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CD20⁺ B Cell Depletion Alters T Cell Homing

Yolanda S. Kap,^{*,†,‡} Nikki van Driel,^{*} Jon D. Laman,^{†,‡} Paul P. Tak,^{§,1} and Bert A. 't Hart^{*,†,‡,¶}

Depleting mAbs against the pan B cell marker CD20 are remarkably effective in the treatment of autoimmune-mediated inflammatory disorders, but the underlying mechanisms are poorly defined. The primary objective of this study was to find a mechanistic explanation for the remarkable clinical effect of the anti-CD20 mAbs in a representative nonhuman primate autoimmune-mediated inflammatory disorder model, experimental autoimmune encephalomyelitis (EAE) in common marmosets, allowing detailed analysis of secondary lymphoid organs (SLO). We observed that the depletion of CD20⁺ B cells creates a less immunostimulatory environment in the SLO reflected by reduced expression of MHC class II, CD40, CD83, and CD80/CD86. APCs isolated from SLO of B cell-depleted EAE monkeys were also less responsive to mitogenic stimulation. The depleted B cell areas were replenished by T cells, of which the majority expressed CD127 (IL-7R) and CCR7. Such effects were not detected in EAE marmosets treated with mAb against BLYS or APRIL, where B cell depletion via withdrawal of essential survival cytokines was not associated with a marked clinical effect. We propose that at least part of the efficacy of anti-CD20 mAb therapy is attributable to the sustained CCR7 expression on T cells within SLO, limiting their release into the circulation. *The Journal of Immunology*, 2014, 192: 4242–4253.

B cell targeting mAbs have emerged as a remarkably effective treatment for a broad spectrum of autoimmune diseases (1). Two categories can be discerned, that is, mAbs that deplete CD20⁺ cells, a ubiquitously expressed surface marker in the B cell lineage (e.g., rituximab, ofatumumab, ocrelizumab), and mAbs that capture essential B cell growth factors, such as BLYS (e.g., belimumab and tabalumab) or APRIL. Regarding the latter approach, also a recombinant fusion protein (atacept) has been generated combining the binding sites of human TACI, the joint receptor for BLYS and APRIL, with the C region of human IgG. Anti-CD20 mAb are broadly effective in autoimmune diseases mediated by T cells and B cells, such as rheumatoid arthritis (RA) (2, 3) and multiple sclerosis (MS) (4–6), as well as perhaps in diseases primarily mediated by autoantibodies, such as systemic lupus erythematosus (SLE) or Sjögren's syndrome (7, 8). However, there is currently no undisputed evidence for a robust clinical effect in the latter conditions. By contrast,

belimumab has been approved for the treatment of SLE (9, 10), whereas treatment with tabalumab and atacept did not result in significant clinical improvement in MS (11) or RA (12–14).

Further development and refinement of B cell targeting therapies requires a deeper understanding of their mode of action. We have previously reported on the biological and clinical effect of anti-CD20, anti-BLYS, and anti-APRIL mAbs in the experimental autoimmune encephalomyelitis (EAE) model in the common marmoset (*Callithrix jacchus*). Throughout two decades, EAE in marmosets has emerged as a representative preclinical model of the autoimmune neuroinflammatory disease MS (15). As seen in MS, we observed a distinct clinical effect of mAbs against CD20 compared with neutralization of BLYS and APRIL in the EAE model induced with recombinant human myelin oligodendrocyte glycoprotein (rhMOG) in CFA. Whereas anti-CD20 mAb was highly effective, suppressing clinical and pathological aspects of the disease (16, 17), mAbs against BLYS or APRIL exerted only moderate effects (18). Nevertheless, all three mAbs induced depletion of B cells from the circulation, whereas anti-BLYS, but not anti-APRIL, induced B cell depletion from secondary lymphoid organs (SLOs) (16–18).

B cells have multiple functions in autoimmunity, which may all be affected by B cell depletion (6). The classical role of B cells in autoimmune disease is the production of autoantibodies that injure targeted tissues by complement- and cell-dependent cytotoxicity. However, because terminally differentiated B cells (plasma cells) do not express CD20, they are not directly depleted by anti-CD20 mAb. Indeed, clinical evaluation of the chimeric anti-CD20 mAb rituximab in relapsing-remitting MS showed that the remarkable clinical effect was not associated with alteration of IgG levels in serum or cerebrospinal fluid (4, 5). In contrast, the clinical improvement after rituximab treatment in RA was associated with reduced autoantibody levels and reduced plasma cell infiltration into the inflamed tissue (19). B cells may also be engaged as APCs in T cell activation. Reduction of T cell proliferation and cytokine production was indeed detected in peripheral blood of MS patients treated with rituximab (20), confirming a contribution of B cells at this level. Studies in mouse EAE pointed at a possible regulatory role of B cells, which was abrogated by anti-CD20 mAb treat-

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Abbreviations used in this article: ALN, axillary lymph node; APRIL, a proliferation-inducing ligand; BLYS, B lymphocyte stimulator; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; LCV, lymphocryptovirus; MNC, mononuclear cell; MS, multiple sclerosis; PD-1, programmed cell death protein 1; qPCR, quantitative PCR; RA, rheumatoid arthritis; rhMOG, recombinant human myelin oligodendrocyte glycoprotein; SLE, systemic lupus erythematosus; SLO, secondary lymphoid organ; Treg, regulatory T cell.

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ment, leading to aggravated disease (21, 22). Little is known about regulatory B cells in humans and whether they are changed by B cell targeting therapies (23, 24). IL-10–producing B cells were found to be reduced in MS (23), but it is unknown whether these were naive or memory B cells (23, 25).

The primary objective of this study was to find a mechanistic explanation for the profound clinical effect of the anti-CD20 mAb in T cell–mediated autoimmune disorders, such as MS and RA. In human clinical trials, a major obstacle is the lack of access to SLO where the kinetics of B cell depletion and the functional effects may be distinct from the peripheral blood compartment. Therefore, we undertook an in-depth immune profiling of the SLOs in the marmoset model of MS. This profiling comprised expression analysis of panels of T cell markers, APC markers, and B cell markers by flow cytometry, immunohistochemistry, and quantitative PCR (qPCR). We previously reported that although proliferation and proinflammatory cytokine production by T cells from SLO were reduced, the T cell percentages were increased (16). Our current results confirm that the emptied B cell areas in SLO are replenished by T cells that express CD127 and CCR7, suggesting that they are retained within SLO. We also observed that APCs in the SLO had a lower expression of MHC class II and costimulatory molecules (CD80/CD86), limiting their capacity to activate T cells. These changes were found in marmosets treated with the clinically effective anti-CD20 mAb, but not with the only moderately effective anti-BLyS and anti-APRIL mAbs.

