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Expansion of IL-6$^+$ Th17-like cells expressing TLRs correlates with microbial translocation and neurological disabilities in NMOSD patients

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ABSTRACT

Different microbial antigens, by signaling through toll-like receptors (TLR), may contribute to Th17-mediated autoimmune diseases, such as neuromyelitis optica spectrum disorder (NMOSD). The objective of this study was to determine the proportion of different Th17-like cell subsets that express TLR in NMOSD patients. For this study, the frequency of different Th17 cell subsets expressing TLR subsets in healthy individuals (n = 20) and NMOSD patients (n = 20) was evaluated by cytometry. The peripheral levels of soluble CD14 (sCD14) and cytokines were determined by ELISA. Our results demonstrated that the proportion of peripheral CD4$^+$ T cells expressing TLR2, 4 and 9 was significantly higher in NMOSD samples than in healthy subjects. In NMOSD, these cells are CD28$^+$ PD-1$^-$ CD57$^-$ and produce elevated levels of IL-17. Among different TLRs$^+$ Th17-like subsets, the proportion of those that co-express IL-17 and IL-6 was significantly higher in NMOSD patients, which was positively correlated with sCD14 levels and EDSS score. By contrast, the percentage of TLRs$^+$ Treg17 cells (IL-10$^+$ IL-17$^+$) was negatively related to sCD14 and the severity of NMOSD. In conclusion, the expansion of peripheral IL-6-producing TLR$^+$ Th17-like cells in NMOSD patients was associated with both bacterial translocation and disease severity.

1. Introduction

Neuromyelitis optica spectrum disorder (NMOSD), like Devic's disease, is an autoimmune inflammatory disease of the central nervous system (CNS), mostly characterized by severe simultaneous or sequential episodes of optic neuritis and/or transverse myelitis (Wingerchuk et al., 2015). Differently from multiple sclerosis (MS), autoantibodies appear to play a central role in NMOSD pathogenesis, such as IgG1 against aquaporin-4 (AQP4) (Lennon et al., 2004). However, despite the involvement of B cells, the production of pathogenic IgG1 depends on CD4$^+$ T cells, and recent evidence has indicated the involvement of Th17 cells in NMOSD (Lin et al., 2016).

Like other autoimmune diseases, the pathogenesis of NMOSD is influenced by a complex interaction between genetic and environmental factors, such as infections. Some evidence has indicated that viral and bacterial infections may precede or occur in temporal association with NMOSD (Mori et al., 2011; Smyk, 2014; Masuda et al., 2015). Interestingly, high responsiveness to *E. coli*, commonly associated with urinary tract infections in NMOSD, was detected in cultured CD4$^+$ T cells from NMOSD patients (Barros et al., 2013). Moreover, this phenomenon was associated with IL-23 and IL-6 production by lipopolysaccharide (LPS)-activated monocytes and plasmatic levels of LPS (Barros et al., 2013). Gut microbiota appears to also play a role in autoimmunity. In this context, a recent study demonstrated NMO patients have higher levels of *Clostridium perfringens* in the gut (Cree et al., 2016), bacteria that was suggested to be involved in NMOSD by a mechanism of molecular mimicry (Varrin-Doyer et al., 2012).

Classically, pathogens mediate their pro-inflammatory actions through ligation of pathogen-associated molecular patterns (PAMPs) to the pattern recognition receptors (PRRs), particularly *via* toll-like receptors (TLRs), expressed on innate immune cells, particularly dendritic cells (DC) (Jiménez-Dalmaroni et al., 2016). Additionally,

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damage-associated molecular patterns (DAMPs) may also trigger DC activation through TLRs (Jin and Flavell, 2013). Due to the ability of breaking tolerance of self-reactive T cells, PAMP-activated DCs could be involved in triggering and/or further exacerbating autoimmune diseases (Marsland and Kopf, 2007).

Although TLRs are typically expressed in innate immune cells, these receptors are also detected in activated human T cells, mainly in the context of autoimmunity (Jiménez-Dalmaroni et al., 2016). High expression of TLR-3 and -9 on T cells was seen in systemic lupus erythematosus (SLE) and was related to disease activity (Klonowska-Szymczyk et al., 2014). In MS patients, a recent study by Nyirenda et al. (2015) demonstrated high TLR2 expression on regulatory $CD4^+$ $CD25^{hi}$ $FoxP3^+$ T cells, and the addition of TLR2 agonist (Pam3Cys) not only inhibited the *in vitro* suppressive action of these cells, but also skews them toward a Th17 phenotype. Collectively, these findings suggest that different PAMPs may directly modulate human T cell behavior. In the context of NMOSD, however, no study evaluating TLR expression patterns on $CD4^+$ T cells and their relationship with disease severity has so far been carried out.

2. Material and methods

2.1. Patients

For our study, 20 patients (2 males and 18 females) with a diagnosis for neuromyelitis optica spectrum disorder (NMOSD), according to Wingerchuk et al. (2015) criteria, were recruited between 2014 and 2016 from Lagoa and Clementino Fraga Filho Hospitals (Rio de Janeiro, Brazil) during clinical remission (Table 1). The disability status was evaluated by two neurologists (R.A. and S.V.A.L) at the time of the study, according to the expanded disability status scale (EDSS) (Kurtzke, 1983). None of the patients had received any immunomodulating treatment, or had been submitted to corticosteroid treatment in the last 30 days. The patients included into the present study were not treated because either they had just defined the final diagnosis of NMOSD or had failed the medication at the moment of blood sampling. Furthermore, no patient had a clinical diagnosis of any infection at the time of blood draw. As control, 20 healthy subjects, matched by age, sex, and racial background were recruited to participate in this study.

