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The expansion of circulating IL-6 and IL-17-secreting follicular helper T cells is associated with neurological disabilities in neuromyelitis optica spectrum disorders

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ABSTRACT

Due to their function in assisting B cells, T_{FH} cells may be involved in the production of pathogenic IgG in neuromyelitis optica spectrum disorder (NMOSD). In the present study, the proportion of IL-6^+ and IL-17^+ T_{FH} cell subsets was higher in NMOSD patients than healthy individuals. The frequency of both T_{FH} cell subsets were directly associated with disease activity. By contrast, NMOSD patients with a higher proportion of IL-10^+ T_{FH} cell subsets showed a lower neurological disabilities score. In summary, all findings suggest that expansion of peripheral IL-6^+ and IL-17^+ T_{FH} cells may be involved in the severity of NMOSD.

1. Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune inflammatory disease of the central nervous system (CNS), which is mostly characterized by severe simultaneous or sequential episodes of optic neuritis and/or transverse myelitis (Wingerchuk et al., 2015a, 2015b), but also affects other areas in the CNS (*e.g.*, brainstem and hypothalamus) (Kinoshita et al., 2009). In terms of immunopathogenesis, growing evidence has suggested the involvement of autoantibodies, mostly the presence of anti-aquaporin 4 (AQP4) antibody (Ab), in spinal cord lesions (Kinoshita et al., 2009; Wingerchuk et al., 2015a, 2015b). AQP4 is a water channel protein expressed in astrocyte foot processes in association with excitatory aminoacid transpoter-2 (EAAT2) (Hinson et al., 2008). The anti-AQP4 Ab is present in approximately 90% of patients with classical NMO and > 50% of patients with NMOSD (Wingerchuk et al., 2015a, 2015b). In addition, an average of 20% of NMOSD patients negative for anti-AQP4 Ab have IgG against myelin oligodendrocyte glycoprotein (MOG) (Wingerchuk et al., 2015a, 2015b). Some evidence has indicated that seropositivity for anti-AQP4 Ab, but not for anti-MOG Ab, correlates with disease severity (Jarius et al., 2014; Sato et al., 2014). This deleterious relationship between presence of anti-AQP4 Ab and neurological disabilities should be associated with astrocyte damage, induced by complement deposition (Hinson et al., 2008) and neutrophil-mediated antibody-dependent cytotoxicity (Jasiak-Zatonska et al., 2016), as well as glutamate-mediated neurotoxicity due to lower EAAT2 availability (Hinson et al., 2008). Although the involvement of autoantibodies in NMOSD is recognized, $CD4^+$ T cells might also be implicated in the disease, particularly follicular $CD4^+$ T cells, named T_{FH} cells (Qi, 2016).

Human T_{FH} cells represent a distinct subset of $CD4^+$ T cells found in secondary lymphoid organs and constitutively express the chemokine receptor CXCR5, which allows them to migrate into the lymphoid follicles (Qi, 2016). These cells are also characterized by a high expression of the transcription factor B cell lymphoma-6 (Bcl-6), programmed cell death receptor-1 (PD-1), inducible T-cell co-stimulator (ICOS), CD40 ligand (CD40L/CD154) and the production of IL-21 (Qi, 2016). T_{FH} cells provide signals for GC formation by inducing B cell proliferation, survival and the differentiation of B lymphocytes into heavy chain switched and affinity maturated antibody-producing plasma cells.

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Moreover, long-lived memory B cells are also generated from GC reactions (Qi, 2016).

After supporting CG development, T_{FH} cells can leave secondary follicles and join the pool of circulating memory T cells (Qi, 2016; Tangye et al., 2013). In the periphery, these cells are Bcl-6 negative and only a very low fraction (< 1%) co-express PD-1 and ICOS. When activated, these cells produce high levels of IL-21, the signature cytokine. In the context of autoimmunity, an increase in IL-21-secreting $CXCR5^+$ $CD4^+$ T cells has been observed in patients suffering from rheumatoid arthritis (AR) and systemic lupus erythematous (SLE) (Rasmussen, 2008). Besides producing IL-21, a study by Morita et al. (2011) demonstrated the existence of different circulating T_{FH} cell subsets able to produce other pro-inflammatory cytokines, such as IFN-γ ($T_{FH}1$) and IL-17 ($T_{FH}17$).

Using PD-1, ICOS and CXCR5 markers to define T_{FH} cells, some authors have demonstrated a higher frequency of these cells in NMOSD, mainly among relapsing patients (Li et al., 2015; Fan et al., 2016; Zhao et al., 2017). Nevertheless, the contribution of different cytokine-secreting T_{FH} cell subsets in NMOSD had not been determined to date, and, in the present study, we observed that an expansion of $IL\text{-}17^+$ and $IL\text{-}6^+$ T_{FH} cell subsets in NMOSD with seropositivity for anti-AQP4 was associated with disease activity.

