B-Cell Activation Influences T-Cell Polarization and Outcome of Anti-CD20 B-Cell Depletion in Central Nervous System Autoimmunity

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Objective: Clinical studies indicate that anti-CD20 B-cell depletion may be an effective multiple sclerosis (MS) therapy. We investigated mechanisms of anti-CD20-mediated immune modulation using 2 paradigms of experimental autoimmune encephalomyelitis (EAE).
Methods: Murine EAE was induced by recombinant myelin oligodendrocyte glycoprotein (rMOG), a model in which B cells are considered to contribute pathogenically, or MOG peptide (p)35-55, which does not require B cells.
Results: In EAE induced by rMOG, B cells became activated and, when serving as antigen-presenting cells (APCs), promoted differentiation of proinflammatory MOG-specific Th1 and Th17 cells. B-cell depletion prevented or reversed established rMOG-induced EAE, which was associated with less central nervous system (CNS) inflammation, elimination of meningeal B cells, and reduction of MOG-specific Th1 and Th17 cells. In contrast, in MOG p35-55-induced EAE, B cells did not become activated or efficiently polarize proinflammatory MOG-specific T cells, similar to naive B cells. In this setting, anti-CD20 treatment exacerbated EAE, and did not impede development of Th1 or Th17 cells. Irrespective of the EAE model used, B-cell depletion reduced the frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg), and increased the proinflammatory polarizing capacity of remaining myeloid APCs.
Interpretation: Our study highlights distinct roles for B cells in CNS autoimmunity. Clinical benefit from anti-CD20 treatment may relate to inhibition of proinflammatory B cell APC function. In certain clinical settings, however, elimination of unactivated B cells, which participate in regulation of T cells and other APC, may be undesirable. Differences in immune responses to MOG protein and peptide may be important considerations when choosing an EAE model for testing novel B cell-targeting agents for MS.

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The central nervous system (CNS) has traditionally been viewed as an immune-privileged compartment with limited and well-controlled access for immune cells. B cells and plasma cells, however, are commonly found in active multiple sclerosis (MS) lesions,[1] and the presence of oligoclonal antibodies within the cerebrospinal fluid remains a hallmark finding in the diagnosis of MS. Myelin-specific antibodies have been identified in areas of vesicular

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demyelination,[2] suggesting that they directly promote CNS damage. The observation that plasma exchange was beneficial in MS patients with histologic evidence of CNS antibody deposition[3] provided further support for a pathogenic role of antibodies. Besides serving as the source for antibody-secreting plasma cells, B cells express major histocompatibility complex (MHC) class II molecules constitutively and may participate as antigen-presenting cells (APCs). B cells are capable of processing native antigen and are very efficient APCs when they recognize the same antigen as the responding T cells.[4,5] As processing of native myelin antigen by CNS resident or infiltrating APCs is required for initiation of CNS autoimmune inflammation and clinical disease,[6,7] myelin-specific B cells may have an important role in the activation of encephalitogenic T cells in the pathogenesis of CNS autoimmune disease.

With greater appreciation that B cells may have dual humoral and cellular roles in MS pathogenesis, interest in use of selective B cell-depleting agents for therapy has intensified.[8,9] Promising results were obtained in clinical trials testing a monoclonal antibody targeting CD20 (Rituxan), a cell surface protein that is expressed on immature and mature B cells, but not on differentiated plasma cells. Treatment with Rituxan was beneficial in patients with relapsing-remitting MS[8] and in a subgroup of primary progressive MS patients with evidence of active CNS inflammation.[9] Anti-CD20–mediated B-cell depletion was also clinically beneficial in a small open-label study in patients with neuromyelitis optica (NMO),[10] a CNS demyelinating disease associated with aquaporin-4–specific antibodies. The purpose of our investigation was to elucidate the immunologic consequences of anti-CD20 therapy in 2 related models of experimental autoimmune encephalomyelitis (EAE).[11] In 1 model, EAE was induced by immunization with recombinant myelin oligodendrocyte glycoprotein (rMOG), which generates a population of antigen-activated B cells and promotes development of antibodies against MOG protein. B-cell depletion prevented rMOG-induced EAE and reversed paralysis when treatment was initiated after EAE onset. In established EAE, anti-CD20–depleted B cells within the CNS. B-cell depletion decreased the frequency of peripheral and CNS encephalitogenic Th1 and Th17 cells and was associated with reduced serum titers of myelin-specific antibodies. These findings highlight the pathogenic role of activated B cells in CNS autoimmune disease, and provide mechanisms of action in support of B-cell depletion for treatment of MS.

In the second model, EAE was induced by immunization with MOG peptide (p) 35-55, which binds MHC II directly on lymphoid APC without processing,[6] and leads to peripheral activation of encephalitogenic T cells.[6,12] Using this protocol, considered B-cell independent, MOG protein-specific B cells were not activated. In contrast to the benefit observed in EAE elicited by MOG protein, B-cell depletion exacerbated clinical and histologic EAE in this model, and development of Th1 and Th17 cells was not dampened. In both rMOG and peptide-induced EAE, CD20-mediated B-cell depletion reduced the frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) and augmented the proinflammatory function of remaining myeloid APCs. These observations indicate that in the absence of proinflammatory B cell function, depletion of unactivated (naive) B cells may not be advantageous. The results of this study highlight key differences between MOG protein and MOG peptide EAE models, and underscore the importance of B-T crosstalk in pathogenesis and regulation of CNS autoimmunity.

Materials and Methods

Mice

C57BL/6 female mice, 5 to 8 weeks of age, as well as μMT mice were purchased from Jackson Laboratories (Bar Harbor, MN). hCD20 transgenic (Tg) C57BL/6 mice[13,14] were used for anti-CD20–mediated B-cell depletion. In these mice, hCD20 recapitulates the expression of endogenous murine CD20,[15] and treatment with the murine antihuman CD20 antibody (clone 2h7) results in rapid depletion of B cells (Gong et al,[13] Supplementary Fig 1A). Untreated or isotype control-treated hCD20 Tg mice developed EAE indistinguishable from wild-type mice (see Supplementary Fig 1B). C57BL/6 MOG 35-55–specific T-cell receptor (TCR) Tg mice[16] were kindly provided by V. K. Kuchroo (Harvard). JHT mice[17] were obtained from K. Rajewsky (Harvard).

Peptides

Mouse MOG p35-55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Auspep (Parkville, Australia). Recombinant mouse MOG (1-117) was synthesized, purified and refolded as previously described.[18] Ovalbumin (OVA) p323-339 (ISQAVHAAHAEINEAGR) was synthesized by Abgent, Inc. (San Diego, CA). Intact OVA was purchased from Sigma-Aldrich (St. Louis, MO).

EAE Induction

Eight to 12-week-old female C57BL/6 or hCD20 Tg C57BL/6 mice were injected subcutaneously with 25μg MOG p35-55 or 100μg rMOG 1-117 in complete Freund adjuvant (DIFCO Laboratories, Detroit, MI). After immunization and 48 hours later, mice received an intravenous injection of 200ng pertussis toxin. Individual animals were observed daily and clinical scores were assessed as follows: 0 = no clinical disease, 1 = loss of tail tone only, 2 = mild monoparesis or paraparesis, 3 = severe paraparesis, 4 = paraplegia and/or quadraparesis, and 5 = moribund or death.

Anti-CD20 Treatment

To ensure maximal B-cell depletion when examining anti-CD20 in prevention of EAE, anti-CD20 mice received weekly intraperitoneal (i.p.) injections of 200μg of a-hCD20 monoclonal antibody

(m2h7) or antiragweed immunoglobulin (Ig)G2a-isotype control monoclonal antibody starting 21 days prior to immunization. For evaluation of anti-hCD20 treatment in established EAE, mice were randomized to weekly treatment once they developed an EAE disease score ≥ 2.

Detection of Anti-MOG Antibodies

Serum was obtained from mice treated with a-hCD20 or isotype control (IgG2a) prior to treatment onset and weekly thereafter. 96-Maxisorb plates (Costar, Corning, NY) were coated with MOG p35-55 or rMOG 1-117 (10μg/ml in phosphate-buffered saline) and then blocked with bovine serum albumin (Sigma-Aldrich). Plate-bound antibodies were detected with horseradish-peroxidase–conjugated anti-mouse IgG (cross-reactive with all Ig isotypes; 1:6,000; Sigma-Aldrich). The antibody titers were quantified at the serum dilution indicated using commercially available anti-MOG 8.18C-5 (Millipore, Bedford, MA) as the standard. Plates were read at 450nm wavelength. SOFTmax enzyme-linked immunosorbent assay (ELISA) plate reader and software (Molecular Devices Corporation, Sunnyvale, CA) were used for data analysis.

