Long-term Effects of IL-6 Receptor Blockade Therapy on Regulatory Lymphocytes and Neutrophils in Neuromyelitis Optica Spectrum Disorder

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Abstract

Background and Objectives

Neuromyelitis optica spectrum disorder (NMOSD) is a disabling autoimmune neurologic disease. Anti–IL-6 receptor (IL-6R) therapy prevents relapses in patients with anti–aquaporin 4 (AQP4)-IgG-positive NMOSD; however, it remains unclear how cellular immune components are altered by anti–IL-6R therapy. In this study, we examined the long-term effects of the anti–IL-6R monoclonal antibody tocilizumab (TCZ) on immune cell profiles in patients with NMOSD.

Methods

Monthly IV injections of TCZ (8 mg/kg) were administered as an add-on therapy to 19 anti–AQP4-IgG-positive patients, who had been refractory to corticosteroids and immunosuppressive drugs. Peripheral blood was collected before infusion of TCZ for flow cytometry analysis of lymphocyte subsets. Seven patients provided whole blood samples for gene expression profiles.

Results

Patients with NMOSD had reduced numbers of lymphocyte subsets with regulatory functions, including transitional B cells, CD56<sup>high</sup> NK cells, and CD45RA<sup>−</sup>FoxP3<sup>high</sup> regulatory T cells. However, after initiating TCZ therapy, the numbers increased to normal levels within 1 year. Gene expression analysis revealed that neutrophil granule–related genes, predominated by those related to azurophil granules, were significantly upregulated in patients with NMOSD. Such alterations suggestive of accelerated myeloid turnover were not observed 1 year after TCZ therapy, and the effects of TCZ on some neutrophil genes were observed as early as 5 days after starting TCZ. In vitro analysis demonstrated that naïve T-cell division was impaired in the enrolled patients, which was fully recovered after 18 months of therapy.

Discussion

In patients with active NMOSD not treated with molecular targeting drugs, we observed reduction or deficiency in lymphocytes with regulatory potentials and activation of neutrophils. However, introduction of anti–IL-6R therapy accompanied by tapering concomitant drugs corrected such abnormalities, which might contribute to persistent relapse prevention. The recovery in the naïve T-cell division after starting TCZ may underlie the relatively low risk of infection in patients under anti–IL-6R therapy.
Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune disease of the CNS, causing inflammatory damage to the optic nerve, spinal cord, brainstem, and the cerebral white matter.1,2 Anti–aquaporin 4-antibody (AQP4-Ab)1,3,4 is a surrogate marker for a core group of NMOSD with predominant astrocyte pathology. For such anti–AQP4-Ab-positive patients, disease-modifying drugs for multiple sclerosis (MS), such as interferon β, are not efficacious but rather harmful,5-7 indicating the difference between NMOSD and MS in the immunopathogenesis. Until recently, corticosteroids and off-label immunosuppressive drugs or plasmapheresis were the only available treatment options for NMOSD. However, clinical trials based on the pathophysiology of NMOSD have provided several high-efficacy drugs, including the B-cell depleting drugs rituximab8,9 and inebilizumab,10 the anticomplement C5 drug eculizumab,11 and the anti–IL-6R drug satralizumab.12,13 Currently, the mechanism of action of each drug in vivo remains poorly understood, and no theoretical background is offered to select a drug for each patient. This study was initiated before the approval of such drugs. We and studies from Germany have shown that monthly infusion of the anti–IL-6R monoclonal antibody tocilizumab (TCZ) reduces relapses, neurogenic pain, and fatigue in patients with NMOSD who have been refractory to immunosuppressive interventional treatments.14-17 Recent phase 3 clinical trials revealed that the recycling anti–IL-6R monoclonal antibody satralizumab as add-on therapy12 or as monotherapy13 would significantly reduce relapses in anti–AQP4-Ab-positive NMOSD. However, because IL-6 is a pleiotropic cytokine involved in numerous biological responses, the mechanisms of IL-6R blockade therapy remain to be investigated further, particularly regarding the cellular immune profiling pretherapy and posttherapy, to clarify the important features of the therapy in comparison with other approved drugs.