2. Materials and methods

2.1. Patients

For our study, 26 patients (3 males and 23 females) with a diagnosis for remittent recurrent NMOSD, according to Wingerchuk et al. (2015a, 2015b), were recruited between 2016 and 2017 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.A.L) at the time of the study, according to the expanded disability status scale (EDSS) (Kurtzke, 1983). All patients were receiving immunosuppressive drugs (Table 1). After recruitment and blood sampling, the occurrence of clinical relapses were determined during a 1-year follow-up. Relapse was defined as a sudden appearance of new neurological symptoms and signs, or worsening of existing symptoms, lasting for at least 24 h. As a control, 25 healthy subjects, matched by age, sex, and racial background were recruited to participate in this study. Of note, no subject had a clinical diagnosis of any infection at the time of blood extraction.

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).

Table 1
Subjects characteristics.

	Control[1] (*n* = 25)	NMOSD[2] (*n* = 26)
Mean age in years (sd)	39.4 (15.9)	41.1 (18.1)
Male (%)	12	12
Disease duration (in years)	NA[4]	6.7 (3–15)
Positive anti-AQP4 Ab (%)[5]	NA[4]	62
Positive anti-MOG Ab (%)[5]	NA[4]	0
EDSS[3] [mean (range)]	NA[4]	4.2 (1–7)
Clinical relapses (%)[6]	NA	46
NMOSD therapy (%)[7]	NA	100

[1] Healthy individuals.
[2] Relapsing-remitting NMO patients in clinical remission.
[3] Expanded Disability Status Scale.
[4] Not analyzed.
[5] Positivity for serum anti-aquaporin-4 (AQP4) Ab or anti-MOG Ab was determined by CBA assay.
[6] The occurrence of relapses one year after blood sampling.
[7] Immunossupressor therapy at the moment of study: cyclophosphamide (*n* = 3), azathioprine (*n* = 13), mycophenolate mofetil (*n* = 10).

2.2. Flow cytometry analysis

Whole peripheral blood from healthy subjects and NMOSD patients were briefly stimulated in 24-well flat bottom microtiter plates with phorbol myristate acetate (20 ng/mL; Sigma-Aldrich) plus Ionomycin (600 ng/mL; Sigma-Aldrich) at 37 °C in a humidified 5% CO_2 incubator for 4 h. For cytokine measurement optimization, Brefeldin A (10 μg/mL; Sigma-Aldrich) was also added to the culture. To determine the circulating percentage of cytokine-secreting T_{FH} cell subsets, mouse anti-human monoclonal antibodies (mAbs) against CD4-FITC, CXCR5-PECy7/PE, IL-21-PE/APC, IFN-γ-APC, IL-10-APC, IL-17-PECy7, IL-6-PE and all isotype control antibodies were purchased from BioLegend (San Diego, CA, USA). Briefly, blood samples were incubated with the aforementioned mAbs against superficial molecules for 30 min at room temperature in the dark, according to manufacturer's instructions. The red blood cells were lysed, washed and, then, the cells were permeabilized by incubating cells with Cytofix/Cytoperm solution (BD Pharmigen, San Diego, CA). After washing, the mAbs for intracellular staining (IL-21-PE/APC, IFN-γ-APC, IL-10-APC, IL-17-PECy7, IL-4-APC, IL-6-PE) were added in different combinations. The different T_{FH} cell subtypes were determined using Accuri C6 flow cytometer (Accuri™, Ann Arbor, MI, USA) and analyzed using Cflow (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 200,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells and doublets. Circulating T_{FH} cells were defined as $CD3^+CXCR5^+IL\text{-}21^+$.

2.3. ELISA technique

The circulating levels of different cytokines were quantified by ELISA technique using OptEIA ELISA kits (BD, Pharmigen, San Diego, CA), according to manufacturer's instructions. Each ELISA was performed using pairs of antibodies against IL-6, IL-17, IL-21 and IL-10. The reaction was revealed with streptavidin-horseradish peroxidase, using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate. Recombinant human cytokines, at concentrations ranging from 3.5–500 pg/mL, were used to construct standard curves.

2.4. Cell-based assays

The presence of plasma anti-AQP4 and anti-MOG antibodies from NMOSD patients was evaluated by cell-based assay (CBA). Briefly, plasmas (diluted at 1/100 for AQP4-Ab and 1/640 for MOG-Ab) were incubated at 4 °C for 20 min, with HEK293T cells previously transfected with the respective plasmids (MOG or AQP4-M23) (Marignier et al., 2013; Cobo-Calvo et al., 2016). The cells were fixed with paraformaldehyde (PFA) for 15 min and stained with a secondary goat antibody against human IgG, conjugated with allophycocyanin (APC) (Jackson Immuno Research Inc.). Results were analyzed using an Accuri C6 cytometer (Accuri™, Ann Arbor,MI, USA) and FlowJo v10 software (FlowJo, LLC).