In conclusion, we demonstrate that the elimination of B cells with anti-CD20 mAb has profound systemic effects. Within the B cell–depleted SLO, an immune environment exists where autoreactive T cells are less activated and have a reduced tendency to exit SLO for homing to the CNS.

Materials and Methods

Animals

Adult common marmosets were purchased from the purpose-bred colony of the Biomedical Primate Research Centre. All study protocols and experimental procedures were reviewed and approved according to the Dutch law on animal experimentation.

Tissue samples

For this study, we have used stored frozen tissues and mononuclear cell (MNC) suspensions from three previous studies (see also Table I).

Study 1. Study 1 (16) used nonimmunized marmosets ($n = 12$) from a pharmacokinetic/dose-finding experiment. Group 1 comprised four marmosets, which all received one dose of PBS (1 ml/kg i.v.). The animals were sacrificed 7 ($n = 2$) or 62 d ($n = 2$) after injection. Group 2 comprised four marmosets, of which two were i.v. injected with one dose of 10 mg/kg and two others with 20 mg/kg anti-CD20 mAb (HuMab 7D8). All four animals were sacrificed 7 d after the injection (indicated as treated day 7). Group 3 comprised four marmosets, of which two were i.v. injected with one dose of 10 mg/kg and two others with 20 mg/kg anti-CD20 mAb (HuMab 7D8). All four animals were sacrificed 62 d after the injection (indicated as treated day 62).

Study 2. Study 2 (16) included rhMOG/CFA-immunized marmosets treated with PBS ($n = 6$) or the anti-CD20 mAb HuMab 7D8 ($n = 7$) by weekly i.v. injections from postimmunization day 21 until the end of the experiment. They first received a dose of 20 mg/kg to induce rapid and profound B cell depletion, followed by a weekly maintenance dose of 5 mg/kg. PBS-treated animals were sacrificed at the peak of the disease. Anti-CD20 mAb–treated animals were sacrificed 100 d after immunization without EAE symptoms.

Study 3. Study 3 (18) included rhMOG/CFA-immunized marmosets treated with PBS ($n = 6$), 10 mg/kg anti-BLyS mAb ($n = 6$), or 10 mg/kg anti-APRIL mAb ($n = 6$) by weekly i.v. injections from postimmunization day 21 until the end of the experiment. All animals were sacrificed at the peak of the disease.

The clinical and pathological consequences of the treatments have been reported in the indicated publications. For reasons of clarity, nonimmunized marmosets are indicated as “naive animals,” whereas marmosets immunized with rhMOG/CFA are indicated as “EAE animals” irrespective of their treatment.

Cell culture and stimulation

For the flow cytometry analysis, spleen and axillary lymph node (ALN) samples were used. Other lymph nodes have been used for previous analysis (16) and were too small for further extensive analysis. In addition, the ALN is the lymph node draining the immunization sites in this model. MNCs isolated from venous blood or from SLOs were cultured for 48 h with LPS (50 ng/ml, *Escherichia coli* strain 0111:B4; Sigma Aldrich, Zwijndrecht, the Netherlands), Con A (2 μ g/ml), and PHA (10 μ g/ml).

Flow cytometry

The procedures and reagents used for the phenotypical characterization of marmoset MNC by flow cytometry have been described previously (26). In brief, cells were stained with the violet viability stain (Invitrogen, Molecular Probes, Carlsbad, CA) to exclude dead cells. Aspecific staining was blocked by FcR blocking reagent (Miltenyi Biotec, Leiden, The Netherlands). Subsequently, cells were incubated with Abs against CD1d (LY38), CD3 (SP34-2), CD9 (ML13), CD27 (MT721), CD56 (NCAM 16.2), CD80 (L307.4), CD86 (IT2.2), HLA-DR (to stain Caja-DR, clone L243) (all from BD Biosciences, San Diego, CA); CD21 (HB5), CD127 (ebioDR5), CD279 (J105), Foxp3 (PCH101), IgM (SA-DA4) (all from eBioscience, San Diego, CA); CD20 (H299) and CD83 (HB15a) (both from Beckman Coulter, Fullerton, CA); CCR4 (205410) and CCR7 (150503) (both from R&D Systems, Minneapolis, MN); CD40 (B-B20; Abcam, Cambridge, U.K.); CD4 (MT310; Dako, Glostrup, Denmark); and CD8 (LT-8; Serotec, Düsseldorf, Germany). Cells were fixed in 1% cytofix (BD Biosciences). Flow cytometric analysis was performed on a FACS LSRII (BD Biosciences) using FlowJo software (Tree Star, Ashland, OR).

qPCR

The isolation of RNA and the qPCR assay were performed as described previously (26). In brief, RNA was isolated using RNeasy minikit (Qiagen, Hilden, Germany), and cDNA was synthesized using RevertAid First Strand cDNA synthesis Kit (Fermentas, St. Leon-Rot, Germany). Expression levels of mRNA were determined by qPCR using iTaq supermix and CFX96 Real-Time system (Bio-Rad, Hercules, CA). Transcript levels were normalized against the reference gene *Abelson* (*ABL*) (27).

Immunohistochemistry

Spleen and lymph nodes 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 air-drying of the slides for 1 h, they were fixed in fresh acetone containing 0.02% (v/v) H_2O_2 . 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 rabbit anti-human CD3 (Dako), mouse anti-human CD20 (clone L26; Dako), mouse anti-human CD21 (B-Ly4; BD), mouse anti-human CD40 (B-B20; Chemicon, Billerica, MA), mouse anti-human IgM (Ni179; Nordic Immunology, Susteren, the Netherlands), and mouse anti-human TFG- β (TB21; AbD Serotec, Oxford, U.K.); recognizes active and latent TGF- β). The anti-CD20 mAb used for immunohistochemistry recognizes an intracellular epitope of CD20, distinct from the epitope recognized by the treatment anti-CD20 mAb HuMab 7D8. Incubations with secondary and tertiary reagents were performed for 1 h at room temperature. Between incubation steps, the slides were washed twice with PBS/0.05% Tween. Incubation with primary unlabeled Ab was followed by incubation with donkey anti-mouse or goat anti-rabbit, both biotin labeled. This was followed by incubation with HRP-labeled avidin-biotinylated-complex (Vectastain ABC; Vector Laboratories, Burlingame, CA). Peroxidase activity was revealed by incubation for 10 min at room temperature with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma) containing 0.03% hydrogen peroxide. Incubation with isotype-matched primary Ab of irrelevant specificity and omission of the primary Ab served as negative staining control.

