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Research

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A type II anti-CD20 monoclonal antibody efficiently depletes CNS B cells in a mouse model of multiple sclerosis

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Running head: CNS B-cell depletion by obinutuzumab
Abstract

Background: Successful therapy with anti-CD20 monoclonal antibodies (mAbs) has reinforced the key role of B cells in the immunopathology of multiple sclerosis. While treatment with currently available anti-CD20 mAbs results in rapid and robust elimination of vascular B cells, B cells residing within compartments of the central nervous system (CNS) are not well targeted. The aim of this study was to determine the effects of a novel class of anti-CD20 mAbs on vascular and extravascular CNS-infiltrating B cells in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis.

Methods: Male double transgenic hCD20xhIgR3 mice and male wild-type C57BL/6 (B6) mice were injected with human myelin oligodendrocyte glycoprotein (MOG)_{1-125} to induce chronic EAE. On days 19, 22, and 25 after immunization, the hCD20xhIgR3 mice were injected intravenously with an anti-human CD20 mAb (5 mg/kg), either rituximab (a type I anti-CD20 mAb) or obinutuzumab (a type II humanized anti-CD20 mAb). The B6 mice received a dose of the murine anti-mouse CD20 antibody 18B12. Development of EAE was assessed daily. Seven days after the last injection, mice were euthanized, splenic B-cell subsets were analyzed by flow cytometry, and differential gene expression determined by single-cell RNA sequencing. Total serum immunoglobulin (Ig)G and anti-MOG_{1-125} IgG titers were measured by enzyme-linked immunosorbent assay. Reduction in CNS-infiltrated CD19^{+} and CD3^{+} cells was analyzed by immunohistochemistry, and ultrastructural CNS pathology was studied by transmission electron microscopy.

Results: Treatment with either anti-CD20 mAb had no effect on the clinical course of the disease, animal weight, or serum antibody levels. Obinutuzumab was superior to rituximab in reducing both splenic and CNS-infiltrated B cells. At the single-cell level, obinutuzumab showed pronounced effects on germinal center B cells as well as on CD4^{+} T cells, which acquired a regulatory-gene signature. In addition, obinutuzumab had beneficial effects on spinal cord myelination. B-cell depletion rates in the 18B12/B6 model were comparable with those observed in obinutuzumab-treated hCD20xhIgR3 mice.
Conclusions: Our results demonstrate differential effects of anti-CD20 mAbs on peripheral immune response and CNS pathology, with type II antibodies potentially being superior to type I in the depletion of tissue-infiltrating B cells.

Word count: 350 (max 350)

Keywords: B cells; B-cell therapy; CD20; experimental autoimmune encephalomyelitis; multiple sclerosis; obinutuzumab; rituximab; 18B12
Background

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and the most common cause of irreversible neurologic disability in young adults [1]. MS is considered to be a heterogeneous disease, with complex genetics and multiple environmental factors playing a role in individual susceptibility to disease development [2, 3]. The clinical course of MS is dependent on the subtype of the disease, with the classical variants including: (i) relapsing-remitting MS (RRMS), which accounts for 80–90% of all MS cases; (ii) secondary progressive MS (SPMS), which follows 70–80% of RRMS cases 10–15 years after disease onset; and (iii) primary progressive MS, which is the rarer variant accounting for 10–15% of all cases [4, 5].

Although the detrimental role of inflammation in MS is undisputed, the pathogenic cell populations and the precise roles they play remain controversial [6, 7]. Furthermore, another level of complexity is added by the diverse mechanisms driving the development of the disease, not just among the different subtypes but also among patients within one type of MS [8, 9]. It is generally agreed that the inflammatory reaction in MS results from the cumulative effect of a number of factors, including activities of cells of the innate and adaptive immune systems and their mediators, and effector molecules such as cytokines and antibodies [10, 11].

One of the best-characterized animal models capable of reflecting important aspects of MS, among which are both the autoimmune inflammatory and the neurodegenerative components, is experimental autoimmune encephalomyelitis (EAE) [12, 13]. EAE can be induced in susceptible animal strains by active immunization with components of the myelin sheath or by the passive transfer of autoreactive T cells [14]. Traditional EAE models are mostly dependent on T cells and macrophages; in particular, T-helper cells have been the focus of research. This “T-cell paradigm” was subsequently also assumed for human MS.

Nevertheless, despite being dubbed a “T cell-mediated disease,” B cells have increasingly gained importance and attention as key components of the pathogenesis of MS [15, 16].
The updated conceptual understanding of the involvement of B cells in the immunopathophysiology of MS has mainly emerged on the basis of the success of anti-CD20 therapy in treating patients with RRMS [17]. In initial studies, depletion of circulating B cells by rituximab, a chimeric anti-CD20 monoclonal antibody (mAb), led to a rapid reduction in gadolinium-enhancing lesions and magnetic resonance imaging lesion load, as well as a decrease in relapse activity in patients with RRMS [10]. More recent studies involving humanized (ocrelizumab) and human (ofatumumab) anti-CD20 mAbs confirmed a high level of efficacy in RRMS in the clinic [18-21]. Furthermore, in patients with primary progressive MS, ocrelizumab is associated with lower rates of clinical and magnetic resonance imaging progression compared with placebo [22]. Despite these therapeutic developments, SPMS – in which one of the characteristic features is chronic CNS-compartmentalized inflammation – remains difficult to treat [23, 24].

One of the factors contributing to sustained neuroinflammation within the CNS has been identified as the presence of large B-cell aggregates that are present in the inflamed meninges of a substantial portion of patients with SPMS [25-29]. These meningeal B cell-rich aggregates of immune cells are considered as potential mediators of “sequestered” inflammation that spreads the continuous CNS injury underlying the secondary progressive form of the disease [16, 30].