T-Cell Coculture Assays

For B cell–T cell coculture assays, B cells were magnetically activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany)-separated from lymph nodes or spleens. Following separation, B cells were evaluated for purity (>99%) by fluorescence-activated cell sorting (FACS) staining for B220. For T-cell coculture assays using remaining splenocytes as APCs, hCD20 Tg mice received weekly injections of 0.2mg of a-hCD20 or IgG2a-isotype starting 21 days prior to immunization with rMOG protein or MOG p35-55 peptide. Twelve days after immunization, spleens were isolated, and $B220^+$ B cells and $CD3^+$ T cells were removed by MACS separation. In the presence of rMOG 1-117 or MOG p35-55, 5×10^5 B cells or remaining splenocytes were cocultured with 1×10^4 naive T cells isolated from MOG TCR Tg mice. After 72 hours, T-cell differentiation was evaluated by FACS or ELISA.

FACS Analysis

B cells were examined by FACS analysis after staining with antibodies specific for B220, CD95 (FAS), GL7, or CD21. B220, CD95, and FAS antibodies were purchased from Pharmingen (San Diego, CA), and anti-CD21 was purchased from eBioscience (San Diego, CA). Proinflammatory T cell differentiation was evaluated by surface staining for CD3 (Pharmingen) and intracellular cytokine staining (ICS) for interferon (IFN)-γ and interleukin (IL)-17 (eBioscience). Activation of monocytic cells was evaluated by surface staining for CD11b (Pharmingen) and ICS for tumor necrosis factor (TNF) or IL-10 (eBiosience). Induction of Treg was evaluated by FACS staining for CD4 (GK1.5), CD25, and Foxp3 (eBiosience).

Cytokine Analysis

Culture supernatants were collected for cytokine analysis at 48 hours (IFN-γ) or 72 hours (IL-17), respectively, and analyzed by ELISA (Pharmingen). The results for ELISA assays are expressed as an average of triplicate wells $\pm$ standard error of the mean (SEM). SOFTmax ELISA plate reader and software (Molecular Devices Corporation, Sunnyvale, CA) were used for data analysis.

Histopathology and Immunohistochemistry

Brains and spinal cords of mice were fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned. Representative sections were stained with Luxol fast blue (LFB) or hematoxylin and eosin (H&E), or evaluated for B-cell or T-cell infiltration by B220 or CD3 immunohistochemistry, respectively. H&E-stained sections (inflammation) and LFB-stained sections (demyelination) were scored on a scale of 0 to 4. B220- and CD3-stained sections were evaluated by morphometric image analysis. Final results were reported as B220- or CD3-stained cells per mm^2 of spinal cord area.

Statistical Analysis

Data are presented as mean $\pm$ SEM. For clinical scores, significance between groups was examined using the Mann-Whitney U test. A value of $p < 0.01$ was considered significant. All other statistical analysis was performed using a 1-way multiple-range analysis of variance test for multiple comparisons. A value of $p < 0.01$ was considered significant.

Results

Naive and MOG-Primed B Cells Differ in Their Capability to Serve as APCs

Two different EAE models were examined in this report. In EAE induced by immunization with MOG protein (rMOG 1-117), internalization and processing by APCs is required for presentation of its encephalitogenic determinant to pathogenic $CD4^+$ T cells.[6] In this model, B cells become activated through recognition of MOG protein via B-cell receptor (BCR) engagement. As shown in Fig 1A, when used as APCs for presentation of MOG protein, B cells isolated from MOG protein-immunized mice efficiently stimulated MHC II-restricted $CD4^+$ T cells that recognize the encephalitogenic MOG p35-55. Following activation, B cells developed into plasma cells that secreted antibodies directed against MOG (Marta et al[19]; see Fig 1B). Therefore, immunization by this protocol activates both cellular and humoral components of B-cell immunity.

Unlike antigen presentation of rMOG, MHC II-restricted T-cell recognition of the MOG p35-55 does not require internalization and processing by APCs.[6] Instead, naive B cells, independent of their BCR specificity, are capable of presenting short peptides through direct binding to their cell surface MHC II molecules. As shown in Figure 1A, B cells from mice immunized with MOG p35-55, like naive B cells, were capable of presenting MOG p35-55, but not MOG protein, to MOG-specific T cells. Further, immunization with MOG p35-55 did not efficiently lead to expansion

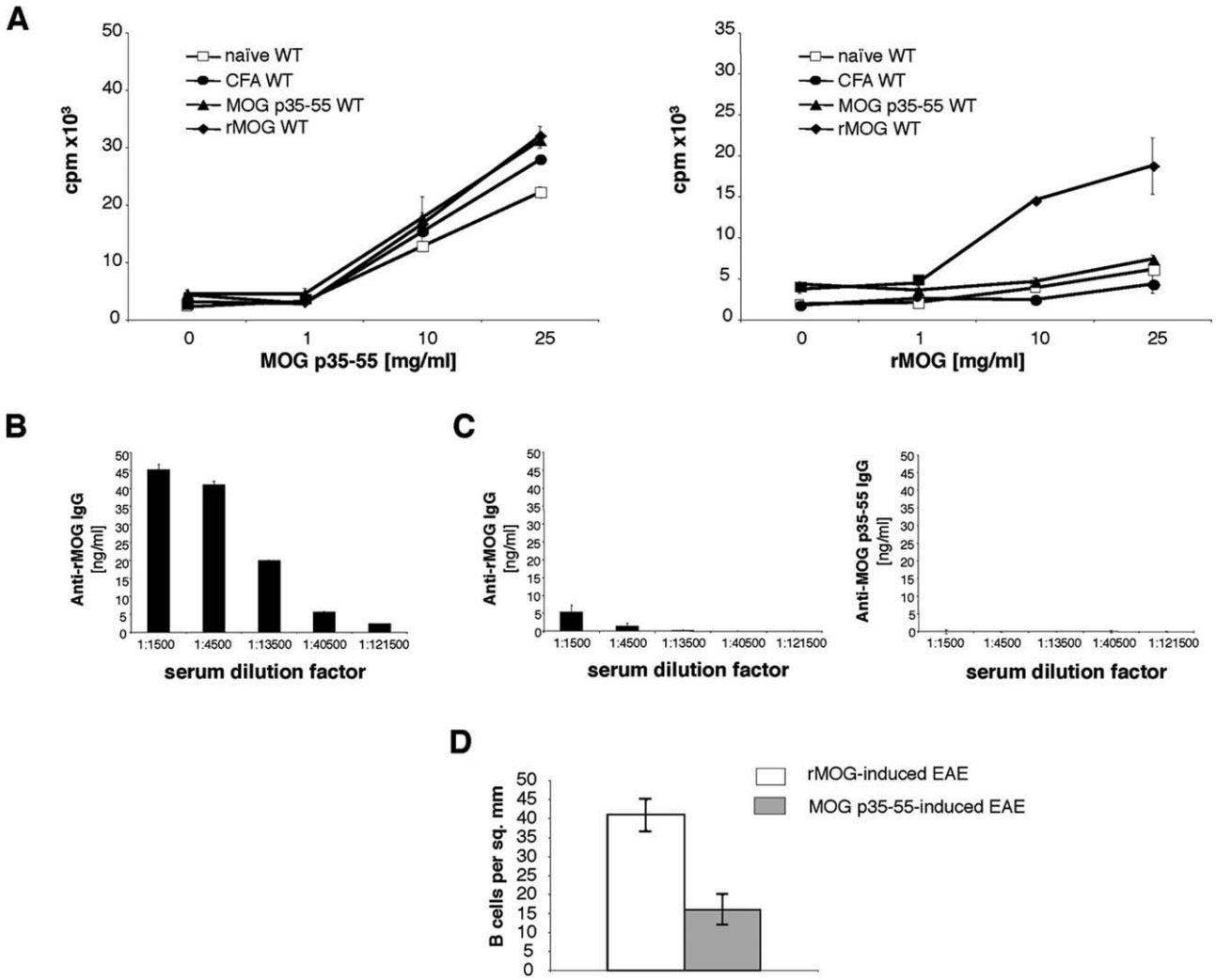

FIGURE 1: Immunization with myelin oligodendrocyte glycoprotein (MOG) protein, but not MOG p35-55, promotes efficient B-cell antigen-presenting cell function and development of myelin-specific antibodies. (A) Magnetically activated cell sorting-separated B cells (purity >95%) isolated from unimmunized (naive) C57BL/6 mice or mice that had been immunized with complete Freund's adjuvant (CFA), MOG p35-55, or recombinant MOG (rMOG) protein were cocultured with naive T cells isolated from MOG T cell receptor transgenic mice in the presence of MOG p35-55 (left panel) or rMOG protein (right panel). T-cell proliferation was evaluated by H^3-Thymidine-incorporation. C57BL/6 mice immunized with (B) rMOG or (C) MOG p35-55 were bled 55 days after immunization. Serum titers against rMOG (B and C, left panel) or MOG p35-55 (C, right panel) were evaluated. (D) Greater numbers of B cells were detected within the central nervous system (CNS) in EAE induced by rMOG than in experimental autoimmune encephalomyelitis (EAE) induced by MOG p35-55. EAE was induced in C57BL/6 mice by immunization with rMOG (100μg) or MOG p35-55 (25μg). CNS B cells in mice with EAE (10/group) were examined by immunohistochemical staining for B220 on day 25 after immunization. cpm = count per minute; WT = wild type; CFA = complete Freund's adjuvant; Ig = immunoglobulin.

of MOG-specific B cells, and was not associated with a significant antibody response (Lyons et al[20]; see Fig 1C).

