Mechanism of regulatory B cells during relapse and remission in multiple sclerosis and neuromyelitis optica spectrum disorder patients

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Directed by Professor Eunjig Lee

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Medical Science

Yeseul Kim

December 2021

This certifies that the Doctoral Dissertation of Yeseul Kim is approved.

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The Graduate School
Yonsei University
December 2021

ACKNOWLEDGEMENTS

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people.

I would first like to express my sincere gratitude to my advisor Professor Ho Jin Kim for his guidance and endearing support. I have been amazingly fortunate to have a mentor like him who gave me the freedom to explore on my own, and at the same time the guidance to recover when my steps faltered. It was an honour to have been his student. I would also like to thank Professor Eunjig Lee for his continuous support throughout the course of my PhD. It would have been impossible to start and finish this journey without him.

Beside my advisors, I would like to thank the rest of my thesis committee, Professor Seung Min Kim, Professor Chae Gyu Park, and Professor Sun Och Yoon for their insightful comments and encouragements.

I wish to pay my special appreciation to Tae-Sik Kim in the flow cytometry suites of National Cancer Center Korea for the operation of LSRFortess and FACSAria. Without him, executing this study would not have been possible.

I would also like to thank and acknowledge the many current and former colleagues and friends of our team that I have had incredible joy and pleasure of working with throughout this journey. Thank you for all your assistance and support. I would not have been able to complete this journey alone.

Last but not least, I would like to thank my family for their endless support and love.

Yeseul Kim

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ABSTRACT

Mechanism of regulatory B cells during relapse and remission in multiple sclerosis and neuromyelitis optica spectrum disorder patients

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The role of B cells in immune response regulation is context-dependent, where bystander B cell activation leads to IL-10 production, suppressing inappropriate immune responses. However, the role of B cells in immune regulation in autoimmune diseases, including multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD), is incompletely understood. MS and NMOSD are autoimmune demyelinating diseases of the central nervous system (CNS), typically following a relapsing and remitting course. Alemtuzumab provides a long-lasting suppression of disease activity in MS. In contrast to its documented efficacy, alemtuzumab's mechanism of action is not fully understood and information about the composition of repopulating B cell pool is scarce. B cell depletion therapy (BCDT) has shown clinical efficacy in NMOSD by eliminating pathogenic B cells; however, its effect on regulatory B cells (Bregs) remains elusive. Our aim was to evaluate the B cell subsets, Breg

cell function, and the effect of immunosuppressive therapy on these cells in patients with central nervous system demyelinating disorders.

Here we pinpoint deficiency of $CD24^{hi}CD38^{hi}$ B cells during relapse in MS and subsequent expansion following alemtuzumab infusion. We also highlight the possible clinical implication of $CD24^{hi}CD38^{hi}$ B cells in MS. A deficient Breg cell number was also observed in NMOSD patients and cell-intrinsic deficit in IL-10 production, specifically in response to B cell bystander activation, was observed. Moreover, we showed that such impairments are responsible for functional defect in $CD24^{hi}CD38^{hi}$ B cells. $CD24^{hi}CD38^{hi}$ B cells from patients with NMOSD did not inhibit $CD4^{+}$ T cell production of IFN-γ, IL-17, and IL-21 and failed to inhibit follicular helper T cell expansion and induce regulatory T cells.

Using cross-sectional and three-year longitudinal studies, we showed that BCDT treatment restored the numerical deficiency of Bregs. Moreover, the post-BCDT repopulated $CD24^{hi}CD38^{hi}$ B cells restored IL-10 production and suppressed IFN-γ and IL-17 production by $CD4^{+}$ T cells.

Oure results suggest that deficiency of $CD24^{hi}CD38^{hi}$ B cells in relapsing patients are restored following immunosuppressive therapy. Moreover, we show that both numerical deficiency of $CD24^{hi}CD38^{hi}$ B cells and their impaired regulatory function contribute to the NMOSD pathophysiology and that such impairment is restored following BCDT.

Key words: autoimmune disease, regulatory B cells, neuromyelitis optica spectrum disorder, multiple sclerosis, B cell depletion therapy, immunosuppressive therapy

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I. OVERALL INTRODUCTION

The delicate balance between effector functions against pathogens and immune tolerance mechanisms to self-antigens is critical to avoid autoimmunity. In the majority of antibody-mediated autoimmune diseases, B cells are generally considered to be pathogenic because of their ability to secrete autoantibodies.

B cells can participate in autoimmune diseases not only through the production of autoantibodies but also by presenting antigens to autoreactive T cells, production of various cytokines, and involvement in T cell-mediated immune responses (*1*).

Following the emergence of regulatory B cells (Bregs) in animal studies, these cell types have been extensively studied. Regulatory cells are important for the maintenance of immune homeostasis and tolerance to self. During bystander activation of B cells through T cell stimulation mediated by CD40, without antigen or B cell receptor (BCR) engagement, B cells fail to produce proinflammatory cytokines and instead secrete significant amounts of the

regulatory cytokine IL-10 that suppresses the local inappropriate inflammatory response.

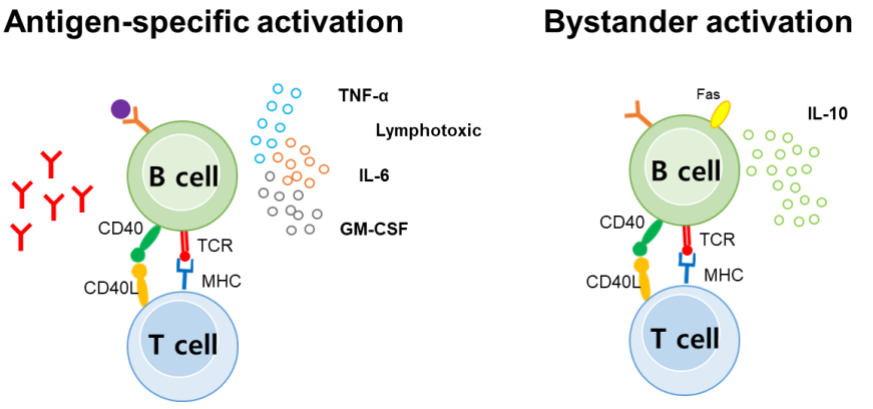


Figure 1. Role of B cells during antigen-specific and antigen non-specific activation.

B cell effector function requires binding of specific antigens to the B cell receptor and T cell help through CD40-CD40L interaction. During antigen-specific activation, B cells produce pro-inflammatory cytokines which result in enhancement of T-B responses. In addition, B cells proliferate and differentiate into antibody secreting cells and produce antibodies. On the other hand, with respect to IL-10 production, B cell regulatory function occurs during bystander activation where B cells do not receive specific B cell receptor stimulation before meeting CD40L. During such bystander activation, IL-10 produced by B cells suppress inappropriate and early inflammatory responses and upregulation of Fas and apoptosis occurs.

Currently, human Bregs have been identified at different stages of B cell development and consist of various B cell surface markers; these include $CD19^{+}CD24^{hi}CD38^{hi}$ immature transitional B cells, $CD19^{+}CD24^{+}CD27^{+}$ memory B cells (*2*), and $CD19^{+}CD27^{int}CD38^{+}$ regulatory-like plasmablasts (*3*). Human $CD19^{+}CD24^{hi}CD38^{hi}$ cells were initially known as bone marrow-derived immature transitional B cells, and CD40 stimulation is required for their regulatory function. Their regulatory functions include inhibition of autoreactive $CD4^{+}$ T cell proliferation and interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production by activated $CD4^{+}$ T cells. $CD19^{+}CD24^{hi}CD38^{hi}$ B cells also prevent T helper (Th)1, Th17, and Tfh cell differentiation and promote the conversion of $CD4^{+}$ T cells into $CD4^{+}FoxP3^{+}$ regulatory T cells (Tregs) (*4, 5*). With the finding that $CD19^{+}CD24^{hi}CD38^{hi}$ cells have immune regulatory functions, the relevance of $CD19^{+}CD24^{hi}CD38^{hi}$ cells in the maintenance of tolerance in several immune-related disorders has begun to emerge (*4, 6-8*).

MS is a disease primarily affecting myelin and oligodendrocytes and no disease-specific autoantigen has been identified. Tissue damage in MS results from a complex and dynamic interplay between the immune system, glia, and neurons. Although there is debate about whether the root cause of MS is intrinsic to the CNS or extrinsic, studies in animal models, particularly EAE in mice, together with analysis of immune cells and their products in CSF and blood of humans, have disclosed a critical role for adaptive immunity. Both helper CD4+ and cytotoxic CD8+ T cells have been described in MS lesions. CD4 T cells are more concentrated in the perivascular cuff, whereas CD8 T cells are widely distributed within the parenchyma. Drugs that limit T-cell access to the CNS can reduce or eliminate new MS lesions. However, T cells reactive to myelin antigens have been observed in similar proportions in individuals with and without MS, suggesting either that these cells are dysfunctional in MS or that other immune factors also play critical roles.

Due to the early and dramatic success of B-cell-depleting antibodies in limiting MS lesion formation and clinical disease activity, there is renewed attention on the role of B cells. It has long been known that the CSF of most people with MS harbors unique antibodies (oligoclonal bands) produced within the CNS. There is evidence that the antibody-producing function of B-lineage cells is important in some MS lesions. However, due to the rapidity of the clinical response to B-cell depletion (as early as 8-12 weeks), even before the reduction of circulating immunoglobulin, it seems more likely that other functions of B cells, including antigen presentation to helper T cells and cytokine production, are more relevant.

Treatments to reduce likelihood of developing new white matter lesions, clinical relapses, and stepwise accumulation of disability have been developed. Alemtuzumab is a highly effective treatment in relapsing MS. It provides a long-lasting suppression of disease activity by altering the proportion of lymphocyte subsets with preferential increase of regulatory T cells. In contrast to alemtuzumab's documented efficacy, alemtuzumab's mechanism of action is not fully understood and information about the composition of the repopulating B cell pool, especially Breg, is scarce.

NMOSD is an autoimmune inflammatory disease of the central nervous system (CNS) characterized by lesions predominantly occurring in the optic nerves and spinal cord. NMOSD pathogenesis involves autoantibodies against water channel aquaporin 4 (AQP4-IgG). AQP4 is highly concentrated along astrocytic end-feet in the CNS, and binding of AQP4-IgG leads to astrocyte damage by complement-dependent cytotoxicity. Complement activation leads to an inflammatory response that further disrupts the blood-brain barrier (BBB), leading to neutrophil, eosinophil, and macrophage infiltration, ultimately causing oligodendrocyte damage, demyelination, and neuronal death (*9, 10*).

Furthermore, evidence indicating the involvement of T cells in the development of NMOSD is accumulating. AQP4-specific T cell responses were found to be

amplified in NMOSD patients, whose T cells exhibited T helper 17 (Th17) polarization (*11*) and Th17-related markers have shown correlation with disease activity and severity in NMOSD. Th17 cells disrupt BBB tight junctions and promote additional $CD4^+$ cell recruitment from the systemic circulation into the CNS (*12*). The release of interleukin (IL)-6 and IL-21 by polyclonally activated $CD4^+$ T cells derived from NMOSD patients correlated with neurological disability (*13*). T follicular helper (Tfh) cells are required for B cell differentiation into antibody-producing cells, and a recent study found that untreated patients with NMOSD show Tfh polarization toward excessive B-helper Tfh subsets (*14*).

NMOSD shows a relapsing-remitting course in which attacks of NMOSD often produce severe disability with incomplete recovery (*15*). Clinical relapse is directly related to disability in NMOSD (*16*); hence, preventing relapse is the primary treatment goal. The anti-CD20 B cell depleting therapy (BCDT), which depletes immature and mature B cells, has exhibited astounding clinical efficacy for the treatment of NMOSD (*17-19*). It is hypothesized that BCDT exerts its clinical efficacy by eliminating pathogenic B cells. The antigen-specific $CD27^+$ memory B cells augment antibody production upon re-stimulation and are characterized by isotype switching and affinity maturation (*20, 21*). They also contribute to autoimmunity through cytokine production and antigen presentation to T cells (*22, 23*). Repeated treatment with BCDT when $CD27^+$ memory B cells reemerged in the peripheral blood mononuclear cells, as maintenance therapy in NMOSD, produced consistent and sustained efficacy (*24-26*). Despite BCDT, an incomplete depletion or rapid recovery of memory B cells is associated with the occurrence of relapse in autoimmune diseases (*24, 27-30*). Following BCDT, a shift in the B cell subset toward a naïve phenotype occurs (*24, 25*).

To date, only a few studies have explored the role of $CD19^+CD24^{hi}CD38^{hi}$ B cells in NMOSD (*31, 32*) and MS. The precise contribution of B cells to the disease pathogenesis and the possible plasticity of B cell function depending on the

environment and the mechanism of action of immunosuppressive therapies are incompletely understood. Most importantly, the contribution of B cell regulatory functions in MS and NMOSD remains unknown. Given that naïve B cells are dominant among those reappearing after immunosuppressive therapies, it is tempting to speculate that a shift in the predominant B cell subsets causes a shift in the immune balance following treatment. To address these questions, the balance in B cell subsets and function of Breg cells and the effect of immunosuppressive therapies on these cells in patients with MS and NMOSD were evaluated in the present study.

Part I

Regulatory capacity of $CD24^{hi}CD38^{hi}$ B cells in healthy individuals

I. INTRODUCTION

Currently, human Bregs have been identified at different stages of B cell development and consist of various B cell surface markers; these include $CD19^{+}CD24^{hi}CD38^{hi}$ immature transitional B cells, $CD19^{+}CD24^{+}CD27^{+}$ memory B cells (*2*), and $CD19^{+}CD27^{int}CD38^{+}$ regulatory-like plasmablasts (*3*). Human $CD19^{+}CD24^{hi}CD38^{hi}$ cells were initially known as bone marrow-derived immature transitional B cells, and CD40 stimulation is required for their regulatory function. Their regulatory functions include inhibition of autoreactive $CD4^{+}$ T cell proliferation and interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production by activated $CD4^{+}$ T cells. $CD19^{+}CD24^{hi}CD38^{hi}$ B cells also prevent T helper (Th)1, Th17, and Tfh cell differentiation and promote the conversion of $CD4^{+}$ T cells into $CD4^{+}FoxP3^{+}$ regulatory T cells (Tregs) (*4, 5*). With the finding that $CD19^{+}CD24^{hi}CD38^{hi}$ cells have immune regulatory functions, the relevance of $CD19^{+}CD24^{hi}CD38^{hi}$ cells in the maintenance of tolerance in several immune-related disorders has begun to emerge (*4, 6-8*).

II. MATERIALS AND METHODS

1. Study population

Twenty-two healthy individuals (15 women and 7 men; mean age: 39.1 ± 10.5 years) were included. The study was approved by the Institutional Review Board of the National Cancer Center. Written informed consent was obtained from all participants.

2. Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated from the fresh blood using a Ficoll-Paque Plus (GE Healthcare) gradient centrifugation. 1% phosphate-buffered saline (PBS) containing 2mM ethylenediaminetetraacetic acid (EDTA) were used as separation buffer. Collected bloods were mixed with 2mM EDTA-PBS 1:1 ratio into 50ml conical tube (Falcon). 15ml of Ficoll-Paque solution was added to another 50ml conical tube and blood-separation buffer mixture was transferred onto Ficoll-Paque gently. Solutions were centrifuged at 1680 rotation per minute (rpm) for 30 minutes with brakes off. The buffy coat layer containing PBMC were collected and mixed with 2mM EDTA-PBS and centrifuged at 1500 rpm for 15 minutes. This step was repeated two times. Freshly isolated PBMCs were either directly assayed, or cryopreserved.

3. $CD19^+$ B cell MACS isolation

$CD19^+$ B cells were isolated by magnetic column purification with anti-CD19-coated beads (Miltenyi Biotec). The PBMC suspension was centrifuged at 300xg for 10 minutes and supernatant was aspirated. The cell pellet was resuspended in 80uL of buffer (0.5% bovine serum albumin + 1X phosphate-buffered saline (PBS)) per 10^7 total cells. 20uL of CD19 MicroBeads (Miltenyi Biotec) per 10^7 total cells was added and incubated for 15 minutes in the refrigerator (2-8 ‘C). Cells were washed and centrifuged at 300xg for 10 minutes. The supernatant was aspirated and cells were resuspended up to 10^8 cells in 500uL buffer. Cells suspensions were applied to MS column that was placed in the magnetic field of

MiniMACS Seprator (Miltenyi Biotec). The unlabeled cells that passed through was collected and labeled CD19+ B cells were collected by removing column from the separator and flushing out labeled cells by firmly pushing the plunger into the column.

4. Fluorescence-activated cell sorting (FACS)

Isolated PBMCs were labelled with fluorochrome-conjugated antibodies below. Cells were resuspended in 500uL of cold Pre-Sort Buffer (BD Biosciences) and 1uL of fluorochrome-conjugated antibodies per 1 x 10^7 cells were added and incubated for 30 minutes on ice protected from light. Cells were washed with Pre-Sort Buffer and resuspended with cold Pre-Sort Buffer to a final concentration of ~3 x 10^6 cells /mL. $CD19^+$, $CD19^+CD24^{hi}CD38^{hi}$, $CD19^+CD24^{int}CD38^{int}$, $CD19^+CD24^{hi}CD38^-$ and $CD4^+CD25^-$ T cells were isolated from PBMCs using FACSAria II (BD Biosciences) (Fig. 1A).

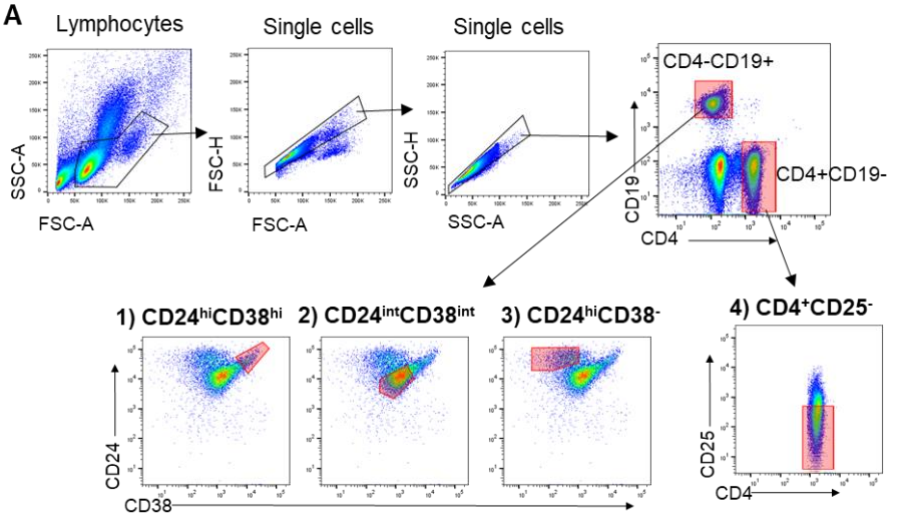


Figure 2. Flow cytometry soring of B and T cells.

PBMCs were surface stained for CD19, CD24, CD38, CD4, and CD25 and sorted by flow cytometry (FACSAria sorter). CD19+ B cells and CD4+ T cells were gated from lymphocytes and single-cell population.

A. The target populations 1) $CD19^{+}CD24^{hi}CD38^{hi}$, 2) $CD19^{+}CD24^{int}CD38^{int}$, 3) $CD19^{+}CD24^{hi}CD38^{-}$, and 4) $CD4^{+}CD25^{-}$ cells were sorted.

Table 1. List of fluorochrome-conjugated antibodies used for co-culture assay

Target	**Fluorochrome conjugation**	**Source**
Anti-human CD19	FITC	BD Biosciences
Anti-human CD24	PE-CF594	BD Biosciences
Anti-human CD38	PE	BD Biosciences
Anti-human CD25	APC-cy7	BD Biosciences
Anti-human CD4	BUV496	BD Biosciences

5. Cell culture

Purified cells were cultured in complete RPMI 1640, L-glutamine, and $NaHCO_3$ supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (100 U/mL) (Hyclone) in 96-well U-bottom plates (Nunc). For detection of cytokine produced by $CD19^+$ B cells and $CD24^{hi}CD38^{hi}$ B cells, purified B cells were stimulated with soluble CD40L (1 μg/mL, Enzo Life Sciences) alone or in combination with goat anti-human IgM BCR cross-linking antibody (XAb) (10 μg/mL, Jackson ImmunoResearch) for 24 h. For co-culture assays, flow cytometry sorted $CD4^+CD25^-$ T cells alone or with B cell subsets were co-cultured 1:1 and stimulated with plate-bound anti-CD3 mAb (0.5 μg/mL; HIT-3a, BD Pharmigen) for 72 h. For the IL-10 blocking assay, anti-IL-10 (5 μg/mL; JES3-9D7, Miltenyi Biotec) and anti-IL-10 receptor (5 μg/mL; 3F9, Biolegend) antibodies were added.