Clinical studies have demonstrated that B cells residing in these specific CNS compartments are protected from depletion following conventional anti-CD20 therapy [31, 32]. Intrathecal injection of rituximab in patients with low-inflammatory SPMS resulted in incomplete depletion of B cells within the cerebrospinal fluid compartment and insufficient dampening of neuroinflammation [31]. Certain B cell-depleting characteristics of the anti-CD20 mAb (rituximab) used in these studies could be one of the reasons for the partial resistance of B cells found within the CNS of these patients. Briefly, depending on their B cell-depleting characteristics, anti-CD20 mAbs are grouped into type I and type II [33]. While type I mAbs (including rituximab, ocrelizumab, and ofatumumab) activate the classical pathway of the complement cascade, and induce complement-dependent cytotoxicity by inducing the
reorganization of CD20 into lipid rafts, type II mAbs trigger direct cell death upon binding CD20 without cross-linking by secondary antibodies [33-35]. Antibody-dependent cell-mediated cytotoxicity and antibody-dependent phagocytosis can be induced by both antibody types in the presence of immune effector cells. However, type II mAbs exert more potent natural killer cell-mediated antibody-dependent cell-mediated cytotoxicity and increased monocyte- and macrophage-mediated antibody-dependent phagocytosis compared with type I mAbs, particularly when glycoengineered [36-38].

Obinutuzumab is a humanized type II anti-CD20 mAb that is currently approved as a first-line treatment for chronic lymphocytic leukemia and rituximab-refractory follicular lymphoma [39-41]. Compared with rituximab, obinutuzumab has demonstrated an increased depletion rate of peripheral B cells in healthy donors, as well as a more efficient reduction of malignant B cells in patients with chronic lymphocytic leukemia [39, 42]. The elbow hinge modification of obinutuzumab results in an alternative binding conformation of the CD20–obinutuzumab complex and is responsible for the more potent induction of direct cell death compared with rituximab and ofatumumab [34, 43]. Furthermore, complement-dependent cytotoxicity and hence FcγRIIb-mediated CD20 internalization are reduced compared with rituximab, resulting in sustained availability and activity of obinutuzumab [38, 44].

In the current study, we tested the therapeutic efficacy of the type II mAb obinutuzumab compared with that of the type I mAb rituximab in a B cell-dependent EAE model using a double transgenic (dbtg) mouse line that expresses both murine and human CD20 on B cells and tolerates the administration and presence of human IgGs [45, 46].
Methods

Mice

Male 6 week-old wild-type (WT) C57BL/6 (B6) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and dbtg hCD20xhIgR3 mice were obtained from Taconic (Ejby, Denmark). The dbtg mice expressed both human and murine CD20 on their B cells, in addition to an immunoglobulin (Ig) mini-repertoire composed of the secreted forms of H-\(\gamma_1\), IgL-\(\kappa\), and IgL-\(\lambda\) chains [45, 46]. Mice were housed under pathogen-free conditions with a 12 h light/dark cycle and free access to standard rodent diet (ssniff Spezialdiäten, Soest, Germany) and water. Special care such as softened food and ClearH\(_2\)O HydroGel (ClearH\(_2\)O, Portland, ME, USA) was provided for paralyzed animals. All animal experiments were approved by the Regierung von Unterfranken (file number 55.2-2532-2-577) and performed according to the criteria outlined by the German Animal Welfare Law, and complied with the German Law on the Protection of Animals, the “Principles of Laboratory Animal Care” (NIH publication no. 86-23, revised 1985), and the ARRIVE guidelines for reporting animal research [47].

Active EAE induction and clinical assessment

All animals were 15 weeks old at the time of immunization. Mice were immunized subcutaneously into both sides of the flank with a total dose of 200 µg human recombinant myelin oligodendrocyte glycoprotein (MOG\(_{1-125}\)) emulsified in complete Freund’s adjuvant (induction kit from Hooke Laboratories, Lawrence, MA, USA). Pertussis toxin (100 ng; Hooke Laboratories) diluted in 100 µL of sterile phosphate-buffered saline (PBS) was injected intraperitoneally on day 0 and again 24 h later. Signs of EAE were monitored daily, starting at day 5 after immunization, using the standard EAE scoring system with the following grades: 0, no signs of disease; 1, limp tail; 2, hindlimb weakness; 3, nearly complete or complete hindlimb paralysis; 4, complete hindlimb and partial forelimb paralysis; 5, moribund. Increments of 0.5 were used to account for intermediate scores not clearly demarcated by the five categories.
Anti-CD20 mAb treatment

The anti-CD20 IgG1 mAbs obinutuzumab and rituximab, the isotype-matched control for obinutuzumab (hIgG1), and the murine anti-CD20 IgG2a antibody 18B12 were kindly provided by F. Hoffmann-La Roche (Basel, Switzerland). The chimeric IgG1 control for rituximab (chIgG1) was purchased from Absolute Antibody (Oxford, UK) and the isotype control antibody for 18B12 (muIgG2a) from Bio X Cell (Lebanon, NH, USA). Mice were grouped \((n = 5–6 \text{ per group})\) according to their EAE course and score, and treatment was started at day 19 after immunization, when all mice had reached an EAE score of ≥2.5. Antibodies were injected intravenously into the lateral tail vein at 5 mg/kg body weight, diluted in 50 µL sterile PBS. Treatment was repeated on days 22 and 25. Animals were euthanized 7 days after the last treatment by administration of CO₂.

