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Late B Cell Depletion with a Human Anti-Human CD20 IgG1κ Monoclonal Antibody Halts the Development of Experimental Autoimmune Encephalomyelitis in Marmosets

Yolanda S. Kap,*†‡ Nikki van Driel,* Erwin Blezer,§ Paul W. H. I. Parren,* Wim K. Bleeker,*† Jon D. Laman,‡‡ Jenny L. Craigen,‖ and Bert A. ’t Hart*†‡*

Depletion of CD20+ B cells has been related to reduced clinical activity in relapsing–remitting multiple sclerosis. The underlying mechanism is not understood, because serum IgG levels were unaltered by the treatment. We report the effect of late B cell depletion on cellular and humoral immune mechanisms in a preclinical multiple sclerosis model (i.e., experimental autoimmune encephalomyelitis [EAE] in the common marmoset). We used a novel human anti-human CD20 IgG1κ mAb (HuMab 7D8) that cross-reacts with marmoset MOG. EAE was induced in 14 marmosets by immunization with recombinant human myelin oligodendrocyte glycoprotein (MOG) in CFA. After 21 d, B cells were depleted in seven monkeys by HuMab 7D8, and seven control monkeys received PBS. The Ab induced profound and long-lasting B cell depletion from PBMCs and lymphoid organs throughout the observation period of 106 d. Whereas all of the control monkeys developed clinically evident EAE, overt neurologic deficits were reduced significantly in three HuMab 7D8-treated monkeys, and four HuMab 7D8-treated monkeys remained completely asymptomatic. The effect of HuMab 7D8 was confirmed on magnetic resonance images, detecting only small lesions in HuMab 7D8-treated monkeys. The infusion of HuMab 7D8 arrested the progressive increase of anti-MOG IgG Abs. Although CD3+ T cell numbers in lymphoid organs were increased, their proliferation and cytokine production were impaired significantly. Most notable were the substantially reduced mRNA levels of IL-7 and proinflammatory cytokines (IL-6, IL-17A, IFN-γ, and TNF-α). In conclusion, B cell depletion prevents the development of clinical and pathological signs of EAE, which is associated with impaired activation of MOG-reactive T cells in lymphoid organs.


Traditionally, B cells were thought to contribute to MS mainly via the production of autoantibodies, which upon binding to myelin sheaths initiate Ab-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (8). Abs directed against myelin proteins, such as myelin oligodendrocyte glycoprotein (MOG), have been isolated from MS patients (9, 10) and healthy controls (11). Autoantibodies were found to amplify demyelination in mouse (12), rat (13), and nonhuman primate (14) experimental autoimmune encephalomyelitis (EAE) models. Furthermore, evidence for Ab and complement-mediated demyelination has been described in type II MS lesions (15). Recently, B cells were detected in ectopic lymphoid structures in the meninges of MS brains, although their putative pathogenic role in these structures is still unknown (16, 17).

The fact that plasma cells do not express CD20 and therefore were not depleted by anti-CD20 mAb likely explains the lack of a reduction in total IgG serum (2, 3). It therefore has been suggested that the clinical effect of depleting B cells in RMS may be due to interference with other B cell functions, such as Ag presentation and cytokine production, leading to impaired activation of T cells and macrophages (2, 3). Reduced numbers of T cells have been reported in the cerebrospinal fluid after B cell depletion, but not in blood (4, 6). Because access to body fluids or organs other than blood for immune profiling is limited in MS patients and healthy controls, we chose to investigate the effect of B cell depletion on T cell activation in lymphoid organs in a relevant animal model of MS, EAE in the common marmoset (18, 19).

The recombinant human MOG (rhMOG)-induced EAE model in the common marmoset is a relevant model for the purpose of this study, because both autoantibodies and anti-MOG T cells are induced. Autoantibodies contribute significantly to the pathogenic process in this marmoset EAE model (14, 20). However, full...
development of evident neurologic disease does not depend strictly on the presence of autoantibodies, because T cells specific for the MOG34–56 epitope can induce demyelination (21, 22). These highly pathogenic T cells, which are activated in the rhMOG EAE model, express CD56, are cytotoxic, and produce IL-17A (21, 22). Cytotoxic CD56+ MOG-specific T cells also are present in MS (23, 24). In marmosets, these T cells can be activated by a very mild immunization protocol that only includes MOG34–56 emulsified in IFA, suggesting that anti-MOG T cells are highly pathogenic and important in the pathogenic process (22).

In recent years, a panel of fully human CD20 Abs was generated in Ig transgenic mice (25). This panel included ofatumumab and HuMab 7D8, which both bind to a unique membrane-proximal CD20 epitope, comprising the small and large extracellular loops. Ofatumumab and HuMab 7D8 display superior CD20 binding and complement-dependent cytotoxicity compared with those of rituximab (25–28).

In the current study, we have induced profound B cell depletion by weekly infusion of the human anti-human CD20 mAb HuMab 7D8 starting 3 wk after the EAE induction by a single immunization with rhMOG formulated in CFA. The treatment was started simultaneously in all of the animals well after immunization (i.e., at week 3) to avoid interference with the induction of the immunopathogenic process but before the onset of overt clinical EAE. Neurological signs in the used model appear between 4 and 16 wk after immunization with rhMOG/CFA (21). The earliest responders display clinical signs already within 4 wk, suggesting that the autoimmune mechanisms needed for the induction of lesions and neurologic deficit can be induced within 4 wk after immunization.