Statistics

Data are presented as individual animals with the mean. Statistical analysis was performed using Prism 6.0b for Mac OS X. A p value <0.05 was considered statistically significant.

Results

Different immune profiles of SLO after anti-CD20 mAb versus anti-BLyS mAb

We determined the effect of B cell targeting therapies on general markers of B cells and T cells. Abs directed against human CD19

are not cross-reactive with marmoset cells, and the anti-CD20 mAb used for flow cytometry is directed against the same epitope as the therapeutic mAb (HuMab 7D8). Therefore, direct analysis of CD20 expression by flow cytometry was a challenge. As reported previously, we could solve this problem by acidic stripping of the therapeutic mAb from the cells. Thus, we could show that CD20⁺ B cells were indeed profoundly depleted from SLO, which was confirmed by using anti-CD40 mAb as an alternative marker for B cells (16).

A single injection of anti-CD20 mAb into naive marmosets induced a significant reduction of CD3⁻ and CD3⁻CD40⁺ MNCs, persisting 7 and 62 d (Study 1; Supplemental Fig. 1A, Table I). The same effect was seen after repetitive injection of anti-CD20 mAb into animals in which EAE was induced (Study 2; Fig. 1A). The depletion of B cells was confirmed by the reduced mRNA expression of CD19 (Fig. 1B).

The spleen of naive animals receiving a single dose of anti-CD20 mAb (study 1) contained a higher percentage of CD3⁺ cells than placebo-treated animals, although the increase did not reach statistical significance (Supplemental Fig. 1A). At 7 d after the single dose of anti-CD20 mAb, the percentage of CD8⁺ T cells in the spleen was higher and the CD4/CD8 ratio was lower compared with placebo-treated animals, but this was normalized at 62 d after dosing (Supplemental Fig. 1A).

Similar observations were made in EAE animals receiving repetitive dosing of anti-CD20 mAb (study 2). We detected a higher percentage of CD3⁺ cells in spleen (Fig. 1A) and a higher expression of CD3 mRNA in the ALN (Fig. 1B). We observed that the percentage of CD4⁺ T cells was higher in the spleen and lower in the ALN of anti-CD20 mAb-treated animals compared with placebo-treated animals, but no significant difference in the CD4/CD8 ratio was detected (Fig. 1A). In PBMCs, the

percentage of CD3⁻CD40⁺ cells was significantly lower in anti-CD20 mAb-treated EAE animals compared with placebo-treated animals, but the percentages of CD3⁻ or CD3⁺ cells did not differ (data not shown).

The higher percentage of CD3⁺ cells and the higher CD3 mRNA expression level likely reflects retention of T cells. Immunohistochemical analysis of tissues obtained in study 2 showed that spleen (Fig. 2) and ALN (16) of placebo-treated EAE animals contain substantially fewer CD3⁺ areas compared with naive animals, which is consistent with previously reported data (28). In animals treated with anti-CD20 mAb, this reduction was not found, raising the question whether T cells may be prohibited to exit the lymphoid organs.

Treatment with anti-BLyS mAb (study 3) led to similar results, that is, a higher percentage of CD3⁺ and CD3⁺CD8⁺ cells within the spleen and a lower percentage of CD3⁻CD40⁺ and CD20⁺ cells within both spleen and ALN compared with placebo-treated animals (Supplemental Fig. 1B, 1C, Table II). Treatment with anti-APRIL mAb (study 3) had an opposite effect, as we found a higher percentage of CD3⁻ cells and a lower percentage of CD3⁺ T cells compared with placebo-treated animals (Supplemental Fig. 1B, 1C). In conclusion, the systemic depletion of B cells has a profound effect on the T cell compartment.

Depletion of B cells involves several subpopulations

We performed an immunohistochemical assessment of CD20, CD21, and IgM expression in SLO. In most anti-CD20 mAb-treated monkeys (study 2), we observed profound depletion of CD20⁺ cells from spleen. In the spleen of the anti-CD20 mAb-treated EAE monkey M05073, we found a small cluster of CD20⁺ cells, which were negative for CD21 and IgM. Whether these were residual cells that had not been depleted or cells that had

Table I. Overview of in vivo experiments

Study and Group Name	Treatment	Dosing Regimen	Immunization	No. of Animals ^a	Day of Sacrifice (Mean ± SD; Range)	EAE Score at Sacrifice (No. of Animals; EAE Score)
Study 1						
Placebo	PBS	Once	None	4	7 or 62 d after PBS ^b	n.a.
Treated day 7	Anti-CD20 mAb	Once		4	7 d after anti-CD20 mAb	n.a.
Treated day 62	Anti-CD20 mAb	Once		4	62 d after anti-CD20 mAb	n.a.
Study 2						
Placebo	PBS	Weekly, starting 21 d after immunization	rhMOG/CFA	6	78 ± 19; 55–105	n = 1: 0.5 (3 episodes of score 2) n = 1: 2.25 n = 4: 3
Anti-CD20 mAb	Anti-CD20 mAb	Weekly, starting 21 d after immunization		7	105 ± 1; 104–106	n = 1: 0.5 n = 6: 0
Study 3						
Placebo	PBS	Weekly, starting 21 d after immunization	rhMOG/CFA	6	46 ± 10; 30–57	n = 6: 3
Anti-BLyS mAb	Anti-BLyS mAb	Weekly, starting 21 d after immunization		6	76 ± 20; 52–102	n = 1: 2 n = 5: 3
Anti-APRIL mAb	Anti-APRIL mAb	Weekly, starting 21 d after immunization		6	59 ± 26; 43–109	n = 2: 2 n = 4: 3

Data summarized from Kap et al. (16) and Jagessar et al. (18).

^aThis is the number of animals included in the in vivo study; but for some animals, we did not have enough frozen cells to perform flow cytometry or RNA to perform qPCR analysis.

^bTwo animals of the PBS-treated group were sacrificed together with the animals of treated day 7, and the other two animals at day 62 together with treated day 62. In this article, those four control animals were used as one group, because they were nonimmunized and treated with PBS. No differences were observed between those animals. n.a., not applicable.