Serum collection and antibody enzyme-linked immunosorbent assay (ELISA)

Blood was taken from the inferior vena cava of each mouse and allowed to clot. Serum was then collected by centrifugation at 1000 \(g\) for 15 minutes at 4°C and stored at -80°C until further analysis. For quantification of total serum IgG and human MOG₁₋₁₂₅-specific IgG, ELISAs were performed using the Mouse IgG Total Uncoated ELISA kit (Thermo Fisher Scientific, Waltham, MA, USA) and the SensoLyte anti-human MOG (1–125) mouse IgG-specific quantitative ELISA kit (AnaSpec, Fremont, CA, USA) following the manufacturers’ instructions.

Isolation of splenocytes for flow cytometry and single-cell RNA sequencing

Mouse spleens were dissected and cells isolated by filtering the tissue homogenate through a 70 µm cell strainer. Cells from individual mice were suspended in 30 mL Gibco RPMI 1640 medium (Merck, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 50 µM \(\beta\)-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; Sigma-Aldrich), and centrifuged at 500 \(g\) for 5 minutes. Resuspended cells were treated with 1× red blood cell lysis buffer (BioLegend, London, UK) for 10 minutes to remove red blood cells. The reaction
was stopped by adding PBS and cells were washed twice. Cells were filtered through a 70 µm cell strainer to remove clumps of dead cells. After an additional centrifugation step, cells were resuspended at a concentration of $2.5 \times 10^6$/mL for all further procedures.

**Flow cytometry**

Splenocytes were harvested as described above and $5 \times 10^5$ cells were transferred into a 96-well plate and incubated with 0.2 µL/well of BD Horizon Fixable Viability Stain 780 (FVS780; BD Biosciences, San Jose, CA, USA) for 30 minutes. Cells were centrifuged at 500 g for 5 minutes before incubation with 50 µL master mix containing 0.125 µg of each antibody. Five different antibody panels were used to identify cell types. Allophycocyanin (APC) anti-CD19 (BioLegend) and brilliant violet (BV) 510 anti-CD3e (BD Biosciences) were used for B- and T-cell staining, respectively. BV510 anti-CD19, APC anti-CD93, BV421 anti-CD21/CD35, and phycoerythrin (PE) anti-CD1d (all BioLegend) were used to differentiate between CD19+/CD93−/CD21lo follicular B cells and CD19+/CD93+/CD21hi/CD1dhi marginal zone B cells. Plasma cells were stained with BV605 anti-CD45R as well as BV421 anti-CD138 (both BD Biosciences) and defined as CD45R−/CD138+. BV510 anti-CD19, BV421 anti-CD80 (BD Biosciences), and PE anti-CD73 (BD Biosciences) were used to stain CD19+/CD80+/CD73+ memory B cells. BV510 anti-CD19, APC anti-IgM (BioLegend), PE anti-IgD (BioLegend), and BV421 anti-IgG (BioLegend) marked CD19+/IgM−/IgD+ naïve and CD19+/IgG+ isotype-switched cells. After incubation, stained cells were washed with BD FACSFlow (BD Biosciences) twice and resuspended in a final volume of 150 µL BD FACSFlow.

Flow cytometric acquisition was performed on a CytoFLEX S machine equipped with CytExpert 2.2 software (Beckman Coulter, Brea, CA, USA). Doublets were excluded using the combined width parameter of the forward and side scatter. Viable cells were gated before lymphocyte gates were set for further analysis. Data analysis was performed using FlowJo v10.0.6 (FlowJo, LLC, Ashland, OR, USA).
**Single-cell RNA sequencing**

Splenocytes were isolated as described above. Cells from 3–4 mice per group were pooled and kept on ice until processing. For each sample, 5000 cells were subjected to 10X Chromium Single Cell 3’ Solution v3.1 library preparation according to the manufacturer’s instructions (10X Genomics, Pleasanton, CA, USA). Libraries were sequenced on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) using the recommended read lengths for 10X Chromium v3.1 chemistry to a depth of 20 000 reads per cell. Reads were converted to FASTQ format using mkfastq from Cell Ranger 3.1.0 (10X Genomics). Reads were then aligned to the 10X Genomics mm10-3.0.0 mouse reference genome (mm10, Ensembl annotation release 93). Alignment was performed using the count command from Cell Ranger 3.1.0. Primary analysis, quality control filtering (gene count per cell, unique molecular identifier count per cell, percentage of mitochondrial transcripts), clustering, UMAP projection, and visualization of gene expression were performed using the Seurat v3.2.0 package under R v4.0.2 (Satija Lab, New York, NY, USA). The raw gene expression was scaled using the SCTransform approach. To allow comparison of gene expression between study groups, SCTransform-scaled data sets were integrated using the shared nearest neighbor approach as implemented in Seurat v3.2.0. Cluster marker genes were determined using edgeR v3.30.3. The edgeR model included the cellular detection rate (= sequencing depth per cell) and the sample identity as covariates to account for technical differences between samples.

The same approach was used to find genes differentially expressed between cells of obinutuzumab- versus rituximab-treated mice in one cell type. The model of the differential expression test included the cellular detection rate as a covariate. Cellular pathways were taken from the Molecular Signatures Database (MSigDB v7.2, https://www.gsea-msigdb.org/gsea/msigdb) [48]. We used the “hallmark inflammatory response” signature, which includes 200 genes defining inflammatory response (Institute B [2004–2019] Gene Set: HALLMARK_INFLAMMATORY_RESPONSE. Gene Set Enrichment Analysis). Genes differentially expressed in at least one of the investigated cell types were visualized as heat
maps (pheatmap v1.0.12) using the mean log-normalized expression and gene-wise scaling. Clustering was performed on the Euclidean distances with complete linkage.