In this study, we report on the baseline and posttreatment characteristics of cellular immune phenotypes in a cohort of patients with NMOSD. Our results reveal targets of anti–IL-6R therapy in NMOSD and give us a hint for clinical practice such as in tapering concomitant immunosuppressive agents after adding an anti–IL-6R drug for treatment of NMOSD.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Data were obtained from 2 open-label clinical studies for evaluating the safety and efficacy of TCZ in patients with NMOSD (UMIN000005889 and UMIN000007866, registered on July 8, 2011, and May 1, 2012, respectively). The study protocol and statistical analysis plan for the studies are available in eSAP 1 (links.lww.com/NXI/A921) and eSAP 2 (links.lww.com/NXI/A922), respectively. The first participant was enrolled on November 2, 2011. The inclusion criteria were as previously described.16 Among 19 participants, 13 fulfilled the 2006 revised NMO diagnostic criteria,18 and 6 had limited forms of NMOSD with AQP4-IgG. Consequently, all participants fulfilled the 2015 international consensus diagnostic criteria for NMOSD with AQP4-IgG.19 Three to 14 healthy controls (HCs), who do not have any immunologic disorders, were also recruited. In addition, a total of 36 patients were recruited as “other NMOSD,” to whom TCZ treatment was not introduced. These patients either visited our outpatient clinic or were admitted to the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), between the ages of 20 and 70 years. Written informed consent in accordance with the Declaration of Helsinki was obtained from all participants. This study was approved by the institutional review board at NCNP.

Peripheral Blood Samples

For TCZ treatment, patients were given a monthly IV dose of TCZ (8 mg/kg) in addition to the basal treatment with corticosteroids and immunosuppressive drugs (Table 1), of which doses were gradually reduced during the first year in most cases. Heparinized peripheral blood was collected before infusion from the patients, and control samples were obtained from HCs and other NMOSD patients. Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll centrifugation technique. In the latest 3 cases, polymorphonuclear cells (PMNs) were also isolated in addition to PBMCs using Polymorphoprep (Axis Shield Diagnostics Ltd, UK) according to the manufacturer’s instructions. Lymphocyte subsets among PBMCs were analyzed by a flow cytometer.
PMNs were processed for neutrophil sorting using an ARIA II cell sorter (BD Biosciences). Serum was also collected and stored at −80°C until the analysis.

T-Cell Coculture Assays

Naïve CD4+ T cells (CD3+CD4+CD127+CD45RA+), CD45RA−CD25high regulatory T (Treg) cells (CD3+CD4+CD127−CD45RA−CD25high), and CD56−CD3−APCs were sorted from PBMCs of 5 patients using an ARIA II cell sorter (BD Biosciences) before and 8 and 18 months after starting TCZ and were cocultured, according to the previously published method with minor modifications.20 In brief, carboxyfluorescein diacetate succinimidyl ester (1 μL/mL)–labeled 5,000 naïve CD4+ T cells and irradiated (30 Gy) 50,000 APCs with or without 5,000 CD45RA−CD25high Treg cells were added into 96-well round-bottomed plates, which were precoated with 0.5 μg/mL OKT3 in RPMI 1640 medium supplemented with 10% fetal bovine sera, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin in a volume of 200 μL. The cells were cultured for 7 days in 37°C, 5% CO2 atmosphere and collected and analyzed for the proliferation of naïve CD4+ T cells during the culture period using a CANTO II flow cytometer.

Measurement of Cytokine and Chemokine Concentrations and Anti–AQP4-IgG Titer in Serum

We measured concentrations of 27 cytokines and chemokines (eTable 1, links.lww.com/NXI/A923), using Bio-Plex systems (Bio-Rad). Anti–AQP4-IgG titers were determined as previously described with minor modifications.21

Gene Expression Analysis

We made comparisons between 7 patients with NMOSD and 12 HCs and between before and after the TCZ treatment in the 7 patients. Peripheral blood was collected in PAXgene blood RNA tubes (PreAnalytiX GmBH) before and 12 months after starting TCZ. In brief, total RNA was extracted, and cDNA was synthesized. cDNA (600 ng) was hybridized with SurePrint G3 Human GE microarray (Agilent Technologies) version 2.0 and scanned. The scanned image data were quantified, and the extracted probes were analyzed online using DAVID 6.8.22 The data were normalized using GeneSpring GX 12.0 (Agilent Technologies), and changes in gene expression were calculated.23 We extracted the probes, which fulfilled one of the 2 criteria: (1) the expression rate (normalized value) equal to or greater than 1.5 or equal to or smaller than 0.67 (p < 0.05) and (2) the expression rate greater than 1 and

