Published in final edited form as:
Ann Neurol. 2010 September ; 68(3): 369–383. doi:10.1002/ana.22081.

B cell activation influences T cell polarization and outcome of anti-CD20 B cell depletion in CNS autoimmunity

Martin S. Weber, MD[1,#], **Thomas Prod'homme, PhD**[1], **Juan C. Patarroyo**[1], **Nicolas Molnarfi, PhD**[1], **Tara Karnezis, PhD**[2], **Klaus Lehmann-Horn, MD**[3], **Dimitry M. Danilenko, DVM, PhD**[4], **Jeffrey Eastham-Anderson**[4], **Anthony J. Slavin, PhD**[5], **Christopher Linington, PhD**[6], **Claude C.A. Bernard, PhD**[2], **Flavius Martin, MD**[4], and **Scott S. Zamvil, MD, PhD**[1]

[1]Department of Neurology, University of California, San Francisco, CA USA

[2]Immunology and Stem Cell Laboratories, Monash University, Melbourne, Australia

[3]Department of Neurology, Technische Universität München, Munich, Germany

[4]Genentech, South San Francisco, CA USA

[5]Boehringer-Ingelheim, Ridgefield, CT USA

[6]University of Glasgow, United Kingdom

Abstract

Objective—Clinical studies indicate that anti-CD20 B cell depletion may be an effective multiple sclerosis therapy. We investigated mechanisms of its immune modulation using two paradigms of experimental autoimmune encephalomyelitis (EAE).

Methods—Murine EAE was induced by either recombinant myelin oligodendrocyte glycoprotein (rMOG), a model in which B cells are considered to contribute pathogenically, or MOG peptide (p)35–55, a model that does not require B cells.

Results—In EAE induced by rMOG, B cells became activated and, when serving as antigen presenting cells (APC), 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 CNS inflammation, elimination of meningeal B cells, and reduction of MOG-specific Th1 and Th17 cells. In contrast, in EAE induced by MOG p35–55, B cells did not become activated or efficiently polarize proinflammatory MOG-specific T cells, similar to naïve B cells. In this EAE 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 regulatory T cells, and increased the capacity of remaining APC to promote development of encephalitogenic T cells.

Interpretation—Our study highlights distinct roles for B cells in pathogenesis and regulation of CNS autoimmune disease. Clinical benefit from depletion of antigen-activated B cells may relate primarily to abrogation of proinflammatory B cell APC function. However, in certain clinical settings, elimination of unactivated B cells, which participate in regulation of T cells and other APC, may be undesirable.

Address correspondence to: Scott S. Zamvil, M.D., Ph.D., Department of Neurology, University of California, San Francisco, 513 Parnassus Avenue, S-268, San Francisco, CA 94143-0114, Telephone: (415) 502-7395, Fax: (415) 502-1331, zamvil@ucsf.neuroimmunol.org.
[#]current address: Department of Neurology, Technische Universität München, Germany

Keywords

B cell depletion; CD20; experimental autoimmune encephalomyelitis; multiple sclerosis; antigen presenting cells; humoral immunity

INTRODUCTION

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 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 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 (APC). B cells are capable of processing native antigen and are very efficient APC when they recognize the same antigen as the responding T cells[4, 5]. As processing of native myelin antigen by CNS resident or infiltrating APC is required for initiation of CNS autoimmune inflammation and clinical disease[6, 7], myelin-specific B cells may have an important role for 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 (RituxanR), 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 neuromyelitis optia (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 two related models of experimental autoimmune encephalomyelitis (EAE)[11]. In one 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 $FoxP3^+$ regulatory T cells (Treg) and augmented pro-inflammatory function of

remaining myeloid APC. 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 cross-talk in pathogenesis and regulation of CNS autoimmunity.

MATERIALS AND METHODS

Mice

C57BL/6 female mice, 5–8 weeks of age, as well as μMT mice were purchased from the 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 anti-human CD20 antibody (clone 2h7) results in rapid depletion of B cells ([13], supplementary Figure 1a). Untreated or isotype control-treated hCD20 Tg mice developed EAE indistinguishable from wild-type mice (supplementary Figure 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 myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Auspep (Parkville, Australia). Recombinant (r) 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, USA). Intact OVA was purchased from Sigma-Aldrich (Saint-Louis, MI).

EAE induction

Eight to twelve week-old female C57BL/6 or hCD20 Tg C57BL/6 mice were injected subcutaneously with 25 μg MOG p35–55 or 100 μg rMOG 1–117 in complete Freund's adjuvant (CFA) (DIFCO Laboratories, Detroit, Michigan, USA). After immunization and 48 hrs later mice received an intravenous injection of 200 ng 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

In order to ensure maximal B cell depletion when examining anti-CD20 in prevention of EAE, anti-CD20, mice received weekly intraperitoneal (i.p.) injections of 200 μg of a-hCD20 monoclonal antibody (m2h7) or anti-ragweed IgG2a-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 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) were coated with MOG p35–55 or rMOG 1–117 (10 μg/ml in PBS) and then blocked with BSA (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) as the standard. Plates were read at 450-nm wavelength. SOFTmax ELISA plate reader and software (Molecular Devices Corporation, Sunnyvale, CA) were used for data analysis.

T cell co-culture assays

For B cell – T cell co-culture assays, B cells were MACS (Miltenyi Biotec)-separated from lymph nodes or spleens. Following separation, B cells were evaluated for purity (>99%) by FACS staining for B220. For T cell co-culture assays using remaining splenocytes as APC, hCD20 Tg mice received weekly injections of 0.2 mg of a-hCD20 or IgG2a-isotype starting 21 days prior to immunization with rMOG protein or MOG p35–55 peptide. 12 days after immunization, spleens were isolated and $B220^+$ B cells and $CD3^+$ T cells were removed by MACS separation. 5×10^5 B cells or remaining splenocytes were co-cultured with 1×10^4 naïve T cells isolated from MOG TCR Tg mice in the presence of rMOG 1–117 or MOG p35–55. After 72 hrs, 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, and anti-CD21 was purchased from eBioscience. Proinflammatory T cell differentiation was evaluated by surface staining for CD3 (Pharmingen) and ICS for IFN-γ and IL-17 (eBioscience). Activation of monocytic cells was evaluated by surface staining for CD11b (Pharmingen) and ICS for 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-hr (IFN-γ) or 72-hr (IL-17), respectively and analyzed by ELISA (Pharmingen, San Diego, CA). The results for ELISA assays are expressed as an average of triplicate wells +/− 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), 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 0–4. B220 and CD3 stained sections were evaluated by morphometric image analysis. Final results were reported as B220 or CD3 stained cells per square mm of spinal cord area.