**Immunohistochemistry and histopathology of cerebellum**

To determine the effect of type I and type II anti-CD20 mAbs on the depletion of CNS-infiltrating B cells, the cerebellum was studied during the chronic stage of EAE. In our previous studies [49], the cerebellum showed the highest degree of B-cell infiltration compared with other regions of the CNS. After transcardiac perfusion with 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany), mouse cerebella were dissected and post-fixed in the same fixative overnight, followed by washing with phosphate buffer (PB), then dehydration and embedding in paraffin. A total of 150 serial sections were cut at a thickness of 5 µm, and every fifth section (up to a total of 30 sections) was stained for B and T cells. In preparation for staining, sections were deparaffinized with xylene and rehydrated in an ascending series of isopropanol. For antigen retrieval, sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for 15 minutes. All the following incubation steps were performed at room temperature. After washing with tris-buffered saline (TBS), the cerebellar tissue sections were blocked with 5% milk powder (Heirler, Radolfzell, Germany) in TBS with 0.1% Tween 20 detergent (TBST) for 30 minutes, followed by 1 h incubation with monoclonal rabbit anti-mouse anti-CD3 antibody (SP1629, 1:150; Abcam, Cambridge, UK). Slides were then washed twice with TBST followed by 1 h incubation with a biotinylated goat anti-rabbit IgG antibody (1:500; Abcam). Alkaline phosphatase coupled to streptavidin (1:500; Vector Laboratories, Burlingame, CA, USA) was applied for 30 minutes after washing with TBST. CD3⁺ cells were visualized with the Vector BCIP/NBT substrate kit (Vector Laboratories). Before B220⁺ cells were stained on the same tissues, sections were washed for 15 minutes with TBST. Subsequently, slides were incubated with monoclonal rat anti-mouse anti-CD45R antibody (RA3-6B2, 1:250; Thermo Fisher Scientific) for 1 h, followed by a further 1 h incubation with biotinylated goat anti-rat IgG antibody (1:500; Abcam). Slides were washed three times with TBST between every step. Streptavidin–horseradish peroxidase (1:2000, Abcam) was applied for 35 minutes. 3,3’-diaminobenzidine (Vector Laboratories) was used
as a chromogen and tissues were counterstained with Nuclear Fast Red (Sigma-Aldrich).

Blinded analysis was conducted by counting infiltrates per section using a Leica DM2000 light microscope (Leica Microsystems, Wetzlar, Germany). Perivascular cuffing of cells was defined as dense infiltrates; cells that infiltrated loosely into the parenchyma were categorized as diffuse infiltrates. Infiltrates with fewer than five cells were excluded from the analysis.

Transmission electron microscopy of spinal cord

To assess ultrastructural spinal cord pathology, two segments of the lumbar spinal cord region from each mouse were post-fixed in 2.5% glutaraldehyde (Carl Roth) in 0.1 M sodium cacodylate buffer (pH 7.4; Carl Roth) overnight, followed by washing with PB until further processing. Osmification of tissues was performed with 1% osmium tetroxide (Science Services, München, Germany) and 1.5% potassium ferrocyanide (Merck) for 2 h. After washing with PB overnight, tissues were dehydrated in an ascending ethanol series until 100% ethanol was replaced by 100% acetone, which was eventually replaced by epoxy resin embedding medium. Specimens were polymerized overnight at 60°C in embedding molds filled with 100% epon (Carl Roth) containing 2% glycidether hardener (Carl Roth). Ultrathin sections (50 nm) were cut and placed on copper mesh grids for transmission electron microscopy analysis. Sections were then stained with uranyl acetate for 10 minutes and lead citrate for 10 minutes.

A total of 25 images per mouse of the ventral funiculus surrounding the median fissure were acquired using a Carl Zeiss Leo EM 906 E transmission electron microscope (Zeiss, Oberkochen, Germany) equipped with ImageSP software v1.2.10.2 (Sysprog, Minsk, Belarus and Tröndle Restlichtverstärkersysteme, Moorenweis, Germany). All images were taken at a magnification of 3597×. Quantification of axonal degeneration was performed manually using ImageSP and by categorizing axons into three different groups: (i) abnormally myelinated axons; (ii) degenerating axons; and (iii) already degenerated axons. In addition, the number of myelinated axons per square millimeter (mm²) was calculated on the same images. Nine images and a mean of 594 ± 70 randomly selected axons per animal (n = 5–6 mice per
group) were analyzed. The correlation between myelin sheath thickness and axon diameter was assessed by measuring the axonal and the total nerve fiber diameter maxima, from which the g-ratio was subsequently calculated by dividing the diameter of the axon by the total nerve fiber diameter. A mean of 229 ± 37 randomly selected axons in 6–7 images per mouse (n = 5–6 mice per group) were measured. Analysis was performed blinded.

**Statistical analysis**

Analysis was performed using GraphPad Prism software v9.0 (GraphPad, San Diego, CA, USA) except for analysis of single-cell RNA sequencing data sets (for details see above). Unless otherwise noted, data are presented as mean and minimum/maximum values. A p-value of ≤0.05 was considered statistically significant.
Results

No improvement of clinical disease following anti-CD20 mAb treatment in chronic EAE

B6 and hCD20xhIgR3 mice (n = 5–6 mice per group) were immunized with human MOG_{1–125} and clinical EAE was scored daily. In all mice, immunization caused severe disease with a chronic course that was characterized by complete paralysis of the hind limbs (Fig. 1). Details on disease onset and severity in the different groups are provided in Table 1. To investigate the effect of anti-CD20 mAb treatment in chronic EAE, mice were treated either with obinutuzumab (hCD20xhIgR3), rituximab (hCD20xhIgR3), 18B12 (WT B6), or the matching isotype controls, 19 days after they had reached an EAE score of ≥2.5. Treatment was repeated on days 22 and 25. None of the anti-CD20 mAbs was able to improve clinical disease as determined by EAE score (Fig. 1, Table 1) or total body weight (Table 1).