After a complete description of the study to the participants, written informed consent was obtained from each individual. The study was approved by the Ethics Committee for Research on Human Subjects of the Federal University of the State of Rio de Janeiro (UNIRIO).

2.2. Peripheral blood mononuclear cell cultures and stimulus

Peripheral blood (20 mL) was collected in heparine-containing tubes (BD Vacutainer, Franklin Lakes, NY), and both plasma and peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on Ficoll-Hypaque density gradient. The plasmas were frozen (− 70 °C) for subsequent cytokine and soluble CD14 analysis by ELISA and for detection of anti-AQP4 antibodies. The PBMC were collected, washed three times in HANK's solution, and then suspended in 1 mL of RPMI-1640. Viable PBMC (1×10^6/mL) were cultured in 24-well flat-bottomed microplates with 1 mL of RPMI 1640 medium supplemented with 2 mM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% of fetal calf serum (FCS), 20 U/mL of penicillin, 20 lg/mL of streptomycin and 20 mM of HEPES buffer. To analyze cytokine production of T cells, PBMC (1×10^6/mL) were kept in a 24-well flat bottom microtiter plates and stimulated for 3 days with anti-CD3/anti-CD28 beads (10 μL/mL). At the end of the cell culturing, and to optimize intracellular cytokine detection, an additional short-term activation of these cells was conducted with phorbol-12-myristate-13-acetate (PMA, 20 ng/mL) plus ionomycin (600 ng/mL) in the presence of brefeldin A (10 μg/mL) for 4 h. During culturing, the plates were maintained at 37 °C in a humidified 5% CO_2 incubator. Of note, the assays were paired, that is, fresh PBMC from one patient and one control were analyzed together at same experiment.

Table 1
Demographic and clinical features of the NMOSD patients and controls.

	Control[a]	NMOSD[b]
No. of subjects (n)	20	20
Gender, female/male (n)	2/18	2/18
Age [(years), mean ± SD]	41.1 ± 20.1	43.1 ± 18.1
Positive anti-AQP4 antibody (%)[c]	NA[f]	28
EDSS [mean (range)][d]	NA[f]	5.1 (0–7)

Data from:
[a] Healthy individuals.
[b] Neuromyelitis optica spectrum disorder (NMOSD) patients in remission phase. Age (years) refers to age at the time blood samples were collected.
[c] Determined by CBA in the plasma of NMOSD patients.
[d] EDSS, Expanded Disability Status Scale that was determined at the time point that blood was collected to perform the immune assays.
[f] Not analyzed.

2.3. Immunofluorescence labeling and flow cytometry

The mouse anti-human monoclonal antibodies (mAbs) to CD3-APC, CD4-FITC, TLR2-PE, TLR4-PE, TLR9-PE, PD-1-APC, CD28-PE-Cγ7, CD57-APC, IL-17-PE-Cγ7, IFN-γ-APC, IL-6-APC, IL-10-APC and all isotype-control antibodies were purchased from BD Bioscience (San Diego, CA, USA), and were used to quantify the percentage of different $CD4^+$ T cell subsets. Briefly, freshly purified PBMC (2×10^5/tube) were incubated with various combinations of aforementioned mAbs for 30 min at room temperature, in the dark, according to manufacturer's instructions. After washing with phosphate-buffered saline (PBS), permeabilization was performed by incubating cells with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) at 4 °C for 20 min. After washing, the antibodies for intracellular staining (anti-IL-17, anti-IFN-γ, anti-IL-10, anti-IL-6) or the corresponding isotype control anti-IgG1 were added in various combinations and incubated for 30 min at 4 °C. After washing with PBS and SFB, the analysis was performed using Accuri C6 (Accuri™, Ann Arbor, MI, USA) and CFlow software (Accuri™, Ann Arbor, MI, USA). Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 100,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells, by using propidium iodide, and doublets. Further, the gated cells were negatives for CD14 marker.

2.4. Detection of anti-AQP4 antibodies

The serology for anti-AQP4 IgG was performed through a live cell based assay (CBA), as previously described (Marignier et al., 2013). Briefly, plasma samples were diluted at 1/100 in PBS with 8% of normal goat serum (NGS), incubated for 20 min, in − 20 °C, with live HEK293T cells, previously transfected with M23-AQP4 isoform. After the incubation time, the complex was washed, fixed with paraformaldehyde (PFA) 1%, and goat antibody anti-human IgG was added (Jackson ImmunoResearch, Inc.). The cells were incubated for 30 min at room temperature, in the dark, then washed with PBS and, finally, analyzed in Accuri C6 cytometer (Accuri™, Ann Arbor,MI, USA) with FlowJo v10 software (FlowJo, LLC).

2.5. Quantification of plasmatic levels of cytokines and sCD14

The quantification of plasmatic cytokines was performed using OptEIA ELISA kits (BD, Pharmigen, San Diego, CA), according to manufacturer's instructions. Briefly, each assay was realized using pairs of mAbs targeting human IL-10, IL-1β, IL-6, IFN-γ and IL-17. The reaction was revealed with streptavidin-horseradish peroxidase, using