Statistical analysis

Data are presented as mean ± 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 one-way multiple-range analysis of variance test (ANOVA) 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 antigen presenting cells

Two different EAE models were examined in this report. In EAE induced by immunization with MOG protein (recombinant (r) MOG 1–117)), internalization and processing by APC 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 APC 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 peptide (p) 35–55. Following activation, B cells developed into plasma cells that secreted antibodies directed against MOG ([19], 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 APC[6]. Instead, naive B cells, independent of their BCR specificity, are capable of presenting short peptides through direct binding to cell surface MHC II molecules. As shown in Fig 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 of MOG-specific B cells, and was not associated with a significant antibody response ([20], Fig 1c).

Kinetics of anti-CD20-mediated B cell depletion differs in distinct tissue microenvironments

Anti-CD20 treatment was investigated in human (h) CD20 transgenic (Tg) C57BL/6 mice[13,14]. These mice develop EAE in a manner that is indistinguishable from wild-type C57BL/6 mice (Supplementary Fig 1). Data indicate that kinetics of B cell depletion in different tissue microenvironments may depend upon vascular access of anti-CD20 antibodies[13]. Depletion of mature ($B220^+CD21^+$) B cells was examined in blood, bone marrow, lymph nodes spleen and in 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 three hours, and in lymph nodes and spleen at two days (Fig 2). B cell depletion in the peritoneum was slower; at two days peritoneal B cells were reduced by approximately 30%, and at seven days by 95%. There was more than 99% of depletion $B220^+CD21^+$ B cells in all immune and non-immune tissues examined 14 days post injection. In order to ensure maximal B cell depletion when anti-CD20 treatment was evaluated for EAE prevention, this antibody was administered weekly starting three 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 and 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 (Fig 3b and 3c).

The potential influence of CD20 B cell depletion in MOG protein-induced EAE on proinflammatory T cell 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 Figure 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 (Fig 4b). In addition to serving as a source for antibody-secreting plasma cells and as APC, 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 Treg in peripheral lymphoid organs as well as within the CNS (Fig 4a).

B cell depletion exacerbates EAE induced by MOG peptide 35–55

Anti-CD20 treatment was investigated 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 5a and Table 1). Clinical worsening was associated with more severe CNS inflammation and demyelination (Fig 5b and 5c), despite the fact that anti-CD20 treatment sufficiently depleted B cells within the CNS (Fig 5d). B cell depletion did not dampen pathogenic Th1 and Th17 responses in this disease model (Fig 5e). In fact, clinical worsening in anti-CD20 treatment of MOG p35–55-induced EAE was generally associated with an increase in CNS Th1, 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 up-regulated 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 non-encephalitogenic control protein ovalbumin (OVA), but not its short peptide OVA p323–339, upgregulated 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 (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 peptide onto MHC II molecules expressed on unactivated (naive) B cell APC alone does not efficiently promote differentiation of encephalitogenic T cells.

B cell depletion augments the capability of residual antigen presenting cells to activate encephalitogenic T cells

Data indicate that B cells may communicate with other APC. 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 APC[27]. Thus, we evaluated how B cell depletion influenced the function of remaining APC. In both EAE models used, $CD11b^+$ cells isolated from mice receiving anti-CD20 treatment produced more pro-inflammatory 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 mice, and cultured them with naïve MOG p35–55 specific T cells. When compared to APC 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 APC after B cell depletion occurred in both EAE models (Fig 7b). In summary, in addition to their role in T cell activation, these results suggest that B cells can regulate other APC, and that nonselective depletion of B cells could augment the proinflammatory function of remaining APC.

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 APC and promoted differentiation of naïve 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 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]. While 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 six-month placebo-controlled trial in relapsing-remitting MS was not associated with a reduction in antibodies[8]. Further, 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 pro-inflammatory 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 (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 that 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 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 APC 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 pro-inflammatory differentiation of other APC 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 pro-inflammatory T cell differentiation[37]. In conjunction with our observation that after anti-CD20 B cell depletion remaining myeloid APC secreted more pro-inflammatory TNF and less anti-inflammatory IL-10, these findings collectively indicate B cells can regulate other APC and suggest that this B cell characteristic may be abrogated by nonselective anti-CD20-mediated B cell depletion.

Naïve 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 one of the goals in MS therapy is to correct this imbalance[41, 42]. While 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 $CD25^+Foxp3^+$ Treg in anti-CD20 treatment of EAE induced by either rMOG or 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 $CD25^+FoxP3^+$ 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 $CD4^+CD25^+ForP3^+$ 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 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. Elegantly, 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 four-fold higher dose of p35–55 used for EAE induction in their study. While 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 anti-mouse (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, while 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 pro-inflammatory cytokines by monocytes is in agreement with the observation by Matsushita, *et al.* that certain B cell subsets have regulatory function, while others support 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.

While 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 T cells, respectively, it should be recognized that reduction in Treg and augmentation of proinflammatory cytokine expression by remaining APC 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, more professional APC[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 APC 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, while exacerbation of p35–55-induced EAE relates to depletion of unactivated (naive) B cells that participate in regulation. As was previously observed for myeloid APC, 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 settings[51]. Recently, we created Tg mice that contain B cells that express membrane MOG-specific BCR, but cannot secrete antibodies (Molnarfi, N. et al., unpublished). These BCR Tg mice will permit us to distinguish between certain cellular functions of Ag-specific B cells and the role of antibodies in pathogenesis of MOG-induced EAE.

