase in the expression of Eotaxin was observed during the treatment, suggesting that natalizumab might limit the immune and inflammatory processes in MS patients.

Hepatocyte growth factor (HGF) is a pleiotropic protein that modulates immune cell functions and also plays an inhibitory role in the progression of chronic inflammation(Adams et al. , 1994). HGF

has been found to be increased in CNS diseases, including MS (Tsuboi et al. , 2002), but some other studies have found decreased HGF CSF levels in patients with MS or unaltered levels of plasmatic HGF between MS patients and healthy controls(Benkhoucha et al. , 2010, Muller et al. , 2012). This discrepancy might be attributed to the fact that there is a compartmentalization of the HGF, produced by the lymphocytes present in the CSF, and not in the serum. In line with these last surveys, in our study a significant decrease of HGF concentration in serum of MS patients treated with natalizumab was observed and this finding may be associated with the beneficial effect of natalizumab.

Finally, we showed that IL-1ra levels also decreased in the sera of patients during the natalizumab treatment. It has been previously stated that IL-1ra may have a downregulating potential in the disease course of MS, and elevated serum levels in active as well as in stable disease stages have been found compared to controls (Heesen et al. , 2000). However, data regarding the role of IL1ra are very poor in literature and it is hard to define the significance of these results.

Besides the effect of natalizumab treatment on the concentration of the circulating cytokines/chemokines, we verified whether the monoclonal antibody affected the replication of JCPyV and/or the IgG response to the virus. However, we could not observe any significant change in the viruria of the patients during the course of treatment, neither in the rate of antibodies directed against JCPyV. This is contrast with some previous published data, that showed increase in JCPyV viruria, seropositivity rate and in the antibodies index in different cohorts of MS patients treated with natalizumab for at least 12 months (Delbue et al. , 2015, Outteryck et al. , 2014, Raffel et al. , 2015). We attributed the discrepancy of these findings to the small sample size analyzed in our study.

5. Conclusions

Long-term treatment of natalizumab was associated with reduced levels of some pro-inflammatory cytokines/chemokines, such as IL-7, IL-16, and with increased levels of Eotaxin. These findings may be related to the beneficial effects associated with natalizumab. However, the treatment did not

modify regulatory T cell function or cause a relevant switch in the Th2/Th1 balance, suggesting that natalizumab has marginal impact on the systemic immunity.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1: Serum levels of the seven proteins that were significantly increased (Eotaxin) or decreased (IL-1ra, IL-7, IL-10, PDGFbb, IL-16 and HGF) during the 21 months of natalizumab treatment. The concentrations were measured before starting the treatment (T0), after 12 months (T12) and after 21 months (T21) and are provided as log pg/mL. Data are presented as median and interquartile ranges.

Figure 2: Th2/Th1 ratios were significantly changed during the time of treatment. Comparative Th2/Th1 ratios considering anti-inflammatory IL-10 cytokine and pro-inflammatory cytokine **IFN- γ** (A) or TNF- α (B). Box and whiskers plots showing median and interquartile ranges are presented.

Figure 3: JCPyV viruria (a) and seroprevalence (b) at T0, T12 and T21. (a) JCPyV viral load in the urine is expressed as viral copies/mL, and data are provided as single measurement and median. (b) JCPyV seroprevalence is expressed as nOD, and box and whiskers plots showing median and interquartile ranges are presented.