We report that this semitherapeutic treatment schedule with HuMab 7D8, when autoantibody production is already ongoing, impaired the development of neurologic deficit. The immunological correlates of this remarkable therapeutic effect were arrest of already initiated autoantibody production and impaired activation of autoreactive T cells in lymphoid organs, read out by reduced proliferation and proinflammatory cytokine production in lymphoid organs. These data obtained in a valid preclinical model of MS contribute to a better understanding of the elusive mechanism underlying the clinical effect of B cell depletion in RRMS.

**Materials and Methods**

**Animals**

The common marmoset monkeys used in this study were purchased from two outbred colonies kept at the Biomedical Primate Research Centre (Rijswijk, The Netherlands) or the German Primate Centre (Duiven, The Netherlands). Animals were declared healthy after the veterinarian’s physical, hematological, and biochemical checkup were included. Monkeys were pair-housed in spacious cages and under intensive veterinary care throughout the study. The daily diet consisted of commercial food pellets for New World monkeys (Special Diet Services, Witham, Essex, U.K.), supplemented with rice, raisins, peanuts, marshmallows, biscuits, fresh fruit, and grasshoppers. Drinking water was provided ad libitum. According to the Dutch law on animal experimentation, all of the study protocols and experimental procedures have been reviewed and approved by the Institute’s Ethics Committee.

Twelve marmosets were used to find an effective dose (pharmacokinetics/pharmacodynamics [PK/PD]), and fourteen marmosets were used for efficacies assessment in the EAE model (Table I). The average body weight distribution of the monkeys selected for the EAE experiment was the same for the control (367 ± 30 g) and treatment groups (332 ± 41 g).

**rhMOG-induced EAE**

EAE was induced with a recombinant protein encompassing the extracellular domain of human MOG residues 1–125 (rhMOG), which was produced in Escherichia coli and purified as described previously (29). The inoculum contained 100 μg rhMOG in 300 μl PBS and was emulsified in 300 μl CFA containing Mycobacterium butyricum (Difco Laboratories, Detroit, MI) by gentle stirring for at least 1 h at 4°C. The emulsion was injected at four locations into the dorsal skin under alfaxalone anesthesia (10 mg/kg; Alfaxan; Vetoquinol, Den Bosch, The Netherlands). Clinical signs were scored daily by two independent observers using a previously described semiquantitative scale (30). Briefly, 0 = no clinical signs; 0.5 = apathy, altered walking pattern without ataxia; 1 = lethargy, tail paralysis, tremor; 2 = ataxia, optic disease; 2.25 = monoparesis; 2.5 = paraparesis, sensory loss; and 3 = para- or hemiplegia. Overt neurologic deficit starts at score 2. For ethical reasons, monkeys were sacrificed once complete paralysis of limbs (score ≥ 3.0) was observed or at the pre-determined end point of the study (i.e., post-sensitization day [psd] 106). Body weight measurements of conscious monkeys, which are used as a surrogate disease marker, were performed three times per week.

**CD20 treatment**

HuMab 7D8 is a human IgG1 mAb (25) (Genmab, Utrecht, The Netherlands) directed against human CD20 and cross-reactive with marmoset CD20 (data not shown).

The PK/PD study comprised 12 marmosets. Four monkeys received a single i.v. dose of 10 mg/kg HuMab 7D8 from a 2.86 mg/ml stock solution. Four marmosets received a single i.v. dose of 20 mg/kg HuMab 7D8 from a 12.18 mg/ml stock solution. Four control animals received 1 ml/kg sterile PBS.

The EAE study comprised 14 marmosets. Seven monkeys received a single i.v. dose of 20 mg/kg HuMab 7D8 21 d after immunization. To maintain plasma levels >5–10 μg/ml HuMab 7D8, 5 mg/kg was administered i.v. every week for the duration of the study. Seven control animals received 1 ml/kg sterile PBS.

**Blood sampling, cell numbers, and plasma levels**

For the initial PK/PD study, 50 μl blood was collected in 450 μl citrated PBS at 5, 15, 30, 60, 120, and 240 min. After 1, 3, 7, and 34 d, 500 μl blood was collected in heparinized vacutainers (Greiner, So¨lingen, Germany) under alfaxalone anesthesia (10 mg/kg). After centrifugation, plasmas were collected and stored frozen at −20°C until analysis of test substance levels was performed.

Every two weeks during the EAE study, 1.5 ml venous blood was collected into EDTA vacutainers (Greiner) under alfaxalone anesthesia (10 mg/kg). In alternating weeks, 50 μl blood was collected in 450 μl PBS/EDETA without sedation. The number of RBCs and WBCs was measured on a Sysmex XT-2000iV (Sysmex, Norderstedt, Germany).

Plasma levels of HuMab 7D8 were determined by ELISA, in which an idiotype-specific mouse mAb (Genmab MS0001-009) was used for capture and a mouse anti-human IgG (Fc-specific) HRP-conjugated mAb (MH116-1; Central Laboratory Blood Bank, Amsterdam, The Netherlands) was used for detection. The quantification limit was 1 μg/ml in plasma.

**Necropsy**

Monkeys selected for necropsy were first deeply sedated by i.m. injection of alfaxalone (10 mg/kg). A maximum volume of blood was collected into EDTA or heparanized vacutainers, and subsequently the marmoset was euthanized by infusion of pentobarbital sodium (Euthesate; Apharmo, Duiwen, The Netherlands).

Spleen and lymph nodes from several anatomical locations were collected aseptically and cut into four pieces, which were processed for cell culture, fixed in buffered formalin, snap-frozen in liquid nitrogen, or processed for RNA extraction with RNAlater (Sigma-Aldrich, St. Louis, MO). Half of the brain and spinal cord was stored in formalin, and the other half was snap-frozen in liquid nitrogen. Femur was collected in PBS for isolation of bone marrow cells.