2.5. Statistical analysis

Statistical analysis was performed using Prism 5.0 software (GraphPad Software). Comparisons between immune assays in the cell cultures from the control group and NMOSD patients were made using two-way ANOVA. Within the patient group, Student's *t*-test was applied. Correlations between variables were sought using Pearson's correlation. Significance in all experiments was defined as $p < .05$.

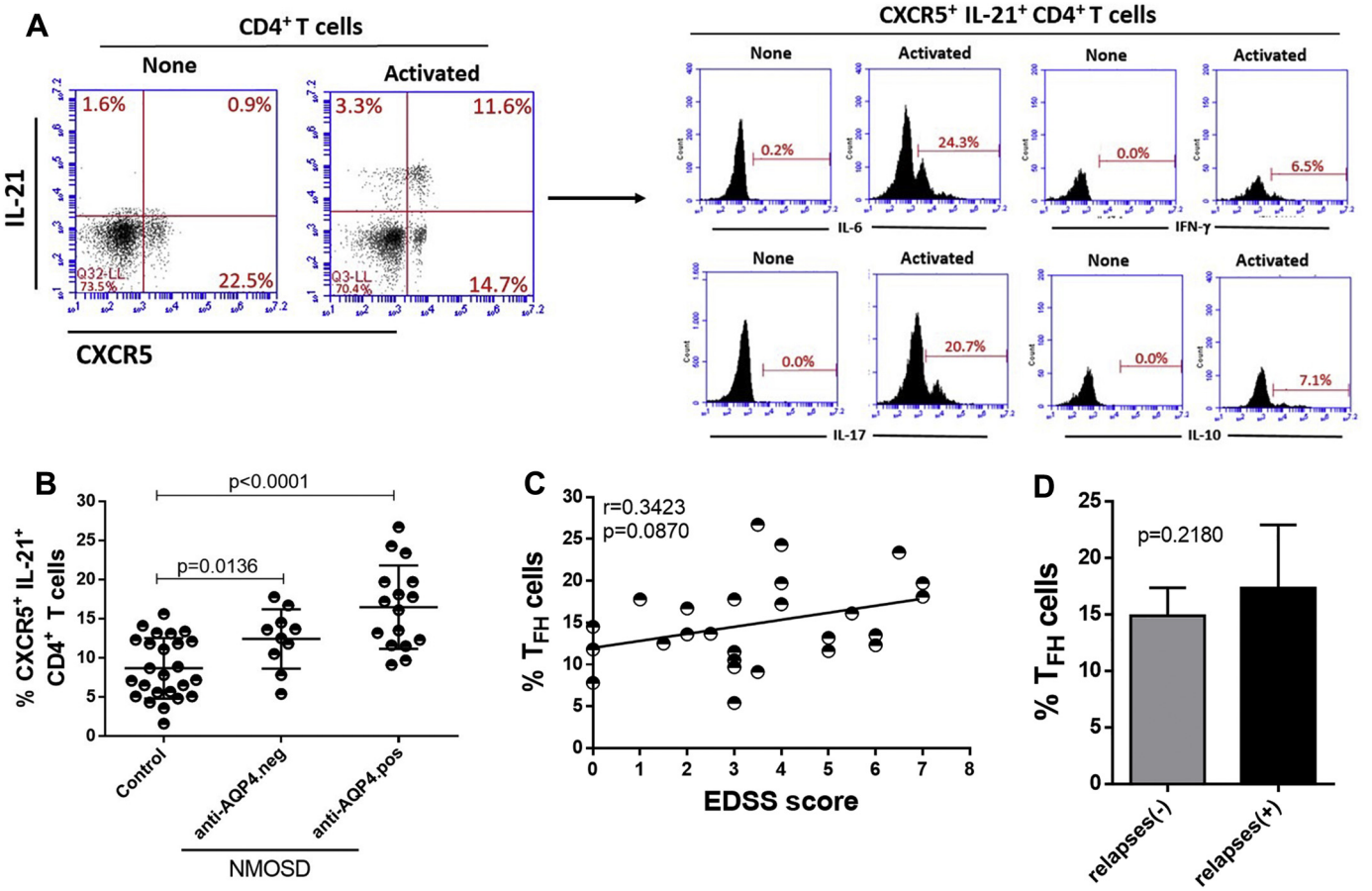

Fig. 1. The percentage of T_{FH} cell subsets in NMOSD patients. In (A), the strategy for gating the T_{FH} (CXCR5$^+$ IL-21$^+$ CD4$^+$) cells, and their cytokines-secreting subsets. (B) Identification of peripheral T_{FH} cells from the control group (n = 25) and NMOSD patients, soronegatives (anti-AQP4neg, n = 9) or soropositives (anti-AQP4pos, n = 16) for anti-AQP4 Ab, was performed after brief stimulation (4 h) with PMA (600 ng/mL) plus Ionomicina (20 ng/mL). In the figure shows the mean (SD) and (**) and (***) indicate $p < .001$ and $p < .0001$ respectively, determined by the two-way ANOVA, In (C) and (D), the frequency of T_{FH} cells from NMOSD patients is demonstrated in function of EDSS score (Pearson's correlation) and relapses (Student's test) occurrence during follow up (1 year), respectively. In (C) and (D), the mean values were compared and the p values are indicated in the figures.

3. Results

3.1. Clinical parameters of individuals

Demographic and clinical features of the NMOSD patients and the age-matched control group are shown in Table 1. As expected, the majority of patients were women. The disability score ranged 1 to 7, and the patients were receiving immunosuppressive therapy. In terms of autoantibody status, 16 from 26 (62%) of NMOSD patients presented antibodies (Ab) against AQP4 while no individual was positive for anti-MOG Ab. Notably, all immune assays were performed during the clinical remission phase (at baseline), and occurrence of clinical relapses was observed in 12 patients during a 1-year follow-up. Regardless of treatment scheme, the majority of patients (10/12) who relapsed had an EDSS score ≥ 5.

3.2. The frequency of different T_{FH} cell subtypes was associated with clinical activity of NMOSD