In this report, we studied two 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]. APC must process MOG protein through the endocytic pathway for MHC class II-restricted presentation of its encephalitogenic determinant to $CD4^+$ T cells, while 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 treatment of CNS autoimmune disease and establishes inhibition of B cell-dependent activation of pathogenic Ag-specific T cells as one 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 non-selective 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Patricia A. Nelson for helpful discussion. We also thank the Canio Refino of Genentech and the Genentech Histology and Immunohistochemistry Laboratories for processing and staining brain and spinal cord specimens. M.S.W. is supported by the National Multiple Sclerosis Society (NMSS, RG 4450A1/T). T.P. received fellowship support from NMSS and Teva Neuroscience. N.M. is supported by an advanced researcher fellowship from the Swiss National Science Foundation (#PA00A—119532). Support for this work was provided to S.S.Z. by the National Institute of Health (NIH) (RO1 AI073737, RO1 AI059709, and RO1 NS063008), the NMSS (RG 3622 and 3913), Dana Foundation, Guthy Jackson Charitable Foundation and Maisin Foundation. C.C.A.B. was supported by grants from the Baker Foundation, the National Health, the Medical Research Council of Australia and the NMSS (CCAB: RG3844A2/1).

References

1. Prineas JW, Connell F. The fine structure of chronically active multiple sclerosis plaques. Neurology. 1978; 28:68–75. [PubMed: 568752]
2. Genain CP, Cannella B, Hauser SL, Raine CS. Identification of autoantibodies associated with myelin damage in multiple sclerosis. Nat Med. 1999; 5:170–175. [PubMed: 9930864]
3. Keegan M, Konig F, McClelland R, et al. Relation between humoral pathological changes in multiple sclerosis and response to therapeutic plasma exchange. Lancet. 2005; 366:579–582. [PubMed: 16099294]
4. Constant S, Schweitzer N, West J, et al. B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo. J Immunol. 1995; 155:3734–3741. [PubMed: 7561077]
5. Constant S, Sant'Angelo D, Pasqualini T, et al. Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of CD4+ T cells. J Immunol. 1995; 154:4915–4923. [PubMed: 7730604]
6. Slavin AJ, Soos JM, Stuve O, et al. Requirement for endocytic antigen processing and influence of invariant chain and H-2M deficiencies in CNS autoimmunity. J Clin Invest. 2001; 108:1133–1139. [PubMed: 11602620]
7. Tompkins SM, Padilla J, Dal Canto MC, et al. De novo central nervous system processing of myelin antigen is required for the initiation of experimental autoimmune encephalomyelitis. J Immunol. 2002; 168:4173–4183. [PubMed: 11937578]
8. Hauser SL, Waubant E, Arnold DL, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med. 2008; 358:676–688. [PubMed: 18272891]
9. Hawker K, O'Connor P, Freedman MS, et al. Rituximab in patients with primary progressive multiple sclerosis: results of a randomized double-blind placebo-controlled multicenter trial. Ann Neurol. 2009; 66:460–471. [PubMed: 19847908]
10. Cree BA, Lamb S, Morgan K, et al. An open label study of the effects of rituximab in neuromyelitis optica. Neurology. 2005; 64:1270–1272. [PubMed: 15824362]
11. Steinman L, Zamvil SS. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann Neurol. 2006; 60:12–21. [PubMed: 16802293]
12. Mendel I, Kerlero de Rosbo N, Ben-Nun A. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity

and T cell receptor V beta expression of encephalitogenic T cells. Eur J Immunol. 1995; 25:1951–1959. [PubMed: 7621871]

13. Gong Q, Ou Q, Ye S, et al. Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. J Immunol. 2005; 174:817–826. [PubMed: 15634903]
14. Hu CY, Rodriguez-Pinto D, Du W, et al. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest. 2007; 117:3857–3867. [PubMed: 18060033]
15. Uchida J, Lee Y, Hasegawa M, et al. Mouse CD20 expression and function. Int Immunol. 2004; 16:119–129. [PubMed: 14688067]
16. Bettelli E, Pagany M, Weiner HL, et al. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. J Exp Med. 2003; 197:1073–1081. [PubMed: 12732654]
17. Chen J, Trounstine M, Alt FW, et al. Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the JH locus. Int Immunol. 1993; 5:647–656. [PubMed: 8347558]
18. Clements CS, Reid HH, Beddoe T, et al. The crystal structure of myelin oligodendrocyte glycoprotein, a key autoantigen in multiple sclerosis. Proc Natl Acad Sci U S A. 2003; 100:11059–11064. [PubMed: 12960396]
19. Marta CB, Oliver AR, Sweet RA, et al. Pathogenic myelin oligodendrocyte glycoprotein antibodies recognize glycosylated epitopes and perturb oligodendrocyte physiology. Proc Natl Acad Sci U S A. 2005; 102:13992–13997. [PubMed: 16172404]
20. Lyons JA, San M, Happ MP, Cross AH. B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide. Eur J Immunol. 1999; 29:3432–3439. [PubMed: 10556797]
21. Korn T, Anderson AC, Bettelli E, Oukka M. The dynamics of effector T cells and Foxp3+ regulatory T cells in the promotion and regulation of autoimmune encephalomyelitis. J Neuroimmunol. 2007; 191:51–60. [PubMed: 17916388]
22. Mann MK, Maresz K, Shriver LP, et al. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. J Immunol. 2007; 178:3447–3456. [PubMed: 17339439]
23. Kroenke MA, Carlson TJ, Andjelkovic AV, Segal BM. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. J Exp Med. 2008; 205:1535–1541. [PubMed: 18573909]
24. Kebir H, Ifergan I, Alvarez JI, et al. Preferential recruitment of interferon-gamma-expressing T H 17 cells in multiple sclerosis. Ann Neurol. 2009; 66:390–402. [PubMed: 19810097]
25. Cervenak L, Magyar A, Boja R, Laszlo G. Differential expression of GL7 activation antigen on bone marrow B cell subpopulations and peripheral B cells. Immunol Lett. 2001; 78:89–96. [PubMed: 11672592]
26. Phan TG, Grigorova I, Okada T, Cyster JG. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. Nat Immunol. 2007; 8:992–1000. [PubMed: 17660822]
27. Fillatreau S, Sweenie CH, McGeachy MJ, et al. B cells regulate autoimmunity by provision of IL-10. Nat Immunol. 2002; 3:944–950. [PubMed: 12244307]
28. Edwards JC, Szczepanski L, Szechinski J, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med. 2004; 350:2572–2581. [PubMed: 15201414]
29. Cambridge G, Stohl W, Leandro MJ, et al. Circulating levels of B lymphocyte stimulator in patients with rheumatoid arthritis following rituximab treatment: relationships with B cell depletion, circulating antibodies, and clinical relapse. Arthritis Rheum. 2006; 54:723–732. [PubMed: 16508933]
30. Anolik JH, Barnard J, Owen T, et al. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. Arthritis Rheum. 2007; 56:3044–3056. [PubMed: 17763423]
31. Lucchinetti C, Bruck W, Parisi J, et al. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol. 2000; 47:707–717. [PubMed: 10852536]