**Magnetic resonance imaging**

One cerebral hemisphere collected at necropsy for MRI was fixed in 4% buffered formalin and 2 wk later transferred into buffered saline containing sodium azide to allow stabilization of magnetic resonance (MR) relaxation time characteristics (31). High-contrast postmortem MR images were recorded on a 9.4 T horizontal bore nuclear magnetic resonance spectrometer (Varian, Palo Alto, CA), equipped with a quadrature coil (RAPID Biomedical, Rimpar, Germany). Brains were submerged in nonmagnetic oil (Fomblin; perfluorinated polyether; Solvay Solexis, Weesp, The Netherlands) to prevent unwanted susceptibility artifacts.

On a sagittal scout image, 41 contiguous coronal slices of 0.75 mm were defined covering the complete brain, with the following characteristics: field of view = 25 × 25 mm; matrix = 256 × 256; zero-filled = 512 × 512;
<table>
<thead>
<tr>
<th>PK/PD Study</th>
<th>EAE Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
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<tr>
<td>2</td>
<td>M06050</td>
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<td>Mi013294</td>
</tr>
<tr>
<td>12</td>
<td>Mi013362</td>
</tr>
</tbody>
</table>

aAge in months at the start of the experiment.

bM06081 died on psd 33 without EAE.

F. female; M. male.
volumetric MRI data sets were collected:

- T2 maps were calculated by a monoexponential fitting of six spin echo images with increasing echo time (TE): repetition time = 2650 ms; TE = 10 + 5 × 10 ms. The second image of this series (i.e., TE = 20 ms) was used for region of interest determination.
- Magnetization transfer ratio (MTR) maps were calculated from two T1-weighted spin echo images with and without a magnetization transfer saturation pulse. MTR values represent the percentage decrease in MR signal intensity as a result of this pulse: repetition time = 1675 ms; TE = 23 ms; MT pulse = 8.19 ms Gaussian-shaped pulse, nominal flip angle 1000, offset = −9.4 kHz.

Calculations of T2 and MTR were done with a homemade software package developed in MATLAB (version 7.2; Mathworks, Natick, MA).

Regions of interest were defined using the freely available Medical Image Processing, Analysis and Visualization package (MIPAV, version 4.3.0; National Institutes of Health, Bethesda, MD). White matter lesions were defined as areas with abnormally high signal intensities in the white matter. The total volume of white matter lesions was determined for the complete hemisphere.

Anti-MOG Ab detection
Ab binding to rhMOG and MOG35-52 was determined using ELISA as described (21). Bound IgM Ab was detected using alkaline phosphatase-conjugated goat anti-monkey IgM (Rockland, Gilbertsville, PA), and bound IgG Ab was detected using polyclonal alkaline phosphatase-conjugated rabbit anti-human IgG (Abcam, Cambridge, U.K.). The results of the Ab assays are expressed relative to a standard curve of pooled necropsy plasma from three rhMOG/CFA-immunized control marmosets of this study. The Ab concentration in pooled plasma was defined arbitrarily as 350 when OD was equal to 1, and ELISA data of 10- and 100-fold diluted test samples were fitted to a four-parameter hyperbolic function, using homemade software ADAMSEL (Dr. E. Remarque, Biomedical Primate Research Centre, The Netherlands).

Procedures and assays for the quantification of a cellular immune response
Mononuclear cell isolation. Mononuclear cell (MNC) suspensions from the PBMCs, spleen, and bone marrow were isolated by density gradient centrifugation over lymphocyte separation medium (Axis-Shield, Oslo, Norway). Cell suspensions from axillary (ALN), inguinal, lumbar (LLN), and cervical lymph nodes were isolated by gently pressing the organ through a nylon mesh and washing with RPMI 1640 medium.

Phenotyping with flow cytometry. To enable visualization of residual CD20-expressing cells in the circulation or lymphoid organs of monkeys treated with HuMab T8D, MNCs were subjected to an Ab stripping protocol aimed at the removal of HuMab T8D. Cells (2 × 107) were incubated with 5 ml acidic buffer (50 mM glycine-HCl, 5 mM KCl, and 130 mM NaCl [pH 3]) for 4 min at room temperature and subsequently washed and used for flow cytometry. The flow cytometry protocol used to phenotype MNCs involved staining of dead cells using violet viability stain (Invitrogen, Carlsbad, CA) and washing with RPMI 1640 medium.

Proliferation.

Proliferation of MNCs was assayed as described previously (32). Briefly, cells were cultured in triplicate at a cell density of 2 × 10^6 per well in 96-well U-bottom plates with rhMOG, MOG peptides, OVA, and Con A. All of the Ags were tested at a concentration of 5 μg/ml. After 48 h of culture, 50 μl supernatant was harvested for cytokine ELISA, and 0.5 μCi per well of tritiated thymidine was added to the cells. Incorporation of radiolabel was determined after 18 h using a Matrix 9600 beta counter (Packard 9600; Packard Instrument, Meriden, CT).

Phenotyping of proliferating cells. This was performed by CFSE vital dye dilution assay as described elsewhere (21).

Cytokine detection with ELISA. Due to the limitation of sample volume and availability of cross-reactive ELISA reagents, we chose to assay for three cytokines. The production of IL-17A, IFN-γ, and IL-12p40/p70 was measured in culture supernatants with commercial ELISA according to the manufacturer’s instructions (U-CyTech, Utrecht, The Netherlands).