Kinetics of Anti-CD20–Mediated B-Cell Depletion Differs in Distinct Tissue Microenvironments

Anti-CD20 treatment was investigated in human (h) CD20 Tg C57BL/6 mice.[13,14] These mice develop EAE in a manner that is indistinguishable from wild-type C57BL/6 mice (see Supplementary Fig 1). Data indicate that kinetics of B-cell depletion in different tissue microenvironments may depend on vascular access of anti-CD20 antibodies.[13] Depletion of mature ($B220^+CD21^+$) B cells was examined in blood, bone marrow, lymph nodes, spleen, and the peritoneal cavity at various time points following a single anti-CD20 treatment of unimmunized hCD20 Tg mice. A hierarchy in tissue susceptibility to CD20-mediated B-cell depletion was evident[13]; reduction of B cells was detected in blood and bone marrow at 3 hours, and in lymph nodes and spleen at 2 days (Fig 2). B-cell depletion in the peritoneum was slower; at 2 days, peritoneal B cells were reduced by approximately 30%, and at 7 days by 95%. There was >99%

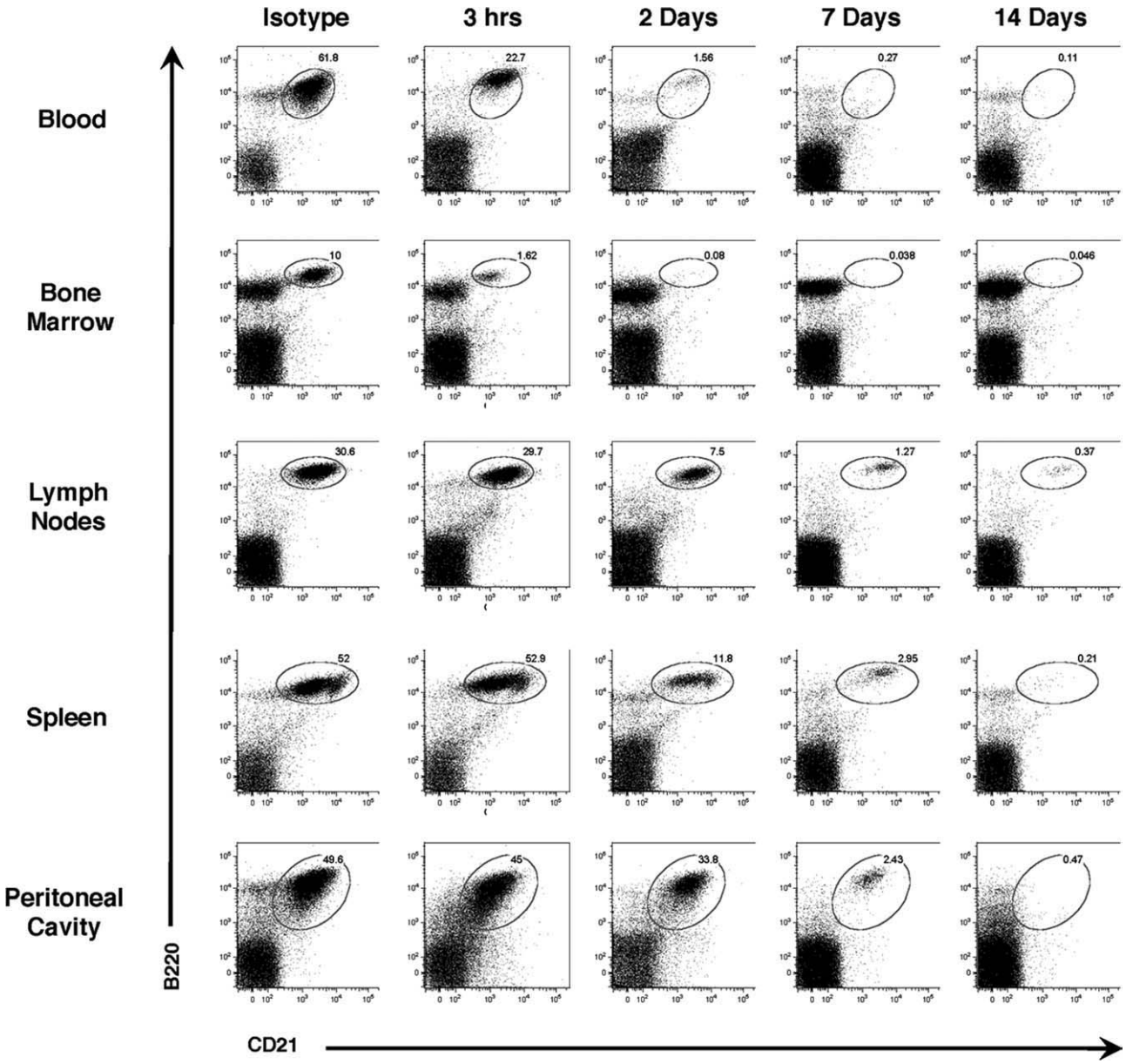

FIGURE 2: Kinetics of anti-CD20–mediated B-cell depletion differs in distinct tissue microenvironments. C57BL/6 hCD20 Tg mice[13,14] were injected intraperitoneally with 200μg murine anti-hCD20 monoclonal antibody (m2H7) or isotype control monoclonal antibody. Cells from blood, bone marrow, lymph nodes, spleen, and the peritoneal cavity were harvested at the indicated time points. Cells were stained with anti-B220 (pan–B-cell marker) and anti-CD21 (a mature B-cell marker), then examined by FACS analysis. Results shown are representative of 2 experiments (2–3 mice/time-point/experiment).

depletion of $B220^{+}CD21^{+}$ B cells in all immune and nonimmune tissues examined 14 days postinjection. To ensure maximal B-cell depletion when anti-CD20 treatment was evaluated for EAE prevention, this antibody was administered weekly starting 3 weeks in advance of immunization.

Anti-CD20 Treatment Depletes B Cells within the CNS and Prevents or Reverses EAE Induced by MOG Protein

Given that B cells responded differently to MOG protein and MOG peptide, we postulated that anti-CD20 treatment might lead to divergent clinical and immunologic outcomes. Anti-CD20–mediated B-cell depletion reduced clinical severity of MOG protein-induced EAE when treatment began prior to disease induction (Fig 3A, Table 1). Similarly, treatment of established EAE reversed paralysis. In these mice, anti-CD20 treatment depleted 60% of B cells within established CNS lesions, which was reflected by a 70% reduction of B cells within meningeal lesions (see Fig 3B, C).

The potential influence of CD20 B-cell depletion in MOG protein-induced EAE on proinflammatory T-cell

TABLE 1: Clinical Responses to Anti-CD20 B-Cell Depletion in rMOG-Induced EAE

	Incidence	Mean Day of Onset (± SEM)	Mean Max Severity (± SEM)	Mean Severity (± SEM)	Mean Severity (± SEM)	Mean Severity (± SEM)
Prevention, days after immunization				13	17	27
Isotype	12/13	11.67 (±0.40)	4.00 (±0.20)	3.13 (±0.35)	2.96 (±0.22)	3.04 (±0.24)
Anti-CD20	13/13	13.00 (±2.85)	3.00 (±0.23)	2.08 (±0.43)	1.75 (±0.25)	1.79 (±0.22)
Treatment, days from start of anti-CD20				0	+4	+14
Isotype	11/11	14.73 (±1.51)	3.86 (±0.18)	2.32 (±0.46)	3.14 (±0.18)	2.60 (±0.21)
Anti-CD20	11/11	14.55 (±1.52)	3.59 (±0.27)	2.32 (±0.26)	2.64 (±0.24)	1.64 (±0.37)

Results are representative of 5 separate experiments (10–13 mice/group/experiment).
rMOG = recombinant myelin oligodendrocyte glycoprotein; SEM = standard error of the mean; Max = maximum.

and humoral responses was examined. In general, in untreated mice with EAE, the frequency of IL-17–producing cells was much lower in the periphery than within the CNS, consistent with observations by other investigators.[21] In anti-CD20 treatment, the frequencies of Th1 and Th17 cells were reduced in the periphery and, to a greater extent, within the CNS (Fig 4A). The absolute numbers of CNS $CD3^+$ T cells were not significantly altered in treatment of established EAE when paralysis was reversed (Supplementary Fig 2), suggesting that anti-CD20 treatment did not initially reduce CNS influx of T cells. Amelioration of established MOG protein-induced EAE by anti-CD20 was associated with a reduction of serum antibody titers directed against rMOG (see Fig 4B). In addition to serving as a source for antibody-secreting plasma cells and as APCs, B cells may participate in homeostasis of regulatory T cells.[22] In this regard, despite the clinical benefit of anti-CD20 treatment, B-cell depletion was associated with reduced frequency of $CD4^+CD25^+Foxp3^+$ Treg in peripheral lymphoid organs as well as within the CNS (see Fig 4A).