32. Serafini B, Rosicarelli B, Magliozzi R, et al. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. Brain Pathol. 2004; 14:164–174. [PubMed: 15193029]
33. Magliozzi R, Howell O, Vora A, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain. 2007; 130:1089–1104. [PubMed: 17438020]
34. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. Immunol Rev. 2008; 224:201–214. [PubMed: 18759928]
35. Evans JG, Chavez-Rueda KA, Eddaoudi A, et al. Novel suppressive function of transitional 2 B cells in experimental arthritis. J Immunol. 2007; 178:7868–7878. [PubMed: 17548625]
36. De Smedt T, Van Mechelen M, De Becker G, et al. Effect of interleukin-10 on dendritic cell maturation and function. Eur J Immunol. 1997; 27:1229–1235. [PubMed: 9174615]
37. Moulin V, Andris F, Thielemans K, et al. B lymphocytes regulate dendritic cell (DC) function in vivo: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. J Exp Med. 2000; 192:475–482. [PubMed: 10952717]
38. Reichardt P, Dornbach B, Rong S, et al. Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. Blood. 2007; 110:1519–1529. [PubMed: 17392507]
39. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med. 2004; 199:971–979. [PubMed: 15067033]
40. Kukreja A, Cost G, Marker J, et al. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest. 2002; 109:131–140. [PubMed: 11781358]
41. Hong J, Li N, Zhang X, et al. Induction of CD4+CD25+ regulatory T cells by copolymer-I through activation of transcription factor Foxp3. Proc Natl Acad Sci U S A. 2005; 102:6449–6454. [PubMed: 15851684]
42. Putheti P, Soderstrom M, Link H, Huang YM. Effect of glatiramer acetate (Copaxone) on CD4+CD25high T regulatory cells and their IL-10 production in multiple sclerosis. J Neuroimmunol. 2003; 144:125–131. [PubMed: 14597106]
43. Sfikakis PP, Souliotis VL, Fragiadaki KG, et al. Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. Clin Immunol. 2007; 123:66–73. [PubMed: 17275413]
44. Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature. 1991; 350:423–426. [PubMed: 1901381]
45. Matsushita T, Yanaba K, Bouaziz JD, et al. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008; 118:3420–3430. [PubMed: 18802481]
46. Pollinger B, Krishnamoorthy G, Berer K, et al. Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. J Exp Med. 2009; 206:1303–1316. [PubMed: 19487416]
47. Hamaguchi Y, Xiu Y, Komura K, et al. Antibody isotype-specific engagement of Fcgamma receptors regulates B lymphocyte depletion during CD20 immunotherapy. J Exp Med. 2006; 203:743–753. [PubMed: 16520392]
48. Kim HJ, Ifergan I, Antel JP, et al. Type 2 monocyte and microglia differentiation mediated by glatiramer acetate therapy in patients with multiple sclerosis. J Immunol. 2004; 172:7144–7153. [PubMed: 15153538]
49. Weber MS, Prod'homme T, Youssef S, et al. Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. Nat Med. 2007; 13:935–943. [PubMed: 17676050]
50. Harris DP, Haynes L, Sayles PC, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol. 2000; 1:475–482. [PubMed: 11101868]
51. Goetz M, Atreya R, Ghalibafian M, et al. Exacerbation of ulcerative colitis after rituximab salvage therapy. Inflamm Bowel Dis. 2007; 13:1365–1368. [PubMed: 17604367]
52. Steinman L, Zamvil SS. Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. Trends Immunol. 2005; 26:565–571. [PubMed: 16153891]

53. Bradl M, Misu T, Takahashi T, et al. Neuromyelitis optica: pathogenicity of patient immunoglobulin in vivo. Ann Neurol. 2009; 66:630–643. [PubMed: 19937948]

54. Bennett JL, Lam C, Kalluri SR, et al. Intrathecal pathogenic anti-aquaporin-4 antibodies in early neuromyelitis optica. Ann Neurol. 2009; 66:617–629. [PubMed: 19938104]