Table 1 Demographics and Baseline Characteristics of 19 Participants With NMOSD and Summary of the Clinical Improvement of the Symptoms After TCZ Treatment

<table>
<thead>
<tr>
<th></th>
<th>Pre-TCZ average (SD)</th>
<th>Post-TCZ (12 mo) average (SD)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at enrollment, average (SD)</td>
<td>44 (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at onset, average (SD)</td>
<td>35 (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male:female</td>
<td>2:17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral corticosteroids</td>
<td>18 (95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZA</td>
<td>9 (47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC</td>
<td>4 (21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX, BUC, and MZB</td>
<td>1 (5) for each drug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concomitant disease/symptoms, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLE</td>
<td>3 (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraine</td>
<td>2 (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>16 (84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>General fatigue</td>
<td>16 (84)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDSS</td>
<td>4.2 (1.7)</td>
<td>3.8 (1.5)</td>
<td>0.1155</td>
</tr>
<tr>
<td>No. of relapses in the adjacent 12 mo</td>
<td>1.9 (1.4)</td>
<td>0.26 (0.65)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Pain scores</td>
<td>4.4 (1.7)</td>
<td>3.1 (3.0)</td>
<td>0.0553</td>
</tr>
<tr>
<td>General fatigue scores</td>
<td>5.5 (1.9)</td>
<td>3.4 (2.4)</td>
<td>0.0094</td>
</tr>
</tbody>
</table>

Abbreviations: AZA = azathioprine; BUC = bucillamine; EDSS = expanded disability status scale; MTX = methotrexate; MZB = mizoribine; SLE = systemic lupus erythematosus; TAC = tacrolimus; TCZ = tocilizumab.
smaller than 1.5 or smaller than 1 and greater than 0.67 ($p < 0.01$).

**Real-Time PCR for Neutrophil RNA**

Whole blood cDNA was synthesized from the extracted RNA as described earlier using a PrimeScript RT-PCR kit (TaKaRa). Neutrophil RNA was extracted from FACSSorted neutrophils (ARIA II, BD Biosciences) using an RNAeasy mini kit (QIAGEN) and cDNA was synthesized. Real-time PCR was performed using commercially available primer pairs. β-actin (ACTB) was used as a standard.

**Statistical Analysis**

Wilcoxon matched pairs test was used for the comparison of before and after TCZ treatment, or 1-way ANOVA followed by a Dunnett multiple comparison test for comparing sequential data of a group. The Mann-Whitney test was used for the comparison of the 2 different groups. All analyses were performed using Prism version 5.01 (GraphPad software, CA). Significant difference was presented as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. For the microarray analysis, a false detection rate was also considered. Poisson distribution was confirmed, and Poisson regression was performed for the analysis of the estimated annual relapse rate (ARR).

**Data Availability**

All data supporting the findings are available within the article and/or in Supplementary materials.

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**Results**

**Effects of IL-6R Blockade by TCZ on Patients With NMOSD**

Since our previous report on the first 7 patients treated with TCZ,$^{16}$ additional 12 patients have enrolled in this clinical study (eFigure 1, links.lww.com/NXI/A920). Collective data from the 19 patients were re-evaluated for the demographics (Table 1) and safety and efficacy of TCZ. Notably, frequency of relapse in these 19 patients had increased year-by-year until the start of TCZ (Figure 1 left). This worsening before TCZ treatment was not unexpected because only patients unresponsive to treatments with corticosteroids and immunosuppressive drugs were recruited. We assume that patients in more serious conditions might have entered this open-label study, compared with the placebo-controlled phase 3 trials for NMOSD.$^{12,13}$ Analysis of the pooled data showed that ARRs and fatigue in numerical rating scores were significantly reduced after 12 months of TCZ treatment (Figure 1 right and Table 1). Eighteen patients were receiving oral prednisolone, and 9 of them were additionally given azathioprine during enrollment. The amount of prednisolone was reduced from a mean of 14 mg to 8.3 mg per day (approximately 41%) and the amount of azathioprine from a mean of 63 mg to 38 mg per day (approximately 40%) after 1 year of TCZ treatment. During this period, 21 adverse events were recorded in 10 patients: 4 were mild (hypotension, abdominal pain with diarrhea, anemia, and thrombocytopenia), 13 moderate (9 cases
of infection, 2 cases of anemia, and 2 cases of leukocytopenia), and 4 severe (3 cases of lymphopenia and 1 case of acute pyelonephritis). In all events, full recovery was achieved after medical care and interventions as required. These adverse events did not result in any patients to withdraw from this study.