6. Cytokine detection through intracellular cytokine staining

Cytokine production was detected by intracellular cytokine staining (ICS). Phorbol 12-myristate 13-acetate (PMA) (20 ng/mL, Sigma-Aldrich), ionomycin (500 ng/mL, Sigma-Aldrich), and Golgistop (BD Biosciences) were added to the last 6 h of cell culture. At the end of 24 h cell culture, cells were harvested and stained with Fixable Viability Stain-Alexa Fluour 700 according to the manufacturer's instructions (BD Biosciences) to determine live/dead cells before staining for surface markers.

Table 2. List of fluorochrome-conjugated antibodies used for B cell ICS

Target	**Fluorochrome conjugation**	**Source**
Anti-human CD19	APC-cy7	BD Bioscience
Anti-human CD24	PE-CF594	BD Bioscience
Anti-human CD38	BV510	BD Bioscience
Anti-human CD27	FITC	BD Bioscience
Anti-human IL-10	PE	BD Bioscience
Anti-human IL-6	BV421	BD Bioscience
Anti-human TNFα	APC	BD Bioscience

7. Co-culture assay

For the co-culture assay, CD19-FITC, CD24-PE-CF594, CD38-PE, CD25-APC-cy7, CD4-BUV496, CD3-BUV395, PD-1-BV786, CXCR5-PerCP-cy5.5, and CD127-BUV (BD Biosciences) were used. Cells were washed, fixed, and permeabilized using Fixation/Permeabilization Solution Kit or Transcription Factor Buffer Set (BD Biosciences) according to the manufacturer's protocol and stained for detection of intracellular cytokines or transcription factors with TNFα-APC, IL-10-PE, IL-6-BV421, GM-CSF-PerCP-cy5.5, IFNγ-BV605, IL-17A-BV655, IL-21-BV421, and FoxP3-AF700 (BD Biosciences). Data were acquired using LSRFortessa (BD Biosciences).

Table 3. List of fluorochrome-conjugated antibodies used for co-culture assay

Target	Fluorochrome conjugation	Source

Anti-human CD19	FITC	BD Bioscience
Anti-human CD24	PE-CF594	BD Bioscience
Anti-human CD38	PE	BD Bioscience
Anti-human CD25	APC-cy7	BD Bioscience
Anti-human CD4	BUV496	BD Bioscience
Anti-human CD3	BUV395	BD Bioscience
Anti-human PD-1	BV786	BD Bioscience
Anti-human CXCR5	PerCP-cy5.5	BD Bioscience
Anti-human CD127	BUV737	BD Bioscience
Anti-human FoxP3	PE	eBioscience
Anti-human IFN-γ	BV605	BD Bioscience
Anti-human IL-21	BV421	BD Bioscience
Anti-human IL-17	BV650	BD Bioscience

8. Statistical analysis

Data were analyzed for significance using Prism (GraphPad) by paired *t-test*, Mann–Whitney *U* test, one-way ANOVA with Tukey's multiple comparison post hoc analysis, or two-way ANOVA with Sidak's multiple comparison test. Repeated measures ANOVA with Tukey's multiple comparison post-hoc analysis was performed for longitudinal analysis. Correlation coefficients and their significance were calculated using a two-tailed Spearman's rank correlation. A confidence interval of 95% or a p-value < 0.05 was considered statistically significant. All values are presented as the mean ± SEM.

III. RESULTS

1. $CD24^{hi}CD38^{hi}$ B cells define brightest IL-10

The inhibitory mechanism of $CD24^{hi}CD38^{hi}$ B cells requires the engagement of CD40–CD40L by activated T cells and subsequent IL-10 production (*4*). $CD24^{hi}CD38^{hi}$ B cells were defined as the brightest $IL\text{-}10^{+}$ cells after CD40 stimulation in B cells derived from HCs (Fig. 2A). In addition, a significantly higher percentage of $CD24^{hi}CD38^{hi}$ B cells was $IL\text{-}10^{+}$ compared with $CD24^{int}CD38^{int}$ and $CD24^{hi}CD38^{-}$ cells in both unstimulated and CD40-stimulated cultures (Fig. 2B).

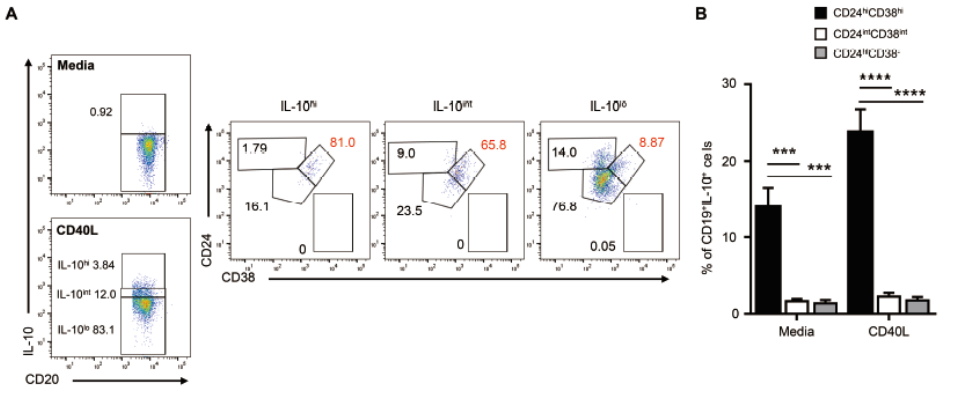


Figure 3. Production of IL-10 by CD24/CD38 B cell subsets

B cells from PBMCs of 8 HCs were purified by magnetic separation. Purified B cells were stimulated with CD40L for 24 hours. PMA+ionomycin + Golgistop was added in the last 6 hour of culture.

A. Representative flow cytometry plots of CD24 and CD38 expression by IL-10^{hi}, IL-10^{+}, and IL-10^{-} HC B cells.

B. Bar graphs showing the mean ± SEM frequencies of IL-10-producing HC CD19^{+}CD24^{hi}CD38^{hi}, CD19^{+}CD24^{int}CD38^{int}, and CD19^{+}CD24^{hi}CD38^{-} cells.

Values are expressed as mean ± SEM. Differences between groups were evaluated by two-way ANOVA. **p < 0.01; ***p < 0.001; ****p < 0.0001.

2. $CD24^{hi}CD38^{hi}$ B cells suppress proinflammatory cytokine production by $CD4^+$ T cells

We first evaluated the regulatory capacity of $CD19^+CD24^{hi}CD38^{hi}$ cells in healthy individuals. Sorted B cell subsets ($CD24^{hi}CD38^{hi}$, $CD24^{int}CD38^{int}$, and $CD24^{hi}CD38^-$) were co-cultured 1:1 with autologous CD4+CD25- T cells and stimulated with plate-bound anti-CD3, which has previously been shown to upregulate CD40L expression on T cells.

The co-culture of $CD24^{int}CD38^{int}$ or $CD24^{hi}CD38^-$ B cells with CD4+ T cells showed no inhibitory effect on IFN-γ, IL-17 and IL-21+ production by CD4+ T cells. However, the co-culture of $CD24^{hi}CD38^{hi}$ B cells with $CD4^+$ T cells significantly suppressed the frequencies of $CD4^+$IFN-γ^+, $CD4^+$IL-17^+, $CD4^+$IL-21^+, and $CD4^+$PD-1^+CXCR5^+ T cells (Fig. 3A and B).

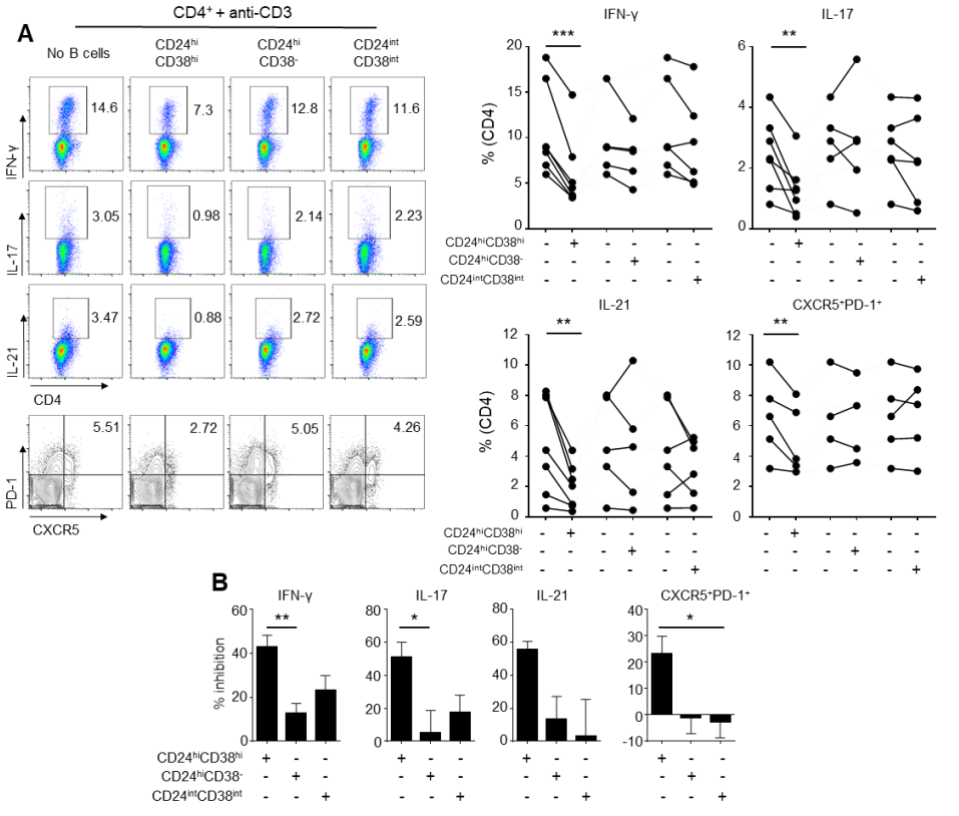


Figure 4. CD24^{hi}CD38^{hi} B cells inhibit proinflammatory cytokine production by CD4^{+} T cells.

CD24^{hi}CD38^{hi}, CD24^{int}CD38^{int} and CD24^{hi}CD38^{-} B cells and CD4^{+}CD25^{-} T cells were sorted by flow cytometry from PBMC of 8 healthy individuals. CD4+ T cells were either cultured alone or with each B cell subsets in 1:1 with plate-bound anti-CD3 mAb for 72 hours. PMA + ionomycin + golgistop was added in the last 6 hours of culture. Cells were surface stained for CD4, PD-1 and CXCR5 and intracellularly stained for IFN-γ, IL-17 and IL-21.

A. Representative flow cytometry plot and scatter plot showing the % of CD4^{+}IFN-γ^{+}, CD4^{+}IL-17^{+}, CD4^{+}IL-21^{+} and CD4^{+}PD-1^{+}CXCR5^{+} in co-culture of each B cell subset and CD4+ T cells.

B. Bar graph showing the % decrease in the production of IFN-γ, IL-17, IL-21 and PD-1^{+}CXCR5^{+} cells by addition of each B cell subsets.

Values are expressed as mean ± SEM. Differences between groups were evaluated by two-way ANOVA (A), or one-way ANOVA (B). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. $CD24^{hi}CD38^{hi}$ B cells induce FoxP3 expression in CD4+ T cells

Previous studies have shown that $CD24^{hi}CD38^{hi}$ B cells suppress not only proinflammatory cytokine production by $CD4^+$ T cells but also employ regulatory functions by inducing Tregs (*7, 33*). Further, the co-culture of $CD24^{hi}CD38^{hi}$ B cells with $CD4^+$ T cells resulted in increased FoxP3 expression in $CD4^+$ T cells (Fig. 4A and B).

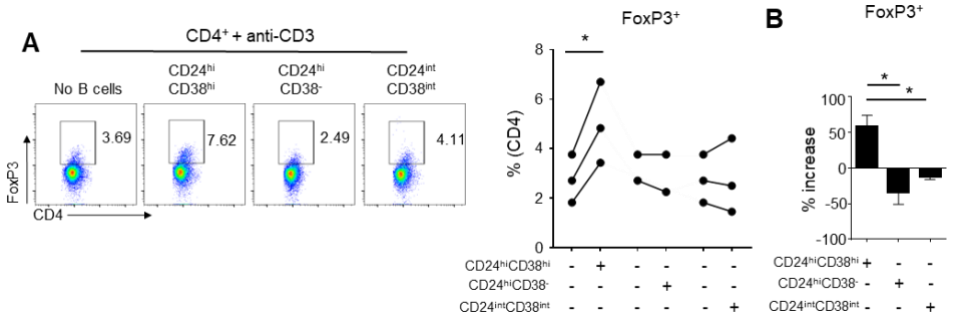


Figure 5. CD24^{hi}CD38^{hi} B cells convert CD4+CD25- T cells into Tregs.

CD24^{hi}CD38^{hi}, CD24^{int}CD38^{int} and CD24^{hi}CD38^{-} B cells and CD4^{+}CD25^{-} T cells were sorted by flow cytometry from PBMC of healthy individuals. CD4^{+} T cells were either cultured alone or with each B cell subsets in 1:1 with plate-bound anti-CD3 mAb for 72 hours. PMA + ionomycin + golgistop was added in the last 6 hours of culture. Cells were surface stained for CD4, CD25 and CD127 and intracellularly stained for FoxP3.

A. Representative flow cytometry plot and graph showing the frequency of CD4+FoxP3+ T cells and CD4+CD25+CD127lo T cells in co-cultures.

B. Bar graphs comparing the % increase in the FoxP3 expression and CD25+CD127lo T cells in co-cultures of each B cell subsets.

Values are expressed as mean ± SEM. Differences between groups were evaluated by two-way ANOVA (A), or one-way ANOVA (B). *$p < 0.05$.

4. The regulatory effect of CD24^{hi}CD38^{hi} B cells are dependent on IL-

10

The inhibitory effect on IFN-γ, IL-21 and $CD4^+PD\text{-}1^+CXCR5^+$ frequencies were reversed when IL-10 and IL-10 receptor blocking antibodies were added to the co-culture (Fig. 5A). However, suppression of $CD4^+IL\text{-}17^+$ by $CD24^{hi}CD38^{hi}$ B cells was not dependent on IL-10.

The increase in FoxP3 expression was partially dependent on IL-10, as Treg induction decreased when IL-10 and IL-10R were blocked.

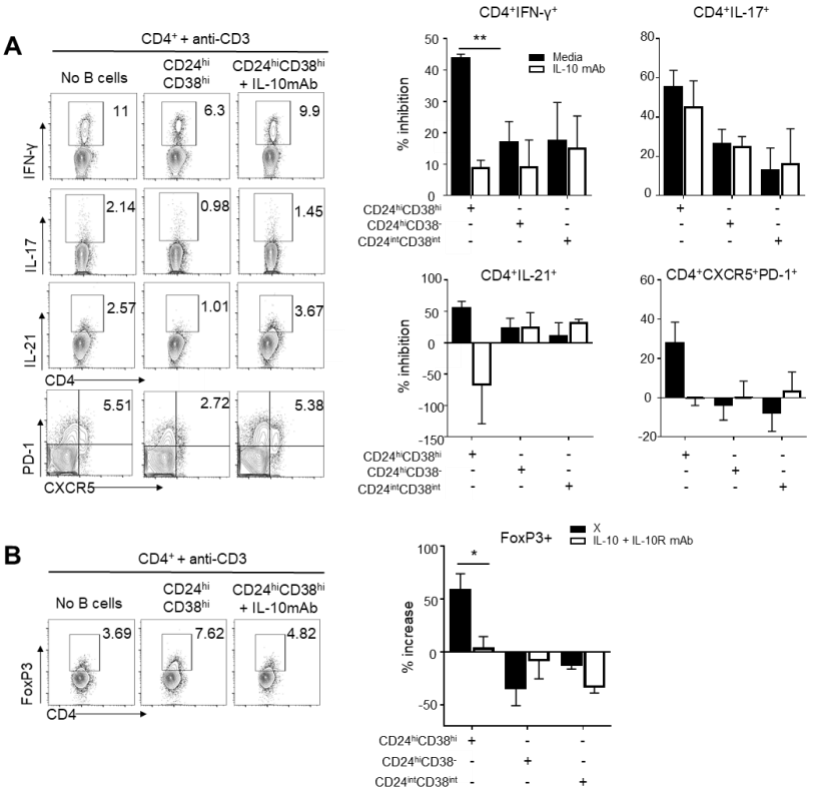


Figure 6. CD24^{hi}CD38^{hi} B cells suppressive effect is dependent on IL-10.
Blocking antibodies to IL-10 (10ug/mL) and IL-10R (2ug/mL) was added to the co-culture.

A. Representative flow cytometry plot and bar graph showing the % decrease in the frequency of CD4$^+$IFN-γ$^+$, CD4$^+$IL-17$^+$, CD4$^+$IL-21$^+$ and CD4$^+$PD-1$^+$CXCR5$^+$ cells were evaluated.

B. Representative flow cytometry plot and bar graphs showing the % increase in frequency of CD4$^+$FoxP3$^+$ and CD4$^+$CD25$^+$CD127^{lo} cells.

Values are expressed as mean ± SEM. Differences between groups were evaluated by two-way ANOVA (A and C), or one-way ANOVA (B). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

IV. DISCUSSION

In the Part I of this study, was showed that $CD24^{hi}CD38^{hi}$ B cells of HC inhibit IFN-γ, IL-17 and IL-21 production by $CD4^+$ T cells and expansion of $CD4^+PD\text{-}1^+CXCR5^+$ cells. The inhibitory effect of HC $CD24^{hi}CD38^{hi}$ B cells on IFN-γ and IL-21 production by $CD4^+$ T cells and increase in FoxP3 expression was largely dependent on IL-10; however, no effect was observed on IL-17. It is conceivable that regulatory B cells, similar to Tregs, exert their inhibitory effects via multiple mechanisms. Thus, while the release of IL-10 is vital in the regulation of Th1 and Tfh cells, other mechanisms, such as cell-to-cell contact could be responsible for the suppression of IL-17. Indeed, the suppressive mechanism of $CD24^{hi}CD38^{hi}$ B cells was also found to depend partially on CD80 and CD86 (*4*). Moreover, blocking either IL-10 or PD-L1 in a co-culture of $CD24^{hi}CD38^{hi}$ B and $CD4^+$ T cells resulted in a significant increase in IL-17 expression by $CD4^+$ T cells (*34*). Nevertheless, the mechanism of inhibition by $CD24^{hi}CD38^{hi}$ B cells, other than the regulation of IL-10 production, remains to be determined.

Part II

Distribution of $CD24^{hi}CD38^{hi}$ B cells and effect of immunosuppressive therapy (alemtuzumab) on $CD24^{hi}CD38^{hi}$ B cell distribution in multiple sclerosis patients

I. INTRODUCTION

The concept that multiple sclerosis (MS) is a T-cell mediated disease has been changed and it is now widely accepted that B cells play a part in the pathogenesis of MS.(*35-37*) Multiple roles of B cells have been elucidated emphasizing that B cells have dual contribution to autoimmunity.(*35, 38-40*) Hence, the concept of regulatory B cells (Breg) has emerged, proving that B cell subset distribution is far more complex than the original concept. A novel Breg subset that was originally known as immature transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}$) has been described to have regulatory capacity through interleukin-10 production.(*4*) Alemtuzumab is a highly effective treatment in relapsing MS. It provides a long-lasting suppression of disease activity by altering the proportion of lymphocyte subsets with preferential increase of regulatory T cells (Treg).(*41, 42*) In contrast to alemtuzumab's documented efficacy, alemtuzumab's mechanism of action is not fully understood and information about the composition of the repopulating B cell pool, especially Breg, is scarce.

Here we pinpoint deficiency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells during relapse and subsequent expansion following alemtuzumab infusion. We also highlight the possible clinical implication of $CD19^{+}CD24^{hi}CD38^{hi}$ cells.