B-Cell Depletion Exacerbates EAE Induced by MOG p35-55

Anti-CD20 treatment was investigated in peptide-induced EAE, a model that does not require B cells for development of EAE. In contrast to anti-CD20 treatment of EAE induced by MOG protein, B-cell depletion initiated either prior to immunization with MOG p35-55, or after onset of paralysis, exacerbated EAE (Fig 5, Table 2). Clinical worsening was associated with more severe CNS inflammation and demyelination, despite the fact that anti-CD20 treatment sufficiently depleted B cells within the CNS. B-cell depletion did not dampen pathogenic Th1 and Th17 responses in this disease model. In fact, clinical worsening in anti-CD20 treatment of MOG p35-55–induced EAE was generally associated with an increase in CNS Th1 cells, Th17 cells, and Th1/Th17 double positive T cells, which may represent a more pathogenic T-cell phenotype.[23,24] Similar to our findings in rMOG-induced EAE, anti-CD20 treatment reduced the frequency of $CD4^+CD25^+FoxP3^+$ Treg in secondary lymphoid organs as well as within the CNS.

B Cells Activated by MOG Protein In Vivo Efficiently Promote Development of Encephalitogenic T Cells

The immunologic mechanisms contributing to the paradoxical clinical outcomes of anti-CD20 depletion in EAE induced by rMOG or MOG p35-55 were examined further. We hypothesized that activated B cells in EAE induced by MOG protein might promote development of proinflammatory T cells, which were eliminated by anti-CD20 treatment. B cells were examined for cell surface expression of FAS, a protein that is upregulated on lymphocytes following antigen receptor engagement, and GL-7, a marker of antigen-primed germinal center B cells.[25] Immunization with MOG protein, but not with p35-55, generated a population of activated B cells that expressed FAS and GL-7 (Fig 6A). Similarly, B cells from mice immunized with the nonencephalitogenic control protein OVA, but not its short peptide OVA p323-339, upregulated FAS and GL7, indicating that B-cell activation was a characteristic associated with immunization with protein. Most importantly, only B cells from MOG protein-immunized mice, but not from unimmunized mice or mice immunized with MOG p35-55, efficiently polarized Th1 and Th17 cells when presenting MOG protein (see Fig 6B). Activated B cells from mice immunized with MOG protein were also more efficient in promoting Th1 and Th17 differentiation of naive MOG p35-55–specific T cells when stimulated with MOG p35-55. Collectively, these results indicate that activated myelin antigen-specific B cells, which are generated in MOG protein-induced EAE, can contribute to encephalitogenic T-cell priming in vivo. Loading of encephalitogenic

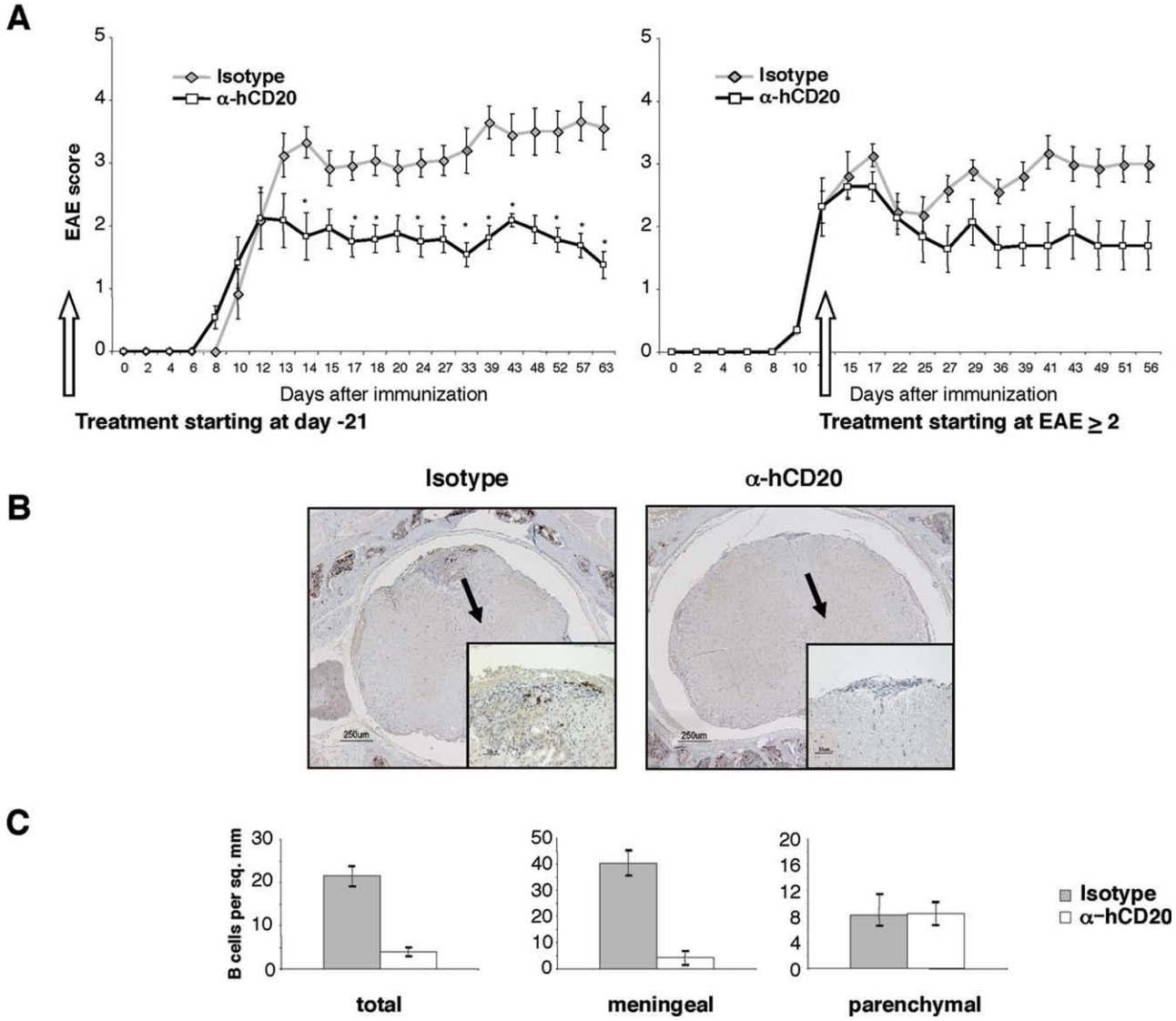

FIGURE 3: Anti-CD20 treatment ameliorates experimental autoimmune encephalomyelitis (EAE) induced by mouse myelin oligodendrocyte glycoprotein. (A) C57BL/6 hCD20 transgenic mice received 200μg anti-hCD20 or isotype control (immunoglobulin G2a) weekly starting 21 days prior to EAE induction (left panel), or after EAE was fully established (EAE score $\geq$2, right panel); white arrows indicate treatment onset. EAE was scored: 0 = no clinical disease, 1 = loss of tail tone only, 2 = mild monoparesis or paraparesis, 3 = severe paraparesis, 4 = paraplegia and/or quadraparesis, and 5 = moribund or death. Results are representative of 5 separate experiments (10–13 mice/group/experiment). (B, C) Mice receiving treatment after EAE was fully established were evaluated for the presence of B cells within spinal cord sections (B220 immunohistochemistry). Shown are (B) representative spinal cord sections and (C) the number of $B220^+$ cells per mm^2 of total (left panel), meningeal (middle panel), or parenchymal (right panel) spinal cord tissue.

peptide onto MHC II molecules expressed on unactivated (naive) B-cell APCs alone does not efficiently promote differentiation of encephalitogenic T cells.