**Reduction of Basophils and Increase of Neutrophils Were Corrected After Anti–IL-6R Treatment**

Before TCZ treatment was commenced, the cell counts of total lymphocytes, eosinophils, and basophils were significantly decreased in the peripheral blood of patients with NMOSD, when compared with HCs (Figure 2, A–C). Similar reductions were also observed in other NMO patients. The number of basophils increased after 1 year of TCZ treatment, whereas the total lymphocyte and eosinophil counts did not change significantly. On the contrary, the number of neutrophils was significantly increased in the peripheral blood of the other NMOSD patients and patients with NMOSD before TCZ treatment, when compared with HCs, and it was significantly reduced after 1 year of TCZ treatment (Figure 2D). There was no difference in the number of monocytes between NMOSD and HC groups, or before and after TCZ treatment (eFigure 2, links.lww.com/NXI/A920). These results suggest that the decrease in the number of basophils and the increase in the number of neutrophils in NMOSD may be driven by activation of IL-6R signaling.

**Reduced Numbers of Transitional B Cells Were Partially Corrected by Anti–IL-6R**

We also evaluated the alterations in various lymphocyte subsets in the peripheral blood before and 1 year after TCZ treatment. Flow cytometric analysis showed that the total number of CD19⁺ B lymphocytes was comparable between the NMOSD and the HC groups, or before and after TCZ treatment (eFigure 3A, links.lww.com/NXI/A920). We focused on analyzing B-cell subsets that are identified by surface expression levels of CD19, CD24, CD27, CD38, IgD, and IgM (eFigure 3B).²³ Transitional B cells (CD19⁺IgD⁺CD24⁻CD38high),
an immature naïve B-cell subset assumed to be the precursors of regulatory B cells,24,25 were significantly reduced in the NMOSD group at pre-TCZ treatment (eFigure 3C). This subpopulation, the precursor of regulatory B cells, increased after 1 year of TCZ treatment, suggesting an involvement of IL-6R signaling in the reduction of the cells. We did not see any difference between NMOSD and HC in the proportion of other cells (eFigure 3C).

**CD45RA FoxP3high Effector Treg Cells Are Increased After IL-6R Blockade Therapy**

We next investigated the effects of TCZ on Treg cells. When we examined the total Treg cells (CD4+FoxP3+ Treg) in HCs and other NMOSD patients, and before and after TCZ treatment in NMOSD, no differences were found among the groups (eFigure 4A, links.lww.com/NXI/A920). Human Treg cells can be divided into 3 subpopulations by the differential expression pattern of FoxP3 and CD45RA (eFigure 4B),20 i.e., effector Treg (CD45RA−FoxP3high), naïve Treg (CD45RA+FoxP3low), and non-Treg (CD45RA−FoxP3low). In the peripheral blood of patients with NMOSD, we observed a significant reduction of CD45RA−FoxP3low naïve Treg cells. This naïve Treg population, which acquires a suppressive phenotype (CD45RA−FoxP3high) upon T-cell receptor stimulation, did not significantly change in number 1 year after TCZ treatment (Figure 3A). On the contrary, the number of CD45RA−FoxP3high effector Treg cells, which are known to exhibit strong immunoregulatory activities, was marginally reduced in NMOSD and was significantly increased after TCZ treatment (Figure 3B). We found no differences in the number of non-Treg (CD45RA−FoxP3low) cells between HCs and patients with NMOSD, or between before and after TCZ treatment (eFigure 4C).

We further conducted a T-cell coculture assay of naïve T cells and APC with the effector Treg cells before and 8 and 18 months after TCZ treatment in 5 patients (Figure 3C, eFigure 4D, links.lww.com/NXI/A920). In this assay, we used CD45RA−CD25 as an alternative surface marker for the effector Treg cells.20 Unexpectedly, the proliferative ability of naïve T cells was reduced in all the pre-TCZ samples. In cases 16 and 17, the responder naïve T cells did not divide at all before TCZ treatment; however, 8 months later, the responder cell division started to be regained and markedly recovered at 18 months. In cases 15, 18, and 19, proliferation of naïve T cells was significantly depressed before TCZ therapy, but almost fully recovered at 18 months. We sequentially reduced concomitant drugs prednisolone and azathioprine over time. However, the reductions we made were very slow and modest (approximately 40%) (eFigure 5),
allowing us to consider the effects of TCZ therapy on the recovery of naïve T-cell proliferation. The recovery of proliferative functions of T cells after introduction of TCZ treatment may coincide with that anti–IL-6R therapy provides a good safety profile in spite of the remarkable effects for relapse prevention.