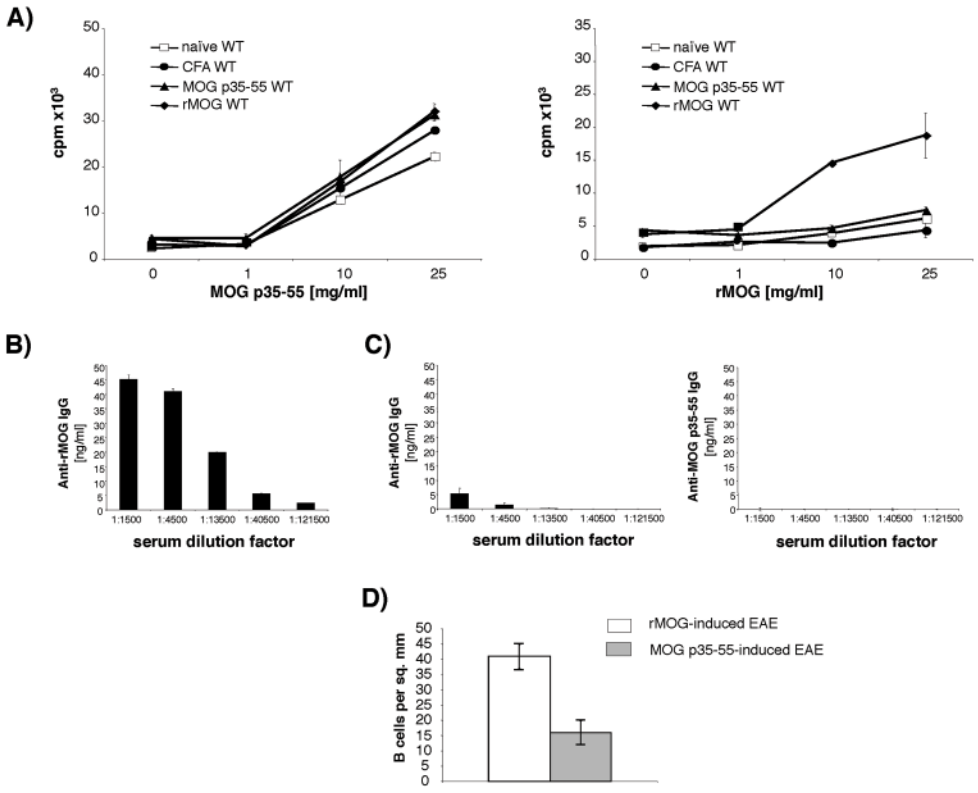

Figure 1. Immunization with MOG protein, but not MOG p35–55, promotes efficient B cell APC function and development of myelin-specific antibodies

a) MACS-separated B cells (purity >95%) isolated from unimmunized (naïve) C57BL/6 mice or mice which had been immunized with CFA, MOG p35–55 or MOG protein (rMOG) were co-cultured with naïve 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^3Thymidin-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 are detected within the CNS in EAE induced by rMOG than in EAE induced by MOG p35–55. EAE was induced in C57BL/6 mice by immunization with rMOG (100 μg) or MOG p35–55 (25 μg). CNS B cells in mice with EAE (10/group) were examined by immunohistochemistical staining for B220 on day 25 after immunization.

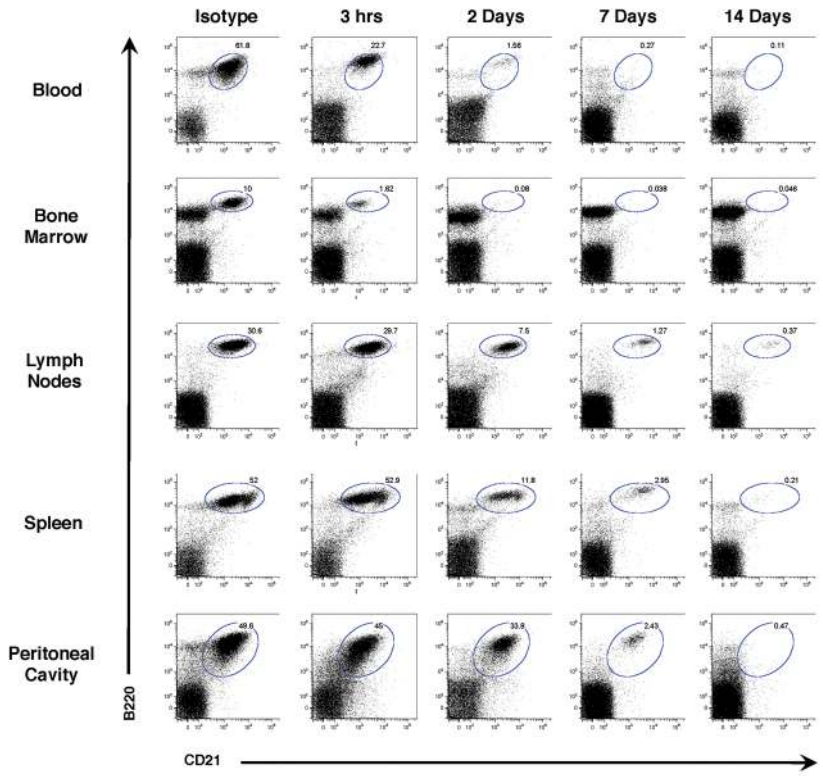

Figure 2. Kinetics of anti-CD20-mediated B cell depletion differs in distinct tissue microenvironments

C57BL/6 hCD20 Tg mice[13,14] were injected i.p. with 200 μg 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 anti-B220 (pan-B cell marker) and anti-CD21 (a mature B cell marker), then examined by FACS analysis. Results shown are representative of two experiments (2–3 mice/time-point/experiment).