B-Cell Depletion Augments the Capability of Residual APCs to Activate Encephalitogenic T Cells

Data indicate that B cells may communicate with other APCs. For example, it was observed that B cells can capture antigen from lymph node subcapsular macrophages via their BCR, and deliver it to follicular dendritic cells, establishing a role for B cells in antigen transport.[26] Through secretion of anti-inflammatory cytokines, B cells may also locally regulate other APCs.[27] Thus, we evaluated how B-cell depletion influenced the function of remaining APCs. In both EAE models used, $CD11b^+$ cells isolated from mice receiving anti-CD20 treatment produced more proinflammatory TNF and less anti-inflammatory IL-10 (Fig 7A). We then investigated whether this cytokine shift could translate into altered APC function. For this purpose, we isolated spleen cells from CD20 B cell-depleted or isotype (control)-treated

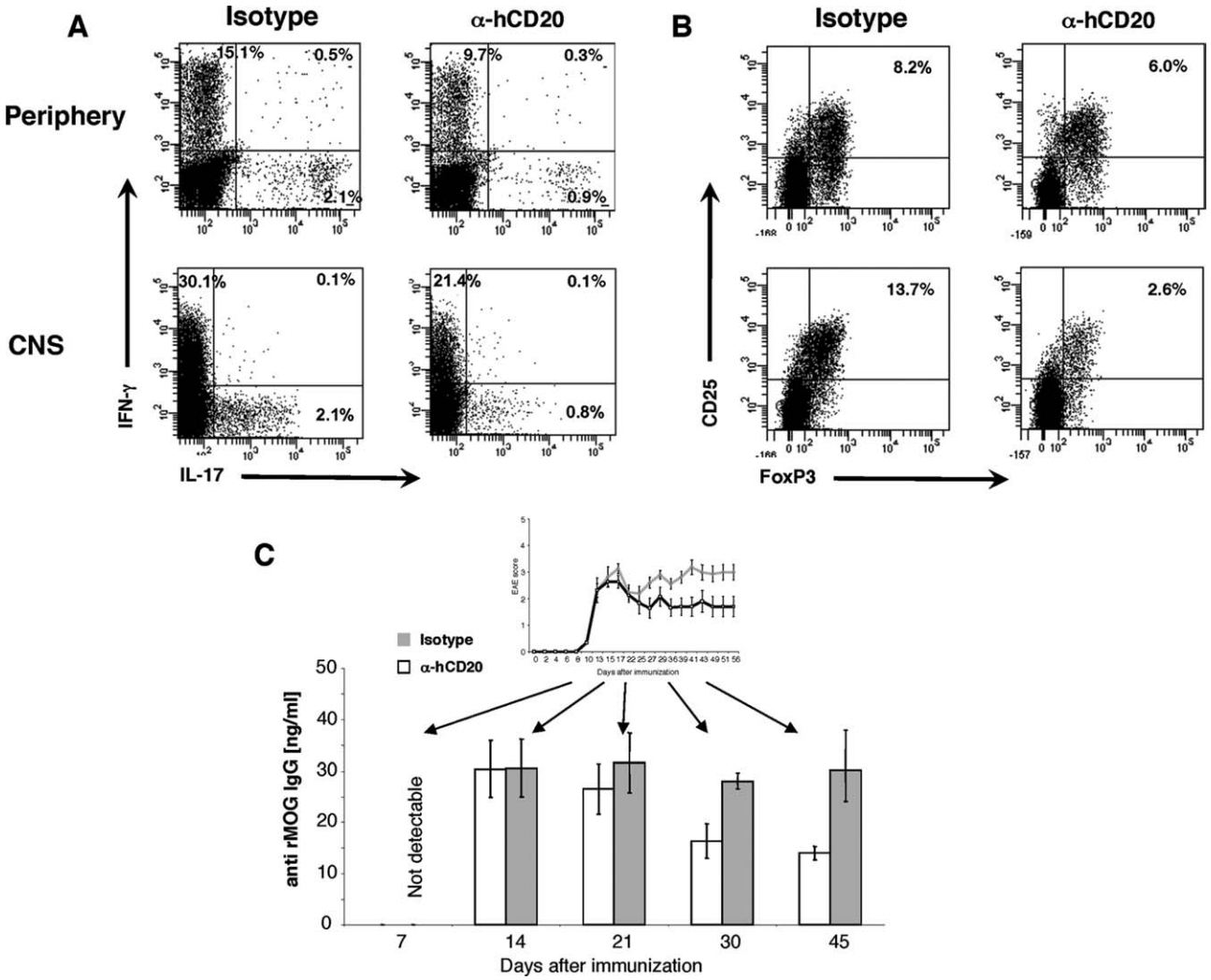

FIGURE 4: In experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG), anti-CD20 B-cell depletion is associated with a reduced frequency of Th1, Th17, and $CD4^+CD25^+Foxp3^+$ regulatory T cells and decreased anti-MOG antibody titers. C57BL/6 hCD20 transgenic mice received 200μg of anti-hCD20 or isotype (immunoglobulin [Ig]G2a, control) weekly after EAE was fully established (EAE score $\geq$2). (A) Proinflammatory differentiation of peripheral (upper panel) and central nervous system (CNS)-infiltrating T cells (lower panel) was evaluated by intracellular fluorescence-activated cell sorting staining for interleukin (IL)-17 and interferon (IFN)-γ (gated on $CD3^+$ T cells) 14 days after onset of treatment. Frequency of peripheral (upper panel) and CNS-infiltrating $FoxP3^+$ regulatory T cells (lower panel) was investigated by CD4/CD25/Foxp3 triple staining (gated on $CD4^+$ T cells). (b) Mice were bled weekly and evaluated for anti-MOG protein antibodies (total IgG; dilution factor 1:13,500). rMOG = recombinant MOG.

mice, and cultured them with naive MOG p35-55–specific T cells. When compared to APCs from isotype-treated mice (after in vitro removal of B cells), APC remaining after in vivo depletion of B cells exhibited an increased capacity to promote development of encephalitogenic Th1 and Th17 cells. Again, this proinflammatory gain of function by remaining APCs after B-cell depletion occurred in both EAE models (see Fig 7B). In summary, in addition to their role in T cell activation, these results suggest that B cells can regulate other APCs, and that nonselective depletion of B cells could augment the proinflammatory function of remaining APCs.

Discussion

Recent studies suggest that CD20-mediated B-cell depletion may be effective in reducing CNS inflammation in MS.[8,9] In this report, we investigated the immunological consequences of anti-CD20 B-cell depletion in EAE induced by MOG protein and MOG p35-55. In MOG protein-induced EAE, but not in EAE induced by MOG p35-55, activated MOG-reactive B cells participated as APCs and promoted differentiation of naive MOG-specific T cells into proinflammatory Th1 and Th17 cells in vitro. Anti-CD20–mediated B-cell depletion ameliorated EAE induced by MOG protein and suppressed

TABLE 2: Clinical Responses to Anti-CD20 B-Cell Depletion in MOG p35-55–Induced EAE

	Incidence	Mean Day of Onset (± SEM)	Mean Max Severity (± SEM)	Mean Severity (± SEM)	Mean Severity (± SEM)	Mean Severity (± SEM)
Prevention, days after immunization				15	21	28
Isotype	11/12	17.88 (±1.27)	3.63 (±0.25)	0.88 (±0.27)	1.88 (±0.34)	2.44 (±0.19)
Anti-CD20	9/11	15.22 (±0.78)	4.44 (±0.24)	1.83 (±0.40)	3.78 (±0.21)	3.00 (±0.19)
Treatment, days after start of anti-CD20				0	+6	+12
Isotype	12/12	13.67 (±0.45)	2.88 (±0.31)	2.42 (±0.33)	1.46 (±0.16)	1.33 (±0.16)
Anti-CD20	11/11	13.40 (±0.40)	3.25 (±0.27)	2.45 (±0.31)	2.15 (±0.12)	2.20 (±0.21)

Results are representative of 4 separate experiments (10–12 mice/group/experiment).
MOG = myelin oligodendrocyte glycoprotein; EAE = experimental autoimmune encephalomyelitis; SEM = standard error of the mean.

development of Th1 and Th17 cells in vivo. Anti-CD20 treatment initiated after MOG-specific antibodies were generated led to subsequent reduction in titers. Investigations in rheumatoid arthritis[28,29] and systemic lupus erythematosus[30] indicated that administration of anti-CD20 similarly dampened humoral responses, although plasma cells, which do not express CD20, were not eliminated.[13] Although reduction of myelin-specific antibodies may potentiate the therapeutic effect of B-cell depletion in a subgroup of MS patients with CNS antibody deposition,[3,31] it should be recognized that the benefit of anti-CD20 B-cell depletion observed in a 6-month placebo-controlled trial in relapsing-remitting MS was not associated with a reduction in antibodies.[8] Furthermore, in EAE antibodies elicited by immunization with mouse MOG protein, although self-reactive, are not considered pathogenic.[19] Thus, the clinical benefit of anti-CD20 treatment observed in this EAE model more likely reflects a reduction in proinflammatory cellular function of MOG-specific B cells.