II. MATERIALS AND METHODS

1. Study population

The present study is a controlled laboratory experiment performed with human peripheral blood samples. For cross-sectional study, 20 MS patients during relapse (MS-relapse) and 17 MS patients in remission (MS-remission) and 11 healthy controls (HC) were included. For longitudinal analysis, 11 patients who were treated with alemtuzumab were include. All MS patients fulfilled the 2010 McDonald's criteria. Demographic and clinical characteristics of participants are summarized in table 1.

Table 4. Demographic and clinical characteristics of MS patients in relapse and

remission

Characteristic	MS-relapse	MS-remission
Age (years, mean±SD)	34.5±9.4	36.9±5.2
Women:men (n:n)	16:4	10:7
Onset age (years, mean±SD)	30.4±22.0	29.8±7.3
Disease duration (years, mean±SD)	12±24.9	6±5.9
EDSS (mean±SD)	2.9±1.9	1.8±1.9

2. Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated from the fresh blood using a Ficoll-Paque Plus (GE Healthcare) gradient centrifugation. 1% phosphate-buffered saline (PBS)

containing 2mM ethylenediaminetetraacetic acid (EDTA) were used as separation buffer. Collected bloods were mixed with 2mM EDTA-PBS 1:1 ratio into 50ml conical tube (Falcon). 15ml of Ficoll-Paque solution was added to another 50ml conical tube and blood-separation buffer mixture was transferred onto Ficoll-Paque gently. Solutions were centrifuged at 1680 rotation per minute (rpm) for 30 minutes with brakes off. The buffy coat layer containing PBMC were collected and mixed with 2mM EDTA-PBS and centrifuged at 1500 rpm for 15 minutes. This step was repeated two times. Freshly isolated PBMCs were either directly assayed, or cryopreserved.

3. Ex-vivo analysis using Flow cytometry

For cross-sectional study, fresh PBMCs were surface-stained with mAbs against CD19-APC-cy7, CD27-FITC, CD24-BV421, and CD38-BV510 (BD Biosciences). For longitudinal study, frozen PBMCs, collected from 11 RRMS patients undergoing alemtuzumab at baseline and 6, 9, and 12 months, were surface stained with monoclonal antibodies as stated above. Data were acquired using FACSVerse (BD Biosciences).

4. Statistical analysis

We performed analysis of significance in Prism (GraphPad, La Jolla, USA). For cross-sectional data, one-way ANOVA analysis with Tukey's multiple comparison post-hoc analysis was performed to compare the frequency of Bregs between HC, MS-relapse and MS-remission. Unpaired t-test was used to compare the absolute number of Bregs between MS-relapse and MS-remission. Repeated measures ANOVA with Tukey's multiple comparison post-hoc analysis was performed for longitudinal analysis. A p-value of <0.05 was considered statistically significant. All values show mean ± SEM.

III. RESULTS

1. Regulatory B cells are deficient in MS patients during relapse

In order to evaluate the relationship between the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells with disease activity of MS, the frequency and the

absolute number was measured in total $CD19^+$ B cells in MS-relapse and MS-remission and HC. The frequency of $CD19^+CD24^{hi}CD38^{hi}$ cells (Fig. 1A, B)) was significantly reduced in MS-relapse compared to MS-remission and HC. The average frequency of Bregs in MS-remission was lower than those of HC, but no statistical difference was observed. The absolute number of $CD19^+CD24^{hi}CD38^{hi}$ cells was significantly reduced in MS-relapse compared to MS-remission (Fig. 1C).

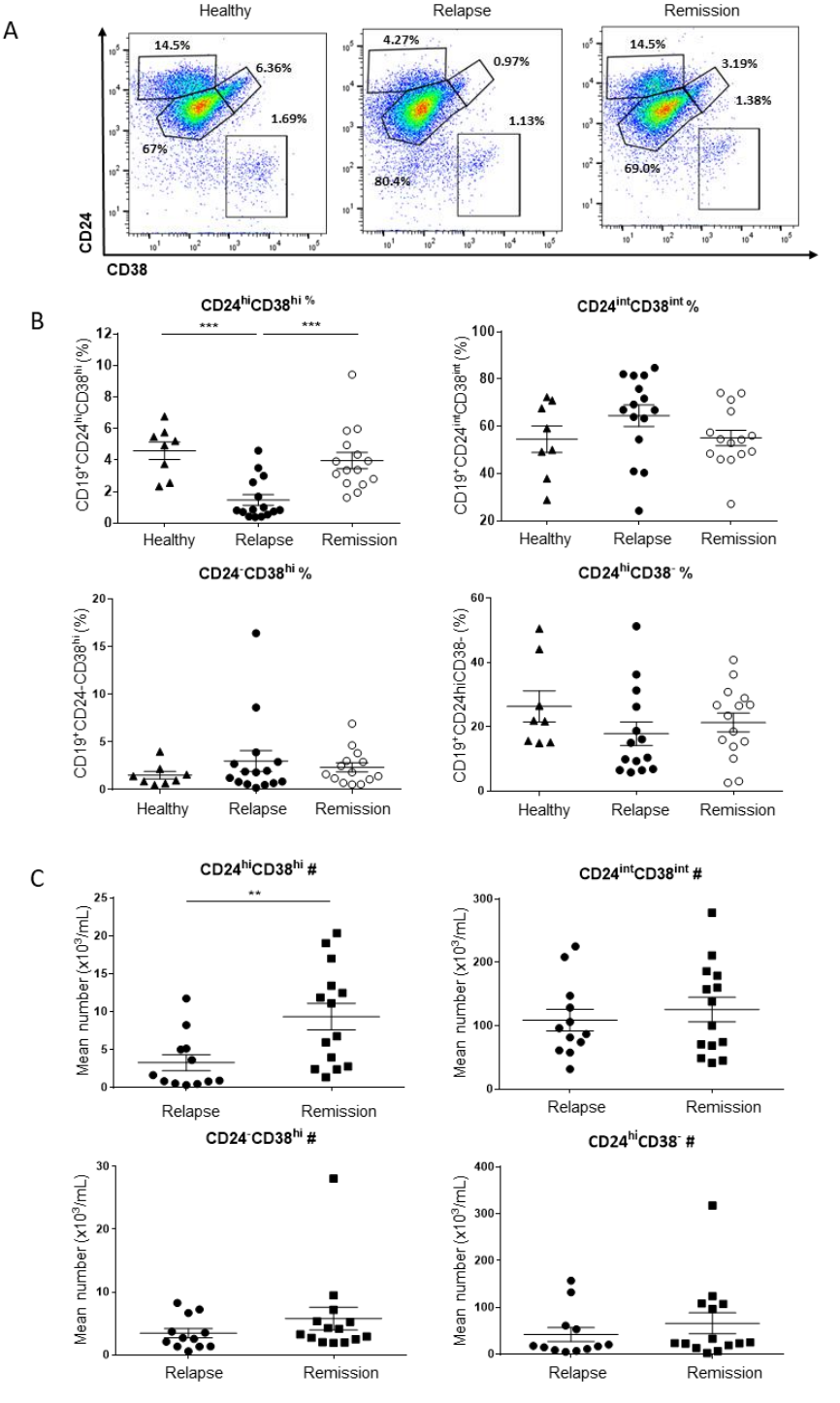


Figure 7. MS patients show deficiency of CD19+CD24hiCD38hi cells during relapse.

The percentage and the absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells were measured in MS patients undergoing relapse and patients in remission and healthy controls. PBMC was isolated from fresh peripheral blood and surface stained for flow cytometry.

A. Representative flow cytometry dot plot of CD24 and CD38 expression in total CD19+ B cells.
B. Scatter plots showing the percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in MS-relapse (n=15), MS-remission (n=15) and HC (n=8). A significant reduction in the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells was observed in MS-relapse compared to MS-remission and HC (relapse vs remission: $p = 0.0006$, relapse vs healthy: $p = 0.0004$). All values show mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc analysis. ***P < 0.001.
C. The absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in MS-relapse (n=15) was significantly reduced compared to MS-remission (n=15) ($p = 0.009$).

All values show mean ± SEM. Data were analyzed by unpaired t-test. **P < 0.01. Ex vivo data were collected from peripheral blood samples taken during the time course of this study.

2. Preferential reconstitution of naïve B cells following alemtuzumab

As expected, the frequency and absolute number of total lymphocytes was decreased at 6 months and gradually increased up to 12 months post alemtuzumab (Fig. 2A). The frequency and the absolute number of memory B cells and plasmablasts were significantly decreased compared to pre-treatment level and naïve B cells comprised the majority of repopulated $CD19^+$ B cells (Fig. 2B).

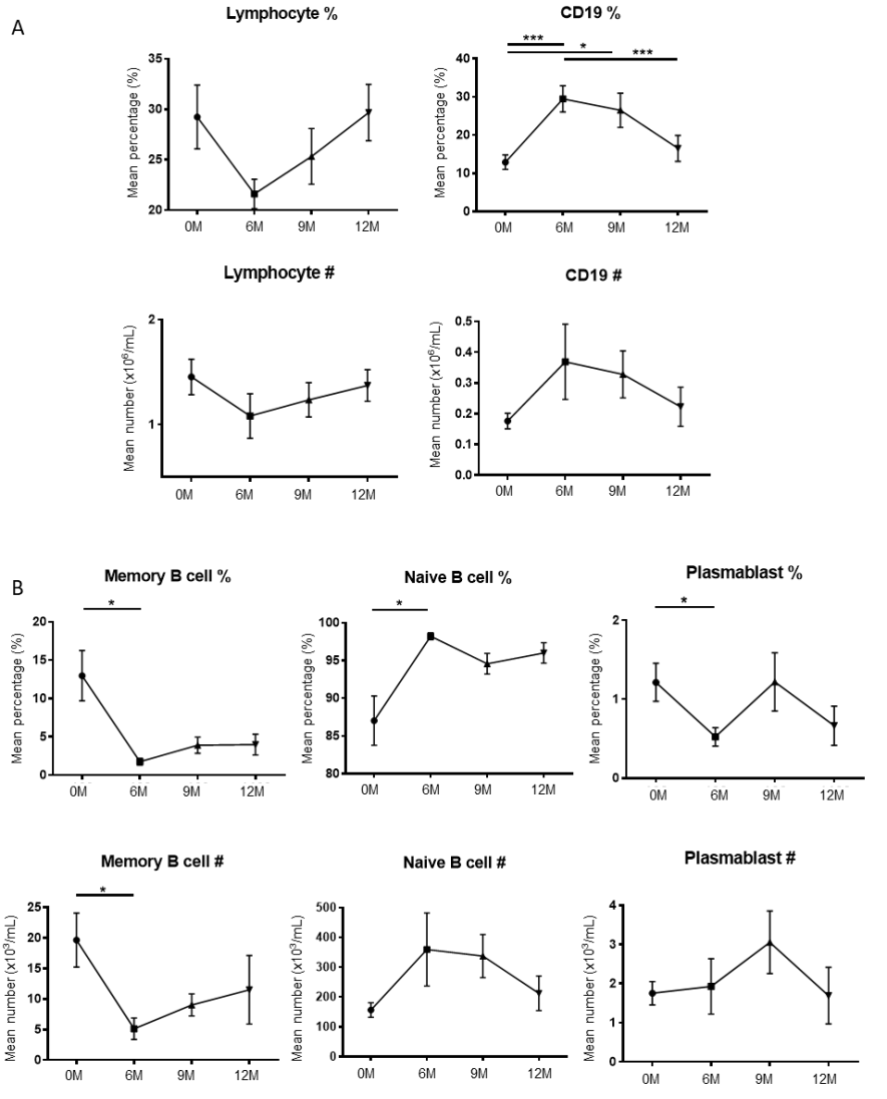


Figure 8. Naive B cells predominate repopulated CD19+ cells following alemtuzumab treatment.

In order to evaluate the B cell subset distribution post-alemtuzuamb, thawed PBMCs of alemtuzumab treated patients (n=11) were evaluated up to 12 months after induction.

A. Cumulative data for the frequency and the absolute number of total

lymphocytes and $CD19^+$ B cells. Successful depletion and reconstitution of lymphocytes and $CD19^+$ B cells was confirmed.

B. Cumulative data for the frequency and the absolute number of $CD19^+CD27^+$ memory B cells, $CD19^+CD27^-$ naïve B cells and $CD19^+CD27^+CD38^{hi}$ plasmablasts. Following alemtuzumab, significant reduction in the frequency of memory B cells (6M vs 0M: $p = 0.0278$) and plasmablasts (6M vs 0M: $p = 0.0448$) was observed and dominance of naïve B cells was observed (6M vs 0M: $p = 0.0269$). The absolute number of memory B cells was significantly decreased compared to 0M (6M vs 0M: $p = 0.0112$).

All values show mean ± SEM. Data were analyzed by repeated measures ANOVA with Tukey's multiple comparison post-hoc analysis.

3. Breg deficiency in MS is restored following alemtuzumab

The frequency and the absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ cells were markedly increased at 6 and 9 months following alemtuzumab treatment compared to pre-treatment level. By the end of the cycle (12M), both the frequency and number were decreased, although did not reach pre-treatment level. The frequency and absolute number of $CD19^{+}CD24^{int}CD38^{int}$ mature naïve B cells were increased at 6 and 9 months post-alemtuzuamb and at 12 months post-alemtuzumab, the frequency of $CD19^{+}CD24^{int}CD38^{int}$ cells was lower than baseline level. A significant decrease in the frequency and absolute number of $CD19^{+}CD24^{hi}CD38^{-}$ memory B cells was observed following alemtuzumab treatment (Fig. 3A-C).

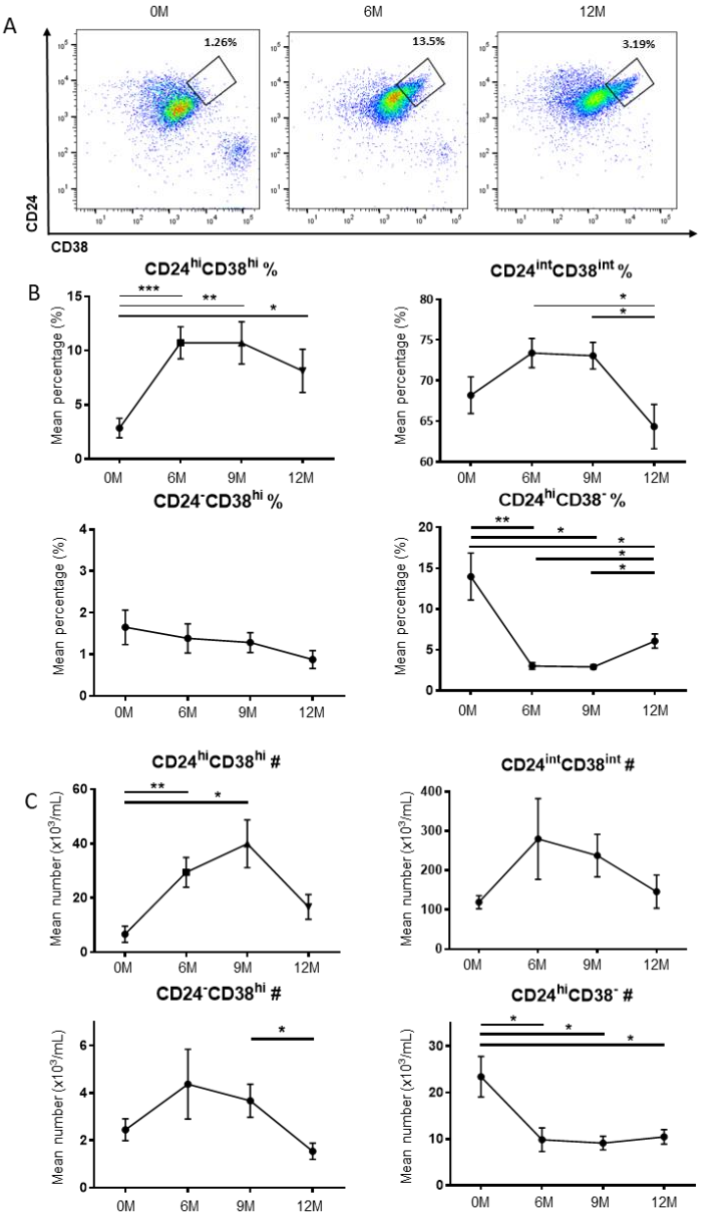


Figure 9. Alemtuzumab treatment restores CD19^{+}CD24^{hi}CD38^{hi} cells.

In order to evaluate the B cell subset distribution post-alemtuzuamb, thawed PBMCs of alemtuzumab treated patients (n=11) were evaluated up to 12 months

after induction.

A. Representative flow-cytometry dot plot of CD24 and CD38 in total $CD19^{+}$ B cells.
B. Cumulative data for the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells. The frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells were significantly increased at 6M and 9M compared to pre-treatment level (6M vs 0M: $p = 0.0004$, 9M vs 0M: $p = 0.0079$). At 9M, the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells started to decrease and by 12M, the frequency was reduced compared to 6M, although it was significantly increased than baseline level (12M vs 0M: p=0.0257).
C. The absolute number was significantly increased at 6M and 9M post-alemtuzumab (6M vs 0M: $p = 0.0063$, 9M vs 0M: $p = 0.02$).

All values show mean ± SEM. Data were analyzed by repeated measures ANOVA with Tukey's multiple comparison post-hoc analysis. *P < 0.05; **P < 0.01; ***P < 0.001.

4. Clinical implications

Among 11 patients treated with alemtuzumab, one patient experienced 2 severe relapses within 2 cycles of alemtuzumab infusion (Fig 4A and B). During his first attack, overshoot of total B cellswas observed while the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells was remarkably decreased to 0.45% compared to previous follow-up (6M: 11.1%). Four months later, the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells increased to 6.5%. Three months post second alemtuzumab infusion, the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells was 10.5%. However, at his second attack, 10M after second cycle infusion, the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells was again markedly decreased to 1.93% accompanied by overshooting B cell response. Interestingly, the reduction in the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells (3.98%) 20 days preceding second attack was observed, which is an immense reduction from previous follow-up (15M: 10.5%). The absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ cells was also reduced during relapses.

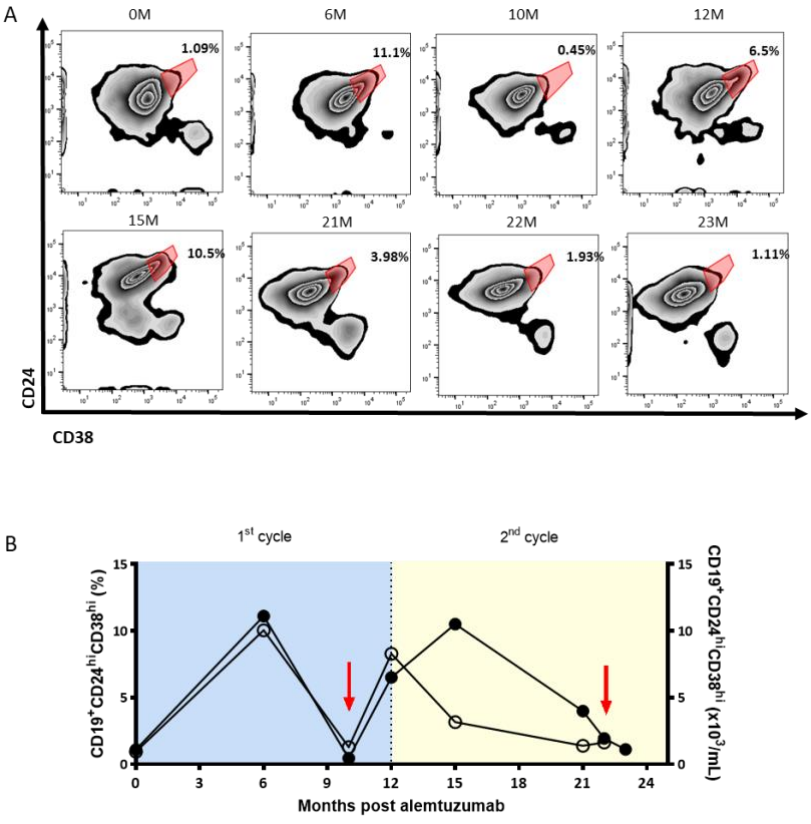


Figure 10. The frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells is decreased during relapse post alemtuzumab treatment.