Cytokine detection with quantitative PCR. RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instructions. Random hexamer primers were used for cDNA synthesis. Expression levels of mRNA were determined by quantitative PCR (qPCR) using iTaq Supermix with ROX and CFX96 Real-Time PCR Detection System (both from Bio-Rad, Hercules, CA). Primer and probes used are listed in Table II. Probes were obtained from the Universal Probe Library set for human (Roche, Indianapolis, IN). Transcript levels were normalized against the reference gene abelson (ABL) (33).

Immunohistochemistry
Spleen and lymph node sections obtained at necropsy were snap-frozen in liquid nitrogen and stored at −80°C. Frozen spleen and lymph node sections of 6 μm were cut and thaw-mounted on gelatin/chrome alum-coated glass slides. Slides were kept overnight at room temperature in a humidified atmosphere. After the slides were air-dried for 1 h, they were fixed in fresh acetone containing 0.02% (v/v) H2O2. Acetone-fixed slides were air-dried for 10 min and subsequently washed in PBS. Tissue sections were incubated with primary Ab overnight at 4°C in a humidified atmosphere. Primary Abs were mouse anti-human CD20 (clone L26; DakoCytomation, Glostrup, Denmark), mouse anti-human CD40 (clone B-B20; Chemicon International, Billerica, MA), and rabbit anti-human CD3 (DakoCytomation). Incubations with secondary and tertiary reagents were performed for 1 h at room temperature. Between incubation steps, the slides were washed twice with PBS. Detection of primary unlabeled Ab was followed by incubation with donkey anti-mouse or goat anti-rabbit, both labeled with biotin. This was followed by incubation with HRP-labeled avidin–biotin-complex (Vectastain ABC, Vector Laboratories, Burlingame, CA). HRP activity was revealed by incubation for 10 min at room temperature with 3-aminol-9-ethyl-carbazole (Sigma-Aldrich, Zwijndrecht, The Netherlands), leading to a bright red precipitate. CD40 expression was enhanced with the tyramide signal amplification kit (Invitrogen, Carlsbad, CA). Incubation with isotype-matched primary Ab of irrelevant specificity and omission of the primary Ab served as negative controls.

Statistical analysis
Data are presented as mean ± SEM of six control and seven treated marmosets. Statistical analysis was performed using Pearson 5.0b for Mac

Table II. Primer and probe combinations for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
<th>Probe</th>
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OS X (GraphPad Software, La Jolla, CA). Survival was analyzed using a log-rank test. Other data were analyzed using the Mann-Whitney $U$ test. A $p$ value $<0.05$ was considered statistically significant.

**Results**

**Dose finding and PK/PD of HuMab 7D8**

The effective Ab dose for maximum B cell depletion from blood and lymph nodes was determined in 12 marmosets. Groups of four monkeys each received a single i.v. injection with placebo (PBS) or HuMab 7D8 at a dose of 10 or 20 mg/kg (Table I). At the indicated time points during the first (Fig. 1A) and subsequent days (Fig. 1B), small venous blood volumes were withdrawn for plasma isolation in which HuMab 7D8 levels were determined. As expected, all of the placebo-treated monkeys scored negative in the assay (data not shown). The average HuMab 7D8 levels in the 10 and 20 mg/kg dose groups measured in the 5-min blood sample were 56 and 181 mg/ml, respectively. The plasma concentration was maintained at trough level $>10$ mg/ml during the following 7 d.

Predictably, the CD20 molecule on B cells in the treated group may be bound by HuMab 7D8 and therefore could not bind the anti-CD20 Ab conjugate used in flow cytometry. Because anti-CD19 mAb cross-reacted only poorly with marmosets, we also have determined CD40-expressing cells to be a surrogate marker for B cell depletion. CD20 is a pan-B cell marker expressed throughout the B cell lineage with the exception of plasma cells. CD40 is expressed besides on activated APCs (dendritic cells and macrophages) also on resting and activated mature B cells but not on plasma cells.

Seven days after the single administration of HuMab 7D8, two animals of each group were sacrificed (Table I), and the extent of B cell depletion from spleen and ALN was assessed by flow cytometry. The profound depletion of CD20$^+$ cells by both doses of HuMab 7D8 is depicted in Fig. 1C. The remaining six animals were sacrificed 62 d after HuMab 7D8 administration (Table I). Fig. 1D shows that even 2 mo after the administration of a single HuMab 7D8 dose reduced percentages of CD20$^+$ and CD40$^+$ cells were found in all of the lymphoid organs. However, the figure also shows that in one monkey of each group (no. 8/Mi013067 and no. 12/Mi013362) insufficient B cell depletion or partial repletion of B cells had occurred in the spleen and ALN at that time (Fig. 1D).

**Anti-CD20 mAb effectively depletes B cells throughout the EAE experiment**

Fourteen unrelated marmosets were immunized with rhMOG in CFA. Seven animals were selected randomly for treatment with...
HuMab 7D8 from psd 21, and also seven animals were selected for placebo treatment with PBS (Table I). On the basis of the PK/PD data, we chose to induce complete B cell depletion with a single loading dose of 20 mg HuMab 7D8 per kilogram of body weight followed by a weekly i.v. maintenance dose of 5 mg/kg. This dosing regimen aimed at a plasma trough level of 5–10 μg/ml, which was described previously to be sufficient for sustained biological activity in vivo (34).