Taking into consideration the CXCR5 and IL-21 markers (Fig. 1A), the proportion of circulating T_{FH} was significantly higher in NMOSD patients in comparison with the control group, notably among those patients positive for anti-AQP4 Ab (Fig. 1B). No difference was observed in the patient subgroups (p = .4340). Additionally, the frequency of these cells did not correlate with either neurological disabilities or occurrence of further relapses after blood sampling (Fig. 1C and D). On the other hand, the proportion of IL-6$^+$ and IL-17$^+$ T_{FH} cell subsets was higher in NMOSD patients than control group, mainly among those seropositive for anti-AQP4 Ab (Fig. 2A). Moreover, the frequency of those T_{FH} cell subsets were directly associated with EDSS score (Fig. 2B). Further, the occurrence of new relapses during follow up (1 year) was observed among patients with a higher frequency of IL-6$^+$ T_{FH} cells (Fig. 2C). No difference was observed with regard to IFN-γ-secreting T_{FH} cells (Fig. 2A). Additionally, these IFN-γ$^+$ T_{FH} cell subset was not associated with clinical parameters (Fig. 2B and C). By contrast, a lower proportion of IL-10-producing T_{FH} cells was identified in NMOSD with anti-AQP4 antibodies (Fig. 2A), and their percentage was positively associated with neurological disabilities (Fig. 2B).

3.3. In vivo IL-6 and IL-17 levels are correlated with T_{FH} cells and disease severity

Plasma levels of IL-6, IL-10, IL-17 and IL-21 were significantly higher in NMOSD patients, regardless of anti-AQP4 antibody status, as compared with the control group (Fig. 3A). Moreover, *in vivo* IL-21, IL-6 and IL-17 levels were directly correlated with the frequency of T_{FH} cells (IL-21$^+$), as well as those cells positives for IL-6 and IL-17 cytokines, respectively (Fig. 3B). No significant correlation was observed between IL-10 and IL-10$^+$ T_{FH} cells. Finally, concerning plasma cytokines, while IL-6 levels were positively associated with EDSS (Fig. 3C), IL-6 and IL-17, but not IL-21 or IL-10, were significantly higher among NMOSD patients who relapse during the follow up (Fig. 3D).