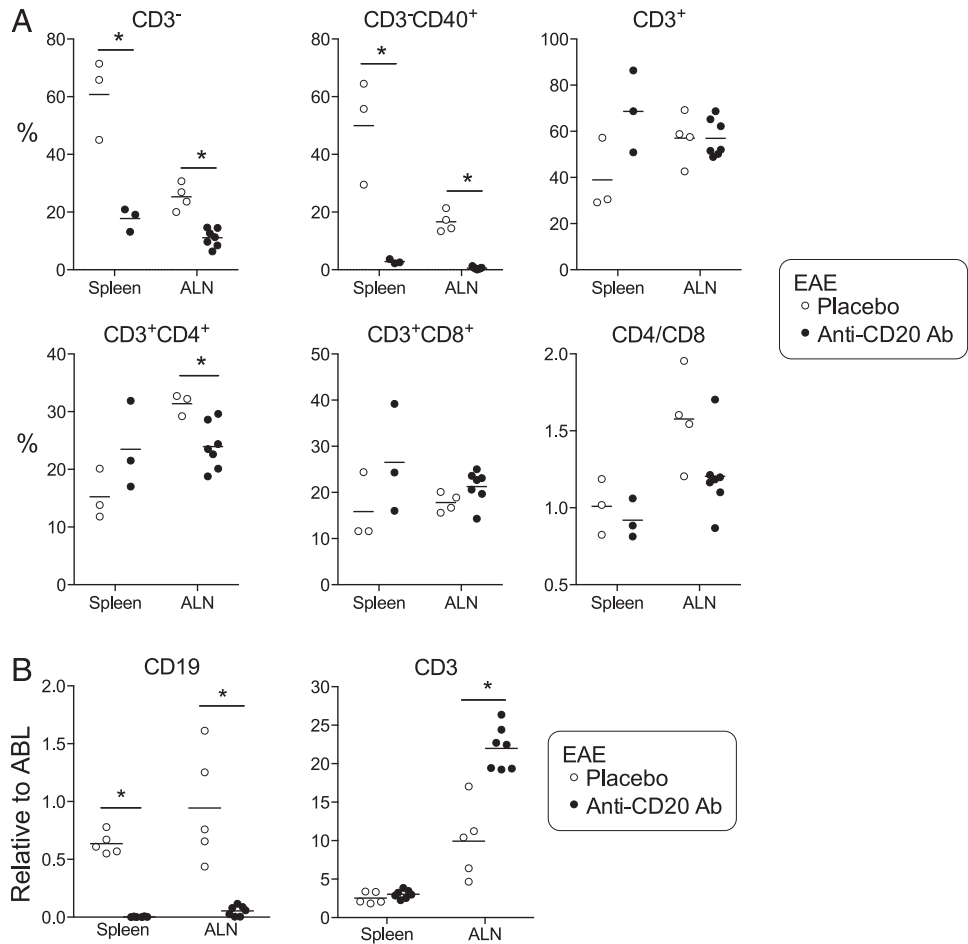


FIGURE 1. B cell depletion induces an increase in the percentage T cells. **(A)** MNCs from spleen and ALN were analyzed by flow cytometry. The percentages of non-T cells ($CD3^-$), B cells ($CD3^-CD40^+$), and T cells ($CD3^+$) are shown for the animals of study 2. **(B)** CD19 and CD3 mRNA levels were determined by qPCR in spleen and ALN of EAE animals. Statistical significance is indicated by an asterisk: $*p < 0.05$.

replenished the spleen is unknown. Splensens from anti-CD20 mAb-treated animals (study 2) contained some IgM^+ cells, but substantially less than splensens from placebo-treated EAE animals (Fig. 3A). In ALN of some anti-CD20 mAb-treated animals (study 2), we found small clusters of $CD20^+$ B cells, which were $CD21^+$ and IgM^+ . In animals without $CD20^+$ follicles, IgM -expressing cell numbers were also reduced, but not completely absent, as just

like in the spleen, few scattered cells could be detected (data not shown).

For a more in-depth phenotypical analysis, we used flow cytometry. Besides CD3 and CD40, cells were stained for CD21, the complement receptor 2 expressed by immature transitional and mature B cells; CD27, a marker for memory B cells, which upon engagement with its ligand CD70 promotes the development of

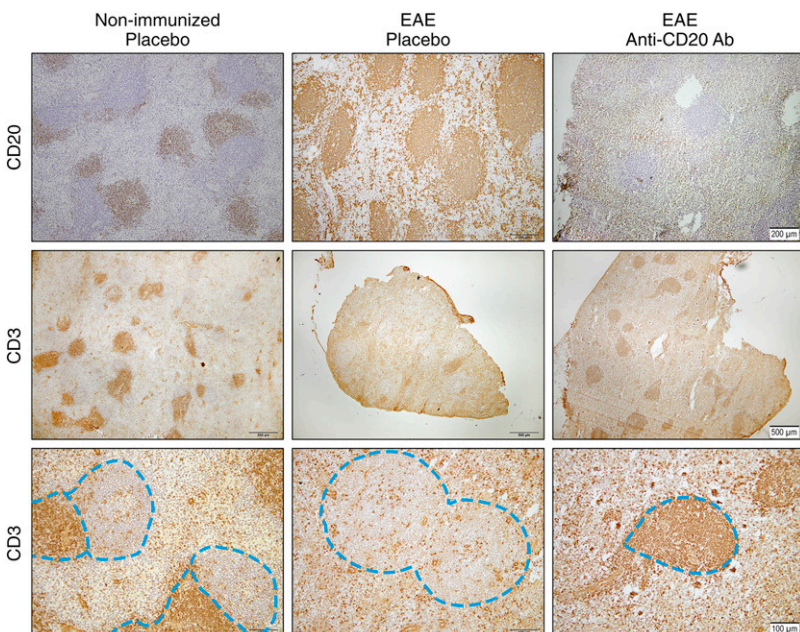


FIGURE 2. T cells remain in the SLO after B cell depletion. Spleen sections were stained with 3,3'-diaminobenzidine tetrahydrochloride for CD3 and CD20. Scale bars, $\times 200 \mu m$ (upper row); $\times 500 \mu m$ (middle row); $\times 100 \mu m$ (bottom row). Representative images are shown. The dotted line in the lower row indicates B cell-T cell areas. In placebo-treated nonimmunized animals, this area consists of both T cells and B cells. In placebo-treated EAE animals, no T cells are present in these areas, whereas in anti-CD20 mAb-treated EAE animals, the whole area consists of T cells.