Plasma levels of HuMab 7D8 were measured every week at 7 d after i.v. administration of HuMab 7D8 or PBS. The plasma level of HuMab 7D8 remained >5 μg/ml, with the exception of one time point (day 42) in one monkey (M07075) (Fig. 2A). Every 2 wk, percentages of CD20+ and CD40+ expressing cells in PBMCs were determined by flow cytometry to confirm B cell depletion by HuMab 7D8. Seven days after the first dose of HuMab 7D8 (i.e., 28 d after immunization), CD20+ and CD40+ cells were almost completely depleted compared with those of the control group (Fig. 2B, Supplemental Fig. 1). Throughout the study, the percentage of CD20+ remained at a very low level in the treatment group, except at psd 84. At this time point, six of the seven animals, M07095 being the only exception, contained elevated numbers of CD20+ cells in the PBMCs. However, because CD40 expression remained low at this time point, we assume that these might be immature B cells repleting the peripheral compartment (Fig. 2B, Supplemental Fig. 1). At necropsy, the percentage of CD20+ and CD40+ cells present in the PBMCs, spleen, bone marrow, and lymph nodes were determined by flow cytometry. In the HuMab 7D8 treatment group, CD20- and CD40-expressing cells were detected, but the levels were still significantly lower than those in the control group (Fig. 2C, Supplemental Fig. 2). Also, the expression of CD19, assessed in spleen and ALN by qPCR, was significantly lower in the treatment group (Fig. 2D).

In conclusion, the chosen HuMab 7D8 dosing regimen induced profound and persistent B cell depletion from blood and lymphoid organs.

**B cell depletion prevents the development of clinical signs**

Six of the seven control animals developed clinically evident EAE characterized by overt neurologic signs above EAE score 2 from psd 55 onward (Fig. 3A). One control animal died unexpectedly at psd 33 without any detectable neurologic signs (M06081). The most likely cause of death deduced from pathology examination was cardiac failure. Therefore, this monkey was excluded from further analysis. Four of the six control animals were sacrificed with at least one paralyzed limb (para-/hemiplegia; score = 3). M02052 was sacrificed with a clinical score of 2.25, because of persistent optic neuritis, which started at psd 48, and excessive weight loss. M07048 was sacrificed at the predetermined end point psd 106 after three short-lasting episodes of ataxia (Fig. 3A).

In contrast, four of the seven animals treated with HuMab 7D8 failed to develop detectable neurologic signs (Fig. 3A). The remaining three treated animals developed neurologic signs only for one day (M07075 and M07095) or two separate days (M04096). Paralysis was observed in none of the treated animals, and all of the animals were sacrificed with a clinical score of 0 or 0.5 at the predetermined end point psd 106 (Fig. 3A). The curves for disease-free survival (i.e., time to EAE score 2.0) and overall survival (time to end point) show that the clinical differences between the two groups are highly significant (Fig. 3B). In conclusion, late B cell depletion abrogated the development of neurologic signs.

**FIGURE 2.** B cells are depleted significantly from blood and lymphoid organs in the marmoset EAE model. A, Treatment with HuMab 7D8 was started 21 d after immunization with rMOG in CFA. Plasma levels of circulating HuMab 7D8 were determined at the indicated time points during the study and are shown in log scale (y-axis). B, CD20 and CD40 expression in the PBMCs was determined by flow cytometry throughout the study. Shown are the percentages of living CD20+ or CD40+ cells of total measured cells. The treatment period is indicated as a gray-shaded box. Open symbols represent the control animals, and closed symbols represent the treated animals. CD20 was not removed with the stripping procedure, because the number of available PBMCs was too small. CD40 was used to confirm B cell depletion. At psd 28, 42, 56, and 70, a significantly lower percentage of B cells was observed in treated animals compared with that in control animals. It is of note that the number of animals decreased during the study: the number of control animals was six until day 42; at psd 56, 70, 84, and 98, CD20 and CD40 expression were analyzed in five, four, two, and one control animals, respectively; the number of HuMab 7D8-treated animals remained seven during the study. C, At necropsy, CD20 and CD40 expression was determined in the PBMCs and lymphoid organs by flow cytometry. White bars are control animals, and black bars are treated animals. Cells underwent a stripping procedure to remove anti-CD20 Ab from cells. Shown are the percentages of viable CD20+ cells and CD40+ cells that were gated from the total analyzed cell number using the live/dead marker (mean ± SEM). B cells were depleted significantly from the PBMCs and lymphoid organs. D, CD19 mRNA expression was determined by qPCR and normalized to expression levels of ABL. Shown are mean ± SEM. The y-axis is in log scale. CD19 mRNA expression was decreased significantly in treated animals, confirming B cell depletion. *p < 0.05 compared with the control group using the Mann-Whitney U test.
FIGURE 3. Late B cell depletion prevents the development of neurologic signs. A. The clinical scores of control (left column) and treated (right column) animals are depicted. The psd is indicated on the x-axis. Dotted lines indicate the percentage of body weight loss compared with the day of immunization (left y-axis). Solid lines indicate clinical score (right y-axis). The treatment period is indicated with a gray-shaded box. The day of sacrifice of individual monkeys is indicated in the figure with ciphers. Monkey M06081 succumbed without neurologic deficit from cardiac failure. Of the six remaining control animals, five developed sustained neurologic signs (clinical score $\geq 2$). Control animal M07048 developed neurologic signs for three independent days, but postmortem MRI analysis confirmed the presence of substantial brain white matter demyelination (Fig. 4). Four of the seven HuMab 7D8-treated animals developed no neurologic signs, and three of the seven animals developed neurologic signs for only 1 or 2 d. B. Survival curves. The upper panel shows the disease-free survival, meaning the time until the animals developed EAE score 2. The lower panel is the survival time until the day of sacrifice, which was the humane or predetermined end point. B cell depletion significantly increased both types of survival ($p < 0.05$ log rank).
B cell depletion prevents (MRI-detectable) CNS pathology

MRI sequences of fixed hemispheres were recorded to confirm that B cell depletion not only prevented the development of neurologic signs but also of brain lesions. Fig. 4A and 4B show representative examples of T2-weighted (T2-W) images and MTR images of two control and two HuMab 7D8-treated animals. White lines encircle the lesions. C, B cell depletion significantly reduced the volume of white matter lesions. Lower T2 and higher MTR within the lesions of the HuMab 7D8-treated animals suggest less damage within the lesions of treated animals. \( p < 0.05 \) compared with the control group using a Mann-Whitney U test.