In both EAE models, anti-CD20 treatment-depleted B cells within the CNS of mice with established EAE. In MOG protein-induced EAE, B cells became activated, and a greater number of B cells infiltrated the CNS (see Fig 1D). The capability to deplete B cells within the CNS is of particular therapeutic relevance in light of the discovery of ectopic B cell follicles[32] within the meninges in some individuals who developed secondary progressive MS, and that formation of these lymphoid follicle-like structures may be associated with elevated risk for irreversible disability.[33] The observation that B cells were efficiently depleted within the meninges suggests that anti-CD20 could be also an attractive candidate for treatment of a subset of patients with secondary progressive MS.

Exacerbation of MOG peptide-induced EAE by anti-CD20 treatment highlights the complexity of B-cell function in CNS autoimmunity. Immunization with MOG p35-55 did not promote B-cell activation. In contrast to anti-CD20 depletion in MOG protein-induced EAE, which was associated with clinical benefit and reduction in proinflammatory Th1 and Th17 cells within the CNS, CD20-mediated depletion resulted in clinical worsening of MOG p35-55–induced EAE and increased numbers of CNS-infiltrating Th1 and Th17 cells. Besides serving as the source for antibody-secreting plasma cells and as APCs for T-cell activation, some B-cell subsets may have an important role in immune regulation of CNS autoimmune disease.[22,27,34] Evidence suggests that antigen-naive B cells exert anti-inflammatory properties,[27,35] which may inhibit maturation and proinflammatory differentiation of other APCs in vivo.[36] In this regard, it has been observed that dendritic cells isolated from B cell-deficient mice produce higher levels of IL-12 and promote proinflammatory T-cell differentiation.[37] In conjunction with our observation that after anti-CD20 B-cell depletion, remaining myeloid APCs secreted more proinflammatory TNF and less anti-inflammatory IL-10, these findings collectively indicate that B cells can regulate other APCs and suggest that this B-cell characteristic may be abrogated by nonselective anti-CD20–mediated B-cell depletion.

Naive B cells may play an important role in development and maintenance of Treg in vivo.[22,38] Deficiencies in the Treg compartment have been identified in several autoimmune conditions, including MS,[39,40] and a goal in MS therapy is to correct this imbalance.[41,42] Whereas some studies suggest that anti-CD20 depletion may be associated with a modest increase of Treg,[14,43] we observed a reduction in numbers of $CD4^+CD25^+Foxp3^+$ Treg in anti-CD20 treatment of EAE induced by either rMOG or

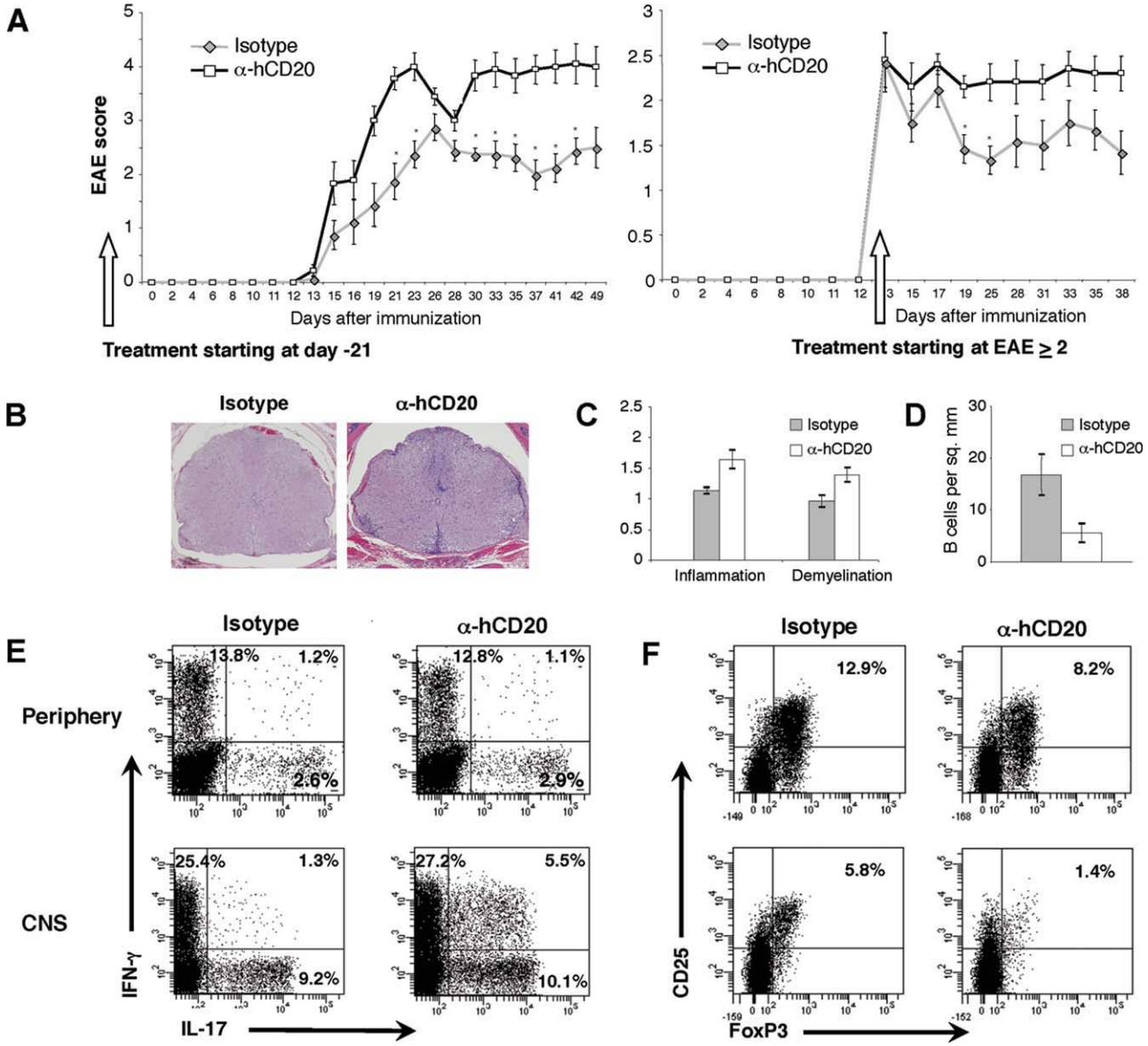

FIGURE 5: Anti-CD20 treatment exacerbates experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein p35-55 peptide. (A) C57BL/6 hCD20 transgenic mice received 200μg anti-hCD20 or isotype control (immunoglobulin G2a) weekly starting 21 days prior to EAE induction (left panel), or after EAE was fully established (EAE score $\geq$2, right panel); white arrows indicate treatment onset. Results are representative of 4 separate experiments (10–12 mice/group/experiment). (B, C) Spinal cords were evaluated for inflammatory infiltration (hematoxylin and eosin) and demyelination, with sections scored on a scale from 0 to 4. (D) Mice receiving treatment after EAE was fully established were evaluated for the presence of B cells within spinal cord sections by immunohistochemistry; shown is the number of B220$^+$ cells per mm^2 of total spinal cord tissue. (E) Proinflammatory differentiation of peripheral (upper panel) and central nervous system (CNS)-infiltrating T cells (lower panel) was evaluated by intracellular fluorescence-activated cell sorting staining for interleukin (IL)-17 and interferon (IFN)-γ (gated on CD3$^+$ T cells) 14 days after treatment onset. (F) Frequency of peripheral (upper panel) and CNS-infiltrating Foxp3$^+$ regulatory T cells (lower panel) was investigated by CD4/CD25/Foxp3 triple staining (gated on CD4$^+$ T cells).

MOG peptide. This finding is further supported by our investigations using B cell-deficient μMT[44] and JHT[17] mice. Similar to unimmunized anti-CD20 B cell-depleted mice, we demonstrated that B cell-deficient μMT or JHT mice contained lower frequencies of CD4$^+$CD25$^+$Foxp3$^+$ Treg (Fig 8), again indicating that B cells participate in Treg homeostasis. There were no obvious qualitative differences in Treg in wild-type and anti-CD20 B cell-depleted mice. In this regard, we did not detect intracellular IL-10 protein production in CD4$^+$CD25$^+$Foxp3$^+$ Treg in either isotype-treated or B cell-depleted mice.