A. Flow-cytometry plot of expression of CD24 and CD38 in total $CD19^{+}$ B cells.

B. Graph showing the clinical course. The frequency and the absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells and infusion of 2 cycles of alemtuzumab is shown. Filled circles indicate the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells and hollow circles indicates the absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells. Red arrow indicate relapse.

IV. DISCUSSION

Little is known about the repopulating B cell pool following alemtuzumab. Cases of severely exacerbated central nervous system inflammation in alemtuzumab treated MS patients have reported B-cell driven pathology, further emphasizing the importance of B cell study in alemtuzumab treated patients.(*43-45*) We show that deficiency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells and $CD19^{+}PD\text{-}L1^{hi}$ cells in the peripheral blood of relapsing MS patients are restored following alemtuzumab and that B cell distribution shifts towards naïve phenotype. In fact, the frequency of Bregs with disparate regulatory mechanisms exceeded baseline level, which may underlie the long-lasting suppression of disease activity.

Interestingly, both frequency and absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ cells are reduced during and prior to relapse in an alemtuzumab treated patient. $CD19^{+}CD24^{hi}CD38^{hi}$ cells are known to maintain Tregs and limit the differentiation of T helper 1 (Th1) and T helper 17 (Th17) cells.(*7*) The deficiency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells could have a significant impact on the regulation of pathology. Indeed, recent studies have found that transitional B cells are impaired in various immune-related disorders,(*4, 7, 46, 47*) although conflicting results were observed in MS.(*48, 49*) In the early phase of almetuzumab therapy, $CD19^{+}$ B cells repopulate earlier than CD4+ T cells and immature B cells dominate the repopulated CD19+ B cells,(*50*) the extensive repopulation of $CD19^{+}CD24^{hi}CD38^{hi}$ cells following alemtuzumab may contribute to the expansion of Tregs while suppressing differentiation of naïve $CD4^{+}$ T cells into Th1 and Th17 cells and hence, contributing to the efficacy of alemtuzumab.

A recent study described $CD19^{+}PD\text{-}L1^{hi}$ cells are capable of suppressing Tfh cell differentiation and expansion through interaction with PD-1 on activated T cells.(*51*) Interaction causes an increase in signal transducer and activator of transcription 5 expression, a known suppressor of Tfh-cell development and expansion. Since Tfh cells aid germinal center formation and hence, involved in the formation of memory B cells and plasma cells, Tfh cells were thought to have

pathogenic role in the B-cell mediated autoimmune diseases. There has been several reports on Tfh cell involvement in MS.(*52-55*) Most of all, there has been report that $CCR7^{+}$ $ICOS^{+}$ circulating memory Tfh cells are increased in MS patients during relapse, but decreased in patients during remission.(*56*) This finding is in line with our results, where $CD19^{+}PD\text{-}L1^{hi}$ cells are decreased in MS patients during relapse, but restored during remission. Hence, suppression of Tfh cell differentiation and proliferation by $CD19^{+}PD\text{-}L1^{hi}$ cells may be impaired due to CD19+PD-L1hi cell deficiency, contributing to enhancement of disease activity.

This study is limited by its small sample size. In addition, we report that the marked reduction of $CD19^{+}CD24^{hi}CD38^{hi}$ cells during relapse in an alemtuzumab treated patient was observed in only 2 relapses of a single case. Therefore, in order to decipher the critical role of $CD19^{+}CD24^{hi}CD38^{hi}$ cells in the long-term disease suppression of MS and in the mechanism of action of alemtuzumab, further longitudinal study with larger number of patients on how $CD19^{+}CD24^{hi}CD38^{hi}$ cells and other lymphocytes (including Tregs) change during relapse, is required. The extensive expansion of $CD19^{+}CD24^{hi}CD38^{hi}$ cells was maintained until 9 months post-alemtuzumab, whereas $CD19^{+}PD\text{-}L1^{hi}$ cells were maintained until the end of the cycle. Further work needs to be established to explain the difference in the results. In addition, a recent study has reported that hyperrepopulation of immature B cells post-alemtuzumab in the absence of adequate regulation by T cells increases the risk of secondary autoimmunity.(*50*) Due to vast composition of immature B cells and lack of understanding of their function, it remains to be further elucidated which specific immature B cell subset would be responsible for the secondary autoimmunity. Lastly, further functional study on the $CD19^{+}CD24^{hi}CD38^{hi}$ cells would clarify the mechanism of action of alemtuzumab.

Part III

Regulatory function of B cells in NMOSD and effect of immunosuppressive therapy (BCDT) on Breg function

I. INTRODUCTION

The delicate balance between effector functions against pathogens and immune tolerance mechanisms to self-antigens is critical to avoid autoimmunity. In the majority of antibody-mediated autoimmune diseases, including neuromyelitis optica spectrum disorder (NMOSD), B cells are generally considered to be pathogenic because of their ability to secrete autoantibodies. NMOSD is an autoimmune inflammatory disease of the central nervous system (CNS) characterized by lesions predominantly occurring in the optic nerves and spinal cord. NMOSD pathogenesis involves autoantibodies against water channel aquaporin 4 (AQP4-IgG). AQP4 is highly concentrated along astrocytic end-feet in the CNS, and binding of AQP4-IgG leads to astrocyte damage by complement-dependent cytotoxicity (*57*). Complement activation leads to an inflammatory response that further disrupts the blood-brain barrier (BBB), leading to neutrophil, eosinophil, and macrophage infiltration, ultimately causing oligodendrocyte damage, demyelination, and neuronal death (*9, 10, 58, 59*).

B cells can participate in NMOSD pathogenesis not only through the production of autoantibodies but also by presenting antigens to autoreactive T cells, production of various cytokines, and involvement in T cell-mediated immune responses (*1*). Furthermore, evidence indicating the involvement of T cells in the development of NMOSD is accumulating. AQP4-specific T cell responses were found to be amplified in NMOSD patients, whose T cells exhibited T helper 17 (Th17) polarization (*11*) and Th17-related markers have shown correlation with disease activity and severity in NMOSD. Th17 cells disrupt BBB tight junctions and promote additional $CD4^+$ T cell recruitment from the systemic circulation into the CNS (*12*). The release of interleukin (IL)-6 and IL-21 by polyclonally activated $CD4^+$ T cells derived from NMOSD patients correlated with neurological disability (*13*). T follicular helper (Tfh) cells are required for B cell differentiation into antibody-producing cells, and a recent study found that

untreated patients with NMOSD show Tfh polarization toward excessive B-helper Tfh subsets (*14*).

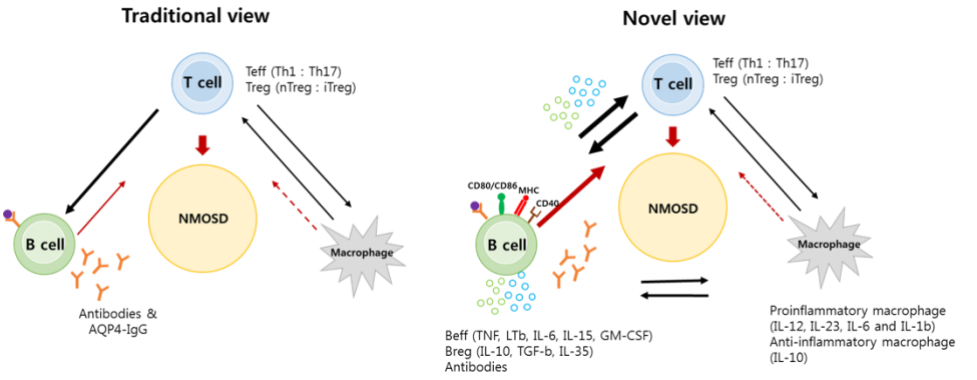


Figure 11. The novel view of immune cell contribution to the pathophysiology of NMOSD.

The traditional view involves T cells as central player in the pathophysiology of NMOSD. An imbalance in proinflammatory Th1and Th17 effector cells and Treg cells was thought to provoke new attacks. Myeloid cells were the main antigen presenting cells that shape the T cell responses. B cells were thought to await the help of T cells to differentiate into antibody-secreting plasmablasts and plasma cells. B cell contribution to NMOSD pathophysiology was generally considered to be related to autoantibody production, AQP4-IgG. In the novel view, the B cell is a full participant in the complex network where macrophages, T cells and B cells are all related. In NMOSD, this complex network is dysregulated. Furthermore, there are two subtypes of B cells, the effector B cells (Beff) that produce proinflammatory cytokines (e.g. TNFα, IL-6, GM-CSF) and regulatory B cells (Bregs) that produce anti-inflammatory cytokines (IL-10, TGF-β). Bidirectional interactions among functionally distinct B cells, T cells, and myeloid cells- and the consequences of such interactions provoke disease exacerbation in NMOSD patients.

Following the emergence of regulatory B cells (Bregs) in animal studies, these cell types have been extensively studied. Regulatory cells are important for the maintenance of immune homeostasis and tolerance to self. During bystander activation of B cells through T cell stimulation mediated by CD40, without antigen or B cell receptor (BCR) engagement, B cells fail to produce proinflammatory cytokines and instead secrete significant amounts of the regulatory cytokine IL-10 that suppresses the local inappropriate inflammatory response. Currently, human Bregs have been identified at different stages of B cell development and consist of various B cell surface markers; these include $CD19^{+}CD24^{hi}CD38^{hi}$ immature transitional B cells, $CD19^{+}CD24^{+}CD27^{+}$ memory B cells (*2*), and $CD19^{+}CD27^{int}CD38^{+}$ regulatory-like plasmablasts (*3*). Human $CD19^{+}CD24^{hi}CD38^{hi}$ cells were initially known as bone marrow-derived immature transitional B cells, and CD40 stimulation is required for their regulatory function. Their regulatory functions include inhibition of autoreactive $CD4^{+}$ T cell proliferation and interferon- γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production by activated $CD4^{+}$ T cells. $CD19^{+}CD24^{hi}CD38^{hi}$ B cells also prevent T helper (Th)1, Th17, and Tfh cell differentiation and promote the conversion of $CD4^{+}$ T cells into $CD4^{+}FoxP3^{+}$ regulatory T cells (Tregs) (*4, 5*). With the finding that $CD19^{+}CD24^{hi}CD38^{hi}$ cells have immune regulatory functions, the relevance of $CD19^{+}CD24^{hi}CD38^{hi}$ cells in the maintenance of tolerance in several immune-related disorders has begun to emerge (*4, 6-8*).

NMOSD shows a relapsing-remitting course in which attacks of NMOSD often produce severe disability with incomplete recovery (*15*). Clinical relapse is directly related to disability in NMOSD (*16*); hence, preventing relapse is the primary treatment goal. The anti-CD20 B cell depleting therapy (BCDT), which depletes immature and mature B cells, has exhibited excellent clinical efficacy for the treatment of NMOSD (*17-19, 60*). It is hypothesized that BCDT exerts its clinical efficacy by eliminating pathogenic B cells. The antigen-specific $CD27^{+}$ memory B cells augment antibody production upon re-stimulation and are

characterized by isotype switching and affinity maturation (*20, 21*). They also contribute to autoimmunity through cytokine production (*23*) and antigen presentation to T cells (*61*). Repeated treatment with BCDT when $CD27^+$ memory B cells reemerged in the peripheral blood mononuclear cells, as maintenance therapy in NMOSD, produced consistent and sustained efficacy (*24-26*). Despite BCDT, an incomplete depletion or rapid recovery of memory B cells is associated with the occurrence of relapse in autoimmune diseases (*24, 27-30*). Following BCDT, a shift in the B cell subset toward a naïve phenotype occurs (*24, 25*).

We have previously shown a deficiency of $CD19^+CD24^{hi}CD38^{hi}$ B cells in the peripheral blood of relapsing multiple sclerosis patients and provided evidence supporting $CD19^+CD24^{hi}CD38^{hi}$ B cells as a potential biomarker for disease activity (*8*). To date, only a few studies have explored the role of $CD19^+CD24^{hi}CD38^{hi}$ B cells in NMOSD (*31, 32*). The precise contribution of B cells to the disease pathogenesis and the possible plasticity of B cell function depending on the environment and the mechanism of action of BCDT are incompletely understood. Most importantly, the contribution of B cell regulatory functions in NMOSD remains unknown. Given that naïve B cells are dominant among those reappearing after BCDT, it is tempting to speculate that a shift in the predominant B cell subsets causes a shift in the immune balance following BCDT. To address these questions, the balance in B cell subsets and function of Breg cells and the effect of BCDT on these cells in patients with NMOSD were evaluated in the present study.

II. MATERIALS AND METHODS

1. Study population

The present study is a controlled laboratory experiment performed with human peripheral blood samples. The study was designed to understand better the function of Breg cells and the effect of BCDT in patients with NMOSD. One hundred and forty-nine patients (125 women and 24 men; mean age: 45.8 ± 10.8 years) who met the 2015 International Panel for NMO Diagnosis criteria (*62*) and were positive for AQP4-IgG (*63*) and negative for MOG-IgG (*64*). The clinical characteristics and demography of NMOSD patients are summarized in Table 1.

The patients were firstly divided into two groups: relapse and remission. The relapse group included all patients with acute clinical attacks regardless of the treatment type for attack prevention. Clinical relapses were defined as newly developed neurological worsening with objective signs that lasted for at least 1 day, with preceding clinical stability for at least 1 month. Blood from the relapse group was collected before the administration of intravenous methylprednisolone. The remission group included patients who are in remission for at least 1 year and was further divided into two groups according to the immunosuppressive therapy: non-BCDT and BCDT. Remission (non-BCDT) included patients receiving immunosuppressive therapies other than BCDT. Remission (BCDT) included patients receiving BCDT. The longitudinal analysis following BCDT was studied in 3 patients. Twenty-two healthy individuals (15 women and 7 men; mean age: 39.1 ± 10.5 years) were also included in this study as a control. None of the patients had received corticosteroids within 6 months of blood collection with the exception of 4 NMOSD patients included in the co-culture study and 3 patients for the longitudinal study as these patients started BCDT 1 month after the acute attack. The study was approved by the Institutional Review Board of the National Cancer Center. Written informed consent was obtained from all participants.

BCDT was administered according to a previously published protocol (*24*, *25*). Briefly, the treatment protocol of the initial 2-years included induction and maintenance phases. Two regimens were used as induction treatment: 1) 375 mg/m^2 infused once weekly for 4 weeks and 2) 1000mg/m^2 infused twice at a 2-week interval. When the memory B-cell frequency was at least 0.05% in PBMCs, patients were given 1 additional infusion of rituximab (375 mg/m^2). After completion of the initial 2-year treatment, the interval for monitoring memory B cells was extended to 10 weeks. As for the maintenance therapy, patients received 1 additional infusion of rituximab (375 mg/m^2) whenever the frequency of reemerging memory B cells in PBMCs exceeded 0.1%. No patient was given concomitant immunosuppressants while receiving rituximab.

Table 5. Demographic and clinical characteristics of patients with NMOSD

Characteristic	Relapse	Remission	BCDT
n	22	40	87
Age (years, mean SD)	42.5 ± 10.4	43.2 ± 7.4	47.2 ± 12.9
Women : men (n:n)	(16 : 6)	(33: 7)	(76 : 11)
Onset age (years, mean SD)	31.8 + 11.6	32.3 + 12.4	33.3 + 12.0
Disease duration (years, mean SD)	10.1 + 7.2	7.68 + 8.1	12.0 + 6.3
EDSS (mean SD)	2.9 + 1.9	2.5 + 1.8	2.8 + 1.8
Treatment duration (years, mean SD)	3.8 + 4.0	4.6 + 4.6	7.9 + 3.5
Medication, n (%)			
Untreated	12 (54.5%)	2 (5%)	
Mycophenolate mofetil	7 (31.8%)	33 (82.5%)	
Azathioprine	1 (4.5%)	5 (12.5%)	
B cell depletion therapy	2 (9.1%)		87 (100%)

One hundred and forty-nine patients with NMOSD, who met the 2015 international Panel for NMO Diagnosis criteria (*62*), were included in this study. Treatment duration was calculated from the initiation date of any immunosuppressive therapy.

2. Peripheral blood mononuclear cell (PBMC) isolation

PBMC were isolated from the fresh blood using a Ficoll-Paque Plus (GE Healthcare) gradient centrifugation. 1% phosphate-buffered saline (PBS) containing 2mM ethylenediaminetetraacetic acid (EDTA) were used as separation buffer. Collected bloods were mixed with 2mM EDTA-PBS 1:1 ratio into 50ml conical tube (Falcon). 15ml of Ficoll-Paque solution was added to another 50ml conical tube and blood-separation buffer mixture was transferred onto Ficoll-Paque gently. Solutions were centrifuged at 1680 rotation per minute (rpm) for 30 minutes with brakes off. The buffy coat layer containing PBMC were collected and mixed with 2mM EDTA-PBS and centrifuged at 1500 rpm for 15 minutes. This step was repeated two times. Freshly isolated PBMCs were either directly assayed, or cryopreserved.

3. $CD19^+$ B cell MACS isolation

$CD19^+$ B cells were isolated by magnetic column purification with anti-CD19-coated beads (Miltenyi Biotec). The PBMC suspension was centrifuged at 300xg for 10 minutes and supernatant was aspirated. The cell pellet was resuspended in 80uL of buffer (0.5% bovine serum albumin + 1X phosphate-buffered saline (PBS)) per 10^7 total cells. 20uL of CD19 MicroBeads (Miltenyi Biotec) per 10^7 total cells was added and incubated for 15 minutes in the refrigerator (2-8 ‘C). Cells were washed and centrifuged at 300xg for 10 minutes. The supernatant was aspirated and cells were resuspended up to 10^8 cells in 500uL buffer. Cells suspensions were applied to MS column that was placed in the magnetic field of MiniMACS Seprator (Miltenyi Biotec). The unlabeled cells that passed through was collected and labeled CD19+ B cells were collected by removing column from the separator and flushing out labeled cells by firmly pushing the plunger into the column.

4. Fluorescence-activated cell sorting (FACS)

Isolated PBMCs were labelled with fluorochrome-conjugated antibodies below. Cells were resuspended in 500uL of cold Pre-Sort Buffer (BD Biosciences) and 1uL of fluorochrome-conjugated antibodies per 1 x 10^7 cells were added and incubated for 30 minutes on ice protected from light. Cells were washed with Pre-Sort Buffer and resuspended with cold Pre-Sort Buffer to a final concentration of ~3 x 10^6 cells /mL. $CD19^+$, $CD19^+CD24^{hi}CD38^{hi}$, $CD19^+CD24^{int}CD38^{int}$, $CD19^+CD24^{hi}CD38^-$ and $CD4^+CD25^-$ T cells were isolated from PBMCs using FACSAria II (BD Biosciences).