In conclusion, these data show that B cell depletion significantly reduced EAE-associated brain pathology.

FIGURE 4. Reduced cerebral white matter lesion load in B cell-depleted EAE marmosets. A and B, Formalin-fixed hemispheres were analyzed with MRI for volume (mm\(^3\)), T2 signal intensity (ms), and MTR (percentage reduction in signal intensity) of white matter lesions. T2-W images were used to calculate the volume (mm\(^3\)). Representative examples of T2-W images (A) and MTR images (B) of two control and two HuMab 7D8-treated animals are shown. White lines encircle the lesions. C, B cell depletion significantly reduced the volume of white matter lesions. Lower T2 and higher MTR within the lesions of the HuMab 7D8-treated animals suggest less damage within the lesions of treated animals. \( p < 0.05 \) compared with the control group using a Mann-Whitney U test.

FIGURE 5. Reduced plasma levels of IgG after B cell depletion. Plasma IgG levels against rhMOG and MOG54–76, being the dominant binding site of anti-MOG IgG Ab, were determined by ELISA. Recorded OD values were transformed into A.U. (y-axis, log scale) using homemade software. A, The upper panel shows the control animals, and the lower panel shows the HuMab 7D8-treated animals with the treatment period indicated as a gray-shaded box. The psd is shown on the x-axis. Shown are the IgG levels of individual animals. B, Area under the curve was calculated for each individual animal and is depicted as mean ± SEM. Plasma IgG levels against rhMOG and MOG54–76 were reduced significantly after B cell depletion. Shown is mean ± SEM. C, Reduced IgG levels also were observed in treated animals at necropsy, which reflects the peak of the disease in control animals. Results are depicted as mean ± SEM (C), \( p < 0.05 \) compared with the control group using a Mann-Whitney U test. A.U., arbitrary units.
B cell depletion results in the reduction of plasma autoantibodies

The immunological hallmark and most widely accepted biomarker of B cell contribution in the EAE model is the production of autoantibodies. To obtain evidence whether B cell functionality is eliminated completely in HuMab 7D8-treated monkeys, we measured plasma levels of IgM and IgG against rhMOG and MOG54-76 every 2 wk and at the start of the treatment. Abs against MOG54-76 were measured, because this peptide comprises a dominant B cell epitope of anti-MOG Abs generated in the rhMOG-induced EAE model (21). IgM levels peaked in both groups at psd 28 and declined after that (data not shown). The levels of rhMOG or MOG54-76-specific IgG were clearly higher in the control group than those in the HuMab 7D8-treated group (Fig. 5A). Until psd 28 (i.e., 7 d after the start of the treatment), IgG levels were comparable between both groups. After psd 28, IgG levels did not increase further in the treatment group, whereas a >10-fold increase was detected in the control group (Fig. 5A). The area under the curves was calculated for each individual animal, and group means were calculated. Fig. 5B shows that IgG autoantibodies against rhMOG and MOG54-76 were significantly lower in the treatment group compared with those in the control group. Also at necropsy, a significant reduction of the autoantibodies was observed (Fig. 5C).

Effect of B cell depletion on cell numbers, T cell phenotype, and proliferation

To test the systemic effect of B cell depletion on the cellular immune compartment in the marmoset EAE model, we first analyzed the distribution of leukocyte and lymphocyte subsets. Fig. 6A shows the variation of WBC, lymphocyte, neutrophil, and monocyte numbers in the PBMCs in both groups. Increased numbers of monocytes were observed in the treatment group (Fig. 6A). At necropsy, we also observed increased absolute numbers of WBCs, lymphocytes, neutrophils, and monocytes in the treatment group, although not significant (Fig. 6B). It is of note that the cell numbers at necropsy are determined at different/individual time points (i.e., the day that each animal is sacrificed with full-blown EAE in the control group and the end of the study for treated animals).

During the study and at necropsy, the phenotype of the lymphocytes was analyzed by flow cytometry to assess the effect of B cell depletion on the T cell compartment. We observed no effect on the CD3 percentage or the CD4/CD8 ratio in the PBMCs throughout the study and at necropsy.
the observation period (Fig. 6C). At necropsy, however, higher percentages of CD3⁺ cells were observed in the PBMCs, spleen, and lymph nodes (Fig. 6D). Although we like to emphasize that this is a percentage and not an absolute number and may be caused by the depletion of the B cell population, CD3 mRNA levels also were increased significantly in the ALN (Fig. 6E). The CD4/CD8 ratio was not changed in the PBMCs or lymphoid organs at necropsy (data not shown).

Next, we analyzed the effect of B cell depletion on proliferation and cytokine production. At necropsy, we observed a higher level of proliferation in the PBMCs from the treatment group stimulated with rhMOG, MOG peptides 24–46 and 34–56, or Con A (Fig. 7A). By contrast, the proliferative responses of MNCs from monkeys in the treatment group to these Ags were lower in spleen, ALN, and LLN. It is of note that also the background proliferation and the response to OVA and Con A were reduced in these organs (Fig. 7A). No differences were observed in inguinal or cervical lymph nodes (data not shown).