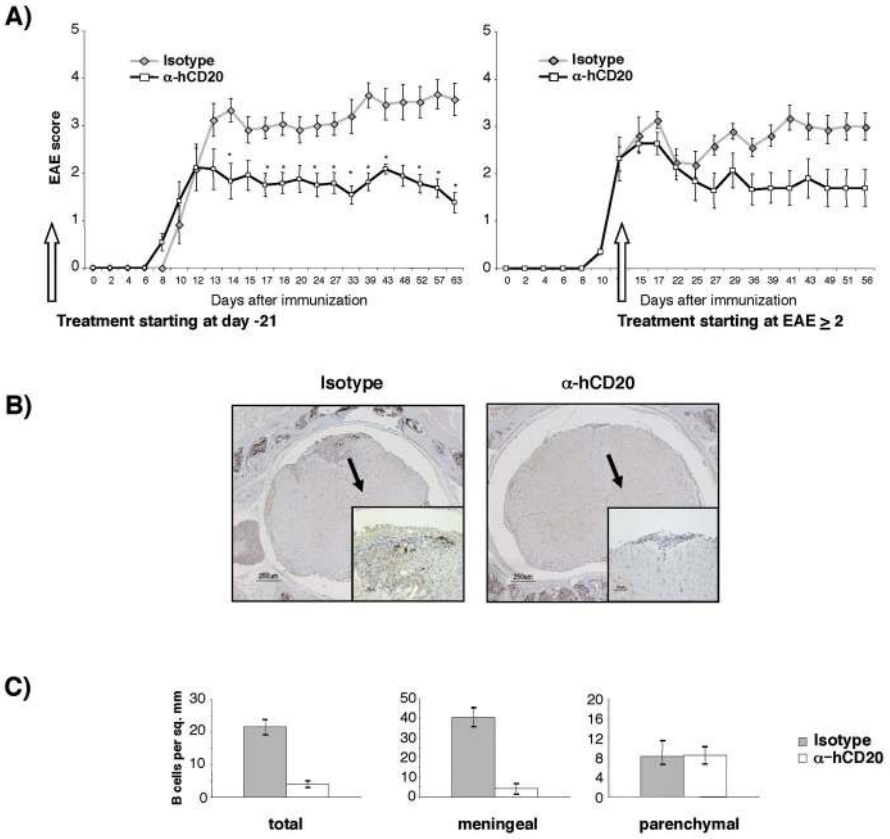

Figure 3. Anti-CD20 treatment ameliorates EAE induced by mouse MOG protein

a) C57BL/6 hCD20 Tg mice received 200 μg anti-hCD20 or isotype control (IgG2a) weekly starting 21 days prior to EAE induction (left panel), or after EAE was fully established (EAE score ≥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 five separate experiments (10–13 mice/group/experiment). **b and 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 square mm of total (left panel), meningeal (middle panel) or parenchymal (right panel) spinal cord tissue.

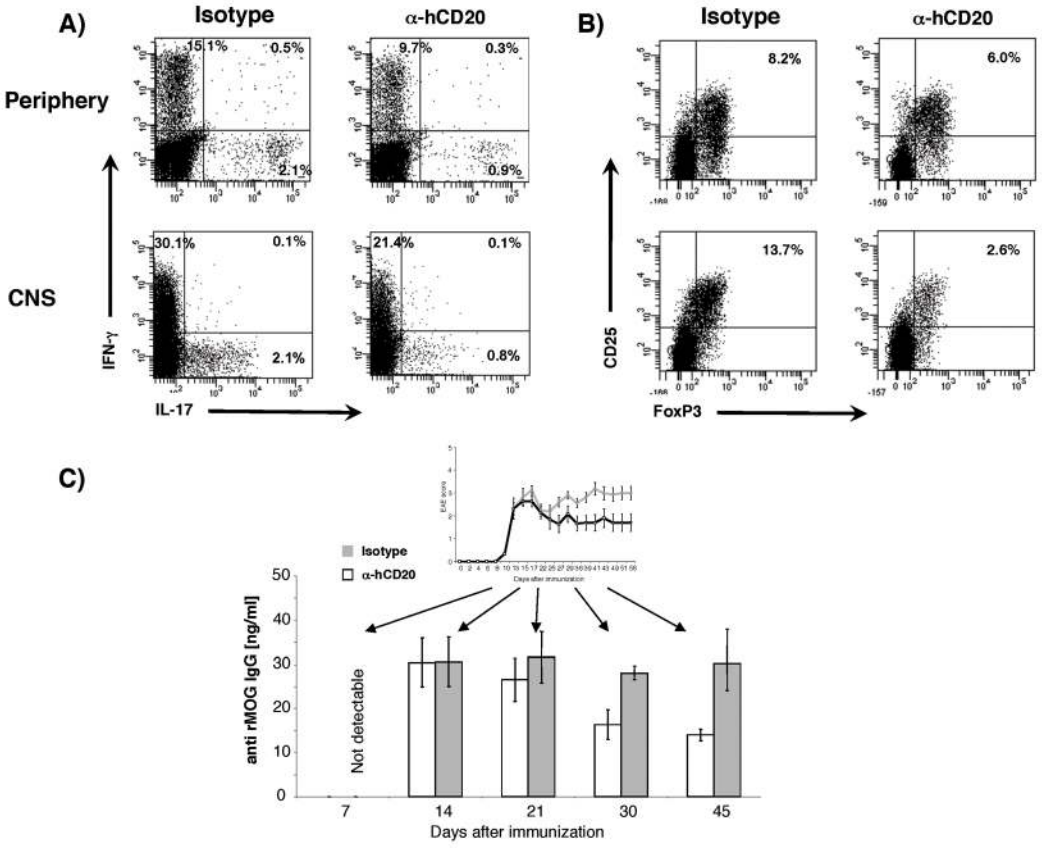

Figure 4. In EAE induced by MOG protein, anti-CD20 B cell depletion is associated with a reduced frequency of Th1-, Th17, and $CD4^+CD25^+FoxP3^+$ regulatory T cells and decreased anti-MOG antibody titers

C57BL/6 hCD20 Tg mice received weekly 200 μg of anti-hCD20 or isotype (IgG2a, control) after EAE was fully established (EAE score ≥2); **a)** Proinflammatory differentiation of peripheral (upper panel) and CNS-infiltrating T cells (lower panel) was evaluated by intracellular FACS staining for IL-17 and 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).