Because the naïve T cells did not proliferate well, we were unable to assess the function of Treg cells in NMOSD before TCZ. However, at 18 months, when naïve T cells proliferated well, CD45RA<sup>-</sup>CD25<sup>high</sup> cells inhibited the proliferation of the naïve T cells quite efficiently.

Recovery of CD56<sup>high</sup> NK Cells in NMOSD by IL-6R Blockade Therapy

NK cells and innate lymphocytes are involved in the pathogenesis of MS. Among NK cell populations, CD56<sup>high</sup> NK cells are regarded as a regulatory subtype because an expansion of this subset correlated with the treatment response of patients with MS to daclizumab, an anti-IL-2Rα monoclonal antibody. We observed that the number of CD56<sup>high</sup> NK cells was significantly reduced in patients with NMOSD before TCZ treatment (Figure 4A). However, the number increased gradually during TCZ treatment, and the increase was statistically significant at 6 and 12 months (Figure 4B). We also examined CD56<sup>low</sup> NK cells and found that they were reduced in number in patients with NMOSD. TCZ treatment did not significantly affect the number of this population (Figure 4C). These results indicate that pathogenesis of NMOSD may be associated with a reduction of both CD56<sup>high</sup> and CD56<sup>low</sup> NK cells. Efficacy of TCZ may be partly mediated through increasing the number of regulatory CD56<sup>high</sup> NK cells. We also analyzed numerical changes in the innate lymphocytes, including γδ T cells, mucosa-associated invariant T (MAIT) cells, and invariant natural killer T cells (eFigure 6A, links.lww.com/NXI/A920). We found a significant reduction of γδ T cells and MAIT cells in patients with NMOSD without TCZ treatment. However, neither γδ T cells nor MAIT cells showed a significant recovery in the cell number during TCZ treatment (eFigure 6B). These results suggest that IL-6R signaling is involved in the reduction of CD56<sup>high</sup> NK cells, but not in CD56<sup>low</sup> NK cells, γδ T cells, or MAIT cells in NMOSD.

Serum Cytokines/Chemokines and Anti-AQP4 Antibodies

Using a cytometric beads assay, we measured cytokines and chemokines in the serum. In the sera of patients with NMOSD before TCZ treatment, the levels of IFN-γ, IL-4, eotaxin, IP-10, and PDGF-bb were increased and G-CSF was decreased when compared with the HC group (eFigure 7A, links.lww.com/NXI/A920). These changes were not corrected after TCZ treatment. On the contrary, serum IL-5 and IL-6 showed a tendency for increase in patients with NMOSD before TCZ treatment, and the increase in IL-5 was eased after TCZ treatment (eFigure 7B). The level of IL-6 increased further after TCZ treatment, which is consistent with the pharmacologic effects of TCZ inducing reduced consumption of IL-6. In addition, we found no significant difference in these inflammatory parameters between patients with NMOSD before TCZ treatment and those receiving other treatment (other NMOSD; eFigure 8). The anti-AQP4-Ab titer tended to decrease after TCZ treatment, but the change was not statistically significant in 1 year (eFigure 9).

Overexpression of Neutrophil Genes in NMOSD Is Linked With IL-6R Signaling

The gene expression was analyzed for the whole blood samples obtained from 7 patients with NMOSD before and 1 year after therapy. Of 50,599 probes, 2,219 probes were basally...
upregulated in the NMOSD samples before therapy, compared with those from the HC group. The IL-6R gene expression was upregulated in the pre-TCZ NMOSD samples, compared with those of HCs. This IL-6R upregulation was maintained after 1 year of TCZ therapy (eTable 2, links.lww.com/NXI/A923). When we evaluated the alterations in gene expression by TCZ, we found that expression of 347 probes were downregulated 1 year later in response to the therapy. It seemed to be of particular interest to identify the probes that are basally upregulated in patients with NMOSD (HCs vs patients with NMOSD) and downregulated after TCZ therapy (NMOSD before vs after treatment). We identified 41 such probes, and expression rates of 11 of these were smaller than 0.5 after TCZ treatment (Table 2). Of interest, 9 of the 11 probes encoded functional molecules contained in azurophil granules of immature neutrophils.30 These results suggest that an accelerated myeloid turnover may take place in untreated patients with NMOSD because of upregulated IL-6R signaling.