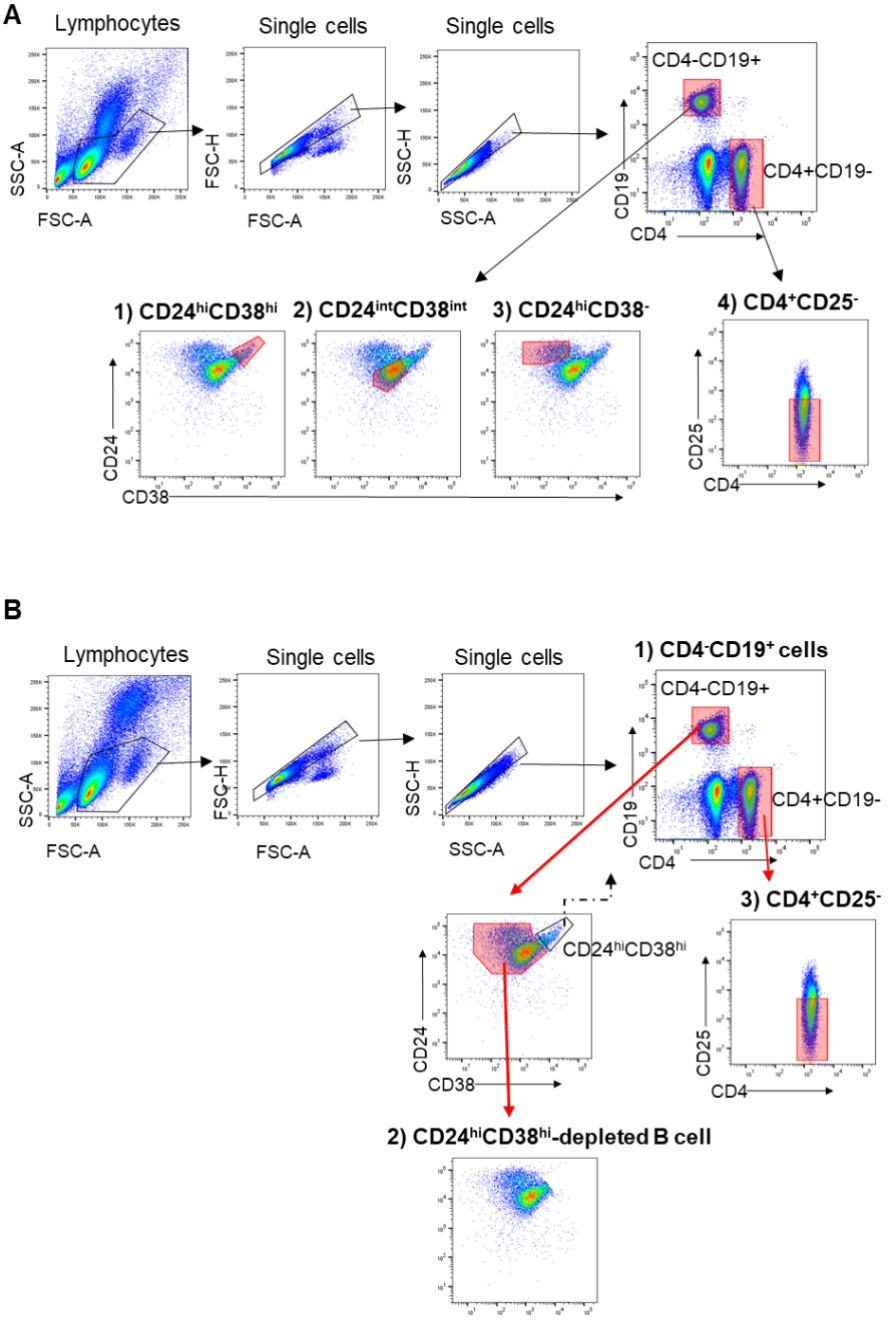


Figure 12. Flow cytometry sorting of B and T cells.

PBMCs were surface stained for CD19, CD24, CD38, CD4, and CD25 and sorted by flow cytometry (FACSAria sorter). CD19+ B cells and CD4+ T cells were gated from lymphocytes and single-cell population.

A. The target populations 1) $CD19^{+}CD24^{hi}CD38^{hi}$, 2) $CD19^{+}CD24^{int}CD38^{int}$, 3) $CD19^{+}CD24^{hi}CD38^{-}$, and 4) $CD4^{+}CD25^{-}$ cells were sorted.

B. The garget populations 1) $CD4^{-}CD19^{+}$ and 3) $CD4^{+}CD25^{-}$ cells were isolated by flow cytometry sorter. $CD19^{+}CD24^{hi}CD38^{hi}$ cells were depleted from $CD19^{+}$ cells and non-depleted $CD19^{+}$ cells passed through cell sorter are defined as 2) $CD24^{hi}CD38^{hi}$-depleted B cell.

Table 6. List of fluorochrome-conjugated antibodies used for co-culture assay

Target	**Fluorochrome conjugation**	**Source**
Anti-human CD19	FITC	BD Biosciences
Anti-human CD24	PE-CF594	BD Biosciences
Anti-human CD38	PE	BD Biosciences
Anti-human CD25	APC-cy7	BD Biosciences
Anti-human CD4	BUV496	BD Biosciences

5. Cell culture

Purified cells were cultured in complete RPMI 1640, L-glutamine, and $NaHCO_3$ supplemented with 10% fetal bovine serum (Hyclone) and penicillin/streptomycin (100 U/mL) (Hyclone) in 96-well U-bottom plates (Nunc). For detection of cytokine produced by $CD19^+$ B cells and $CD24^{hi}CD38^{hi}$ B cells, purified B cells were stimulated with soluble CD40L (1 μg/mL, Enzo Life Sciences) alone or in combination with goat anti-human IgM BCR cross-linking antibody (XAb) (10 μg/mL, Jackson ImmunoResearch) for 24 h. For co-culture assays, flow cytometry sorted $CD4^+CD25^-$ T cells alone or with B cell subsets were co-cultured 1:1 and stimulated with plate-bound anti-CD3 mAb (0.5 μg/mL; HIT-3a, BD Pharmigen) for 72 h. For the IL-10 blocking assay, anti-IL-10 (5 μg/mL; JES3-9D7, Miltenyi Biotec) and anti-IL-10 receptor (5 μg/mL; 3F9, Biolegend) antibodies were added.

6. Ex-vivo analysis using Flow cytometry

For cross-sectional and longitudinal analyses, fresh PBMCs were surface-stained with mAbs against CD19-APC-cy7, CD27-FITC, CD24-BV421, and CD38-BV510 (BD Biosciences). For longitudinal study, frozen PBMCs. Collected from 11 RRMS patients undergoing alemtuzumab at baseline and 6, 9, and 12 months, were surface stained with monoclonal antibodies as stated above. Data were acquired using FACSVerse (BD Biosciences).

7. Cytokine detection through intracellular cytokine staining

Cytokine production was detected by intracellular cytokine staining (ICS). Phorbol 12-myristate 13-acetate (PMA) (20 ng/mL, Sigma-Aldrich), ionomycin (500 ng/mL, Sigma-Aldrich), and Golgistop (BD Biosciences) were added to the last 6 h of cell culture. At the end of 24 h cell culture, cells were harvested and stained with Fixable Viability Stain-Alexa Fluour 700 according to the

manufacturer's instructions (BD Biosciences) to determine live/dead cells before staining for surface markers.

Table 7. List of fluorochrome-conjugated antibodies used for B cell ICS

Target	**Fluorochrome conjugation**	**Source**
Anti-human CD19	APC-cy7	BD Bioscience
Anti-human CD24	PE-CF594	BD Bioscience
Anti-human CD38	BV510	BD Bioscience
Anti-human CD27	FITC	BD Bioscience
Anti-human IL-10	PE	BD Bioscience
Anti-human IL-6	BV421	BD Bioscience
Anti-human TNFα	APC	BD Bioscience

8. Co-culture assay

For the co-culture assay, CD19-FITC, CD24-PE-CF594, CD38-PE, CD25-APC-cy7, CD4-BUV496, CD3-BUV395, PD-1-BV786, CXCR5-PerCP-cy5.5, and CD127-BUV (BD Biosciences) were used. Cells were washed, fixed, and permeabilized using Fixation/Permeabilization Solution Kit or Transcription Factor Buffer Set (BD Biosciences) according to the manufacturer's protocol and stained for detection of intracellular cytokines or transcription factors with TNFα-APC, IL-10-PE, IL-6-BV421, GM-CSF-PerCP-cy5.5, IFNγ-BV605, IL-17A-BV655, IL-21-BV421, and FoxP3-AF700 (BD Biosciences). Data were acquired using LSRFortessa (BD Biosciences).

Table 8. List of fluorochrome-conjugated antibodies used for co-culture assay

Target	**Fluorochrome conjugation**	**Source**
Anti-human CD19	FITC	BD Bioscience
Anti-human CD24	PE-CF594	BD Bioscience
Anti-human CD38	PE	BD Bioscience
Anti-human CD25	APC-cy7	BD Bioscience
Anti-human CD4	BUV496	BD Bioscience
Anti-human CD3	BUV395	BD Bioscience
Anti-human PD-1	BV786	BD Bioscience
Anti-human CXCR5	PerCP-cy5.5	BD Bioscience
Anti-human CD127	BUV737	BD Bioscience
Anti-human FoxP3	PE	eBioscience
Anti-human IFN-γ	BV605	BD Bioscience
Anti-human IL-21	BV421	BD Bioscience
Anti-human IL-17	BV650	BD Bioscience

9. Statistical analysis

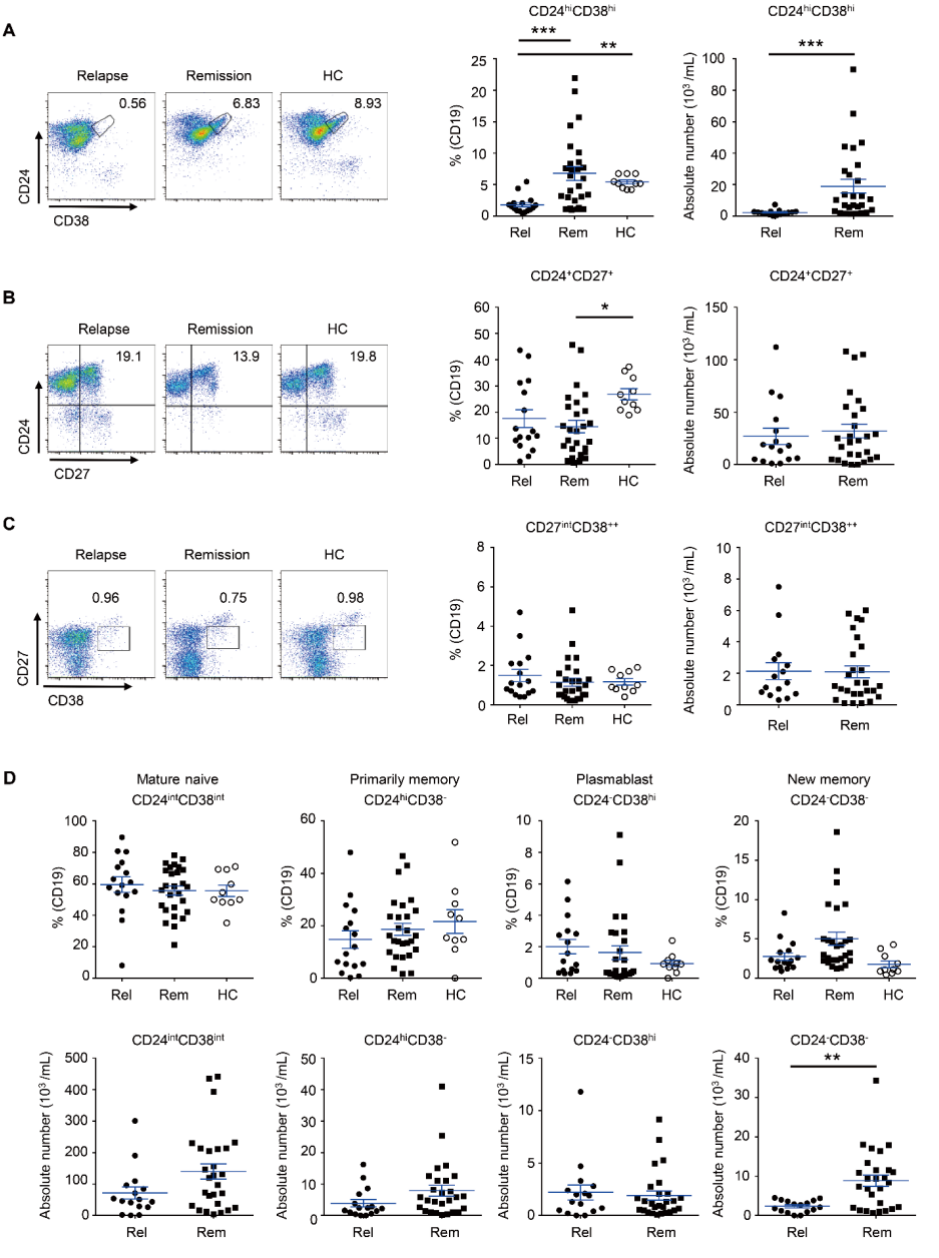
Data were analyzed for significance using Prism (GraphPad) by paired *t-test*, Mann–Whitney *U* test, one-way ANOVA with Tukey's multiple comparison post hoc analysis, or two-way ANOVA with Sidak's multiple comparison test. Repeated measures ANOVA with Tukey's multiple comparison post-hoc analysis was performed for longitudinal analysis. Correlation coefficients and their significance were calculated using a two-tailed Spearman's rank correlation. A confidence interval of 95% or a p-value < 0.05 was considered statistically significant. All values are presented as the mean ± SEM.

III. RESULTS

1. Numerical deficiency of $CD24^{hi}CD38^{hi}$ B cells in the NMOSD relapse group

We evaluated the frequency and the absolute number of the three Breg subsets $CD19^{+}CD24^{hi}CD38^{hi}$ immature transitional B cells, $CD19^{+}CD24^{+}CD27^{+}$ memory B cells, and $CD19^{+}CD27^{int}CD38^{+}$ regulatory-like plasmablasts in the peripheral blood of 16 patients in relapse and 27 patients remission (excluding BCDT patients) and 10 healthy controls (HCs). The frequency and the absolute number of $CD24^{hi}CD38^{hi}$ B cells were significantly decreased in the relapse group compared to remission and HCs (Fig. 2A). Although the frequency of $CD24^{+}CD27^{+}$ memory B cells was significantly lower in the remission group than in HCs, the frequency of $CD27^{+}CD38^{int}$ regulatory-like plasmablasts and the absolute number of $CD24^{+}CD27^{+}$ memory B cells and $CD27^{+}CD38^{int}$ regulatory-like plasmablasts was not significantly different between patients and HCs (Fig. 2B and C). The frequency and absolute number of $CD24^{int}CD38^{int}$ (mature naïve), $CD24^{hi}CD38^{-}$ (primarily memory), $CD24^{-}CD38^{hi}$ (plasmablast), and $CD24^{-}CD38^{-}$ (new memory) B cells showed no difference between NMOSD patients and HCs, except for $CD24^{-}CD38^{-}$ B cells that had significantly decreased absolute number in remission group (Fig. 2D).

Considering that the relapse group included treatment-naïve patients (n = 9) as well as those treated with immunosuppressive therapies (n = 7; azathioprine 1, mycophenolate mofetil 6), and the frequency of $CD24^{hi}CD38^{hi}$ B cells positively correlated with time since last attack, the decreased frequency of $CD24^{hi}CD38^{hi}$ B cells was more reflective of disease activity than treatment effect in patients with NMOSD (Fig. 2E and F). Similarly, both treatment-naïve patients (n = 2) and patients treated with immunosuppressive therapies (n = 25; azathioprine: 5, mycophenolate mofetil: 20) were included in the remission group.



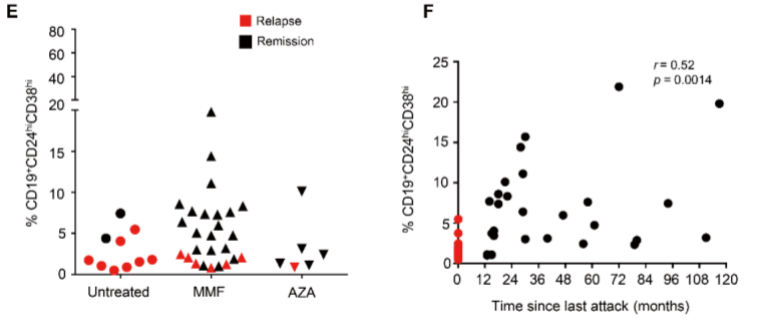


Figure 13. The frequency and absolute number of $CD19^{+}CD24^{hi}CD38^{hi}$ cells are decreased in patients during relapse.

PBMCs isolated from 16 patients during relapse (Rel), 27 patients during remission (Rem), and 10 HCs were stained *ex vivo* for the expression of CD19, CD24, CD38, and CD27. Representative flow cytometry plot of B cell subset gating for patients during relapse and remission and HCs:

A. $CD19^{+}CD24^{hi}CD38^{hi}$ immature transitional B cells,

B. $CD19^{+}CD24^{hi}CD27^{+}$ memory B cells,

C. $CD19^{+}CD27^{int}CD38^{++}$ regulatory-like plasmablasts,

D. $CD19^{+}CD24^{int}CD38^{int}$ mature naïve, $CD19^{+}CD24^{hi}CD38^{-}$ primarily memory, $CD19^{+}CD24^{-}CD38^{hi}$ plasmablast and $CD19^{+}CD24^{-}CD38^{-}$ new memory B cells. Scatter plots showing mean percentages and mean absolute numbers of B cells within each subset.

E. NMOSD patients were grouped according to their treatment status and those patients who are in relapse are shown in red dots and patients in remission are shown in black dots.

F. Scatter plot showing the correlation of the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ cells and time since last attack (months).

Values are expressed as mean ± SEM. Differences between groups were evaluated by one-way ANOVA to compare the frequency of B cell subsets and Mann–Whitney test to compare absolute numbers. Correlation coefficients were calculated using a two-tailed Spearman's rank correlation. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2. $CD24^{hi}CD38^{hi}$ B cell-intrinsic deficit in patients with NMOSD

NMOSD patients during relapse not only have a numerical deficiency in Bregs but also show impairment in IL-10 production during bystander B cell activation (Fig. 3A). During stimulation that mimics antigen engagement, B cells derived from the relapse group produce significantly higher levels of proinflammatory cytokines than HCs (Fig. 3B). These results indicate a possible deficiency in B cell immunomodulation.

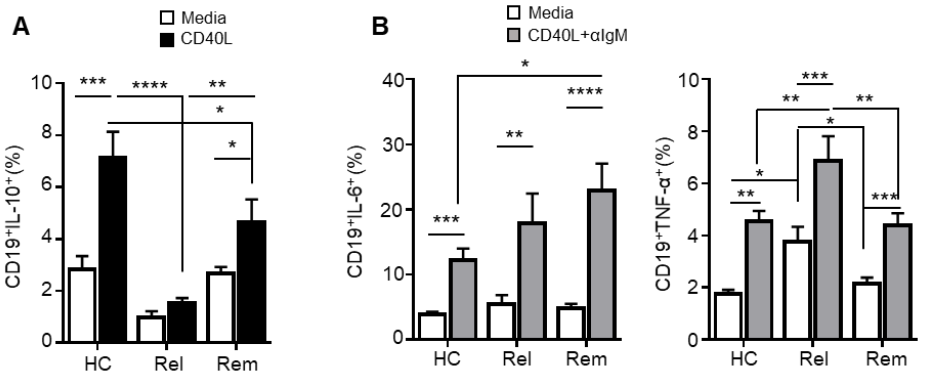


Figure 14. The cytokine production by CD19$^+$ B cells following CD40L and CD40L+αIgM stimulation.

B cells from PBMCs of 7 patients with NMOSD in relapse (Rel), 11 patients in remission (Rem), and 8 HCs were purified by magnetic separation. Purified B cells were stimulated with CD40L alone (**A**) or CD40L + anti-IgM (**B**) for 24 h. PMA + ionomycin + Golgistop was added in the last 6 h of culture. The mean ± SEM frequencies of (**A**) CD19$^+$IL-10$^+$, (**B**) CD19$^+$IL-6$^+$, and CD19$^+$TNF-α$^+$ cells were compared between patients during relapse and remission and HCs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

To determine whether $CD24^{hi}CD38^{hi}$ B cells derived from patients with NMOSD exhibit Breg cell-intrinsic deficits, we cultured isolated $CD24^{hi}CD38^{hi}$ B cells in the presence of CD40L to assess their response to CD40 stimulation. While $CD24^{hi}CD38^{hi}$ B cells from HC and remission group responded to CD40 stimulation by upregulating IL-10 production, $CD24^{hi}CD38^{hi}$ B cells from the relapse group did not induce IL-10 in response to the same stimuli (Fig. 4A and B). The ratios of IL-10/TNF-α and IL-10/IL-6 produced by $CD24^{hi}CD38^{hi}$ B cells were significantly decreased in the relapse group compared to that in HCs and remission group (Fig. 4C). These findings show that following Breg bystander activation, $CD24^{hi}CD38^{hi}$ B cells from relapse patients exhibit impaired IL-10 production and imbalance in pro- and anti-inflammatory cytokine profile.