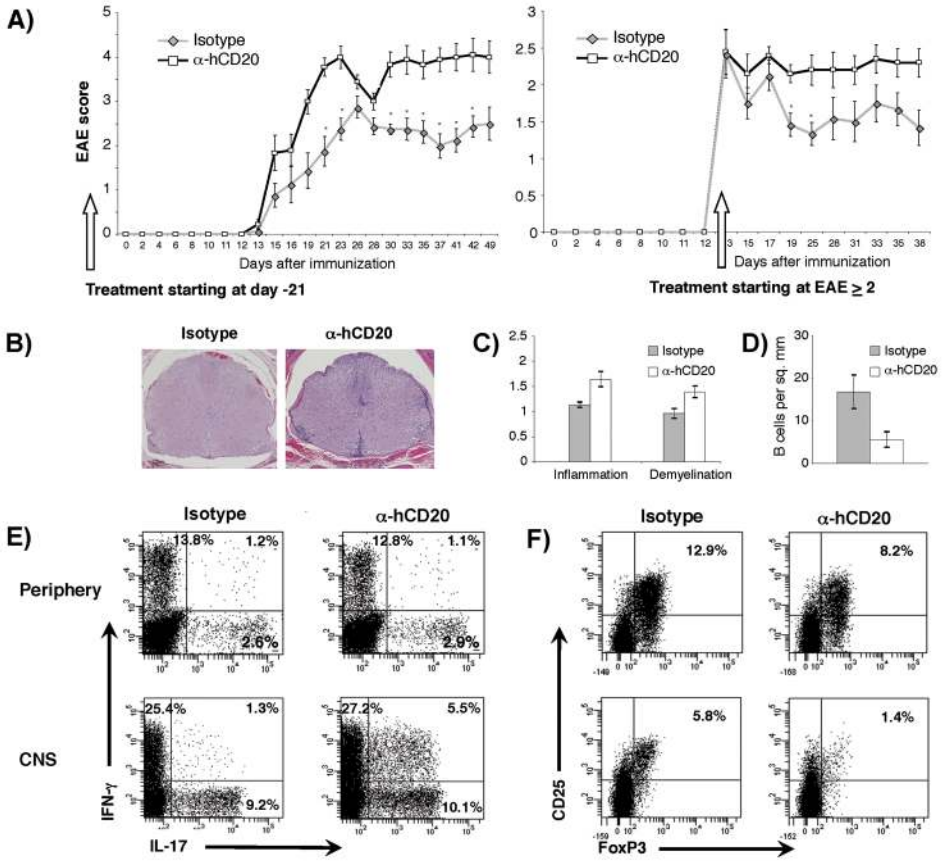

Figure 5. Anti-CD20 treatment exacerbates EAE induced by MOG p35–55 peptide

a) C57BL/6 hCD20 Tg mice received 200 μg anti-hCD20 or isotype control (IgG2a) weekly starting initiated 21 days prior to EAE induction (left panel), or after EAE was fully established (EAE score ≥2, right panel); white arrows indicate treatment onset. Results are representative of four separate experiments (10–12 mice/group/experiment). **b, c)** Spinal cord was evaluated for inflammatory infiltration (H+E) and demyelination with sections scored on a scale from 0–4. **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 square mm of total spinal cord tissue. **e)** Proinflammatory differentiation of peripheral (upper panel) and CNS-infiltrating T cells (lower panel) was evaluated by intracellular FACS staining for IL-17 and 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).

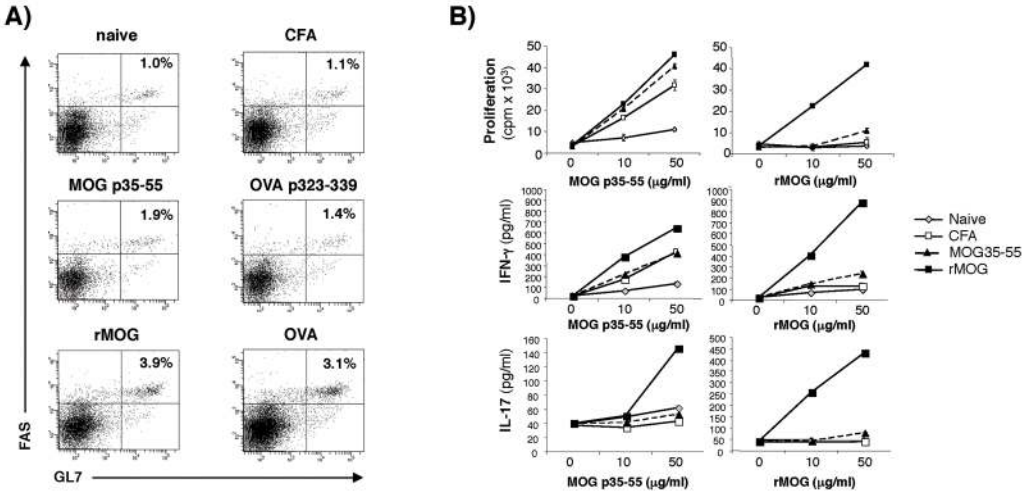

Figure 6. Immunization with MOG protein generates a population of activated antigen-specific B cells that efficiently process and present rMOG protein to MOG 35–55 TCR Tg T cells

a) B cells isolated from C57BL/6 wild-type which had not been immunized (naïve) or immunized with CFA, MOG p35–55, OVA p323–339, MOG protein or OVA protein were evaluated for surface expression of FAS and GL7 (gated on $B220^{+}$). b) MACS-separated B cells (purity >95%) isolated from unimmunized (naïve), CFA-, MOG p35–55-, or rMOG-immunized mice were co-cultured with naïve T cells isolated from MOG T cell receptor Tg mice in the presence of MOG p35–55 or rMOG protein. Proinflammatory T cell differentiation was evaluated by secretion of IFN-γ (upper panel) or IL-17 (lower panel).