The phenotype of the cells in spleen and ALN that proliferated against rhMOG was assessed with the CFSE dilution assay. We observed that depletion of CD20⁺ cells led to reduced proliferation of both CD4⁺ and CD8⁺ T cells from ALN and, albeit to a lesser extent, spleen (Fig. 7B and data not shown).

In conclusion, the late depletion of B cells has profound effects on the T cell compartment. The most obvious effect observed in blood at necropsy was increment of monocyte numbers. The numbers of CD3⁺ T cells also were increased in blood and all of the analyzed secondary lymphoid organs. The activity assessment showed that proliferation was increased in blood but decreased in the secondary lymphoid organs.

Reduced cytokine production after B cell depletion
The rhMOG-induced protein levels of IL-12 (p40/p70), IL-17A, and IFN-γ were assessed in supernatant of rhMOG-stimulated MNC cultures. We observed IL-17A production by splenocytes in four of the six control animals and by MNCs from ALNs and LLNs from two of the six control animals in response to stimulation with rhMOG. By contrast, we observed rhMOG-stimulated IL-17A production only in splenocytes of one of the seven B cell-depleted animals (Fig. 8A). No such obvious differences between control and treated animals were observed for the production of IFN-γ and IL-12 (Fig. 8A).

Cytokine production in spleen and ALN also was analyzed at the mRNA level. In treated animals, mRNA levels of IL-10 and IL-1β were increased in ALN, and IL-7 mRNA levels were reduced in both spleen and ALN compared with those of the control animals. This indicates that B cells are an important source of IL-7 in the rhMOG-induced EAE model. No differences between treated and nontreated animals were observed for IL-17A, IFN-γ, TNF-α, IL-6, and IL-4 (Fig. 8B).

The data in the previous paragraph demonstrated that CD3⁺ T cell numbers are increased in the lymphoid organs of B cell-depleted monkeys, as also was reflected by increased CD3 mRNA levels in the ALN (Fig. 6E). For this reason, we chose to normalize the T cell cytokine mRNA levels (IL-17A, IFN-γ, TNF-α, IL-6, and IL-4) against CD3 instead of ABL. Indeed, the corrected data show reduced IL-17A, IFN-γ, TNF-α, and IL-6 levels in ALN of treated animals, suggesting less cytokine production per T cell (Fig. 8C).

In conclusion, the reduction of T cell proliferation together with the reduced cytokine production in lymphoid organs indicates that the depletion of CD20⁺ B cells impairs the activity and/or pathogenic function of cellular autoimmune mechanisms.

B cell depletion alters the composition of T cell and B cell areas in spleen and lymph nodes
We have investigated in more detail the effect of B cell depletion on the organization of spleen and ALN using H&E staining and immunostaining of cryosections. The anti-CD20 Ab (clone L26) used for immunostaining recognizes an intracellular epitope of CD20 (35). Therefore, this Ab does not compete with the anti-CD20 Ab administered in vivo.

The H&E staining of the spleen shows a similar structural organization in control and HuMab 7D8-treated animals. The spleen of all of the animals, except M04096, is composed of similar areas

FIGURE 7. Increased T cell proliferation in blood versus reduced T cell proliferation in lymphoid organs after B cell depletion. A, Proliferation at necropsy was assessed using the incorporation of [³H]thymidine. Shown are the mean ± SEM in cpm (γ-axis). Proliferation in the spleen was assessed in six of the seven HuMab 7D8-treated animals, because the spleen of M05073 could not be analyzed due to small cell numbers. Proliferation in the LLN was analyzed in four animals of each group. Increased proliferation against rhMOG and MOG peptides was observed in PBMCs of treated animals. In contrast, reduced general (no stimulation, OVA, or Con A) and MOG-specific proliferation was observed in lymphoid organs of treated animals. B, Percentage of dividing CD3⁺CD4⁺ or CD3⁺CD8⁺ cells in the ALN. Percentages of total measured events are shown in mean ± SEM. Reduced proliferation against rhMOG and OVA was observed in both T cell compartments. *p < 0.05 compared with the control group using a Mann-Whitney U test.
of white pulp (WP) and red pulp (Fig. 9). In the control animals, the WP consisted of T cell and B cell areas. In the HuMab 7D8-treated animals, the WP consisted only of T cells, even in the areas where B cells were expected (Fig. 9). M07095 showed a small area of CD20+ and CD40+ cells (data not shown), but in none of the other HuMab 7D8-treated animals CD20- or CD40-expressing B cells were observed in the WP. A few CD40+ cells were detected in the WP of HuMab 7D8-treated animals, but the morphology suggests that these were follicular dendritic cells or macrophages (Fig. 9, inset). The structure of the medulla and the cortex in ALN was also similar in both groups as assessed by H&E staining (Fig. 9). CD3 staining shows normal structural organization in control animals. However, in the HuMab 7D8-treated animals, the number of CD3+ cells appeared to be increased in the cortex (Fig. 9), which is in line with the increased CD3 mRNA expression (Fig. 6E). A few CD20+ cells were observed in M07075 and M07095, but in the other five HuMab 7D8-treated animals, no CD20 staining was found. CD40 was detected in B cell areas of ALN of both control and HuMab 7D8-treated animals. However, in the HuMab 7D8-treated animals, we observed that the B cell areas were hypocellular compared with those of control animals. Furthermore, flow cytometry showed a reduced number of B cells, and also the morphology of the CD40+ cells suggested that these cells were not B cells.

These data demonstrate that the general structure of secondary lymphoid organs is not changed by B cell depletion. In the spleen,
The B cell areas appeared to be filled with T cells. In ALN, T cell numbers seemed to be increased in T cell areas, and the B cell areas were hypocellular and only contained CD40+ non-B cells.