No impact of anti-CD20 mAb treatment on total and anti-MOG_{1–125} serum IgG

To evaluate whether the depletion of CD20⁺ cells by obinutuzumab, rituximab, or the murine mAb 18B12 had an effect on serum Ig levels, total IgG and anti-MOG_{1–125} IgG were measured by ELISA. Blood was taken from n = 5–6 mice per group, 7 days after the end of treatment. As shown in Fig. 2, neither total IgG nor anti-MOG_{1–125} antibody titers were diminished following treatment with anti-CD20 mAbs, compared with the corresponding isotype controls.

More efficient depletion of CD19⁺ B cells in the spleen by obinutuzumab compared to rituximab

Flow cytometric analysis of the spleen was performed to investigate the depleting capacity of the different anti-CD20 mAbs. Splenic B cells were isolated from 5–6 mice per group, 7 days after the end of treatment with anti-CD20 mAbs or corresponding isotype controls. As shown in Fig. 3, obinutuzumab-treated mice displayed decreased numbers of naïve (CD19⁺/IgD⁺/IgM⁻) (Fig. 3B), marginal zone (MZ) (CD19⁺/CD93⁻/CD21^{hi}/CD1d^{hi}) (Fig. 3C), follicular (FO) (CD19⁺/CD93⁻/CD21^{hi}/CD1d^{lo}) (Fig. 3D), and isotype-switched (CD19⁺/IgG⁺) B cells (Fig. 3G). Rituximab treatment was less effective and mainly depleted isotype-
switched B cells (Fig. 3G). 18B12, which was used as murine anti-CD20 mAb in WT B6 mice, was highly potent and depleted approximately 90% of all B-cell subsets except for memory B cells (Fig. 3B-D). Interestingly, although memory (CD19+/CD80+/CD73+) B cells express CD20, they were spared by anti-CD20 mAb-mediated depletion in all three treatment groups (Fig. 3F). As expected, the relative numbers of CD20− plasma cells (CD45R−/CD138+) (Fig. 3E) and CD3+ T cells (Fig. 3H) remained unaffected. The relative depletion compared to the isotype control is provided in Fig. 3I for each group.

**Differential gene expression in obinutuzumab- vs. rituximab-treated mice**

Expression of genes associated with an inflammatory response was analyzed by single-cell RNA sequencing of splenic lymphocytes in both obinutuzumab- and rituximab-treated mice (n = 3–4 mice per group), 7 days after treatment end. As shown in Fig. 4, obinutuzumab and rituximab differentially affected B-cell and T-cell subsets. Most notably, obinutuzumab showed a strong effect on germinal center B cells. In addition, the gene profile of CD4+ T cells in obinutuzumab-treated mice implicated a shift toward a regulatory phenotype, which was reflected by upregulation of *Foxo1, Cbl, Junb, Irf1, Ifnar1, Ccr6, Ccr7*, and *Ccr10*, as well as *Il27ra* [50-60].

**Reduction in the number of perivascular and parenchymal CNS B-cell infiltrates in obinutuzumab-treated mice**

Two types of infiltrates were analyzed: diffuse parenchymal vs. dense perivascular B cell infiltrates (Fig. 5A, B). As above, different groups of mice were treated with obinutuzumab, rituximab, 18B12, or the matched isotype controls and sacrificed 7 days after the end of treatment. Compared with treatment with the isotype controls, the numbers of both diffuse and dense B-cell infiltrates were reduced following treatment with obinutuzumab and 18B12 (Fig. 5B, D). In contrast, rituximab only depleted dense perivascular B-cell infiltrates but did not target B cells infiltrating into the parenchyma (Fig. 5B, D). Obinutuzumab treatment also reduced the number of diffuse T-cell infiltrates, while none of the anti-CD20 mAbs had any effect on dense T-cell infiltrates (Fig. 5C, D).
EM analysis of murine spinal cord sections revealed irreversible and ongoing axonal degeneration and abnormal myelination in all animals (Fig. 6). Analysis was performed during the chronic stage of the disease, 7 days after the last treatment with anti-CD20 mAbs or the corresponding isotype controls. Neither obinutuzumab nor rituximab treatment had an effect on axons that were in the process of degeneration or had already degenerated (Fig. 6B, left and middle panels). However, there was a reduction in the number of axons displaying signs of abnormal myelination, such as splitting or ballooning of myelin lamellae, in mice treated with rituximab ($p = 0.0185$) (Fig. 6B, middle panel). As shown in Fig. 6C, the total number of axons per mm$^2$ (as an indicator of axonal loss) remained unaffected by anti-CD20 treatment, as did the g-ratio (the diameter of the axon divided by the diameter of the nerve fiber including myelin sheath, Fig. 6D). Analysis of the overall degree of myelination demonstrated a significant shift back toward normally myelinated axons in obinutuzumab-treated mice (Fig. 6E, left panel; $p = 0.0208$ for the slope difference between the control and treatment group).
Discussion

Over recent years, treatment with anti-CD20 mAbs has demonstrated compelling evidence of a profound reduction in new brain lesions and relapse rates in patients with RRMS. All of the anti-CD20 mAbs studied have been of type I classification. While these mAbs deplete peripheral B cells efficiently, they do not sufficiently target CD20\(^+\) B cells that reside within the CNS compartments [31, 32]. In this study, we examined the effects of the type I anti-CD20 mAb rituximab and the type II anti-CD20 mAb obinutuzumab on vascular and extravascular CNS-infiltrated B cells in the B cell-dependent human MOG-induced EAE model.