Anti-CD20 therapy has been examined in other EAE settings.[45,46] B-cell depletion prevented exacerbations

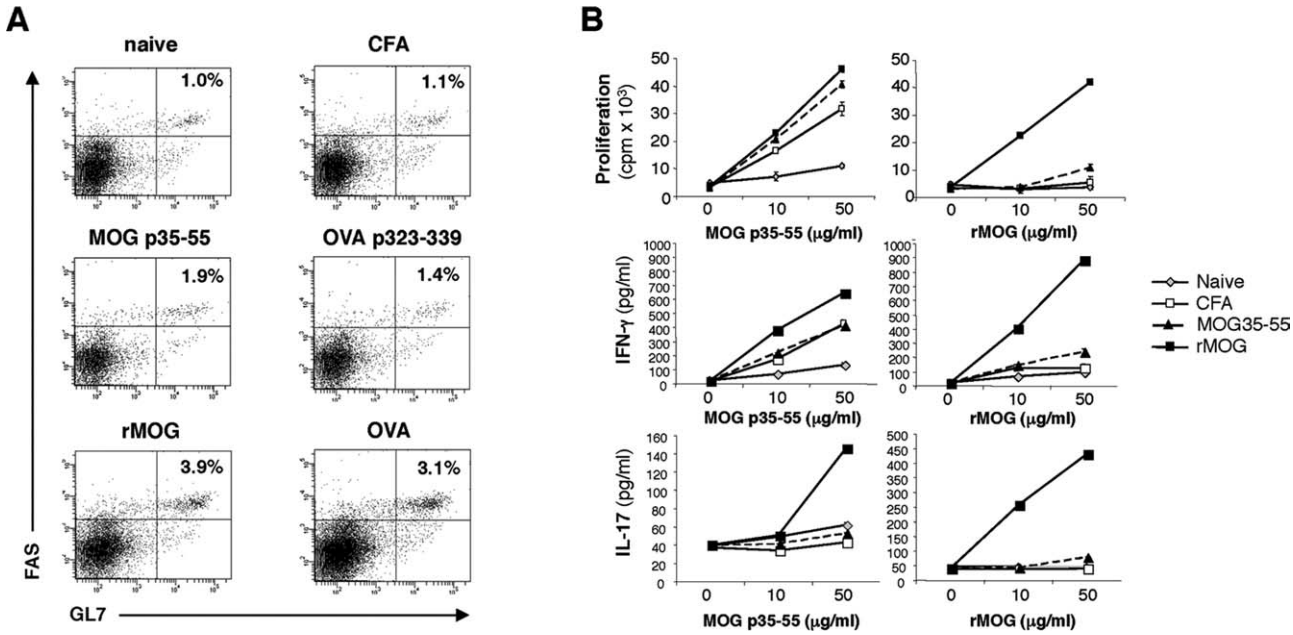

FIGURE 6: Immunization with myelin oligodendrocyte glycoprotein (MOG) generates a population of activated antigen-specific B cells that efficiently process and present recombinant MOG (rMOG) protein to MOG p35-55 T-cell receptor (TCR) transgenic (Tg) T cells. (A) B cells isolated from C57BL/6 wild type mice that had not been immunized (naive) or immunized with complete Freund's adjuvant (CFA) alone, MOG p35-55, ovalbumin (OVA) p323-339, MOG protein, or OVA protein were evaluated for surface expression of FAS and GL7 (gated on $B220^+$). (B) magnetically activated cell sorting-separated B cells (purity >95%) isolated from unimmunized (naive) and CFA-, MOG p35-55-, or rMOG-immunized mice were cocultured with naive T cells isolated from MOG TCR Tg mice in the presence of MOG p35-55 or rMOG protein. Proinflammatory T-cell differentiation was evaluated by secretion of interferon (IFN)-γ (upper panel) or interleukin (IL)-17 (lower panel).

in a murine model of spontaneous relapsing-remitting EAE in which Tg T cells and B cells both recognize MOG.[46] A recent publication by Matsushita and colleagues[45] also demonstrated exacerbation of MOG peptide-induced EAE when B cell-depleting treatment began prior to disease induction. The authors attributed worsening of disease to the absence of an IL-10–producing (B10) regulatory B-cell subset. When anti-CD20 treatment started 14 days after immunization, severity of MOG p35-55–induced EAE was ameliorated, leading the authors to conclude that although protective at the time of disease induction, at a later stage, B cells or B-cell subsets may promote disease progression. The apparent divergence in outcome of B-cell depletion in reversal of MOG peptide-induced disease in our study could reflect differences in experimental procedures, such as dose of MOG p35-55 used for EAE induction, or the nature of the anti-CD20 antibody used.[47] One striking difference, however, is that they detected a peptide-specific antibody response upon immunization with their MOG p35-55 preparation, which could have reflected the 4-fold higher dose of p35-55 used for EAE induction in their study. Although those antibodies did not likely contribute in a pathogenic manner, their appearance may be indicative of B-cell activation and maturation following immunization with MOG peptide, which was not observed in this report. Also, in our investigation, B-cell depletion in hCD20 Tg mice was achieved using a mouse anti-hCD20 monoclonal antibody. More recently, we tested a mouse antimouse (m) CD20 monoclonal antibody for prevention of EAE induced by MOG protein or MOG peptide in non-Tg mice. Consistent with our findings using mouse anti-hCD20, anti-mCD20 treatment suppressed development of proinflammatory T cells and clinical EAE induced by MOG protein, whereas it promoted development of proinflammatory T cells and exacerbated clinical EAE induced by MOG p35-55. Most importantly, our demonstration that B cells regulate secretion of proinflammatory cytokines by monocytes is in agreement with the observation by Matsushita et al that certain B-cell subsets have regulatory function, whereas others support the pathogenesis of CNS autoimmune disease. Unlike the results of Matsushita and colleagues, our data indicate that the immunological and clinical outcome of B-cell depletion is determined by the activation status and antigen-specificity of B cells, rather than the time of treatment initiation.

Although the paradoxical clinical outcomes of CD20-mediated B-cell depletion in EAE induced by MOG p35-55 and MOG protein correlated with increased and decreased frequencies of proinflammatory