Discussion
Clinical trials with a chimeric CD20 mAb have shown that depletion of B cells has beneficial effects in RRMS, while autoantibody levels were unaffected (2, 3). This observation has raised questions on the exact immunopathogenic role of B cells in MS. We have investigated in a nonhuman primate model of MS (i.e., EAE in the common marmoset) immunopathogenic mechanisms of which are altered by B cell depletion. Because the chimeric anti-CD20 mAb (rituximab) does not cross-react with marmoset CD20, we used the fully human CD20 mAb HuMab 7D8 (25, 26). In the first part of the current study, we have analyzed the extent and duration of B cell depletion after a single i.v. dose of HuMab 7D8 at 10 or 20 mg/kg. The results demonstrate the high potency of HuMab 7D8 in the marmoset, because it induced profound and long-lasting depletion of B cells from peripheral blood as well as lymphoid organs. In the second part of the study, we have chosen a dosing regimen with a high loading dose of 20 mg/kg HuMab 7D8 for obtaining a robust B cell depletion and thereby lowering the risk of inducing neutralizing Ab, followed by a 4-fold lower weekly maintenance dose at 5 mg/kg for maintaining maximum efficacy. The data show that the applied dosing schedule completely abrogated the typical MS-like clinical and pathological signs of this EAE model. This demonstrates the high in vivo efficacy of the Ab in this model and emphasizes the important role of B cells in MS.

We observed substantially lower levels of rhMOG-specific IgG in marmosets treated with HuMab 7D8 compared with those of control animals, although the early stage production of IgM antibodies was unaffected. It is important to note that at the time that the HuMab 7D8 treatment was started (i.e., 21 d after immunization) the production of anti-MOG IgG production had already been initiated but had not reached peak levels. Peak levels of IgG autoantibody in the control group were reached about ∼3 wk later. The reported data suggest that the HuMab 7D8 treatment did not completely suppress anti-MOG IgG production but rather arrested the further increased production of anti-MOG IgG. The effect of B cell depletion on autoantibody production in MS is unknown. Interestingly, in systemic lupus erythematosus patients, treatment with rituximab led to suppression of IgG autoantibody production, whereas no effect was observed on total IgG serum levels (36).

The current study shows that depletion of CD20+ B cells induced a few changes in peripheral blood (i.e., increased numbers of monocytes and increased proliferation of MNCs against...
rhMOG and MOG peptides). A more profound effect was found in the lymphoid organs. The histological analysis of spleen and lymph nodes demonstrated that B cell depletion dramatically altered the relative proportions of B cells and T cells. Where complete depletion was expected, we had not anticipated the increase of T cells. It can be assumed safely that the altered T cell/B cell ratio changes the environmental conditions under which the activation of the MOG34–56-reactive T cells, which mediate the expression of neurologic deficit, occurs (21, 22). The immune profiling of spleen and lymph nodes indeed confirmed that although these organs contain higher percentages of CD3+ T cells, proliferation of MOG (peptide)-reactive T cells is impaired and cytokine production is skewed toward a more anti-inflammatory profile characterized by reduced IL-17A and IFN-γ and increased IL-10. The strong reduction of IL-7 production in the B cell-depleted monkeys may be of particular interest, because this is a crucial cytokine for the survival of IL-17A-producing T cells, which are thought to have a central pathogenic role in MS and EAE models (39), including the EAE model in marmosets (22). Impairment of T cell activation has been put forward as a possible explanation for the clinical effect of B cell depletion in RRMS (2, 3). Reduced T cell activity after B cell depletion has been observed in various experimental systems, including naïve mice, NOD mice, two mouse models for arthritis, and mice infected with Listeria monocytogenes (40, 41). Taken together, these data suggest that the beneficial effect of systemic B cell depletion may be mediated by mitigated T cell activation in lymphoid organs.

In view of recent data on the pathogenic function of intra-CNS-localized EBV-infected B cells in MS (42), although not confirmed by others (43, 44), it is tempting to speculate whether the clinical effect of the anti-CD20 Ab can be explained by the depletion of B cells from the CNS. Several lines of evidence indicate that MOG34–56-specific T cells, which have a key role in the clinical expression of EAE in marmosets, can be activated ex vivo by EBV-transformed B cells (21, 22) (K.G. Haanstra, J.A.M. Wubben, D.M. Lopes Estêvão, M. Jonker, R.Q. Hintzen, and B.A. ’t Hart., manuscript in preparation). Indeed, we have observed that the CD20+ cells that are clearly present in the CNS of EAE-affected marmosets cannot be detected in the anti-CD20 Ab-treated monkeys (data not shown). We cannot exclude, however, that this is due to masking of CD20 by the therapeutic Ab. CD40 expression cannot be used as a control, because this marker is upregulated strongly in the EAE-affected CNS and Abs for human CD19 do not cross-react with the marmoset counterpart (45).

In conclusion, we have shown that B cell depletion in the marmoset EAE model mitigates the activation of autoreactive T cells. We like to postulate that this reduction in T cell activation contributes to the remarkable clinical effect of B cell depletion, and we hypothesize that a similar T cell change occurs in MS. It is difficult to prove this directly in MS patients, because the T cell activity in lymphoid organs cannot be examined. Current clinical trials support the idea that B cell depletion is a major step in therapy development for RRMS patients. Future studies are warranted to elucidate further the Ag-presenting function of B cells, especially in the CNS, and to investigate further the organization of lymphoid organs after B cell depletion.

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Disclosures
At the time that the reported study was executed, P.W.H.P. and W.K.B. were employed by Gennab Utrecht, The Netherlands, and J.L.C. was employed by GlaxoSmithKline, Stevenage, U.K.

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