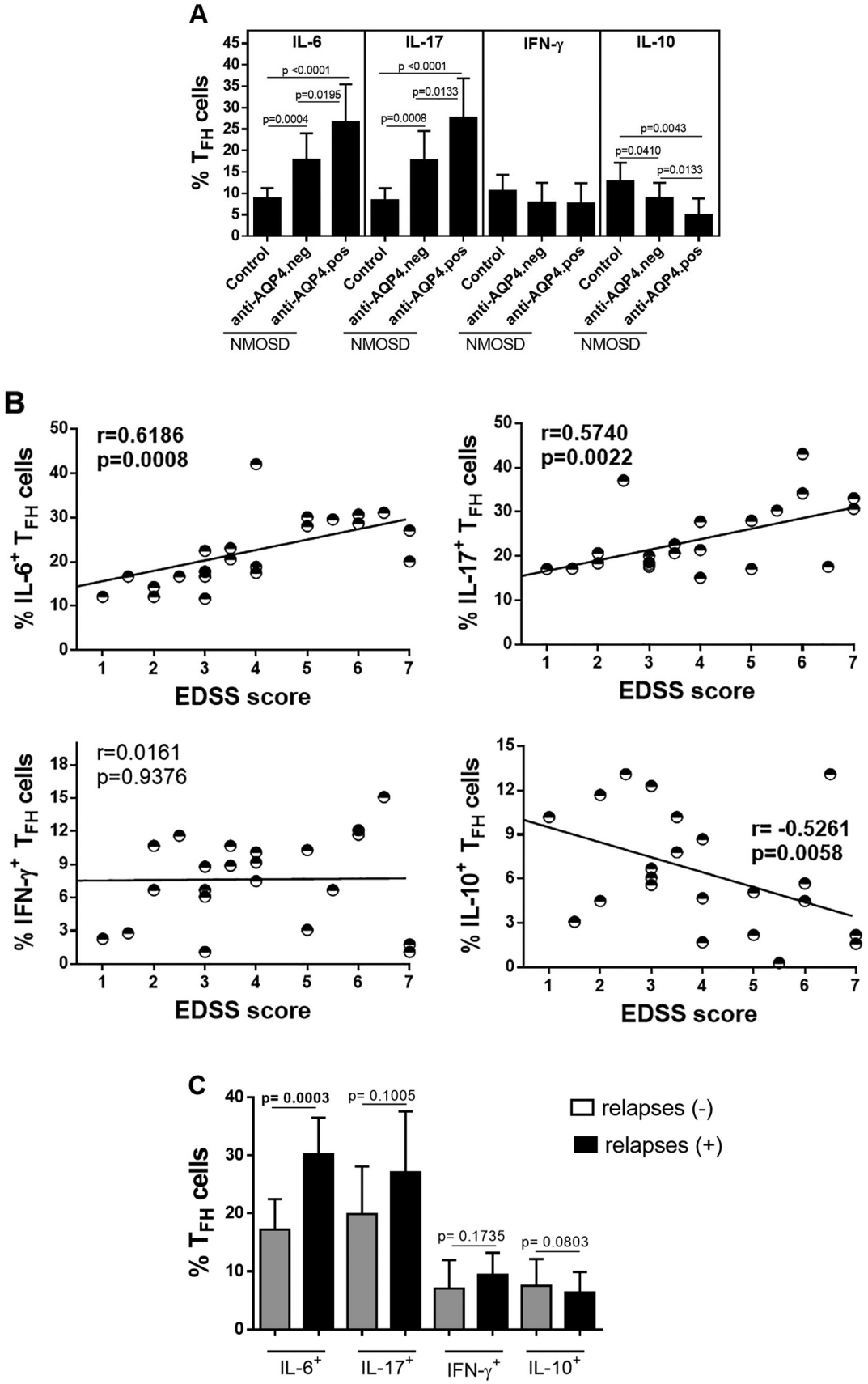

Fig. 2. The proportion of different T_{FH} cell subsets and its relationship with clinical parameters in NMOSD patients. In (A), the percentage of PMA/IO-stimulated T_{FH} cells producers of IL-6, IL-17, IFN-γ and IL-10 was analyzed in healthy subjects (n = 26) and anti-AQP4neg (*n* = 10) and anti-AQP4pos (n = 16) NMOSD patients. In NMOSD (B), the percentage of different cytokine-secreting T_{FH} cells was correlated with neurological disabilities, determined by EDSS score. The Pearson's correlation was applied and the *p* values are indicated at the figure. In (C), the mean percentages of these T_{FH} cell subsets were stratified in function of relapse occurrence during the first year after blood sampling. The mean values were compared by using Student's *t*-test and the p values are indicated in the figures.