Analysis of splenic B-cell subsets revealed more potent depletion of CD19\(^+\) B cells by obinutuzumab compared with rituximab, possibly due to the differentiated effector functions of type II mAbs [34, 38, 61]. Furthermore, our data show that follicular B cells were more efficiently depleted than marginal zone B cells, which is in line with previous findings [62]. Since marginal zone B cells play an important role in the first-line defense against systemic blood-borne infections [63], their preservation under anti-CD20 therapy should be beneficial. Marginal zone B cells may be partially spared from depletion because both obinutuzumab and rituximab are IgG1 antibodies; it was previously shown that this isotype depletes marginal zone B cells inefficiently compared with IgG2a, which depletes 98% of follicular and marginal zone B cells [62]. In addition, the expression of CD20 per cell varies between the different B-cell subsets, which potentially alters their relative susceptibility to antibody-mediated depletion. Plasma cells do not express CD20 and are hence not targeted by anti-CD20 mAbs, which is consistent with observations made in this study, both in flow cytometry experiments and when measuring total serum IgG and anti-MOG\(_{1–125}\) antibody titers. Since bone marrow plasma cells are intrinsically long-lived, and thus mature B cells are not required for maintaining their numbers, short-term CD20 B-cell depletion has no effect on pre-existing antibody levels [64].
Analysis of the expression of genes involved in the inflammatory response revealed pronounced effects of obinutuzumab on germinal center B cells. In particular, there was an upregulation of genes such as Psm and Nlrc5 associated with antigen presentation on MHC class I molecules [65-68]. Other genes that were found to be upregulated in germinal center B cells on obinutuzumab treatment are associated with B-cell development, proliferation, and differentiation. Prkcd, for example, plays an important role in B-cell signaling and autoimmunity, as well as regulation of growth, apoptosis, and differentiation [69-74]; Sppl2a is involved in the cleavage of CD74, which is essential for the development and functionality of B lymphocytes in mice [75]; and Stat5 is involved in promoting the survival of mature lymphocytes and possibly in regulating anti-apoptotic genes in primary B cells [76].

Transforming growth factor (TGF)-β1 expression has been assigned a regulatory role, and it has been shown that TGF-β1−/− EAE mice develop earlier neurologic impairment compared with their littermates [77]. TGF-β1 can downregulate the function of antigen-presenting cells and, in turn, encephalitogenic T-helper type 1/17 responses. Thus, higher expression of Tgfb1 genes in the residual germinal center B cells in obinutuzumab-treated mice might be a favorable effect associated with the dampening of neuroinflammation. Obinutuzumab also seemed to exert beneficial effects on CD4+ T cells. Several genes associated with a regulatory function were upregulated, including Foxo1, Cbl, Junb, Irf1, Ifnar1, Ccr6, Ccr7, and Ccr10, as well as Il27ra [50-60]. Regulatory CD4+ T cells have a suppressive effect on immune responses by limiting the activation, proliferation, survival, and pro-inflammatory function of various immune cells, including subsets of T cells such as T-helper cells [78].

An additional key finding of our study was that both B and T cells that infiltrated into the CNS parenchyma were strongly reduced by obinutuzumab, but spared in rituximab-treated mice. This is consistent with the most recent findings of Roodselaar and colleagues, who demonstrated more pronounced depletion of B and T cells in the brain when using type II anti-CD20 mAbs in a mouse model of SPMS [61]. In patients with MS, B-cell depletion is associated with less CNS inflammation over the months following treatment [10, 18, 22, 79-81]. B cells not only function as antigen-presenting cells but are also an important source of...
soluble inflammatory mediators, which can be toxic to neurons and oligodendrocytes [82, 83]. Consistent with the more potent depletion of parenchymal B cells, electron microscopic analysis revealed a significantly positive effect of obinutuzumab on the myelination status of the nerve fibers in the inflamed spinal cord.

Conclusions

Our results suggest that type II mAbs such as obinutuzumab might be superior to type I mAbs with respect to exerting effects on extravascular B cells. Our data also show that the murine anti-CD20 mAb 18B12 is highly effective in reducing the number of CNS-infiltrating B cells, and might thus be a good murine option for studying the depletion of tissue-infiltrating B cells using WT B6 EAE models.
| Abbreviation | Description |
|--------------|-------------|
| APC          | allophycocyanin |
| B6           | C57BL/6 |
| BV           | brilliant violet |
| CNS          | central nervous system |
| dbtg         | double transgenic |
| EAE          | experimental autoimmune encephalomyelitis |
| ELISA        | enzyme-linked immunosorbent assay |
| FO           | follicular |
| Ig           | immunoglobulin |
| mAb          | monoclonal antibody |
| MOG          | myelin oligodendrocyte glycoprotein |
| MS           | multiple sclerosis |
| MZ           | marginal zone |
| PB           | phosphate buffer |
| PBS          | phosphate-buffered saline |
| PE           | phycoerythrin |
| RRMS         | relapsing-remitting multiple sclerosis |
| SPMS         | secondary progressive multiple sclerosis |
| TBS          | tris-buffered sodium |
| TBST         | TBS with 0.1% Tween 20 |
| TGF          | transforming growth factor |
| WT           | wild-type |
Declarations

Ethics approval

The study was approved by the Regierung von Unterfranken (file number 55.2-2532-2-577).
hCD20xhIgR3 mice were obtained from Taconic as regulated by an agreement between
F. Hoffmann-La Roche and FAU Erlangen-Nürnberg.