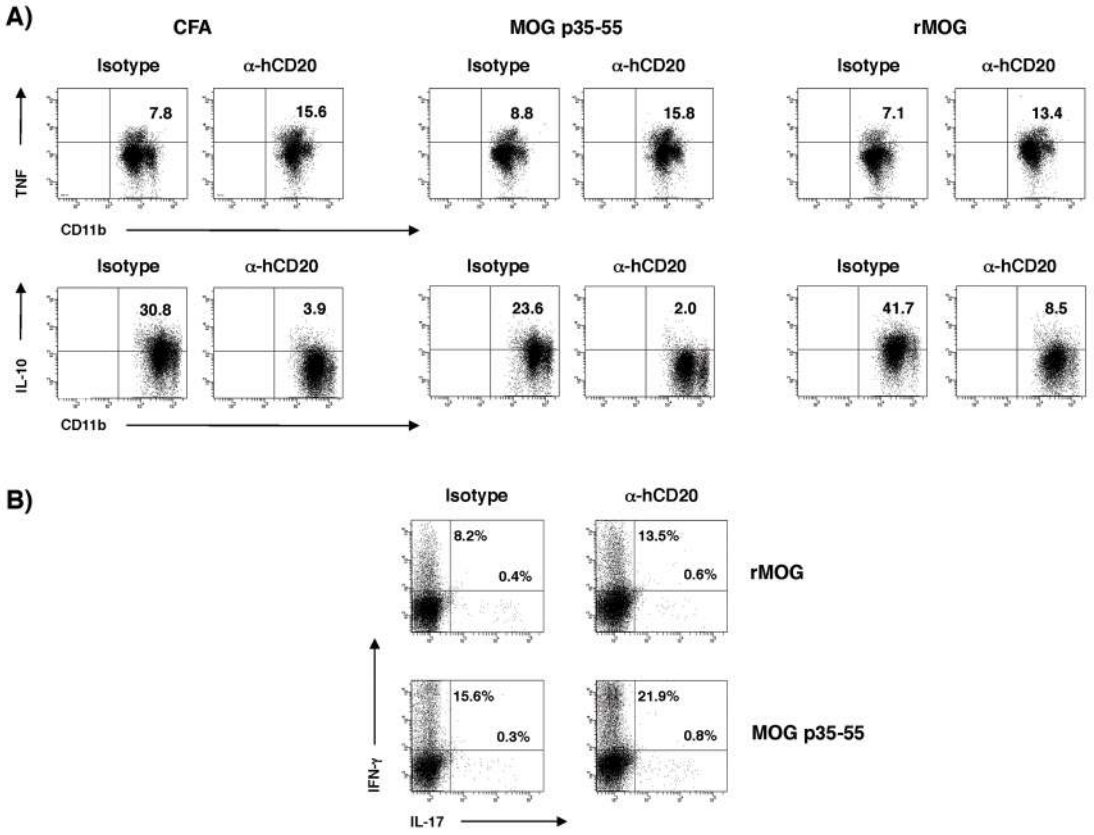

Figure 7. Anti-CD20 B cell depletion increases the capacity of remaining APC to generate encephalitogenic T cells

C57BL/6 hCD20 Tg mice received weekly 0.2 mg of anti-hCD20 or isotype (IgG2a, control) starting 21 days prior to EAE induction with rMOG protein (upper panels) or MOG p35–55 peptide (lower panels). 12 days after immunization, spleens were isolated and $B220^+$ B cells and $CD3^+$ T cells were removed by MACS-separation. **a)** Production of TNF and IL-10 by remaining $CD11b^+$ cells (gated) was evaluated by intracellular FACS staining. **b)** Remaining splenocytes were co-cultured with naïve T cells from MOG p35–55-specific TCR 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 IFN-γ (gated on $CD3^+$ T cells).

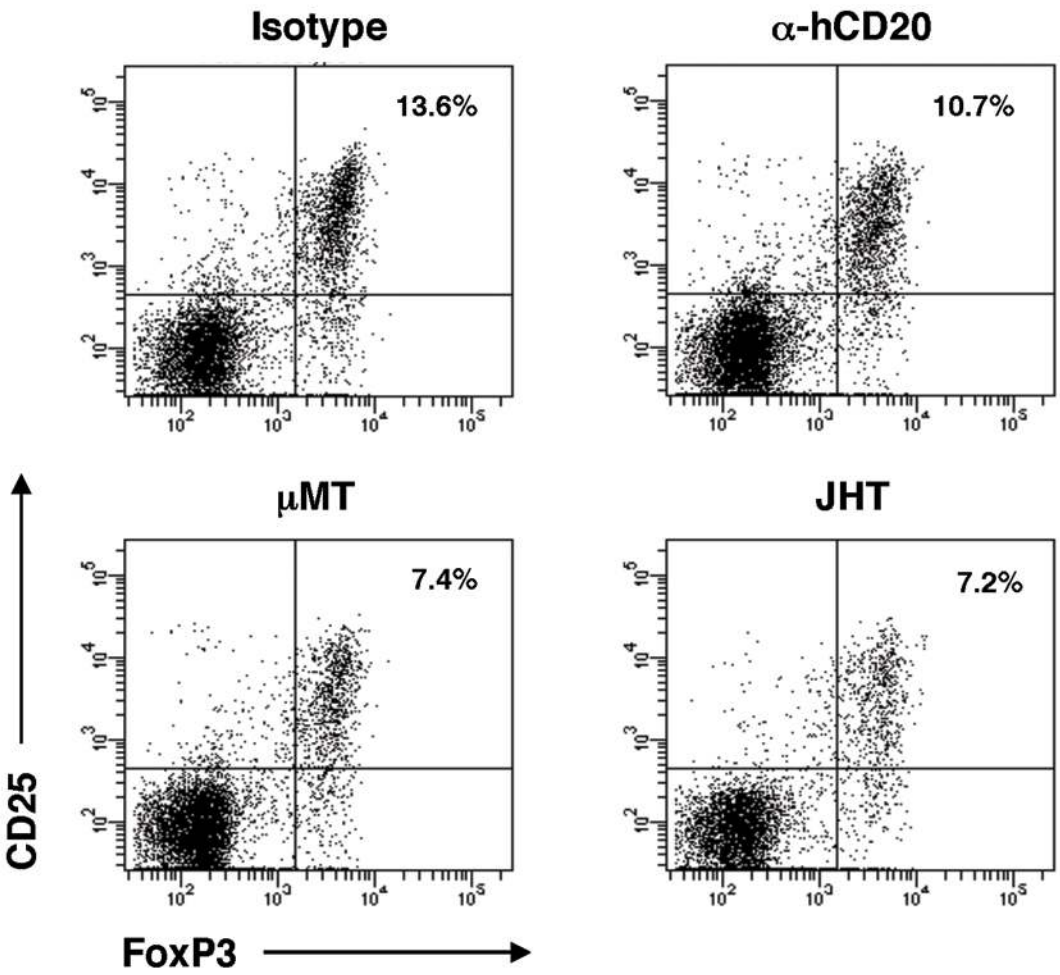

Figure 8. B cell deficiency is associated with reduced frequency of FoxP3$^+$ regulatory T cells

Unimmunized C57BL/6 hCD20 Tg mice that received 200 μg of isotype or anti-hCD20 were compared to un-immunized C57BL/6 B cell-deficient μMT or JHT mice. Frequency of peripheral FoxP3$^+$ regulatory T cells was investigated by CD4/CD25/FoxP3 triple staining (gated on CD4$^+$ T cells).

Ann Neurol. Author manuscript; available in PMC 2012 June 15.

Table 1

Clinical responses to anti-CD20 B cell depletion in EAE induced by MOG protein or MOG p35–55

a) 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)

b) 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 five separate experiments (10–13 mice/group/experiment)

#Results are representative of four separate experiments (10–12 mice/group/experiment)