By using RT-PCR, we confirmed the upregulation of CTSG (cathepsin G), DEFA4 (defensin, alpha 4, corticostatin), and AZU1 (azurocidin 1) in patients with NMOSD, which was reduced after TCZ therapy (Figure 5A, eFigure 10A, links.lww.com/NXI/A923). We further sorted neutrophils from the patients’ blood during the first 5 days - 4 months of treatment in 3 patients and examined the expression of neutrophil-expressing genes CTSG, DEFA4, AZU1, BPI (bacterial/permeability-increasing protein), and LTF (lactotransferrin) by RT-PCR (Figure 5B, eFigure 10B). We noticed that expression of CTSG, DEFA4, AZU1, and LTF was upregulated in only one of the pre-TCZ samples, compared with samples from HCs. Although it is possibly because the sorting procedure was not optimal to isolate entire neutrophils, the results may reflect the variety in neutrophil gene expression in individual cases. Notably, the increased values of CTSG, DEFA4, AZU1, and LTF in the single case were suppressed 5 days after TCZ treatment. Although CTSG and DEFA4 values increased afterward, the expression of AZU1 and LTF in the patient remained to be suppressed at all points of examination. Furthermore, we confirmed a significant and sustainable reduction in LTF mRNA expression in the neutrophil samples during TCZ treatment (eFigure 10B).

Gene function analysis revealed that defense response to bacteria or other organisms is associated with the IL-6 responsive genes; genes that were upregulated before the anti-IL-6R therapy compared with HCs and downregulated after therapy in patients with NMOSD (Figure 5C). This is consistent with the finding that the responsive genes were dominated by neutrophil-related genes.

**Discussion**

In this study, we investigated the mechanisms of anti-IL-6R therapy in NMOSD using flow cytometry and DNA microarray
analysis. We found that the numbers of transitional B cells, CD56\textsuperscript{high} NK cells, effector Treg cells (CD45RA\textsuperscript{−}Fox3\textsuperscript{high} or CD45RA\textsuperscript{−}CD25\textsuperscript{high}), and neutrophils were significantly altered in NMOSD. Notably, such alterations were restored by anti–IL-6R therapy, indicating the role of IL-6R signaling in the regulation of neutrophils and regulatory lymphocytes in NMOSD.

The observed increase of effector Treg cells was consistent with the ability of IL-6 to inhibit the induction of Treg cells.\textsuperscript{31} We then conducted a T-cell coculture assay to evaluate interactions of naïve CD4\textsuperscript{+} T cells (responder) and CD45RA\textsuperscript{−}CD25\textsuperscript{high} cells (Treg). By measuring the proliferation of responder T cells, we tried to evaluate the functions of Treg. Both responder and regulatory cells were isolated from 5 patients before and 8 months and 18 months after TCZ treatment (Figure 3C, eFigure 4D, links.lww.com/NXI/A920). Because responder naïve CD4\textsuperscript{+} T cells had reduced proliferative ability before TCZ therapy in all 5 patients, Treg-mediated inhibition of the responder cell proliferation was not measurable at baseline. At later time points, however, proliferation of naïve CD4\textsuperscript{+} T cells were measurable and Treg cells at 18 months were almost fully functional in the coculture assay. Given these results, one may claim that monitoring Treg cell functions during TCZ treatment should ideally use same responder cells from each donor, if such coculture assays are applied. However, this was not
pressed the AQP4-Ab nosuppressive drugs given to the patients had already sup-
partly because high amounts of corticosteroids and immu-
portion of plasmablasts (eFigure 3C, links.lww.com/NXI/short-term prednisolone.38 Although the e
phibil counts is observed in healthy individuals receiving
arthritis (RA), TCZ treatment reduces neutrophil counts,35,36
roles of neutrophils in the disruption of the blood-brain barrier
fectious disease.