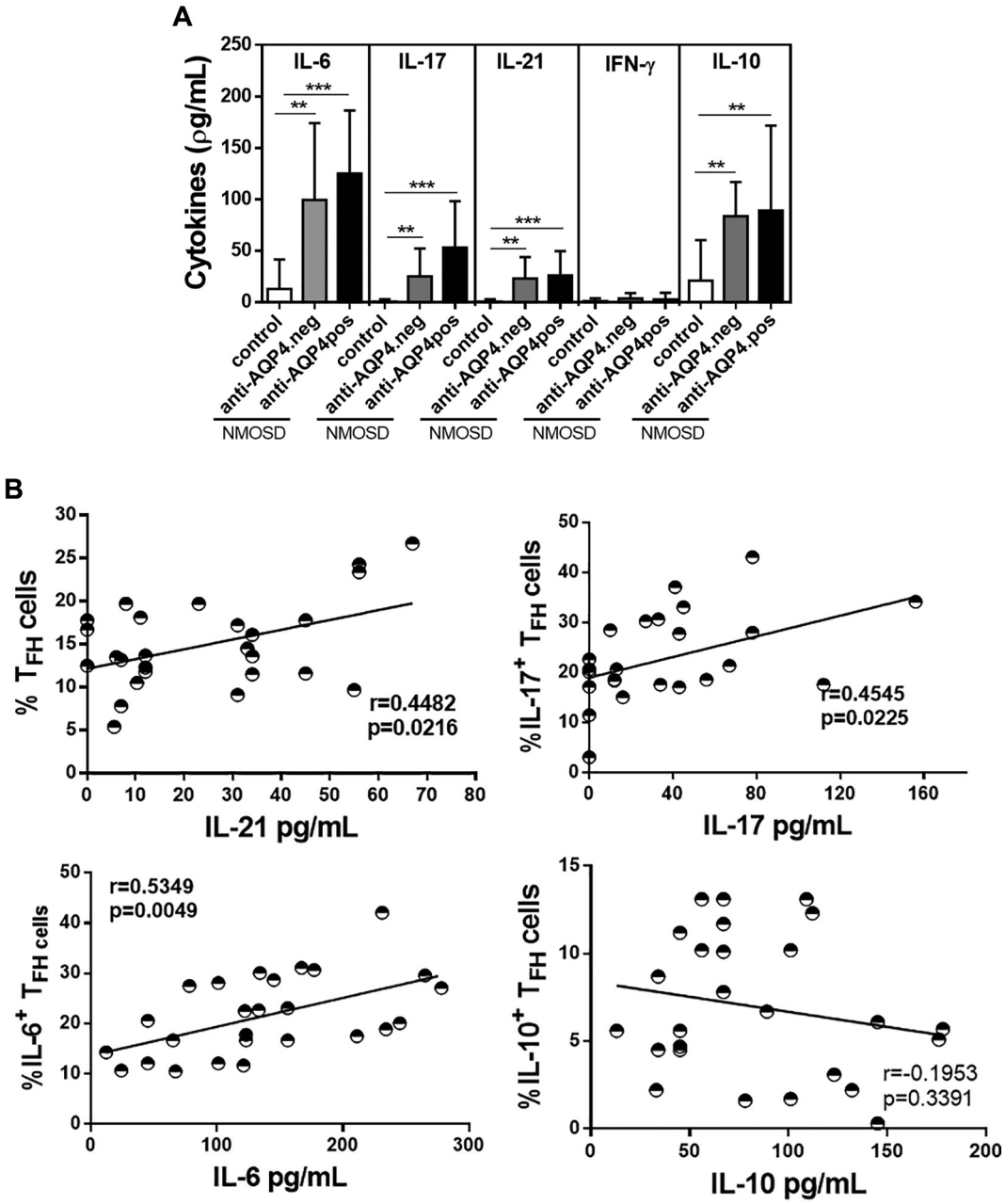

Fig. 3. *In vivo* cytokines and their relationship with circulating T_{FH} cell subsets and clinical parameters in NMOSD patients. In (A), plasma levels of IL-6, IL-17, IL-21, IFN-γ and IL-10 from NMOSD patients (n = 26) were quantified by ELISA in control group and NMOSD samples. The mean values of cytokines between different groups were compared by using the two-way ANOVA, and (**) and (***) indicates $p < .001$ and $p < .0001$, respective. In (B) and (C), the *in vivo* cytokines were correlated with different T_{FH} cell subsets or EDSS score, respectively. In (B and C), the Pearson's correlation was applied and the *p* values are indicated at the figure. In (D), cytokines values were stratified by occurrence of clinical relapses during fallow up (1 year) ly. The (D) shows the mean (SD) and the *p* values were obtained by the Student's *t*-test.

4. Discussion

It is believed that NMOSD immunepathogenesis involves the production of pathogenic, IgG notably directed to AQP4. Nevertheless, the synthesis of affinity matured IgG-producing plasma cells depends on T_{FH} cells, a distinct IL-21-producing $CXCR5^+$ $CD4^+$ T cell subset (Qi, 2016).

Here, a higher percentage of T_{FH} cells, identified as being $CXCR5^+IL\text{-}21^+$, was observed in NMOSD following brief *in vitro* activation, mainly among patients seropositive for anti-AQP4 Ab. Nevertheless, their frequency was not associated with clinical parameters. T_{FH} cells produce IL-21 in association with other cytokines (Rasmussen, 2008; Morita et al., 2011; Tangye et al., 2013; Qi, 2016), and, in the present study, an overpresentation of $IL\text{-}6^+$ and $IL\text{-}17^+$ T_{FH} cells was associated with advanced neurological disabilities and occurrence of further relapses. To our knowledge, this is the first report that suggested an involvement of different cytokine-producing T_{FH} cells in NMOSD.