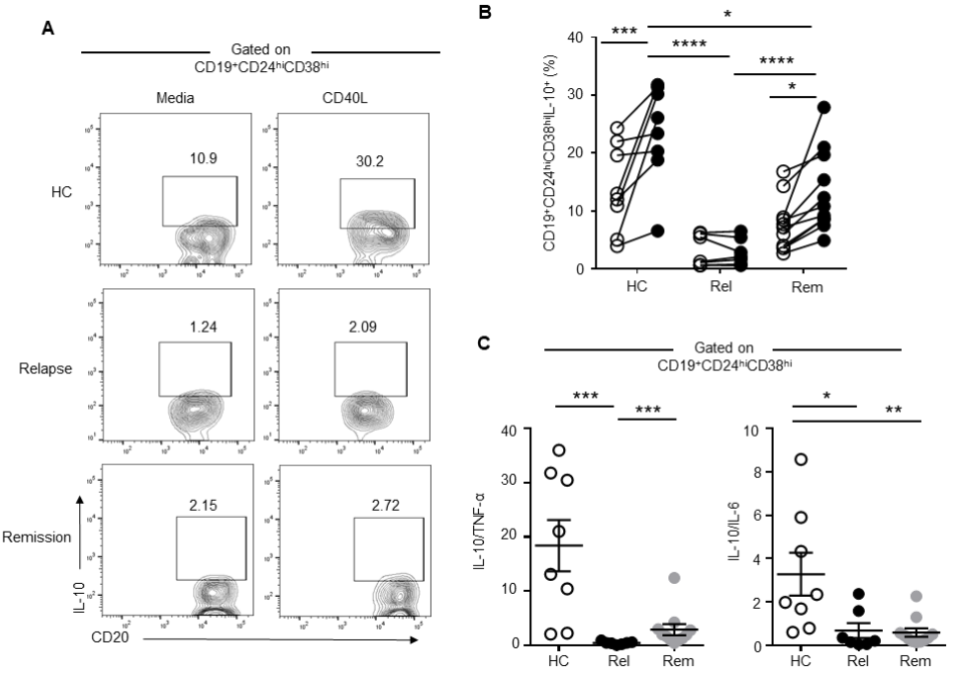


Figure 15. CD24^{hi}CD38^{hi} B cells from NMOSD patients show an abnormal response to CD40 stimulation and decreased IL-10/TNF-α and IL-10/IL-6 ratios compared to HCs.

B cells from PBMCs of 7 patients with NMOSD in relapse (Rel), 11 patients in remission (Rem), and 8 HCs were purified by magnetic separation. Purified B cells were stimulated with CD40L for 24 h. PMA + ionomycin + Golgistop was added in the last 6 h of culture.

A. Representative flow cytometry plots showing the frequency of CD19^{+}CD24^{hi}CD38^{hi}IL-10^{+} cells in HCs and patients in relapse and remission.

B. Graph showing the frequency of CD19^{+}CD24^{hi}CD38^{hi}IL-10^{+} upon CD40 stimulation in NMOSD patients and HCs.

C. Graph showing the ratios of IL-10/TNF-α and IL-10/IL-6 produced by $CD19^{+}CD24^{hi}CD38^{hi}$ cells in response to CD40 stimulation.

Values are expressed as mean ± SEM. Differences between groups were evaluated by two-way ANOVA (B and D) or one-way ANOVA (E). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

3. NMOSD CD24^{hi}CD38^{hi} B cells do not suppress CD4^{+} T cell cytokine production

The numerical and cell-intrinsic deficits in NMOSD CD24^{hi}CD38^{hi} B cells could represent the functional defects of these Bregs in NMOSD. We evaluated whether CD24^{hi}CD38^{hi} B cells isolated from NMOSD patients (n = 4; relapse: 1 and short-term remission: 3) could control the release of proinflammatory cytokines produced by CD4^{+} T cells.

NMOSD CD24^{hi}CD38^{hi} B cells failed to suppress the production of IFN-γ, IL-17, and IL-21 by T cells and CD4^{+}PD-1^{+}CXCR5^{+} cells (Fig. 5A and B). NMOSD CD24^{int}CD38^{int} B cells did not affect CD4^{+} T cells.

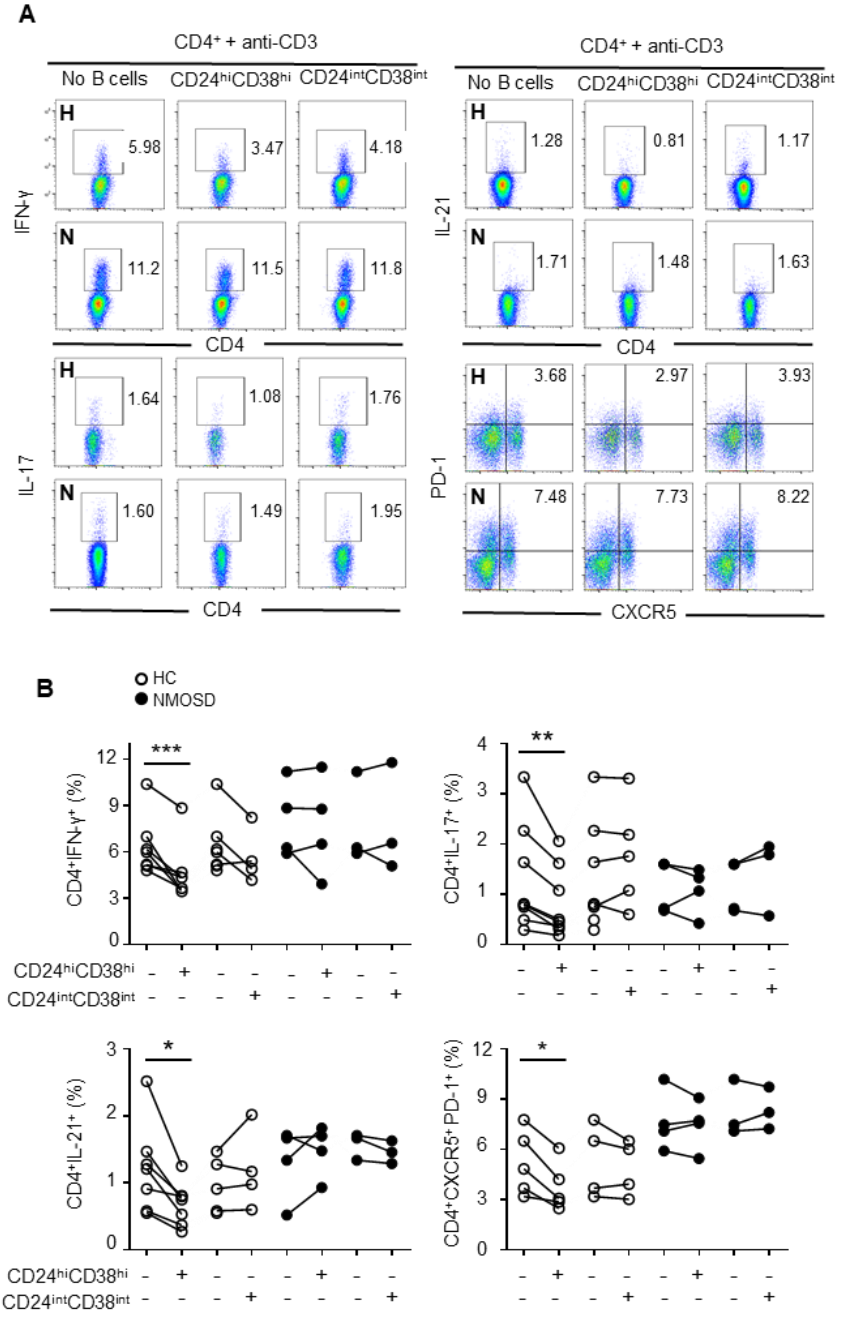


Figure 16. $CD24^{hi}CD38^{hi}$ B cells from patients with NMOSD do not suppress proinflammatory cytokine production by $CD4^+$ T cells

$CD24^{hi}CD38^{hi}$ and $CD24^{int}CD38^{int}$ B cells and $CD4^+CD25^-$ T cells from NMOSD patients and HCs were sorted by flow cytometry. B cell subsets were cultured 1:1 with $CD4^+CD25^-$ T cells for 72 h with plate-bound anti-CD3 mAb (0.5 μg/mL). PMA + ionomycin + Golgistop was added in the last 6 h of culture.

A. Representative flow cytometry and

B. scatter plots showing the frequencies of IFN-γ^+, IL-17^+, IL-21^+, and PD-1^+CXCR5^+ $CD4^+$ T cells when $CD4^+$ T cells were cultured alone and in the presence of B cell subsets. Representative results for 4 patients with NMOSD and 8 HCs are presented.

Mean ± SEM. Differences were evaluated by two-way ANOVA. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Next, we evaluated whether removing $CD24^{hi}CD38^{hi}$ B cells from the co-culture system might abolish this suppressive effect on T cell cytokine production. $CD19^{+}CD24^{hi}CD38^{hi}$ B cells were depleted from $CD4^{-}CD19^{+}$ cells obtained from HCs (n = 4) and NMOSD patients (n = 5) by flow cytometry sorting.

Undepleted $CD19^{+}$ cells or $CD24^{hi}CD38^{hi}$-depleted $CD19^{+}$ cells were cultured with $CD4^{+}$ T cells and stimulated for 72 h with plate-bound CD3 monoclonal antibody (mAb). The depletion of HC $CD24^{hi}CD38^{hi}$ B cells resulted in a significant increase in the frequency of $CD4^{+}IFN\text{-}\gamma^{+}$, $CD4^{+}IL\text{-}17^{+}$, $CD4^{+}IL\text{-}21^{+}$, and $CD4^{+}PD\text{-}1^{+}CXCR5^{+}$ T cells compared with undepleted $CD4^{+}CD19^{+}$ cells in healthy individuals. In contrast, depletion of NMOSD $CD24^{hi}CD38^{hi}$ B cells resulted in no significant change in the frequency of $CD4^{+}IFN\text{-}\gamma^{+}$, $CD4^{+}IL\text{-}17^{+}$, $CD4^{+}IL\text{-}21^{+}$, and $CD4^{+}PD\text{-}1^{+}CXCR5^{+}$ T cells (Fig. 6).

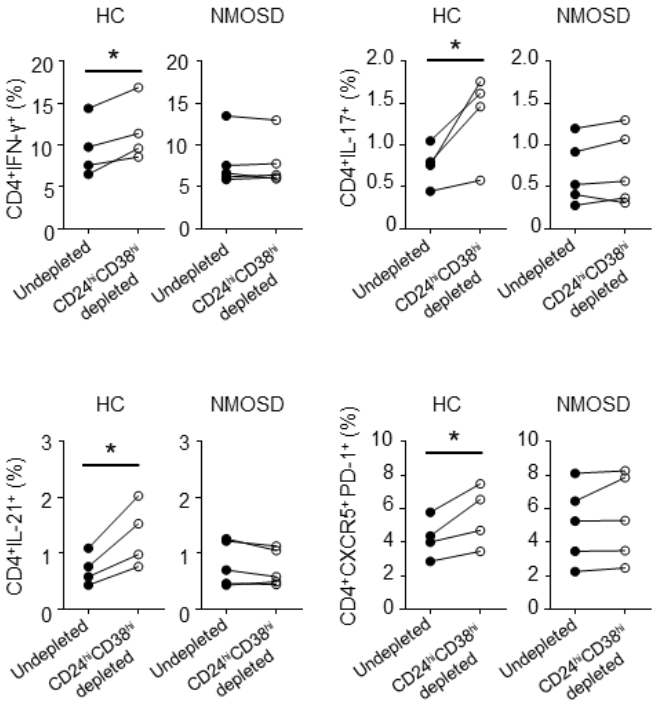


Figure 17. CD24^{hi}CD38^{hi} B cells from patients with NMOSD do not suppress proinflammatory cytokine production by CD4^{+} T cells

CD24^{hi}CD38^{hi} and CD24^{int}CD38^{int} B cells and CD4^{+}CD25^{-} T cells from NMOSD patients and HCs were sorted by flow cytometry. B cell subsets were cultured 1:1 with CD4^{+}CD25^{-} T cells for 72 h with plate-bound anti-CD3 mAb (0.5 μg/mL). PMA + ionomycin + Golgistop was added in the last 6 h of culture. CD19^{+} B cells from 4 HCs and 5 patients with NMOSD were depleted of CD19^{+}CD24^{hi}CD38^{hi} cells by flow cytometry sorting. Scatter plot showing the frequency of CD4^{+}IFN-γ^{+}, CD4^{+}IL-17^{+}, CD4^{+}IL-21^{+}, and CD4^{+}PD-1^{+}CXCR5^{+} cells after CD24^{hi}CD38^{hi} B cell depletion.

4. NMOSD $CD24^{hi}CD38^{hi}$ B cells fail to induce Tregs

To evaluate the contribution of $CD24^{hi}CD38^{hi}$ B cells to the induction of $CD4^{+}FoxP3^{+}$ Tregs, we co-cultured $CD24^{hi}CD38^{hi}$ B cells sorted from HCs (n = 4) or NMOSD patients (n = 3) in 1:1 ratio with autologous $CD4^{+}CD25^{-}$ T cells and stimulated them with plate-bound anti-CD3 for 72 h. We found an increase in FoxP3 expression only after co-culture with HC $CD24^{hi}CD38^{hi}$ B cells, while NMOSD $CD24^{hi}CD38^{hi}$ B cells failed to induce Tregs (Fig. 7A).

Depletion of HC $CD19^{+}CD24^{hi}CD38^{hi}$-depleted B cells resulted in a significant reduction in the frequency of $CD4^{+}FoxP3^{+}$ and $CD4^{+}CD25^{+}CD127^{lo}FoxP3^{+}$ cells compared with that of undepleted B cells co-cultured with $CD4^{+}$ T cells (Fig 7B). These results suggest that NMOSD $CD24^{hi}CD38^{hi}$ B cells have impaired regulatory function as they were unable to inhibit pro-inflammatory cytokine production by $CD4^{+}$ T cells and failed to induce Tregs.

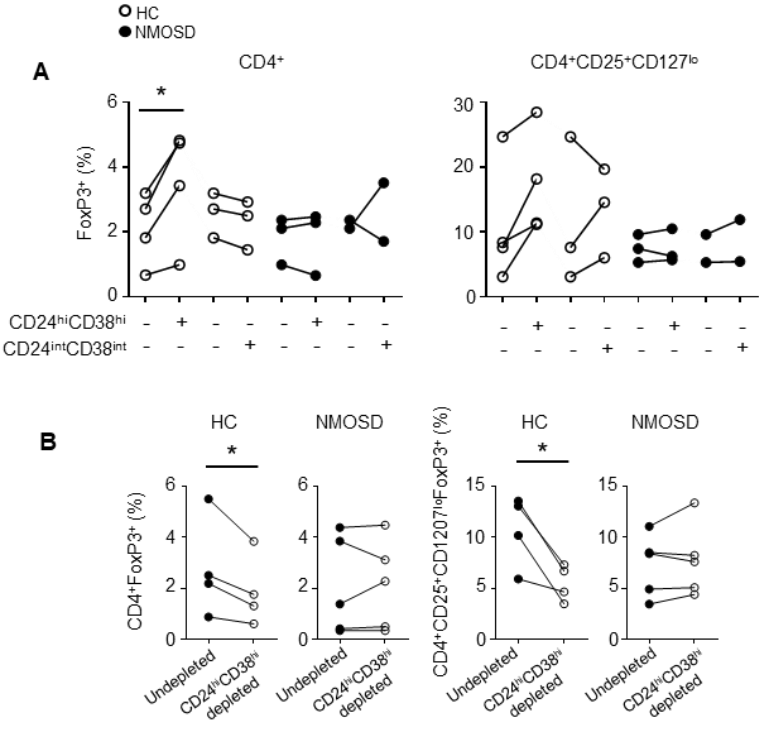


Figure 18. $CD24^{hi}CD38^{hi}$ B cells from patients with NMOSD and fail to convert $CD4^{+}$T cells to Tregs.

$CD24^{hi}CD38^{hi}$ and $CD24^{int}CD38^{int}$ B cells and $CD4^{+}CD25^{-}$ T cells from NMOSD patients and HCs were sorted by flow cytometry. B cell subsets were cultured 1:1 with $CD4^{+}CD25^{-}$ T cells for 72 h with plate-bound anti-CD3 mAb (0.5 μg/mL). PMA + ionomycin + Golgistop was added in the last 6 h of culture.

A. Scatter plot showing the frequency of $CD4^{+}FoxP3^{+}$ and $CD4^{+}CD25^{+}CD127^{lo}FoxP3^{+}$ cells when $CD4^{+}$ T cells were cultured alone and in the presence of B cell subsets. Representative results for 3 NMOSD patients and 4 HCs are presented.

B. Scatter plot showing the frequency of $CD4^+FoxP3^+$ and $CD4^+CD25^+CD127^{lo}FoxP3^+$ cells following co-culture of undepleted B cells or $CD19^+CD24^{hi}CD38^{hi}$-depleted B cells with $CD4^+$ T cells.

Mean ± SEM. Differences were evaluated by two-way ANOVA (B and D) or unpaired t-test (C and E). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

5. $CD24^{hi}CD38^{hi}$ B cells constitute the majority of repopulated B cells

following BCDT

Our results thus far provide important new data that identified a defect in the suppressive activity of a Breg population in NMOSD. We next aimed to determine whether BCDT led to the replacement of defective Bregs with fully functional Bregs. To assess this possibility, we first analyzed the effect of BCDT on the frequency of $CD24^{hi}CD38^{hi}$ B cells.

The *ex vivo* analysis revealed that the frequency of $CD19^{+}$ cells was significantly decreased in the BCDT group (n = 61) compared to the non-BCDT group (n = 44; relapse: 16 and remission: 28) and HCs (n = 10) (Fig. 8A), whereas the naïve/memory B cell ratio displayed an opposite trend (Fig. 8B). The absolute number of $CD19^{+}$ cells and $CD27^{-}$ naïve B cells of BCDT patients were significantly lower than patients in remission non-BCDT, whereas the absolute number of $CD27^{+}$ memory B cells was significantly lower in the BCDT group compared to relapse and remission non-BCDT group (Fig. 9 A and B). The percentage and absolute number of $CD24^{hi}CD38^{-}$ B cells were significantly decreased in the peripheral blood of patients in the BCDT group compared to the non-BCDT group and HCs (Fig. 8C). In contrast, the percentage and the absolute number of $CD24^{hi}CD38^{hi}$ B cells were significantly increased in the BCDT group (Fig. 8D). Interestingly, two patients who experienced relapse during BCDT had low percentages of $CD24^{hi}CD38^{hi}$ B cells (1.34% and 0.81%) and $CD24^{hi}CD38^{-}$ B cells (5.42% and 0.22%; red dots in Fig. 8C and D). The percentage of $CD24^{hi}CD38^{hi}$ B cells was positively correlated with the percentage of naïve B cells and negatively correlated with the percentage of memory B cells (Fig. 8E).

There was no significant difference in the percentage and absolute number of $CD24^{int}CD38^{int}$, $CD24^{-}CD38^{hi}$, and $CD24^{-}CD38^{-}$ B cells in the BCDT group compared to that in the non-BCDT group and HCs (Fig. 9C).

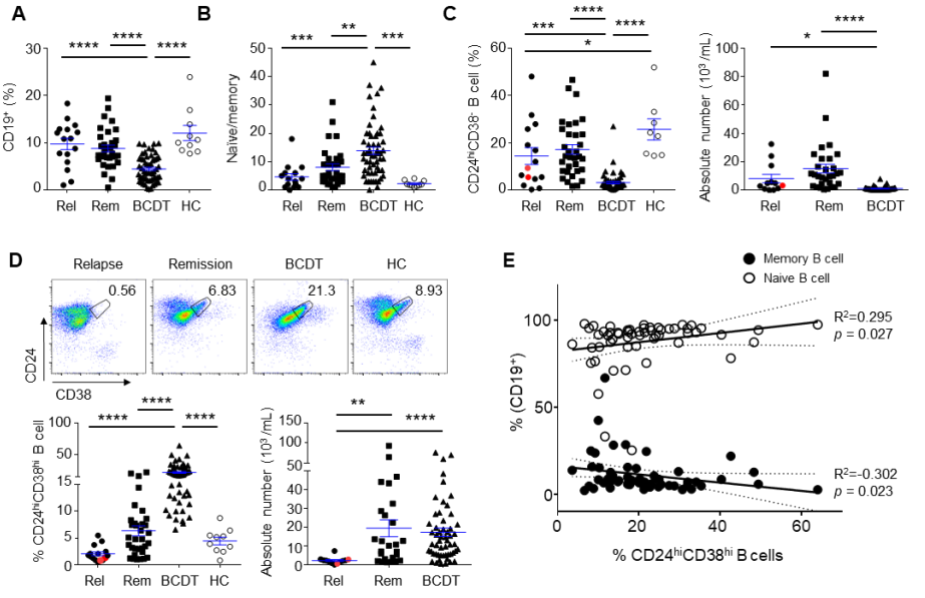


Figure 19. CD24^{hi}CD38^{hi} B cells are restored by B cell depletion therapy (BCDT) and constitute the majority of repopulated B cells.