Table II. Immune changes in SLO induced by treatment of rhMOG/CFA-immunized marmosets with anti-CD20 or anti-BLyS mAbs

Parameter	Anti-CD20	Anti-BLyS
Clinical effect	+++ ^a	+ ^a
APC markers		
MHC class II	↓ ^b	↓ ^b
CD83	↓	— ^b
CD80	↓	—
CD86	↓	—
B cell markers		
IgM	↓	↓
CD9	↓	↓/— ^b
CD20	↓	↓
CD21	↓	↓
CD27	↓	↓/—
CD40	↓	↓
CD127	↑	↑/— ^b
CCR7	↑	↑
T cell markers		
CD3	↑	↑
CD4	— (spleen) ↓(ALN)	—
CD8	—	↑
CD4/8	—	—
CD279 (PD-1)	↓	↓(Only CD4)
CD27	↓(Only CD8)	—
CD127	↑(Only CD8)	—
CCR7	↑	—

For reasons of clarity, the effects of anti-APRIL are not included in this table.

^a+++; 4/7 animals remained free of EAE symptoms, +; 0/5 animals remained symptom free, but survival was significantly delayed compared with placebo-treated animals.

^b↑ ↓: Significantly higher/lower in the treatment group compared with placebo-treated group; ↑/— ↓/—: higher/lower in the treatment group compared with placebo-treated group, although not significant; —: no difference between placebo-treated and mAb-treated group.

plasma cells; surface IgM, expressed by immature and mature B cells; CD127, the IL-7R required for survival, proliferation, and differentiation of B cells; CD9, a marker of marginal zone B cells; and CCR7, a chemokine receptor expressed on T and B cells directing their migration to and residence within SLO.

Within the CD3⁺ and CD3⁺CD40⁺ populations in SLO, we detected reduced expression of CD27, IgM, CD9, and CD21 in EAE monkeys treated with repetitive doses of anti-CD20 mAb (study 2; Fig. 3B), in naive monkeys treated with a single dose of anti-CD20 mAb (study 1; Supplemental Fig. 2A), and in monkeys treated with anti-BLyS mAb (study 3; Supplemental Fig. 2B). In contrast, these populations were slightly increased in monkeys treated with anti-APRIL mAb (study 3; Supplemental Fig. 2B).

Compared with placebo-treated EAE animals, the percentage of CD3⁺ cells expressing IL-7R (CD127) was significantly higher in animals treated with either a single dose or repetitive doses of anti-CD20 mAb (studies 1 and 2); this was also observed after treatment with anti-BLyS mAb (study 3; Fig. 3B, Supplemental Fig. 2).

The percentage of CD3⁺ cells expressing CCR7 was higher in the ALN of anti-CD20 mAb-treated EAE animals (study 1; Fig. 3B) and in the spleen of anti-BLyS mAb-treated animals (study 3; Supplemental Fig. 1) compared with placebo-treated animals. This effect was not observed after a single dose of anti-CD20 mAb or repetitive dosing of anti-APRIL mAb (study 3; Supplemental Fig. 1).

The changes in B cell percentages were less profound in the peripheral blood compartment after repetitive dosing of anti-CD20 mAb (study 1). The percentage of circulating CD3⁺IgM⁺ cells was reduced, but the percentages of CD3⁺CD40⁺CD27⁺, CD3⁺CD9⁺, CD3⁺CD127⁺, and CD3⁺CCR7⁺ cells were not altered (data not shown).

The fraction of regulatory B cells was investigated by analyzing CD3⁺CD5⁺CD1d⁺ cells by flow cytometry. In naive and EAE animals, the percentage of these cells was very low (<0.5%) and

was not significantly altered by the anti-CD20 mAb treatment (studies 1 and 2; data not shown).

Taken together, these data show that treatment with anti-CD20 mAb or anti-BLyS mAb depletes immature, mature, and memory B cells.

B cell depletion does not induce T regulatory cells

We previously reported that T cells isolated from SLO of B cell-depleted EAE marmosets have an impaired capacity to proliferate and generate proinflammatory cytokines compared with T cells from placebo-treated monkeys (16). Nevertheless, T cell percentages and numbers in the SLO of anti-CD20 mAb-treated EAE animals were clearly increased compared with placebo-treated EAE animals (Figs. 1, 2). This warrants the question whether these T cells may have a regulatory function.

Fig. 4 (study 2) shows that mRNA levels in spleen and ALN of Foxp3, CTLA-4, and IL-10 were not significantly different between monkeys treated with placebo or anti-CD20 mAb (Fig. 4A). However, Foxp3 expression was higher in the inguinal and lumbar lymph nodes of anti-CD20 mAb-treated EAE animals compared with placebo-treated animals (data not shown), suggesting regionally different expression of this marker. We observed significantly lower expression of GITR (glucocorticoid-induced TNFR) in the ALN of animals treated with anti-CD20 mAb compared with placebo-treated animals (Fig. 4A). However, Foxp3 and GITR expression alone do not specifically identify Tregs because these markers are also expressed by activated T cells. TGF-β mRNA levels were not significantly altered, but in some anti-CD20 mAb-treated animals, the expression was substantially higher than in placebo-treated animals (Fig. 4A). This observation could be confirmed with immunohistochemistry (Fig. 4B).

Next, we analyzed whether Treg markers (TGF-β, Foxp3, GITR, CTLA-4) could be induced by ex vivo stimulation of the cells with the immunizing Ag rhMOG or the mitogen LPS. We detected no major differences between the placebo-treated and the anti-CD20 mAb-treated group in response to stimulation (data not shown), with the exception of CTLA-4. Stimulation with rhMOG induced a 4-fold higher mRNA expression of CTLA-4 in the ALN of placebo-treated animals, whereas the expression of this marker was increased only 2-fold in anti-CD20 mAb-treated animals (Fig. 4C).

Finally, we determined the expression of programmed cell death protein 1 (PD-1 or CD279), a member of the CTLA-4 family that relays negative regulatory signals. Interestingly, expression of this protein was reduced on CD4⁺ and CD8⁺ T cells in spleen and ALN of anti-CD20 mAb-treated EAE animals compared with placebo-treated EAE animals (Fig. 4D). We observed no change in PBMCs of EAE animals treated with a repetitive dose of anti-CD20 mAb or in SLO after a single dose of anti-CD20 mAb (data not shown). Taken together, these data show that there is no clear effect of B cell depletion on the induction or expansion of Tregs.

B cell depletion induces CCR7 expression on intra-SLO IL-7R⁺ CD8⁺ T cells

Next, we assessed the expression of CD127 and CCR7 on T cell subpopulations in SLO from placebo- and mAb-treated monkeys.