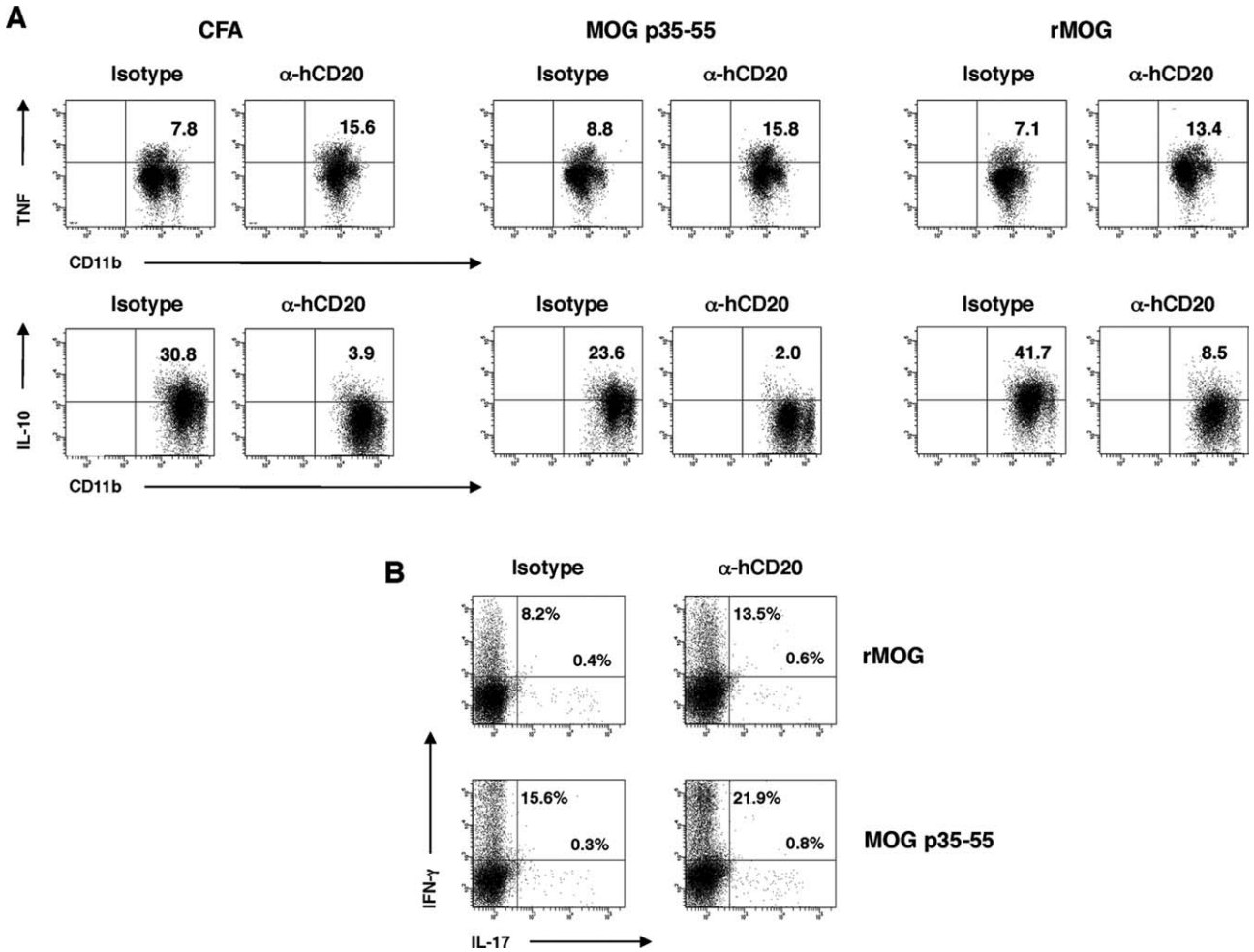

FIGURE 7: Anti-CD20 B-cell depletion increases the capacity of remaining antigen-presenting cells to generate encephalitogenic T cells. C57BL/6 hCD20 transgenic (Tg) mice received 200μg of anti-hCD20 or isotype (immunoglobulin G2a, control) weekly starting 21 days prior to experimental autoimmune encephalomyelitis induction with recombinant myelin oligodendrocyte glycoprotein (rMOG) (upper panels) or MOG p35-55 peptide (lower panels). Twelve days after immunization, spleens were isolated and B220$^+$ B cells, and CD3$^+$ T cells were removed by magnetically activated cell sorting separation. (A) Production of tumor necrosis factor (TNF) and interleukin (IL)-10 by remaining CD11b$^+$ cells (gated on CD11b) was evaluated by intracellular fluorescence-activated cell sorting (FACS) staining. (B) Remaining splenocytes were cocultured with naive T cells from MOG p35-55-specific T-cell receptor Tg mice in the presence of the antigen used for immunization. Proinflammatory T-cell differentiation was evaluated by intracellular FACS staining for IL-17 and interferon (IFN)-γ (gated on CD3$^+$ T cells). CFA = complete Freund's adjuvant.

T cells, respectively, it should be recognized that reduction in Treg and augmentation of proinflammatory cytokine expression by remaining APCs were common features of CD20 B-cell depletion in both models. B cells may undertake additional cellular immune functions, which could have been eliminated by anti-CD20 treatment. It was observed that B cells are capable of capturing protein via their antigen-specific BCR and delivering it to lymph node follicular dendritic cells, which are more professional APCs.[26] Through this mechanism of antigen transport, B cells can contribute indirectly to proinflammatory T-cell polarization. We have demonstrated that activated MOG-specific B cells, but not naive B cells, serve directly as APCs and polarize proinflammatory T cells. Therefore, we favor the possibility that there is a balance, and that the benefit from eliminating MOG protein-activated B cells reflects inhibition of their proinflammatory cellular function, whereas exacerbation of p35-55–induced EAE relates to depletion of unactivated (naive) B cells that participate in regulation. As was previously observed for myeloid APCs, which can be divided into proinflammatory type I or anti-inflammatory type II classes,[48,49] B cells may exhibit proinflammatory Be1 or anti-inflammatory Be2 T cell-polarizing phenotypes.[50] In the absence of antigen-activated Be1 cells, CD20 B-cell depletion may exacerbate autoimmune disease in some

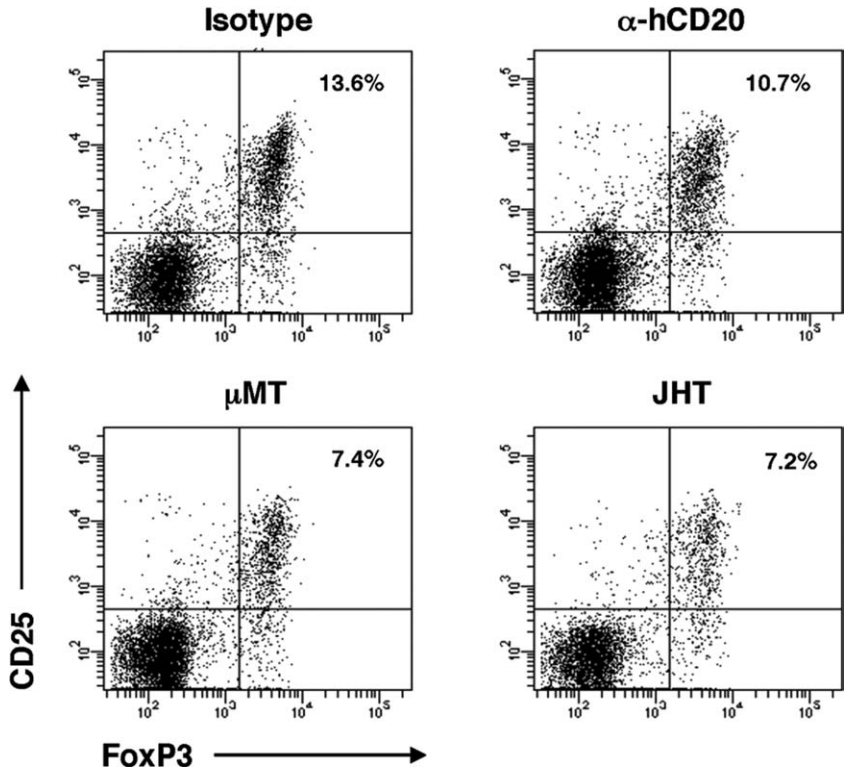

FIGURE 8: B-cell deficiency is associated with reduced frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells. Unimmunized C57BL/6 hCD20 transgenic mice that received 200μg of isotype or anti-hCD20 were compared to unimmunized C57BL/6 B cell-deficient μMT or JHT mice. Frequency of peripheral $Foxp3^+$ regulatory T cells (Treg) was investigated by CD4/CD25/Foxp3 triple staining (gated on $CD4^+$ T cells).

settings.[51] Recently, we created Tg mice that contain B cells that express membrane MOG-specific BCR, but cannot secrete antibodies (N. Molnarfi et al, unpublished data). These BCR Tg mice will permit us to distinguish between certain cellular functions of Ag-specific B cells and the role of antibodies in the pathogenesis of MOG-induced EAE.

In this report, we studied 2 distinct EAE models. One cannot conclude that EAE induced by either MOG protein or MOG peptide more closely reflects MS. Each model has its virtues and may emphasize different aspects of pathogenesis.[52] APCs must process MOG protein through the endocytic pathway for MHC class II-restricted presentation of its encephalitogenic determinant to $CD4^+$ T cells, whereas MOG p35-55 can be loaded onto MHC II molecules directly.[6] We have demonstrated that activated MOG-primed B cells are capable of efficiently presenting MOG protein and promoting differentiation of pathogenic MOG-specific T cells. Immunization with MOG protein elicits a stronger antibody response than does priming to MOG peptide. Our results highlight key differences in cellular and humoral B-cell responses to MOG protein and MOG peptide, which could be important when choosing an EAE model for preclinical testing of other novel B cell-targeting agents for MS.

In summary, this study supports the use of anti-CD20–mediated depletion of activated B cells in the treatment of CNS autoimmune disease and establishes inhibition of B cell-dependent activation of pathogenic Ag-specific T cells as an immunological mechanism that may contribute to its clinical benefit in MS. In addition, the observations in this report may be relevant to B-cell depletion therapy in NMO, which is associated with pathogenic AQP4-specific IgG1, a T cell-dependent antibody subclass.[53,54] Our study cautions that nonselective elimination of B cells may prevent unactivated or regulatory B cells from exerting their beneficial anti-inflammatory influence on other immune cells. Selective depletion of antigen-activated B cells may be a valuable strategy to further improve efficacy of B cell-targeted therapies in MS and other inflammatory CNS demyelinating diseases.

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Authorship

M.S.W. and T.P. contributed equally to this study.

Potential Conflicts of Interest

Dmitry Danilenko is an employee of Genentech. Christopher Linington received a grant from the Multiple Sclerosis Society. Flavius Martin is an employee of Genentech, as well as an owner of stock. Anthony Slavin was an employee of Amgen, Inc. and Novartis and is currently an employee of Boehringer-Ingelheim. Dr. Slavin was paid money for two patents - WO 99/41247 Treatment of multiple sclerosis using COP-1 and Th2-enhancing cytokines EP 105488020010007758 Treatment of multiple sclerosis using COP-1 and Th2-enhancing cytokines (Brigham and Women's Hospital, Boston, MA) and US 2005152896 Anti-galanin antibodies and uses thereof WO 2005/058961 Antibodies specific for human Galanin, and uses thereof (Amgen, Inc.). Dr. Slavin also owns stock in Amgen, Novartis, Gilead, Elan, Merck, Prana Biotechnology, MetLife Inc. and Natus Medical Inc. as part of a mutual fund/IRA account. Scott Zamvil was paid an honoraria, as well as had travel and accommodations paid for by Genentech to attend a Genentech PPMS Advisory Board Meeting in November 2006, as well as a Genentech meeting on B cells and B cell depletion in MS in September, 2007.

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