PBMCs isolated from 18 patients during relapse (2 patients in relapse following BCDT included) (Rel), 27 patients during remission but not receiving BCDT (non-BCDT), 61 patients in remission and treated with BCDT (BCDT), and 10 HCs were stained *ex vivo* for the expression of CD19, CD24, and CD38. Scatter plot showing the

A. frequency of CD19^{+} cells within lymphocyte gate,

B. ratio of naïve/memory B cells,

C. frequency and absolute number of CD24^{hi}CD38^{-} B cells,

D. frequency and absolute number of CD24^{hi}CD38^{hi} B cells.

E. Scatter plot showing the percentage of naïve or memory B cells on the y-axis and percentage of CD24^{hi}CD38^{hi} B cells on the x-axis.

Mean ± SEM. Differences were evaluated by one-way ANOVA (A–D) or Spearman's correlation coefficient (E). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

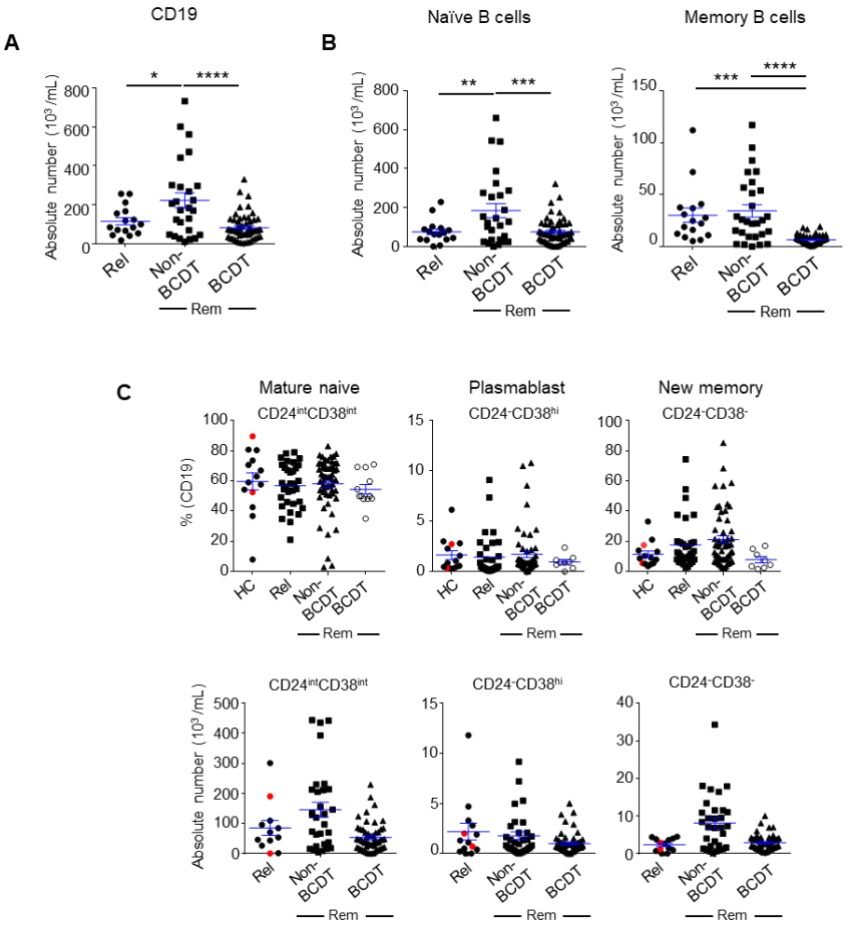


Figure 20. Composition of repopulated B cells after BCDT.

PBMCs isolated from 18 NMOSD patients during relapse (2 patients in relapse following BCDT included), 27 patients during remission non-BCDT, 61 patients treated with BCDT and 10 healthy individuals were stained ex-vivo for the expression of CD19, CD24 and CD38. Scatter plot showing the frequency and the absolute number of

(**A**) $CD19^{+}$, (**B**) $CD27^{-}$ naïve B cells and $CD27^{+}$ memory B cells, (**C**) $CD24^{int}CD38^{int}$, $CD24^{hi}CD38^{-}$ and $CD24^{-}CD38^{hi}$ B cells.

In long-term (n = 3; 3 years) follow-up of BCDT, the frequency of memory B cells and $CD24^{hi}CD38^{hi}$ B cells were analyzed in samples before B cell depletion and every 6 weeks since initiation of treatment. In the first BCDT cycle, memory B cells were the dominant cell type in the early repopulation phase, whereas $CD24^{hi}CD38^{hi}$ B cells were dominant during the late repopulation phase. In the subsequent BCDT cycles, $CD24^{hi}CD38^{hi}$ B cells were dominant throughout the entire B cell repopulation phase (Fig. 10).

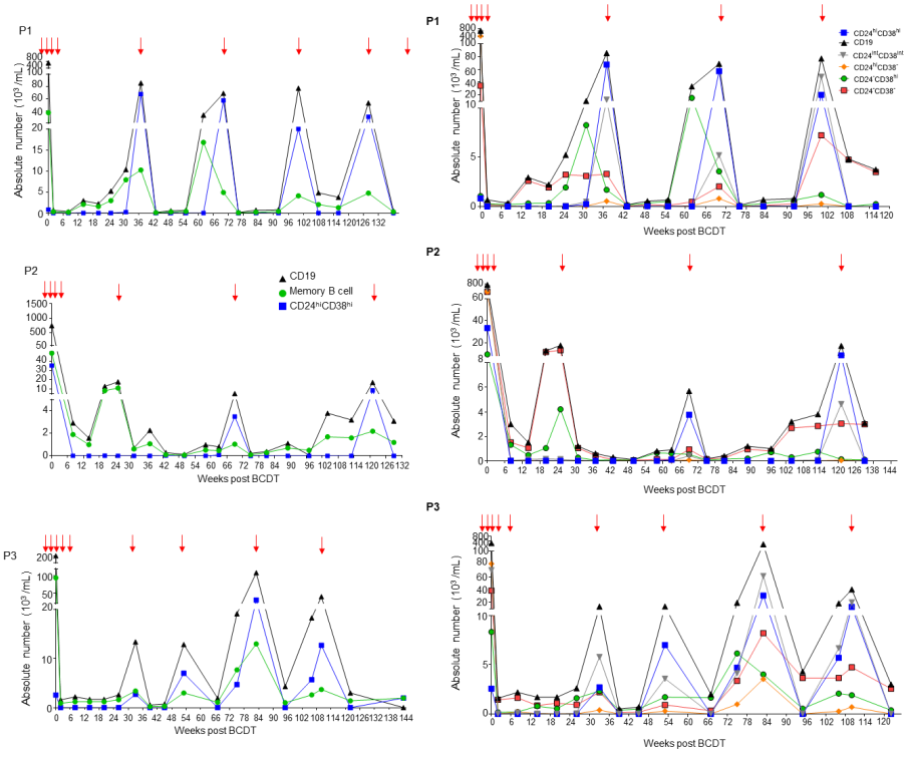


Figure 21. Repopulation kinetics of B cells subsets after BCDT.

A 3 year follow up of number of $CD19^{+}$ (black line), $CD24^{hi}CD38^{hi}$ B cells (blue line), $CD24^{int}CD38^{int}$ B cells (gray line), $CD24^{hi}CD38^{-}$ cells (orange line), $CD24^{-}CD38^{hi}$ (green line) and $CD24^{-}CD38^{-}$ (red line) in 3 patients treated with BCDT. BCDT injection is represented as red arrows.

Mean ± SEM. Differences were evaluated by one-way ANOVA (A–D) or Spearman's correlation coefficient (E). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

6. BCDT reverses abnormal NMOSD B cell response to CD40 stimulation

A vital question that remains to be addressed is whether the benefit of BCDT is directly mediated by the expanded $CD24^{hi}CD38^{hi}$ B cells with normalized Breg function or whether it reflects the absence of pathogenic B cells or a combination of both. In addition, the restoration of $CD24^{hi}CD38^{hi}$ B cells following BCDT does not necessarily reflect restoration of Breg cell type, as this could be due to alterations in the distribution of naïve and memory B cells since $CD24^{hi}CD38^{hi}$ B cells are originally immature naïve cells. Hence, we evaluated the cytokine profiles of repopulated B cells following BCDT.

Stimulation of B cells derived from BCDT group (n = 19; mean treatment duration: 8.7 ± 2.97 years) with CD40L resulted in significant production of IL-10, and CD40 + BCR stimulation resulted in significant production of IL-6 and TNF-α (Fig. 11A and B). The frequency of $CD19^{+}IL\text{-}10^{+}$ cells was significantly higher in the BCDT group than relapse group (n = 7), and no significant difference was observed between the BCDT group and remission group (n = 11) and HCs (n = 10) (Fig. 11C). Moreover, treatment with BCDT resulted in a significant decrease in the frequency of $CD19^{+}TNF\alpha^{+}$ cells compared to the non-BCDT group and HCs. The frequency of $CD19^{+}IL\text{-}6^{+}$ cells was similar between the BCDT and non-BCDT groups but significantly higher than HCs.

Surprisingly, unlike $CD24^{hi}CD38^{hi}$ B cells derived from the relapse group, the repopulated $CD24^{hi}CD38^{hi}$ B cells responded to CD40 stimulation by upregulating IL-10 (Fig. 11D). The ratio of IL-10/TNF-α was significantly higher in the BCDT group than the relapse group, and no significant difference was observed when compared with HCs and remission group (Fig. 11E). The IL-10/IL-6 ratio was still significantly lower in the BCDT group than HCs,

suggesting that Breg function may be recovered in repopulated B cells following BCDT.

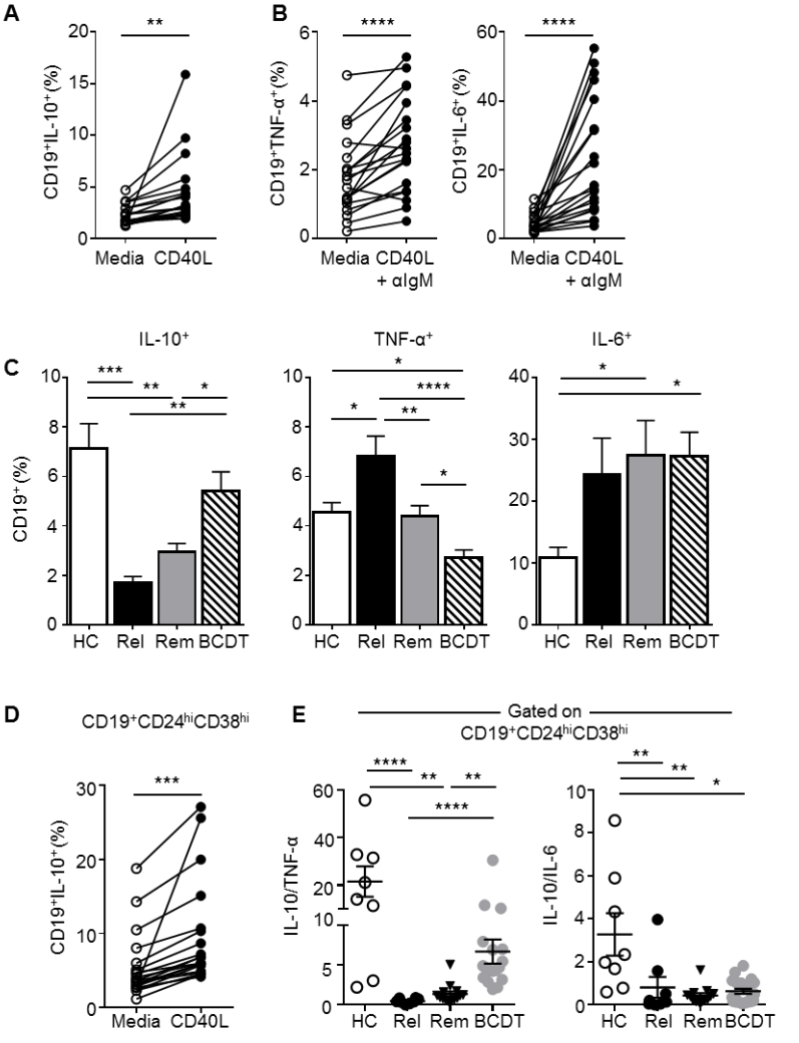


Figure 22. CD24^{hi}CD38^{hi} B cells of B cell depletion therapy (BCDT)-treated patients show normal response to CD40 stimulation and increased IL-10 levels.

B cells from PBMCs of 19 patients treated with BCDT were purified by magnetic separation. Purified B cells were stimulated with CD40L alone or CD40L + anti-IgM for 24 h. PMA + ionomycin + Golgistop was added in the last 6 h of culture.

A. Graph showing increase in the frequency of $CD19^{+}IL\text{-}10^{+}$ cells following CD40 stimulation.

B. Graph showing the increase in the frequency of $CD19^{+}TNF\text{-}\alpha^{+}$ and $CD19^{+}IL\text{-}6^{+}$ cells following CD40 + B cell receptor (BCR) stimulation.

C. Bar graph comparing the frequencies of induced $CD19^{+}IL^{-}10^{+}$ cells following CD40 stimulation and induced $CD19^{+}TNF\text{-}\alpha^{+}$ and $CD19^{+}IL\text{-}6^{+}$ cells following CD40 + BCR stimulation in patients treated with and without BCDT (non-BCDT) and HCs.

D. Graph showing the production of IL-10 by $CD24^{hi}CD38^{hi}$ B cells following CD40 stimulation in BCDT-treated patients.

E. Graph showing the ratios of IL-10/TNF-α and IL-10/IL-6 produced by $CD19^{+}CD24^{hi}CD38^{hi}$ cells in response to CD40 stimulation.

Values are presented as mean ± SEM. Differences between groups were evaluated by paired t-test (A, B, and D) or one-way ANOVA (C and E). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

7. Repopulated $CD24^{hi}CD38^{hi}$ B cells exhibit regulatory function

recovery

To evaluate how altered CD40 responsiveness and cytokine production of Bregs affect $CD4^+$ T cells, we sorted $CD24^{hi}CD38^{hi}$ B cells from patients who received BCDT (n = 8) and cultured them with autologous $CD4^+CD25^-$ T cells in the presence of CD3 mAb for 72 h.

We found that repopulated $CD24^{hi}CD38^{hi}$ B cells suppressed IFN-γ and IL-17 production by $CD4^+$ T cells but not IL-21 production and had no effect on the frequency of $CD4^+PD\text{-}1^+CXCR5^+$ cells (Fig. 12A). The percentage inhibition of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells by the repopulated $CD24^{hi}CD38^{hi}$ B cells was similar between patients who received BCDT and HCs. The percentage inhibition of $CD4^+IL\text{-}21^+$ and $CD4^+PD\text{-}1^+CXCR5^+$ cells by the repopulated $CD24^{hi}CD38^{hi}$ B cells was significantly lower than that of HC $CD24^{hi}CD38^{hi}$ B cells (Fig. 12B). Furthermore, the repopulated $CD24^{hi}CD38^{hi}$ B cells did not induce FoxP3 expression when cultured with $CD4^+$ T cells, and the percentage increase in $CD4^+FoxP3^+$ cells was significantly lower than HC $CD24^{hi}CD38^{hi}$ B cells (Fig. 12C and D). We found that the ability of $CD24^{hi}CD38^{hi}$ B cells to suppress IFN-γ and IL-17 production by $CD4^+$ T cells was restored following BCDT. However, the ability of $CD24^{hi}CD38^{hi}$ B cells to induce FoxP3 was not restored by BCDT. Hence, these results suggest that BCDT leads to the repopulation of B cells with partial recovery in their regulatory function.

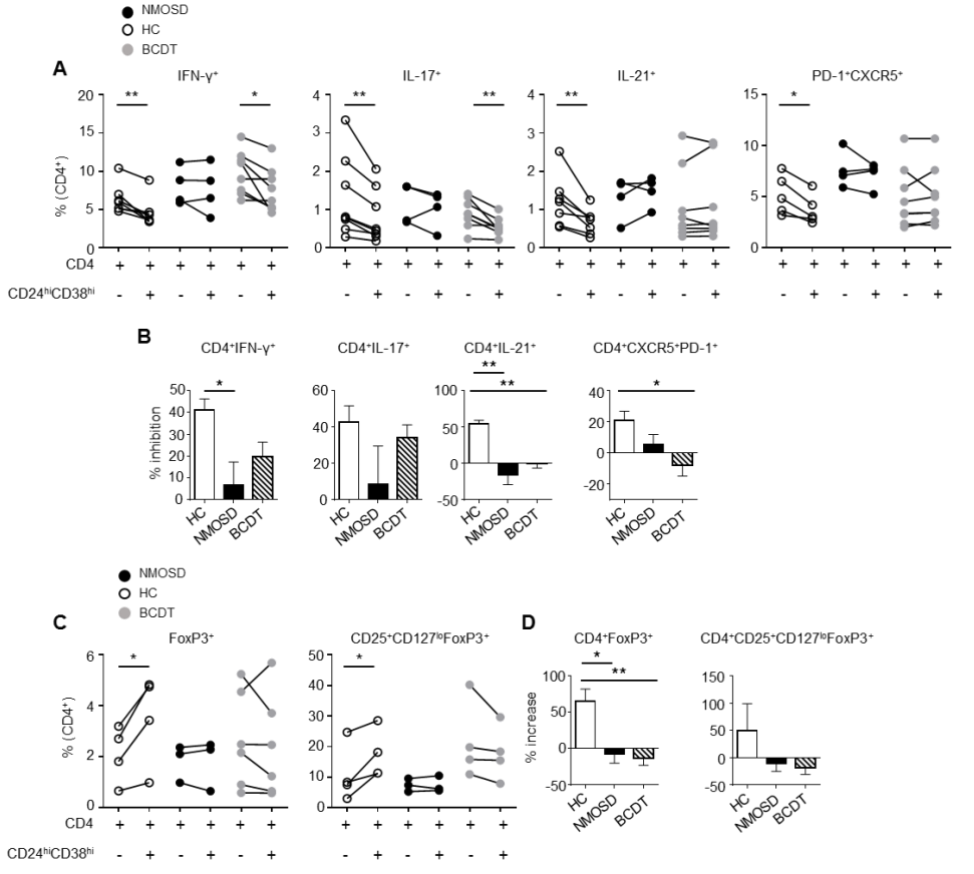


Figure 23. CD24^{hi}CD38^{hi} B cells of B cell depletion therapy (BCDT) treated patients inhibit IFN-γ and IL-17 production by CD4^{+} T cells.

CD24^{hi}CD38^{hi} B cells and CD4^{+}CD25^{-} T cells from 4 NMOSD patients (non-BCDT patients), and 8 BCDT-treated patients, and 8 HCs were sorted by flow cytometry. CD24^{hi}CD38^{hi} B cells were cultured 1:1 with CD4^{+}CD25^{-} T cells for 72 h with plate-bound anti-CD3 mAb (0.5 μg/mL). PMA + ionomycin + Golgistop was added in the last 6 h of culture.

A. Graph showing the frequency of IFN-γ^+, IL-17^+, IL-21^+, and PD-1^+CXCR5^+ $CD4^+$ T cells when $CD4^+$ T cells were cultured alone and in the presence of $CD24^{hi}CD38^{hi}$ B cells.

B. Bar graph showing the percentage decrease in cytokine production by $CD4^+$ T cells and in the frequency of Tfh cells post addition of $CD24^{hi}CD38^{hi}$ B cells to $CD4^+$ T cell culture.

C. Graph showing the frequency of $CD4^+FoxP3^+$ and $CD4^+CD25^+CD127^{lo}FoxP3^+$ cells when $CD4^+$ T cells were cultured alone and in the presence of $CD24^{hi}CD38^{hi}$ B cells. Representative results for 3 NMOSD patients (non-BCDT patients), 6 BCDT-treated patients, and 4 HCs.

D. Bar graph showing the percentage increase in FoxP3 expression by addition of $CD24^{hi}CD38^{hi}$ B cells to $CD4^+$ T cells.

Values are expressed as mean ± SEM. Differences were evaluated by two-way ANOVA (A and C) or one-way ANOVA (B and D) *$p < 0.05$; **$p < 0.01$.