It is widely acknowledged that neutrophils are present in the
acute lesions of NMOSD1,2,30 and IL-6 enhances neutrophil
migration.32 Moreover, recent works have indicated pathogenic
roles of neutrophils in the disruption of the blood-brain barrier
in NMOSD33 or in the production of cell-free DNA, which
triggers the type 1 IFN pathway.34 In patients with rheumatoid
arthritis (RA), TCZ treatment reduces neutrophil counts,35,36
and our study demonstrated that the neutrophil count was
decreased after TCZ treatment in NMOSD. We also reduced
the prednisolone and azathioprine doses slowly and modestly
(approximately 40%) in patients with NMOSD (Table 1). Glucocorticoids are known to cause detachment of neutrophils
from endothelial cells through induction of Anxa1, which
reduces the expression of L-Selectin,37 and the increase in neu-
trophil counts is observed in healthy individuals receiving
short-term prednisolone.38 Although the effects of tapering concomitant drugs cannot be excluded, our data support the
major role of TCZ in neutrophil reduction because such effects
de of TCZ on neutrophils were confirmed in RA.35,36

Gene expression analysis showed that genes upregulated
in NMOSD peripheral blood are dominated by neutrophil-
related genes, most of which are detected in azurophil granules
(Table 2). These genes, including cathepsin G, are usually
transcribed in the early developmental stage of neutrophils, at
the promyelocyte phase in the bone marrow.39,40 Thus, our observation may reflect an accelerated myeloid turnover in
NMOSD at the baseline. Moreover, after starting TCZ, ex-
pression of these neutrophil genes was significantly reduced.
Such changes included both rapid and slow decline, which
cannot be explained by an increased attachment of neutrophils
to endothelial cells due to corticosteroid reduction.

As a potential mechanism of anti–IL-6R therapy in NMOSD,
we previously indicated that plasmablasts producing the anti-
AQP4 antibody represent an important target.14,21,41 In fact,
survival of plasmablasts derived from patients with NMOSD
was inhibited by an anti–IL-6R antibody in vitro.21 Analysis of
a single case showed a rapid disappearance of plasmablasts in
the peripheral blood after a single injection of TCZ.14 In this
study, we did not observe a significant reduction in the pro-
portion of plasmablasts (eFigure 3C, links.lww.com/NXI/A920) or in serum anti-AQP4 antibody titers during the first
year of TCZ treatment (eFigure 9) on average. This can be
partly because high amounts of corticosteroids and immu-
nosuppressive drugs given to the patients had already sup-
pressed the AQP4-Ab–producing plasmablasts. In addition,
we should recall that the patients were in the remission phase
as judged by clinical criteria. It is also arguable that anti-AQP4
antibodies produced by plasma cells in the bone marrow
could not be controlled by anti–IL-6R therapy.42

It is conceivable that effects of TCZ on the regulatory lym-
phocytes could tighten the regulatory immune network for
prevention of relapse, which may explain the delayed effect of
this type of therapy. Though no protocol for appropriate
steroid tapering for NMOSD is available, relatively rapid ta-
pering was shown to induce mild relapse in the satralizumab
trial.43 It is possible that before regulatory cells are sufficiently
recovered, tapering of concomitant drugs should be con-
ducted at a reasonably slow pace.

Although IL-6R blockade is used as an acute phase therapy in
some cases,44 we experienced early relapse in 2 of the 19
patients, within 2 weeks of the first TCZ infusion. The very
early relapse in the patients may be incidental or just reflect
high activity of NMOSD. However, it is also possible that
higher IL-6R blockade might have triggered relapse. Earlier effects of anti–IL-6R should be also anticipated because expression of genes associated with neu-
troph granules was suppressed within 2 months, followed by
numerical recovery of CD56high NK cells at 6 months and of
transitional B cells and effector Treg cells at 12 months.

This study and our previous work indicated therapeutic
effects of TCZ on fatigue and neuropathic pain; however, a
clinical trial with satralizumab12 did not provide supportive
results. In addition, efficacy of satralizumab might be inferior
to TCZ in a small proportion of cases, as we have seen 2
patients who became unstable shortly after switching from
TCZ to satralizumab (unpublished). While TCZ was given 8
mg/kg monthly, satralizumab was given a fixed dose of
120 mg at 0 week, 2 weeks, and 4 weeks and every 4 weeks
thereafter. It would be interesting to conduct a study to
compare treatment regimen for these drugs, which could
provide an important information on the most effective
application of anti–IL-6R therapy.

In the new era, we have multiple therapeutic choices for
NMOSD, requiring the construction of a strategy depending
on each person’s pathophysiology. We believe that our data
would contribute to further understanding of the pathogen-
esis of NMOSD and to inventing a new strategy aiming at the
cure and full recovery of the disease.

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**Disclosure**

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**Appendix**

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