CD127 is the IL-7R α subunit, which forms a functional heterodimeric receptor complex with the common γ-chain (CD132) on naive and memory T cells. CD127 has a critical role in lymphocyte development. The percentage CD127⁺ T cells (Fig. 5A) and the CD127 expression level per cell (Fig. 5B) were increased in the CD8⁺ T cell population of EAE animals treated with repetitive dosing of anti-CD20 mAb compared with placebo-treated EAE animals (study 2). Treatment had no effect on CD127 expression by CD4⁺ T cells (Fig. 5A, 5B). We also observed no effect in naive

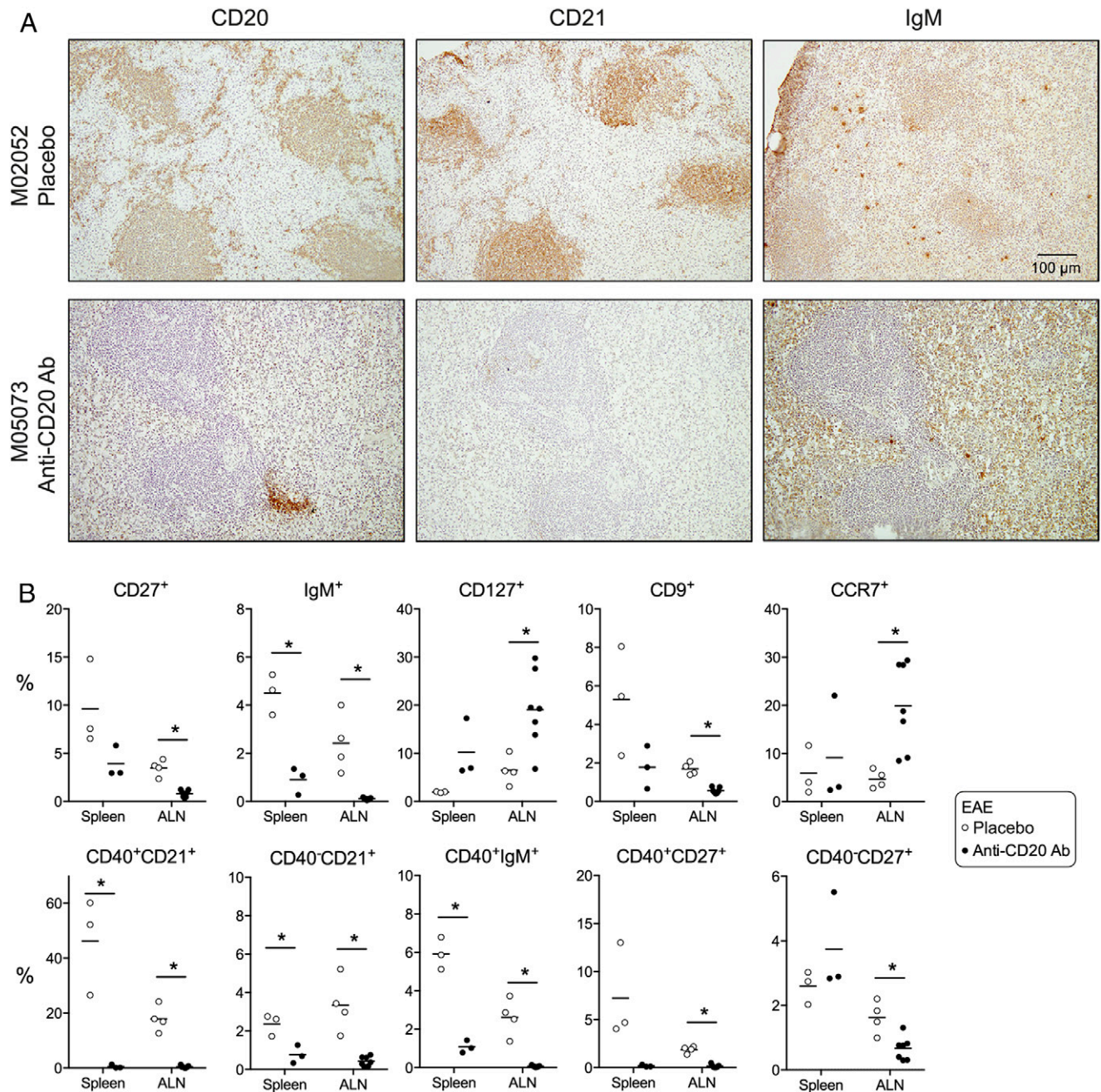


FIGURE 3. Immature, mature, and memory B cells are depleted by anti-CD20 mAb. **(A)** The spleen of placebo-treated and anti-CD20 mAb-treated EAE animals (study 2) was stained with 3,3'-diaminobenzidine tetrahydrochloride for CD20, CD21, and IgM. A representative animal of each group is shown. **(B)** MNCs from spleen and ALN of study 2 were stained for several B cell markers. Cells were gated on CD3⁻ cells within the live lymphocyte population. Shown are the percentages of cells within the lymphocyte population. Statistical significance is indicated by an asterisk: **p* < 0.05.

animals treated with a single anti-CD20 mAb dose (study 1; Supplemental Fig. 3A). Moreover, the effect seemed restricted to the anti-CD20 mAb, because CD127 expression was not changed in EAE animals treated with repetitive doses of anti-BLyS mAb or anti-APRIL mAb (study 3; Fig. 5A, 5B).

The CD8⁺CD127⁺ cells of EAE animals treated with a repetitive dose of anti-CD20 mAb were characterized in more detail. The data depicted in Supplemental Fig. 3B show that the CD8⁺ T cell population of anti-CD20 mAb-treated EAE monkeys contains higher percentages CD127⁺CD27⁺ and CD127⁺CD56⁺ cells than spleen and ALN of placebo-treated EAE monkeys (study 2).

We previously reported that IL-7 mRNA expression is substantially reduced in the spleen and ALN of animals treated with anti-CD20 mAb (16) (study 2; Fig. 5C). We analyzed whether the

expression of this cytokine could be increased by stimulation with rhMOG or LPS. Fig. 5D shows that splenocytes, but not ALN cells, of animals treated with anti-CD20 mAb and stimulated with rhMOG or LPS produced 2-fold less IL-7 mRNA than stimulated cells of placebo-treated animals (study 2; Fig. 5D).