Some studies have demonstrated an expansion of $PD\text{-}1^+CXCR5^+CD4^+$T cells in relapsing NMOSD patients (Li et al., 2015; Zhao et al., 2017). However, none of these studies has demonstrated any relationship between these cells and neurological disabilities. Although Zhao et al. (2017) did not perform intracellular IL-21 staining in these cells, they observed a positive correlation between these cells and

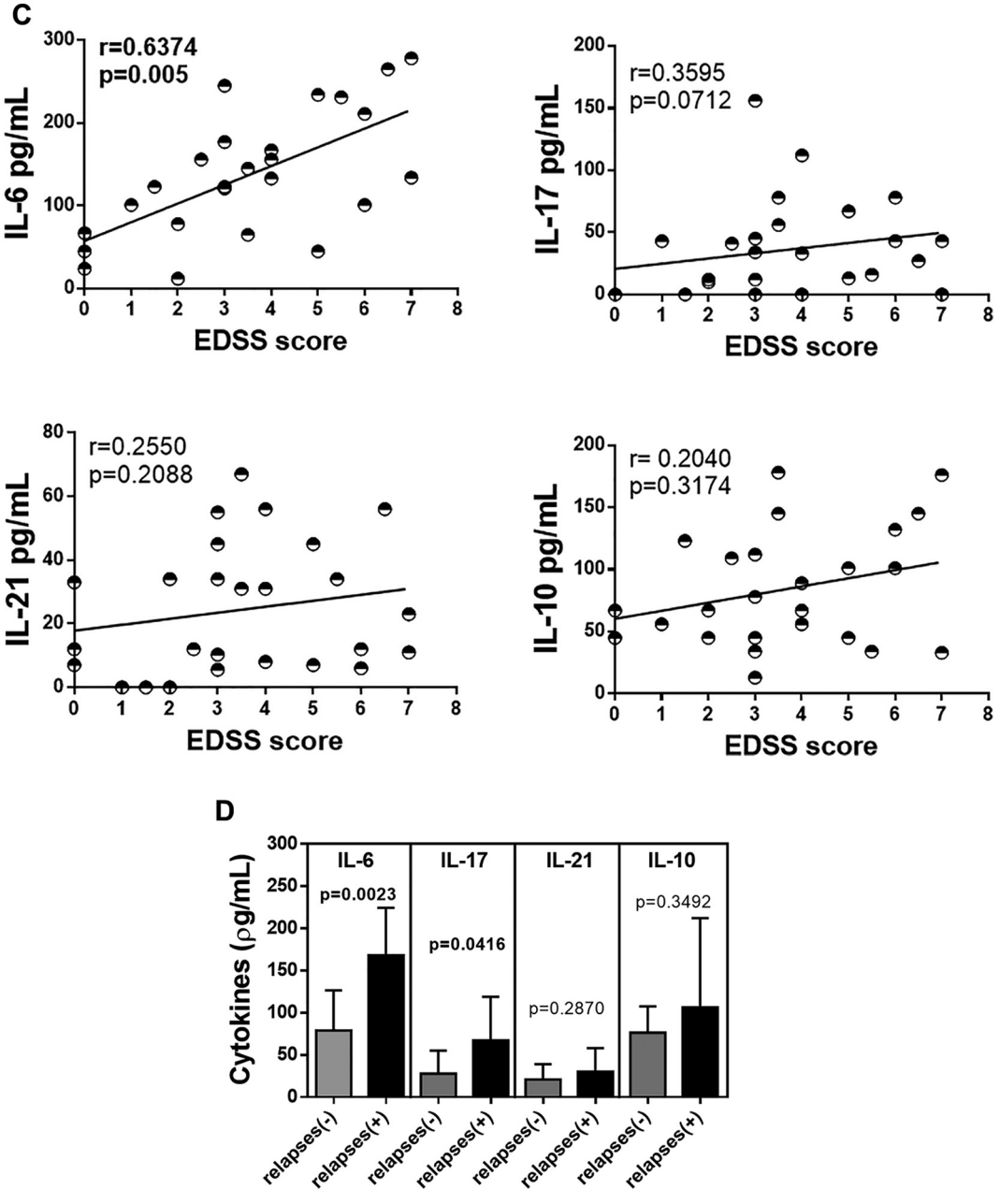

Fig. 3. (*continued*)

