Interleukin 6 signaling promotes anti-aquaporin 4 autoantibody production from plasmablasts in neuromyelitis optica

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Neuromyelitis optica (NMO) is an inflammatory disease affecting the optic nerve and spinal cord, in which autoantibodies against aquaporin 4 (AQP4) water channel protein probably play a pathogenic role. Here we show that a B-cell subpopulation, exhibiting the CD19^{int}CD27^{high}CD38^{high}CD180[−] phenotype, is selectively increased in the peripheral blood of NMO patients and that anti-AQP4 antibodies (AQP4-Abs) are mainly produced by these cells in the blood of these patients. These B cells showed the morphological as well as the phenotypical characteristics of plasmablasts (PB) and were further expanded during NMO relapse. We also demonstrate that interleukin 6 (IL-6), shown to be increased in NMO, enhanced the survival of PB as well as their AQP4-Ab secretion, whereas the blockade of IL-6 receptor (IL-6R) signaling by anti-IL-6R antibody reduced the survival of PB in vitro. These results indicate that the IL-6-dependent B-cell subpopulation is involved in the pathogenesis of NMO, thereby providing a therapeutic strategy for targeting IL-6R signaling.

neuroinflammatory disease | autoimmunity | multiple sclerosis | central nervous system | IL-6 receptor blockade

Neuromyelitis optica (NMO) is an inflammatory demyelinating disorder characterized by recurrent attacks of severe optic neuritis and myelitis. Unlike the conventional form of multiple sclerosis (CMS), the lesions of NMO tend to spare the cerebral white matter, especially during the early stage (1), and even a single episode of attack can cause serious neurological deficits such as total blindness and paraplegia. Accordingly, accumulation of irreversible damage to the central nervous system (CNS) along with rapid progression of disability is more frequently found in NMO compared with CMS (2).

NMO can be distinguished from CMS by clinical, neuroimaging, and serological criteria (3). It is now known that serum anti-aquaporin 4 (AQP4) autoantibodies can be used as a disease marker of NMO (1, 2). AQP4 is the most abundantly expressed water channel protein in the CNS and is highly expressed in the perimicrovessel astrocyte foot processes, glia limitans, and ependyma (4). Emerging clinical and pathological observations suggest that anti-AQP4 antibodies (AQP4-Abs) play a key role in the pathogenesis of NMO. Prior studies have documented a significant correlation of serum AQP4-Ab levels with the therapeutic efficacy of plasma exchange during clinical exacerbations of NMO (2, 5). In the CNS lesions of NMO, reduced expression of AQP4 on astrocytes is evident even during the early stage (6), which is followed by the occurrence of vasculocentric destruction of astrocytes associated with perivascular deposition of complement and IgG (7).

On the other hand, recent studies have suggested that AQP4-Abs alone are incapable of causing the clinical and pathological features of NMO. In fact, Hinson et al. emphasized the role of cellular immunity in combination with AQP4-Abs by showing

that the attack severity of NMO was not correlated with serum AQP4-Ab levels (8). It was also demonstrated that direct injection of IgGs derived from NMO patients into the brains of naïve mice did not cause NMO-like lesions, although brain tissue destruction associated with leukocyte infiltration was elicited by coinjecting human complement (9). Other groups have shown that the passive transfer of IgGs from NMO patients to rats challenged with induction of experimental autoimmune encephalomyelitis (EAE) may cause a decrease in the expression of AQP4 in astrocytes along with worsening of clinical EAE (10–12). In contrast, the transfer of IgGs to unimmunized rats did not cause any pathology. These results suggest that induction of AQP4-Ab-mediated pathology in NMO depends on the presence of complement, leukocytes, and T cells.

Although AQP4-Ab-secreting cells are a potential target for therapy, detailed characteristics of AQP4-Ab-producing cells have not been clarified yet. Because some NMO patients have elevated serum anti-nuclear and anti-SS-A/SS-B Abs (1), as found in patients with systemic lupus erythematosus (SLE) or Sjögren syndrome, NMO might share common pathological mechanisms with these autoimmune diseases. Kikuchi et al. previously reported that CD180[−] B cells are activated B cells capable of producing autoantibodies in SLE (13). CD180 is a member of the leucine-rich repeat family of molecules with homology to Toll-like receptor 4 (14), which is highly expressed by naïve and memory B cells but not by plasma cells (15). Odendahl et al. demonstrated that CD27^{high}CD38⁺ B cells, capable of producing high-affinity IgG (16), are increased in the peripheral blood of SLE patients with some correlation to disease activity (17). Considering the phenotypes of autoantibody-producing cells reported in SLE, we analyzed the expression of CD27, CD38, and CD180 on CD19⁺ B cells in the peripheral blood of NMO patients. We found that CD27^{high}CD38^{high}CD180[−] B cells were significantly increased in AQP4-Ab seropositive patients diagnosed with NMO or NMO spectrum disorder (1) compared with healthy subjects (HS) or CMS patients. Notably, this B-cell subpopulation was found to be a major source of AQP4-Abs in the peripheral blood of AQP4-Ab seropositive patients and depended on interleukin-6 receptor (IL-6R) signaling for survival.