CCR7 (CD197) is the receptor of the chemokines CCL19 and CCL21, and is expressed on lymphocytes. CCR7 is engaged in homing of T cells to SLO. To facilitate the release of activated T cells into the circulation from SLO, CCR7 is downregulated (29). Consistent with this principle, we phenotyped the core pathogenic T cell fraction in the rhMOG/CFA marmoset EAE model as CD3⁺CD4⁺/CD8⁺CD27⁺CD56⁺ and CCR7⁻ (30).

In anti-CD20 mAb-treated animals, the percentage of CCR7-expressing cells and the expression level per cell was higher

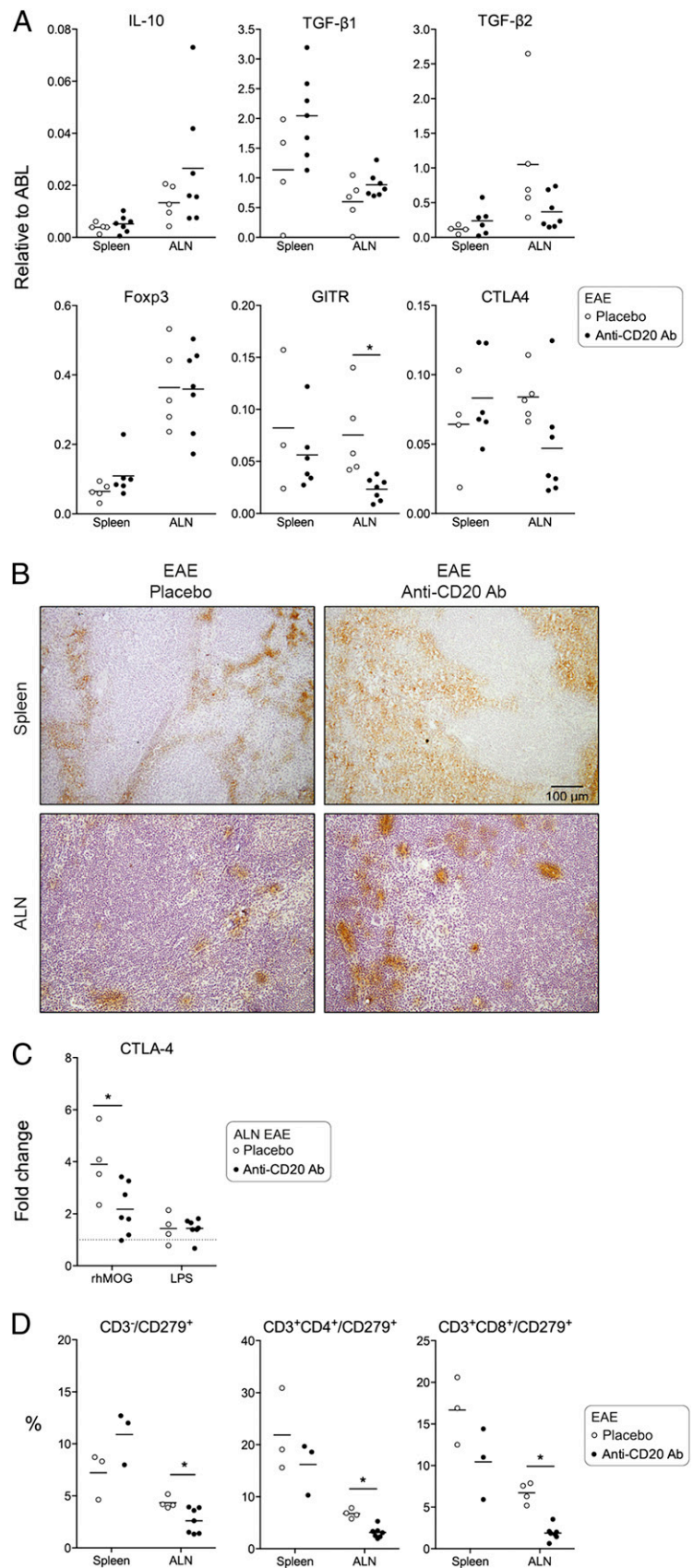
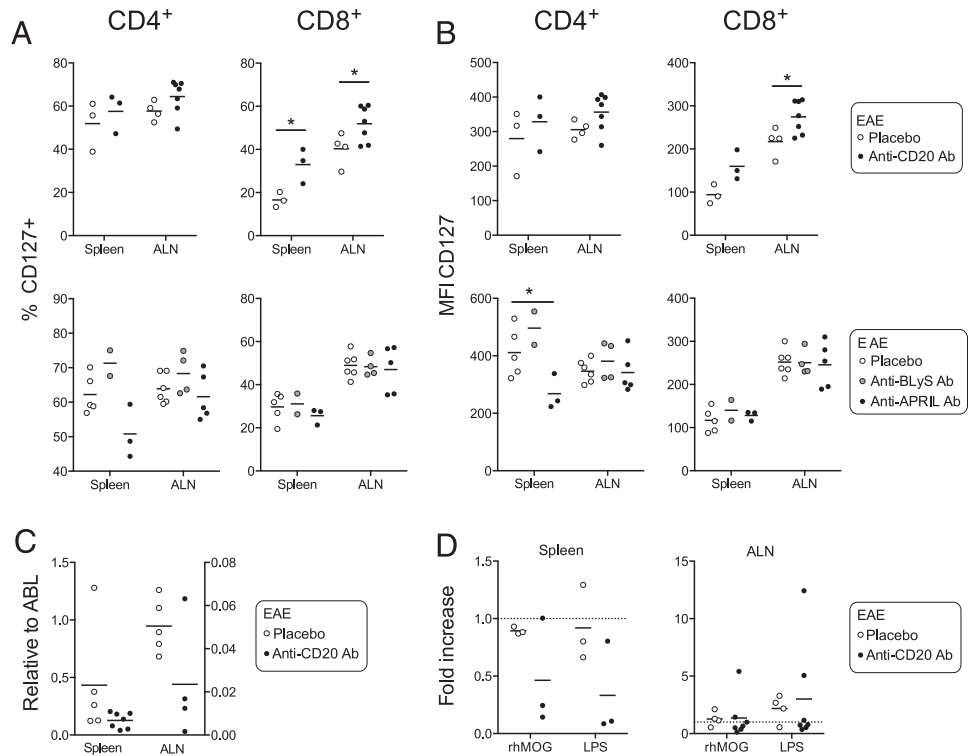