Consent for publication

Not applicable

Availability of data and materials

The data sets used and analyzed during the current study are available from the
corresponding author upon reasonable request.

Competing interests

ST, RC, and VS, PH and ABE declare that they have no competing interests. EU is an
employee of F. Hoffmann-La Roche. SK reports grants from the Deutsche
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Authors’ contributions

ST participated in the study design, performed all experiments, analyzed and interpreted the
data, and wrote the manuscript. RC and VS assisted with experiments. RC drafted and
revised the manuscript for intellectual content. PK and ABE were responsible for single-cell
RNA sequencing and helped with the data analysis and visualization. SK and EU were
responsible for conceptualization, funding acquisition, study design, and supervision, and
drafted and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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### Table 1. Clinical disease characteristics in mice treated with anti-CD20 mAbs

| Mouse strain | hCD20xhIgR3 hlgG1 | hCD20xhIgR3 obinutuzumab | WT B6 chlgG1 | WT B6 rituximab | WT B6 muIgG2a | WT B6 18B12 |
|--------------|--------------------|---------------------------|--------------|-----------------|---------------|-------------|
| **Treatment** |                    |                           |              |                 |               |             |
| Number of mice (n) | 6 | 6 | 6 | 5 | 6 | 5 |
| **Day of EAE onset** | 9.7 ± 0.5 | 9.8 ± 0.4 | 9.3 ± 0.8 | 9.0 ± 1.0 | 9.0 ± 0.0 | 9.0 ± 0.0 |
| **Maximum EAE disease score** | 3.5 ± 0.3 | 3.6 ± 0.5 | 3.6 ± 0.6 | 3.5 ± 0.4 | 3.7 ± 0.3 | 3.8 ± 0.3 |
| **Disease score before start of treatment** | 2.8 ± 0.5 | 3.0 ± 0.5 | 2.7 ± 0.3 | 2.7 ± 0.3 | 3.0 ± 0.4 | 2.9 ± 0.2 |
| **Final disease score** | 2.9 ± 0.4 | 3.0 ± 0.8 | 2.9 ± 0.9 | 2.6 ± 0.5 | 3.0 ± 0.3 | 2.7 ± 0.3 |
| **Score difference\(^a\)** | 0.1 ± 0.4 | 0.0 ± 1.0 | 0.3 ± 0.9 | -0.1 ± 0.4 | 0.0 ± 0.4 | -0.2 ± 0.2 |
| **Weight before start of treatment (g)** | 26.8 ± 1.7 | 26.1 ± 0.8 | 26.4 ± 1.8 | 26.5 ± 2.1 | 26.0 ± 1.4 | 26.9 ± 1.7 |
| **Final weight (g)** | 27.0 ± 2.1 | 25.8 ± 0.8 | 27.2 ± 1.7 | 26.7 ± 2.6 | 25.8 ± 2.0 | 26.6 ± 2.6 |
| **Weight difference\(^a\) (g)** | 0.3 ± 0.8 | -0.3 ± 0.4 | 0.7 ± 0.6 | 0.2 ± 0.9 | -0.2 ± 1.7 | -0.3 ± 1.5 |

\(^a\)Vs. value before start of treatment

Data shown are mean values ± standard deviation.

EAE, experimental autoimmune encephalomyelitis; WT, wild-type.
Figure legends

**Fig. 1. Effect of anti-CD20 mAb treatment on clinical disease in hCD20xhIgR3 and WT B6 mice**

EAE was assessed daily in all mice (n = 5–6 per group) and scores are shown as means ± standard error of the mean. Dotted lines mark the three time points (T1, day 19; T2, day 22; T3, day 25) of treatment with 5 mg/kg anti-CD20 mAb: (A) obinutuzumab, (B) rituximab, or (C) 18B12, or their respective isotype control antibodies (hIgG1, chIgG1, or mIgG2a). Note that treatment started after all animals had reached an EAE score of ≥2.5, i.e. on day 19 after immunization. Mice were scored using the standard EAE scale ranging from 0 to 5: 0, no signs of disease; 1, limp tail; 2, hindlimb weakness; 3, nearly complete or complete hindlimb paralysis; 4, complete hindlimb and partial forelimb paralysis; 5, moribund.

**Fig. 2. Serum levels of total and anti-MOG_{1–125} IgG in hCD20xhIgR3 and WT B6 mice**

Mice were each injected three times with 5 mg/kg anti-CD20 mAbs or the corresponding isotype controls. Serum IgG levels were determined by ELISA in 5–6 mice per group, 7 days after the last injection. Each circle represents the mean value for an individual mouse. The bars indicate the group mean value as well as minimum and maximum values.

**Fig. 3. Depletion of splenic B cell subsets by anti-CD20 mAbs in hCD20xhIgR3 and WT B6 mice**

Spleen cells were harvested from 5–6 animals per group, 7 days after injection with 5 mg/kg of obinutuzumab, rituximab, 18B12, or the corresponding isotype controls. Using flow cytometry, cells were gated on single live lymphocytes followed by the identification of different B-cell subsets using five separate antibody panels. (A) B cells were defined as CD19⁺, (B) naïve B cells as CD19⁺/IgM⁺/IgD⁺, (C) marginal zone B cells as CD19⁺/CD93⁻/CD21^{hi}/CD1d^{hi}, (D) follicular B cells as CD19⁺/CD93⁻/CD21^{hi}/CD1d^{lo}, (E)
plasma cells as CD45R<sup>-</sup>/CD138<sup>+</sup>, (F) memory cells as CD19<sup>-</sup>/CD80<sup>-</sup>/CD73<sup>+</sup>, and (G) isotype-switched cells as CD19<sup>-</sup>/IgG<sup>+</sup>. (H) T cells were defined as CD3<sup>+</sup>. Each circle represents the mean value of an individual mouse. The bars indicate the group mean value as well as minimum and maximum values. Student’s t test was used for normally distributed data and the Mann–Whitney U test for non-parametric data. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Relative depletion of the different B cell subsets in anti-CD20 mAb- compared to isotype control-treated groups. FO, follicular; MZ, marginal zone.