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Results

CD27^{high}CD38^{high}CD180[−] B Cells Were Increased in the Peripheral Blood of NMO Patients. Although AQP4-Abs are identified as IgGs (18), no prior study has focused on proportional changes of B-cell subsets in NMO. We therefore performed multicolor flow cytometric analysis of peripheral blood mononuclear cells (PBMC) derived from patients and controls. After starting the study, we soon noticed a remarkable expansion of a distinct B-cell subset in some patients with NMO. The expanded B cells were identified as a population of CD27^{high}, CD38^{high}, and CD180[−], and showed lower expression of CD19 than other B cells (Fig. 1A). Notably, this population did not express the B-cell marker CD20 (Fig. S1). First, we collected samples from patients in remission and analyzed the pooled data. We found that the proportion of this subpopulation among CD19⁺ B cells was significantly increased in AQP4-Ab seropositive patients with NMO or NMO spectrum disorder (Fig. 1B) compared with HS or CMS patients. There was no significant difference in the proportion of this B-cell subpopulation between those with typical NMO and those with NMO spectrum disorder. Furthermore, the frequency of this B-cell subpopulation was correlated with the serum AQP4-Ab titer (Fig. S2). Comparison of paired samples obtained from the same patients during relapse and in

remission showed that the CD27^{high}CD38^{high}CD180[−] B cells further increased during relapse (Fig. 1C). In contrast, the frequencies of CD27[−] naïve B cells (nB) and CD27⁺CD38[−] memory B cells (mB) were not altered in AQP4-Ab seropositive patients compared with controls (Fig. S3). The large majority of seropositive patients were treated with corticosteroids. However, the frequency of CD27^{high}CD38^{high}CD180[−] cells among CD19⁺ B cells was not correlated with the daily corticosteroid dose given to patients (Fig. S4). Moreover, the increase in cells in NMO patients was still evident compared with that in CMS patients similarly treated with corticosteroids (Fig. S5). Taken together, the selective increase in CD27^{high}CD38^{high}CD180[−] B cells in seropositive patients was thought to reflect their role in the pathogenesis of NMO but not to be an effect of the corticosteroid treatment.

Expanded Cells Resemble Early Plasma Cells in Gene Expression and Morphology. To gain insights into the developmental stage of the CD27^{high}CD38^{high}CD180[−] B cells, we quantified the mRNA expression of B-cell-associated transcription factors in sorted cell populations. Compared with nB and mB, this population showed much higher expression of B-lymphocyte-induced maturation protein 1 (Blimp-1) and IFN regulatory factor 4 (IRF4), which are essential for the regulation of plasma cell differentiation (19, 20) (Fig. 2A). In contrast, the expression of paired box gene 5 (PAX5), known to be down-regulated in early plasma cell differentiation (21), was reciprocally reduced in the B-cell subset. However, it was notable that the cells of interest expressed CD19, which is not detected in mature plasma cells. Moreover, only 40% of this population expressed the most reliable plasma cell marker CD138 (22). Morphological analysis also confirmed the similarity of this population to plasma cells: they exhibit eccentric nucleus, perinuclear hof region, and abundant cytoplasm. However, they possess a larger nucleus with a lower extent of chromatin clumping compared with CD138⁺ plasma cells derived from HS (Fig. 2B). Notably, the CD138⁺ population among CD27^{high}CD38^{high}CD180[−] cells in NMO patients was

Fig. 1. CD27^{high}CD38^{high}CD180[−] B cells increased in NMO patients. (A) A flow cytometric scheme for the analysis of B-cell subpopulations. PBMC from HS and NMO in remission were stained with fluorescence-conjugated anti-CD19, -CD27, -CD38, and -CD180 mAbs. CD19⁺CD27⁺ cells were partitioned (Upper) and analyzed for expression of CD38 and CD180 (Lower). Values represent the percentage of CD38^{high}CD180[−] cells within CD19⁺CD27⁺ cells. (B) Analysis of the pooled data derived from patients in clinical remission. This shows the percentages of CD27^{high}CD38^{high}CD180[−] cells within CD19⁺ cells from HS, seropositive patients, and CMS patients (***P* < 0.01; Tukey's post hoc test). (C) Comparison of remission and relapse of NMO. Data obtained from the same patients are connected with lines (**P* < 0.05; Wilcoxon signed rank test).