IV. DISCUSSION

The recent discovery of Bregs has drawn attention, and many studies have focused on deciphering their role in autoimmune diseases. In this study, we showed that Bregs derived from patients with NMOSD have impaired regulatory function. Unlike HCs, $CD24^{hi}CD38^{hi}$ B cells derived from NMOSD patients lacked suppressive effects on IFN-γ, IL-17, and IL-21 production by $CD4^{+}$ T cells. In addition, they failed to inhibit Tfh cell expansion and effectively induce Tregs. We also demonstrated that both numerical deficiency of $CD24^{hi}CD38^{hi}$ B cells and their cell-intrinsic impairment in response to CD40 stimulation, leading to impaired IL-10 production, underly such defects. Consistent with our results, previous studies have reported a lower percentage of $CD24^{hi}CD38^{hi}$ B cells in NMOSD patients than healthy individuals (*32*). We further showed that repeated BCDT treatment restores Breg function along with restoration of numerical deficiency and IL-10 production.

A previous study has shown that reappeared B cells following BCDT were significantly enriched in $CD24^{hi}CD38^{hi}$ B cells in NMOSD (*65*). However, a small number of patients (n = 9) were involved, and a longitudinal study was followed for only 12 months. In cross-sectional studies with a larger number of patients (n = 84) and long-term, 3-year longitudinal studies, we showed that $CD24^{hi}CD38^{hi}$ B cells constitute the majority of repopulated B cells following BCDT.

In our data, B cells that appear during the early stage (6-12 weeks post injection) of repopulation are $CD27^{+}$ memory B cells. These memory B cells were not $CD24^{hi}CD38^{-}$ subsets, well-known as primarily memory type, but $CD24^{-}CD38^{-}$ which are newly defined memory B cells (*66*). Other B cell subset observed during the early repopulation phase of BCDT was $CD24^{-}CD38^{hi}$ B cells, which are known as plasmablasts. $CD24^{-}CD38^{-}$ B cells were significantly increased in the elderly, but more interestingly, this population is constituted of $IgD^{-}CD27^{+}$

switched memory and $IgD^{-}CD27^{-}$ double negative B cells, which are antigen-experienced types. In addition, they are positive for CD5 whose expression is associated with the presence of the BCR, suggesting a marker of antigen exposure.

Several studies have shown residual B cells following BCDT were of memory type (*67-69*). In line with our findings, other studies have observed expansion of memory B cells in the repleting B cell pool (*67, 70, 71*). Induction of EAE with MOG $protein_{1-117}$ represents a model in which B cells are activated and MOG $peptide_{35-55}$-induced EAE represents a purely T cell-mediated model (*61*). An important study on B cell repletion kinetics post BCDT in MOG $protein_{1-117}$-induced EAE and MOG $peptide_{35-55}$-induced EAE showed that the environment in which B cells repopulate is critical as the reemerging B cells post BCDT in MOG protein-induced model showed considerable increase in the frequency of differentiated, antigen-experienced B cells with a reduced BCR diversity (*72*). Hence, just as suggested in animal studies, CNS autoantigen exposure in humans may promote expansion of memory B cells from reemerging B cells.

B cells can modulate immune responses by regulating their cytokine profiles, depending on different physiological conditions. When B cells are appropriately stimulated through both CD40 and BCR, they produce proinflammatory cytokines, such as TNF-α, IL-6, and lymphotoxin, to amplify T and B cell responses. On the other hand, activation through CD40 alone leads to significant production of IL-10 to suppress the early immune response and reduce inappropriate bystander activation of B cells (*73*). We showed that NMOSD B cells fail to induce IL-10 upon CD40 stimulation and showed significantly lower IL-10 production compared with HCs. Upon CD40 + BCR activation, NMOSD B cells showed excessive production of IL-6 and TNF-α compared with HC B cells. These results suggest that NMOSD B cells respond inappropriately to different immune stimuli.

$CD24^{hi}CD38^{hi}$ B cells require CD40 stimulation to produce IL-10 and exert their regulatory functions (*4*, *7*). We demonstrated that NMOSD $CD24^{hi}CD38^{hi}$ B cells showed an abnormal response to CD40 stimulation, and hence, could not induce IL-10. We further highlight the post-BCDT recovery of impaired IL-10 production by $CD24^{hi}CD38^{hi}$ B cells in response to CD40 stimulation in patients with NMOSD. Consistent with this, CD40 stimulation significantly increased the $CD24^{hi}CD38^{hi}$ B cell-produced IL-10/TNF-α in BCDT-treated patients compared with relapse patients. IL-6 production by $CD24^{hi}CD38^{hi}$ B cells was not altered by BCDT. The studies that evaluated cytokine production by reemerging B cells in NMOSD post BCDT are scarce, however, in a study on the evaluation of maturation/activation stage of repopulating B cells following BCDT in MS patients, the majority of returning B cells were $CD24^{hi}CD38^{hi}$ B cells but highly activated and produced more IL-6 compared to B cells pre-BCDT, although there was no difference in the IL-10 and TNF-α level (*70*).

A previous study reported that induced IL-10 production is transient, as IL-10 producing B cells lose their capacity to produce IL-10 after re-stimulation. Moreover, during the acute relapse phase, $CD4^+$ T cells show elevated CD40L levels in patients with NMOSD (*74*). Hence, continuous exposure of $CD24^{hi}CD38^{hi}$ B cells to CD40L *in vivo* may render them hyporesponsive, leading to a loss of their capacity to produce IL-10 in NMOSD. In other autoimmune diseases, CD40 downregulation in B cells was observed within a week after BCDT initiation (*75*). In addition, the mRNA level of CD40L on T cells was found to be reduced following BCDT (*76*), implying that a reduction in the expression of CD40 and CD40L following BCDT could lead to reversion in the CD40–CD40L desensitization, allowing normal responsiveness of repopulated $CD24^{hi}CD38^{hi}$ B cells to CD40 stimulation. Another possibility is that newly repopulated $CD24^{hi}CD38^{hi}$ B cells may not have received adequate CD40 stimulation during the acute relapse phase, resulting in normal IL-10 production in response to CD40 stimulation.

Further, we observed that HC $CD24^{hi}CD38^{hi}$ B cells produced TNF-α and IL-6 in addition to considerable production of IL-10 upon CD40 stimulation. In line with our findings, previous studies have observed that $CD24^{hi}CD38^{hi}$ B cells can produce proinflammatory cytokines (TNFα and IL-6) (*39*, *40*), arguing against the entirely anti-inflammatory capacity of these cells. The ratios of IL-10/TNF-α and IL-10/IL-6 produced by $CD24^{hi}CD38^{hi}$ B cells from the relapse group were significantly reduced compared to those of HCs, suggesting an imbalance in the $CD24^{hi}CD38^{hi}$ B cell cytokine production during relapse. The CD40–CD40L interaction promotes B cell proliferation, maturation, and antibody production. This interaction also plays a critical role in the development of autoimmune diseases (*77*). An animal study showed that CD40L-knockout mice did not develop experimental autoimmune encephalomyelitis (EAE) after immunization (*78*). Therefore, the imbalance between the pro- and anti-inflammatory cytokines produced by NMOSD $CD24^{hi}CD38^{hi}$ B cells in response to CD40–CD40L interaction may affect their function in an autocrine manner. Indeed, NMOSD $CD24^{hi}CD38^{hi}$ B cells failed to suppress IFN-γ, IL-17, and IL-21 production by $CD4^{+}$ T cells as well as the expansion of $CD4^{+}PD\text{-}1^{+}CXCR5^{+}$ cells. $CD24^{hi}CD38^{hi}$ B cells derived from healthy individuals were found to inhibit IFN-γ (*4*), IL-17 (*34*), and IL-21 production by $CD4^{+}$ T cells, as well as Tfh cell expansion (*5*, *79*).

A previous study found that healthy $CD24^{hi}CD38^{hi}$ B cells suppressed Tfh cell differentiation through IL-10, whereas this suppressive effect was significantly reduced in $CD24^{hi}CD38^{hi}$ B cells from patients with primary Sjögren's syndrome with an impaired IL-10-producing capacity (*5*). The same study found Breg cells to suppress both Th1 and Th17 cells in the central lymph node of experimental Sjögren's syndrome mice. IL-6 is a multifunctional cytokine that restricts the differentiation of $CD4^{+}$ T cells into Tregs but promotes the differentiation of Th1 cells, Th17 cells, and plasmablasts (*80*, *81*). IL-6 is also a critical component of Tfh cell differentiation (*5*). Hence, a shift in IL-10/IL-6 ratio in NMOSD

$CD24^{hi}CD38^{hi}$ B cells may have created an environment favorable for Th1, Th17, and Tfh cell maintenance rather than suppression.

The inhibitory effect of HC $CD24^{hi}CD38^{hi}$ B cells on IFN-γ and IL-21 production by $CD4^+$ T cells was largely dependent on IL-10; however, no effect was observed on IL-17. It is conceivable that regulatory B cells, similar to Tregs, exert their inhibitory effects via multiple mechanisms. Thus, while the release of IL-10 is vital in the regulation of Th1 and Tfh cells, other mechanisms, such as cell-to-cell contact could be responsible for the suppression of IL-17. Indeed, the suppressive mechanism of $CD24^{hi}CD38^{hi}$ B cells was also found to depend partially on CD80 and CD86 (*4*). Moreover, blocking either IL-10 or PD-L1 in a co-culture of $CD24^{hi}CD38^{hi}$ B and $CD4^+$ T cells resulted in a significant increase in IL-17 expression by $CD4^+$ T cells (*34*). Nevertheless, the mechanism of inhibition by $CD24^{hi}CD38^{hi}$ B cells, other than the regulation of IL-10 production, remains to be determined.

In the present study, the induction of Tregs by $CD24^{hi}CD38^{hi}$ B cells seen in healthy individuals was not observed in patients with NMOSD. It has been found that the frequency of $CD4^+FoxP3^+$ cells was not significantly different between NMOSD patients and HCs (*11*), although a significantly decreased FoxP3 mRNA expression was found in PBMCs of patients with NMOSD (*82*). Further studies are required to determine whether B cell-extrinsic deficit in FoxP3 levels in patients with NMOSD is responsible for the lack of Treg induction by $CD24^{hi}CD38^{hi}$ B cells.

As our results suggest that both Breg numerical deficiency and cell-intrinsic deficit in IL-10 production may account for the failed suppressive function of $CD24^{hi}CD38^{hi}$ B cells, we hypothesized that the mechanism underlying BCDT involves repopulation of Bregs with competent regulatory function as well as the elimination of pathogenic B cells. In this regard, the effect of BCDT on activated B cell-mediated EAE model reduced the severity of clinical symptoms, whereas

disease exacerbation was observed in purely T cell-mediated EAE model (*72, 83*). With the reemergence of B cells, a gradual exacerbation was observed in B cell-mediated model while disease severity decreased in T cell-mediated model, suggesting the balance between regulatory and pathogenic B cells is critical in the maintenance of disease activity.

Subsequently, we showed that the suppressive function of $CD24^{hi}CD38^{hi}$ B cells was partially recovered following BCDT as repopulated $CD24^{hi}CD38^{hi}$ B cells suppressed IFN-γ and IL-17 production by $CD4^+$ T cells but failed to induce Tregs, despite the unaltered production of IL-6 by $CD24^{hi}CD38^{hi}$ B cells. This could be because $CD24^{hi}CD38^{hi}$ B cells suppress $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells through IL-10 and PD-L1 (*34*). PD-L1 was highly expressed on B cells following BCDT in EAE and following *ex vivo* B cell depletion in human PBMCs (*51*). Hence, enhanced production of IL-10 and PD-L1 upregulation in repopulated $CD24^{hi}CD38^{hi}$ B cells could have contributed to the restoration of suppressive capacity of $CD24^{hi}CD38^{hi}$ B cells against $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells while overcoming the effect of IL-6. In contrast, the repopulated $CD24^{hi}CD38^{hi}$ B cells did not suppress Tfh cells and Tregs. Previous studies have found that BCDT decreases the frequency of circulating Tfh cells while increasing the frequency of Tregs (*14, 84*). Such features following BCDT could have affected Breg function. Evaluation of possible T cell-intrinsic deficit specific to NMOSD patients would help to explain the lack of suppressive effects of NMOSD $CD24^{hi}CD38^{hi}$ B cells.

There are some potential limitations in this study. Although the strength of this study was a long-term longitudinal analysis of B cell repopulation at multiple time points (every 6 weeks), the sample size is small. We lack data on how the frequency of $CD24^{hi}CD38^{hi}$ B cells changes when samples are obtained on time points longer than 1 year of depletion and following BCDT discontinuation. Moreover, it would be interesting to compare the frequency of $CD24^{hi}CD38^{hi}$ B

cells and their function between patients in the 1st cycle of BCDT and patients who have undergone long-term BCDT for more than 10 years, which will provide the effect of long-term BCDT on $CD24^{hi}CD38^{hi}$ B cells. In addition, our functional study on $CD24^{hi}CD38^{hi}$ B cells was performed with a limited number of relapse patients. An interesting finding highlighted in our *ex vivo* analysis is that patients who experienced relapse following BCDT had low percentages of $CD24^{hi}CD38^{hi}$ B cells (1.34% and 0.81%). This finding may imply that the numerical restoration of Bregs following BCDT may not be simply due to the B cell repopulation process but also reflects the disease activity of patients. In patients who experience relapse following BCDT, it remains to be elucidated whether functional impairment along with the numerical deficiency in Bregs occurs. Lastly, evaluation of presence of Bregs in the brain or spinal cord lesions in NMOSD patients would address the role of Bregs at the site of inflammation. However, such studies are extremely difficult to perform as the data can only be obtained from post-mortem tissues.

In conclusion, in NMOSD, where autoreactive T and B cells interact and contribute to disease pathology, the low number of $CD24^{hi}CD38^{hi}$ B cells and their impaired regulatory function fail to restrict inflammation and further contribute to disease exacerbation. Following B cell depletion, the newly repopulated B cells comprise a greater number of functionally competent $CD24^{hi}CD38^{hi}$ B cells, along with the removal of pathogenic B cells, restores the immune balance in favor of tolerance.

V. CONCLUSION

In conclusion, in MS and NMOSD, where autoreactive T and B cells interact and contribute to disease pathology, Breg deficiency was observed during relapse. Following, treatment with CD52- or CD20-monoclonal antibody therapies, such deficiency was restored. In further functional study, the low number of $CD24^{hi}CD38^{hi}$ B cells and their impaired regulatory function fail to restrict inflammation and further contribute to disease exacerbation. Following B cell

depletion, the newly repopulated B cells comprise a greater number of functionally competent $CD24^{hi}CD38^{hi}$ B cells, along with the removal of pathogenic B cells, restores the immune balance in favor of tolerance.

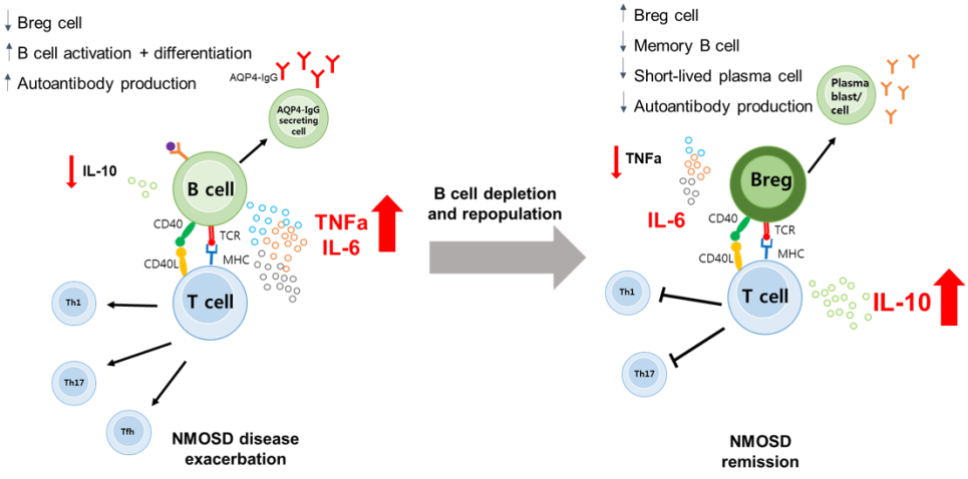


Figure 24. Graphical summary.

Reduced function or numbers of regulatory B (Breg) cells promote pro-inflammatory circuits involving the increased activation of T helper 17 (Th17) cells and Th1 cells and the production of pathogenic antibodies. B cell depletion results in reductions in the numbers of short-lived palsmablasts and some memory B cell subsets, in the production of antibodies and pro-inflammatory cytokines and in antigen presentation to T cells. Following B cell reconstitution with immature B cells, regulatory circuits are re-established, leading to the increased production of IL-10. These effects diminish responses by Th17 cells and Th1 cells, augment the function of regulatory T cells and promote autoimmune disease remission.

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ABSTRACT (IN KOREAN)

조절 B세포의 다발성경화증 및 시신경척수염 재발-완화 조절기전

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김예슬

항원 특이적이지 않은 bystander 활성화는 B 세포가 IL-10을 생성하여 부적절한 면역반응을 억제하게 하므로 면역반응 조절에서 B세포의 역할은 context-depedent 하다. 하지만, 다발성 경화증과 시신경척수염을 포함한 자가면역질환에서 B 세포의 면역 조절에 기여하는 역할은 아직 정확하게 밝혀지지 않았다. 다발성 경화증과 시신경척수염은 중추신경계의 자가면역-탈수초 질환이며 재발-완화 과정을 반복한다. 다발성경화증의 대표적인 면역억제제 중 하나인 알렘투주맙 (Lemtrada, Genzyme, MA. USA)은 CD52에 결합하는 humanized monoclonal IgG1 antibody이다. 알렘투주맙은 CD52를 발현하는 세포들을 선택적으로 표적하여 표적세포를 depletion 시켜 질병활성도를 낮추고 오래 지속되게 한다. 하지만, 명확한 mechanism of action은 아직 밝혀지지 않았으며, repopulation된 B세포의 distribution, 특히 조절 B세포에 관한 연구들은 드물다. 마찬가지로, B cell depletion therapy (BCDT)는 anti-CD20 monoclonal antibody이며 병원성 B세포를 제거하여 시신경척수염 환자들에게서 효능을 보이지만, 조절 B세포에 미치는 영향에 대해서는 연구가 필요한 실정이다. 따라서, 본 연구에서는 B 세포 subsets, 조절 B 세포의 기능과 이러한 세포들에 면역억제제가 미치는 효과를 중추신경계 탈수초 질환 환자들에게서 평가하고자 했다.

재발 상태의 다발성 경화증 환자의 말초에서 CD24hiCD38hi B 세포의 수적 결핍이 알렘투주맙 치료 후 증가함을 관찰하였다. 조절 B세포의 수적 결핍은 시신경 척수염 환자에게서도 관찰되었으며 특히 bystander 활성화에 의한 IL-10 생성에 세포-본질적인 결손 (cell-intrinsic deficit)을 관찰하였다. 시신경척수염 환자의 CD24hiCD38hi B세포는 CD4+ T 세포의 IFN-ɣ, IL-17, IL-21 생산을 억제하지 못하였으며 Tfh 세포

expansion 억제와 조절 T세포의 증가를 유도하지 못했다. 따라서, 앞선 수적 결핍과 B 세포-intrinsic 한 결점이 CD24hiCD38hi B세포의 기능적 결함을 책임지고 있다는 것을 증명하였다.
단면연구와 3년의 종적 연구에서 BCDT 치료가 조절 B세포의 수적 결핍을 회복시켰으며, BCDT 치료 후 repopulate 된 CD24hiCD38hi B 세포는 IL-10 생성 기능이 회복되었고 CD4+ T 세포의 IFN-ɣ 및 IL-17 생성을 억제하였다.
본 연구에서는 중추신경계 자가면역 질환에서 감소 되어있는 CD24hiCD38hi B 세포가 면역억제제 치료를 받은 후 회복됨을 확인하였다. 또한, CD24hiCD38hi B 세포의 수적 결핍과 손상된 조절 기능은 시신경척수염 병태생리에 기여하며 이러한 impairment는 BCDT 치료 후 회복됨을 보여주었다.

핵심되는 말 : 자가면역질환, 조절 B세포, 시신경척수염, 다발성 경화증, B cell depletion therapy, 면역억제제

PUBLICATION LIST

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