**Fig. 4.** Differential expression of inflammatory genes in splenic lymphocytes of obinutuzumab- and rituximab-treated mice as determined by single-cell RNA sequencing

Analyzed subsets comprised mature naïve, marginal zone, follicular, and germinal center B cells, as well as CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Cells were analyzed in 3–4 mice per group, 7 days after the end of treatment with either obinutuzumab or rituximab. Details on the statistics are provided in the Methods section. FO, follicular; GC, germinal center; MN, mature naïve; MZ, marginal zone; OBZ, obinutuzumab; PC, plasma cell; RTX, rituximab.

**Fig. 5.** Effect of anti-CD20 mAbs on CNS-infiltrating B and T cells

The cerebella of 5–6 mice per group were analyzed 7 days after the end of treatment with anti-CD20 mAbs or isotype controls, as indicated in the figure insets. (A) Representative images of a diffuse (left) and dense (right) B-cell infiltrate. (B) B220<sup>+</sup> cells were defined as B cells; (C) CD3<sup>+</sup> cells were counted as T cells. Thirty images per mouse were analyzed. Each circle represents the mean value for an individual mouse. The bars indicate the group mean value as well as minimum and maximum values.
Fig. 6. Anti-CD20 mAb treatment effects on ultrastructural spinal cord pathology in hCD20xhIgR3 and WT B6 mice

Spinal cords of mice (n = 5–6 per group) were dissected 7 days after the final injection of 5 mg/kg anti-CD20 mAbs or corresponding isotype control. (A) Representative electron micrographs showing the different categories of nerve fiber degeneration. Splitting and ballooning of myelin lamellae indicate abnormally myelinated axons; accumulation of organelles and dark cytoplasm indicate degenerating axons; and myelin debris and empty myelin sheaths indicate degenerated axons. (B) Quantification of axonal degeneration. Each circle represents the mean value of an individual mouse. The bars indicate the group mean value as well as minimum and maximum values. *p ≤ 0.05, unpaired t test. (C) Number of axons per mm$^2$. The dotted line marks the baseline value (195 axons/mm$^2$) in healthy mice (n = 7). (D) The g-ratio was calculated by dividing the diameter of the axon by the diameter of the nerve fiber (axon + myelin sheath) in a mean ± SD of 229 ± 37 randomly selected axons/mouse (n = 5–6 mice per group). The dotted line represents the baseline value of normally myelinated axons (0.718) (n = 7; mean ± SD of 13 ± 19 axons/mouse). The bars indicate the group mean value as well as minimum and maximum values. Statistical significance was calculated using Welch’s corrected t test. (E) Scatter plots showing the myelin sheath thickness of individual myelinated axons as a function of the respective axon diameter with simple linear regression analysis (black line: anti-CD20 mAbs; grey line: isotype controls; dashed line: baseline). The baseline was determined from healthy mice (n = 7). The significant slope change of the regression analysis of obinutuzumab-treated mice toward the baseline indicates a positive effect of the therapeutic mAb on myelination (p = 0.0298). Statistical significance was calculated using ANCOVA.
Figure 2

Total IgG

Anti-MOG\textsubscript{1-125} IgG
Figure 3

A. B cells
B. Naïve B cells
C. MZ B cells
D. FO B cells
E. Plasma cells
F. Memory cells
G. Isotype-switched cells
H. T cells

I. Relative depletion

|          | T cells | B cells | Naïve B cells | FO B cells | MZ B cells | Isotype-switched cells | Plasma cells | Memory cells |
|----------|---------|---------|---------------|------------|------------|------------------------|--------------|--------------|
| Obinutuzumab no depletion | 53% (± 22) | 45% (± 46) | 90% (± 13) | 72% (± 28) | 31% (± 53) | no depletion | no depletion | no depletion |
| Rituximab no depletion | 15% (± 27) | no depletion | no depletion | no depletion | 12% (± 56) | no depletion | no depletion | no depletion |
| 18822 no depletion | 94% (± 3) | 98% (± 1) | 97% (± 1) | 83% (± 16) | 88% (± 8) | no depletion | no depletion | no depletion |
Figure 5

A. Diffuse infiltrate vs Dense infiltrate

B. Count of B cells in Diffuse vs Dense infiltrates

C. Count of T cells in Diffuse vs Dense infiltrates

D. Summary of cell counts and depletion:

| Treatment      | Diffuse B cell infiltrates | Dense B cell infiltrates | Diffuse T cell infiltrates | Dense T cell infiltrates |
|----------------|----------------------------|--------------------------|----------------------------|--------------------------|
| Obinutuzumab   | 75 % (± 12)                | 57 % (± 19)              | 51 (± 10)                  | no depletion             |
| Rituximab      | no depletion               | 69 % (± 13)              | no depletion               | no depletion             |
| 188D12         | 50 % (± 19)                | 77 % (± 22)              | no depletion               | no depletion             |
Figure 6