Fig. 2. Resemblance of CD27^{high}CD38^{high}CD180[−] cells to plasma cells. (A) mRNA expression of Blimp-1, IRF4, and PAX5. B-cell subpopulations [CD27^{high}CD38^{high}CD180[−] (PB), CD27[−] naïve (nB), CD27⁺CD38[−] memory (mB)] were sorted by FACS and total RNA was extracted for qRT-PCR analysis. RNA levels were normalized to ACTB for each sample (***P* < 0.01; ****P* < 0.001; Tukey's post hoc test). (B) May-Grünwald-Giemsa staining of B-cell subpopulations. PB (Upper Left), mB (Lower Left), and nB (Lower Right) from NMO are presented along with morphologically identified plasma cells (CD19^{int}CD27^{high}CD38^{high}CD138⁺) from HS (Upper Right).

Fig. 4. Effect of exogenous IL-6 on PB. (A) IL-6 promotes the survival of PB. FACS-sorted PB were cultured in the presence or absence of recombinant IL-6 (1 ng/mL) for 2 d. PI staining of cultured PB showed that exogenous IL-6 increased the percentage of surviving cells (Lower) compared with cells cultured in the medium alone (Upper). Values shown are percentages of unstained cells. (B) Comparison of IL-6 with BAFF and APRIL. Here we show that only IL-6 could promote cell survival. Data are expressed as fold increase of % PI⁺ cells following the addition of each cytokine. At least four independent experiments were performed to obtain the results (** $P < 0.01$; Tukey's post hoc test). (C) Effect of IL-6 on XBP-1 expression. FACS-sorted PB were cultured with or without IL-6 for 24 h, and total RNA was extracted from the cells to quantify expression levels of XBP-1(u) and XBP-1(s) by qRT-PCR. Each line connects values obtained from seven independent experiments (* $P < 0.05$; ** $P < 0.01$; Wilcoxon signed-rank test).

dition of IL-6. These results suggest that IL-6 promoted the survival of PB and enhanced IgG secretion from PB, leading to an increased production of AQP4-Abs in NMO patients (Fig. 4C). In addition, we found that the frequency of PB tended to be increased when serum IL-6 levels were higher than the mean $\pm 2 \times$ SEM of those in HS [PB/PBMC (%) for IL-6 high group 0.62 ± 0.47 (%); PB/PBMC (%) for IL-6 low group 0.15 ± 0.05 (mean $\pm$ SD)]. These observations prompted us to address whether the blockade of IL-6R signaling could exhibit any influence on PB. We cultured PBMC derived from AQP4-Ab seropositive patients in the presence of 20% autologous serum and examined the effect of adding anti-IL-6R antibody by counting the number of surviving PB. We found that the frequency of PB among total B cells decreased significantly in the presence of anti-IL-6R mAb (Fig. 5A and B). Among six patients examined, the PB reduction was remarkable in three patients, but was only marginal in the other patients. Notably, the former group of patients showed higher IL-6 levels in the serum (4.69, 6.47, and 25.5 pg/mL for each patient), compared with the latter (1.42, 1.43, and 2.91 pg/mL). The frequency of other B-cell subpopulations did not change with the addition of anti-IL-6R mAb. These results led us to postulate that in vivo administration of anti-IL-6R mAb may ameliorate NMO.