plasma levels of IL-21. In LES, a classical humoral autoimmune disease, $CXCR5^{+}PD\text{-}1^{+}IL\text{-}21^{+}CD4^{+}$ T cells were directly associated with disease activity (Sawaf et al., 2016). Since PD-1 expression on T_{FH} cells enhances the ability of these lymphocytes to help B cells (Tangye et al., 2013; Onabajo et al., 2013; Qi, 2016), there is a possibility that $PD\text{-}1^{+}T_{FH}$ cells in the aforementioned NMOSD studies (Li et al., 2015; Zhao et al., 2017) are positive not only for IL-21, but for other cytokines as well. Another interesting finding by Zhao et al. (2017) study was the ability of rituximab (RTX), a monoclonal against CD20 that depletes B cells, in attenuating disease activity in NMSO patients. This beneficial effect was related to a significant reduction of the frequency of circulating T_{FH} cells and IL-21 and IL-6 plasma levels. These findings are in line with some clinical studies suggesting RTX as more efficient therapeutic option in the NMOSD treatment (Kessler et al., 2016). Nonetheless, take into account the ability of the bone marrow to restore peripheral B cells, the development of a novel therapeutic drug for depletion of circulating T_{FH} cells may provide long-term protection for clinical attacks in NMOSD patients.

Concerning *in vivo* cytokines, the plasma levels of IL-6, IL-21 and IL-17 were higher in NMOSD patients than in the control group. These findings are in agreement with other studies demonstrating elevated circulating levels of IL-6 and IL-21 in NMOSD patients (Fan et al., 2016; Zhao et al., 2017). In addition, in our cohort, IL-6 and IL-17 concentrations were positively associated with clinical parameters. The higher peripheral levels of IL-10 that could be produced by different immune cells, in our patients showed an attempt to control the production of pro-inflammatory cytokines.

Some evidence has linked IL-6 and IL-17 to NMOSD pathogenesis (Chihara et al., 2011; Wang et al., 2011; Barros et al., 2016). Indeed, a recent study published by our group (Barros et al., 2016) demonstrated an elevated percentage of circulating dual IL-6 and IL-17-secreting $CD4^{+}$ T cells positive for toll-like receptor (TLR) 2 and TLR4 in NMOSD patients with advanced neurological disabilities. There is a possibility that a fraction of these lymphocytes may become T_{FH} cells and thereby,

support germinal center reactions. In the SLE animal model, the production of autoantibodies was dependent on $ICOS^+IL\text{-}17^+IL\text{-}21^+T_{FH}$ cells (Wu et al., 2008). Consistent with this finding, the percentage of IL-6- and IL-17-secreting T_{FH} cell subsets was higher in our NMOSD patients with anti-AQP4 Ab. Since IL-6 enhances the survival of plasmablasts, thereby promoting anti-AQP4 Ab production (Chihara et al., 2011), the expansion of IL-6-producing T_{FH} cells could help to at least partially explain why seropositivity to anti-AQP4 Ab is related to more attacks and diminished recovery in NMOSD patients (Sato et al., 2014). Unfortunately, although it presents elevated sensitivity, anti-AQP4 IgG dosage by CBA is not a classical quantitative. This means therefore, that we are unable to perform any comparison between anti-AQP4 titers and circulating T_{FH} cells.

With regard to IFN-γ-secreting T_{FH} cells, little is known about this follicular subset in autoimmune diseases (Che et al., 2016). In the present study, no difference was observed with regard to peripheral IFN-γ-secreting T_{FH} cells in NMOSD patients when compared to healthy controls. These findings can be explained with previous studies that showed a lower capacity of these cells to support Ab production when compared with $T_{FH}17$ cells (Morita et al., 2011). Finally, a lower percentage of circulating IL-10-secreting T_{FH} cells was identified in our patients, notably among those with advanced neurological disabilities. These findings are in agreement with some observations indicating a critical role in controlling GC responses for regulatory $IL\text{-}10^+$ $FoxP3^+$ $CD4^+$ T cells, named T_{FR} cells, which are found in follicles (Sage and Sharpe, 2016). Although our sample size was small, this data suggests that a lower percentage of $IL\text{-}10^+$ T_{FH} cells in NMOSD with anti-AQP4 Ab may fail to attenuate pathogenic T_{FH} cell subsets in NMOSD.

5. Conclusions

Although the sample size of the present study could be increased, our data suggests that an increase in circulating $IL\text{-}6^+$ and $IL17^+$ T_{FH} cells in NMOSD patients seropositive for anti-AQP4 Ab, associated with lower frequency of $IL\text{-}10^+$ T_{FH} subset, may contribute to the severity of the disease. Although preliminary, our data can help to design new immunotherapeutic tools to treat NMOSD.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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