Discussion

A growing body of evidence suggests that AQP4-Abs play a pathogenic role in NMO (6, 7, 10–12). Here we report that a B-cell subpopulation bearing the CD19^{int}CD27^{high}CD38^{high}CD180[−] phenotype is responsible for the selective production of AQP4-Abs. The cells that we call PB are vulnerable to IL-6R blockade by anti-IL-6R mAb, leading us to propose anti-IL-6R mAb as a therapeutic option for NMO. Bennett et al. recently reported that plasma cells in CSF are a potential source of pathogenic AQP4-Abs (10). However, this study has not excluded a possible role of AQP4-Abs produced in the peripheral blood. It has been

Fig. 5. IL-6R blockade selectively inhibits the survival of PB. (A) PBMC were cultured in a medium containing 20% autologous serum in the presence of IL-6R-blocking antibody or its isotype control mAb for 2 d. The cells were stained and analyzed as described in the experiment in Fig. 1A. Data represent the percentages of PB within CD19⁺CD27⁺ cells. A representative pair of six independent experiments is shown. (B) The percentage of PB within CD19⁺ B cells (PB%) was determined for each pair of cultures either with anti-IL-6R mAb (IL-6R-blocking) or isotype control mAb (isotype) before and after starting the culture. Then, the PB survival ratio was calculated for each culture by dividing the PB% at the end of the culture by the PB% obtained before starting the culture. Lines connect the PB survival ratios of six independent experimental pairs to clarify that IL-6R blockade reduces PB survival (* $P < 0.05$; Wilcoxon signed-rank test).

repeatedly shown that the passive transfer of pathogenic autoantibodies, including AQP4-Abs (10–12, 32), augments the formation of inflammatory lesions in EAE. Therefore, once T-cell-mediated inflammation takes place in the CNS, pathogenic autoantibodies produced outside the CNS are able to enter the CNS compartment. It is also notable that AQP4-Abs are more abundant in the peripheral blood of NMO patients than in their CSF (33). Taken together, we speculate that PB that are expanded in the peripheral blood during relapse may play a critical role in the pathogenesis of NMO by producing AQP4-Abs, although more work is necessary to explore whether PB can enter the CNS.

It is generally thought that circulating IgGs are mainly secreted by long-lived plasma cells residing in healthy bone marrow. It remains unclear how PB secreting AQP4-Abs can differentiate and survive in the peripheral circulation. It has been previously shown that autoantibodies producing plasma cells accumulate in peripheral lymphoid organs (34). It would be interesting to investigate which organs blood PB move to during the course of NMO. The levels of IL-6 in the serum and CSF are elevated in NMO compared with HS or CMS patients (27, 28). In this regard, it is of note that blocking IL-6R signaling was found to dramatically reduce the survival of PB ex vivo, which was dependent on the presence of autologous serum containing IL-6. These results suggest that the increase of PB in AQP4-Ab seropositive patients could be attributed to the increased IL-6 in the serum. We also demonstrated that improved PB survival in the presence of exogenous IL-6 was accompanied by up-regulated expression of XBP-1. It is noteworthy that wild-type and XBP-1^{−/−} B cells start to produce more IL-6 after forced overexpression of XBP-1(s), which results in the operation of a positive feedback loop controlling IgG secretion (31). Treatment with anti-IL-6R is promising because IL-6R blockade could terminate this vicious loop that controls the production of autoantibodies.

It has been reported that NMO patients have higher levels of BAFF in the serum or CSF compared with CMS patients (35). BAFF is also known to support plasma cell differentiation and survival of PB induced in vitro (25). However, in our ex vivo

control (CHO^{VC}) using FuGENE 6 Transfection Reagent (Roche). After 2 wk of geneticin (Invitrogen) selection, stable clones were established by single-cell sorting. The expression of hAQP4 in the established clones was confirmed using anti-human AQP4 antibody and FITC-anti-rabbit IgG antibody. Reactivity of AQP4-Abs to CHO^{AQP4} was confirmed using seropositive NMO patients' sera diluted at 1:1,000 and FITC-anti-human IgG antibody. To measure the AQP4-Ab activity in culture supernatants, these supernatants were concentrated up to 10 times using an Amicon Ultra 0.5 mL 100K device (Millipore), and 10 μ L of the solution was added to 3×10^4 CHO^{AQP4} and CHO^{VC} cells. After incubation on ice for 20 min, cells were washed with sterile PBS containing 1% BSA and stained with FITC-anti-human IgG antibody. After a 10-min incubation on ice, the cells were washed and fixed for 15 min in 2% paraformaldehyde. Then the cells were washed and analyzed by flow cytometry.

Data Analysis. Histogram overlay analysis was performed using Cell Quest software (BD Biosciences). Statistics were calculated using Prism (GraphPad Software). Wilcoxon signed-rank test, Mann-Whitney *U* test, ANOVA, or Spearman's correlation test were also used when appropriate. Post hoc tests were used as a multiple comparison test after confirmation of equal variances by ANOVA.

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