FIGURE 4. B cell depletion does not induce features of Tregs. **(A)** mRNA expression of regulatory markers was determined in spleen and ALN of placebo-treated and anti-CD20 mAb-treated EAE animals of study 2. mRNA levels were determined by qPCR and expressed relative to the household gene Abelson (ABL). IL-10 data were published previously (16). **(B)** Sections of spleen and ALN of study 2 were stained with 3,3'-diaminobenzidine tetrahydrochloride for TGF- β . A representative animal of each group is shown. **(C)** Cells isolated from the spleen and ALN of placebo-treated and anti-CD20 mAb-treated EAE animals (study 2) were stimulated with rhMOG or LPS for 48 h. CTLA-4 mRNA expression was determined by qPCR and related to the household gene Abelson (ABL). The fold change of stimulated cells compared with nonstimulated cells is shown. **(D)** The percentage of CD279 (PD-1)⁺ cells was determined by flow cytometry on spleen and ALN of study 2. Cells were first gated on live lymphocyte population. Shown is the percentage of CD279⁺ cells within the CD3⁻, CD3⁺CD4⁺, or CD3⁺CD8⁺ population. Statistical significance is indicated by an asterisk: * $p < 0.05$.

compared with placebo-treated monkeys (study 2; Fig. 6A, 6B). The increase in CCR7 was observed on several subpopulations, including cells that were either positive or negative for CCR4, CD27, and CD56 (study 2; Supplemental Fig. 3C). The higher

CCR7 expression levels were only detected in SLO and not in PBMCs (study 2; data not shown). Treatment with anti-BLyS mAb or anti-APRIL mAb did not induce altered CCR7 expression (study 3; Fig. 6A, 6B).

FIGURE 5. The frequency of CD8⁺CD127⁺ T cells in the SLO is higher after B cell depletion. **(A)** The percentage of CD127⁺ cells was determined within the CD4⁺ and CD8⁺ T cell populations. **(B)** The mean fluorescence intensity (MFI) of CD127 is shown. **(C)** The IL-7 mRNA levels were determined by qPCR, as published previously (16). **(D)** Cells isolated from the spleen and ALN of placebo-treated and anti-CD20 mAb-treated EAE animals were stimulated with rhMOG or LPS for 48 h. mRNA expression of IL-7 was determined by qPCR and related to the household gene *Abelson* (*ABL*). The fold change of stimulated cells compared with nonstimulated cells is shown. Statistical significance is indicated by an asterisk: **p* < 0.05.



The increase of CCR7 expression seems a direct response to the anti-CD20 mAb as within 7 d after a single dose of the mAb in naive animals, the percentage of CCR7⁺ cells and the expression level was higher in the CD4⁺ T cell population of the spleen. However, the enhancement was transient, because CCR7 expression levels were normalized at postinjection day 62 (study 1; Supplemental Fig. 3A).

The mRNA expression of CCR7 was higher in the ALN, but not in spleen, of anti-CD20 mAb-treated animals compared with placebo-treated animals. CCL19, one of the ligands for CCR7, was lower in the spleen and higher in the ALN of anti-CD20 mAb-treated animals compared with placebo-treated animals. The other ligand for CCR7, CCL21, was equally expressed between anti-CD20 mAb-treated and placebo-treated EAE animals (study 2; Fig. 6C). In conclusion, treatment with anti-CD20 mAb, but not anti-BLyS mAb, affects the expression of CD127 on CD8⁺ T cells and CCR7 on several T cell subsets.

B cell depletion reduces the expression of APC activation markers

The impaired activation of T cells in B cell-depleted animals (16) warrants the question whether B cell depletion affects the expression of APC markers needed for T cell activation. We investigated the expression of MHC class II (Caja-DR), the dendritic cell (DC) maturation marker CD83, and the costimulatory molecules CD80 and CD86 in the CD3⁺ population.

The frequency of CD3⁺Caja-DR⁺ cells, representing all APCs, was significantly lower in animals treated with one (study 1, Supplemental Fig. 4A) or repetitive doses of anti-CD20 mAb compared (study 2; Fig. 7A) with placebo-treated animals. The administration of one dose of the anti-CD20 mAb had a long-lasting effect, as the reduction in CD3⁺Caja-DR⁺ cells was still detectable at 62 d postinjection (study 1; Supplemental Fig. 4A). Treatment with anti-BLyS mAb had a similar effect as anti-CD20 mAb, but treatment with anti-APRIL mAb had an opposite effect and led to a slightly higher percentage of CD3⁺Caja-DR⁺ cells (study 3; Supplemental Fig. 4A).

CD83 is a marker of mature DCs (31). The percentage of CD3⁺CD83⁺ cells is lower after one dose (study 1; Supplemental

Fig. 4A) and repetitive dosing of anti-CD20 mAb (study 2; Fig. 7A) compared with placebo-treated animals, but 62 d after the single dose administration, the frequency had normalized (study 1; Supplemental Fig. 4A). The CD83 expression level per individual cell was not affected by B cell depletion (Fig. 7B, Supplemental Fig. 4A). Anti-BLyS mAb and anti-APRIL mAb had no detectable effect on the percentage of CD83⁺ cells or on the expression level of this marker (study 3; Supplemental Fig. 4A).

CD80 and CD86 are markers of mature APCs that relay costimulation (signal 2) to T cells via interaction with CD28. We observed a lower percentage of CD80⁺ or CD86⁺ cells in the monkeys treated with a single dose (study 1; Supplemental Fig. 4A) or repetitive doses (study 2; Fig. 7A) of anti-CD20 mAb compared with placebo-treated animals. The expression level of CD86 per cell was significantly lower in animals treated with a repetitive dose of anti-CD20 mAb compared with placebo-treated animals (study 2; Fig. 7B). In contrast, the expression level of CD80 was significantly higher in the spleen of anti-CD20 mAb-treated EAE animals compared with placebo-treated animals (study 2; Fig. 7B). When comparing the expression level of CD80 in the spleen of placebo-treated naive and EAE animals (control groups of studies 1 and 2), it may be that CD80 is down-regulated during EAE. This reduction was not observed after repetitive dosing of anti-CD20 mAb treatment (Fig. 7B). The anti-BLyS mAb or anti-APRIL mAbs did not significantly affect the expression of these costimulatory molecules (study 3; Supplemental Fig. 4A).

Next, we assessed whether the impaired expression of MHC class II, CD83, CD80, and CD86 could be restored by ex vivo mitogenic stimulation with LPS, Con A, or PHA. Fig. 8 shows that the percentage of CD3⁺Caja-DR⁺ cells after stimulation relative to nonstimulated cells, expressed as fold increase, was higher in the spleen and ALN of animals treated with a repetitive dose of anti-CD20 mAb compared with placebo-treated animals (study 2; Fig. 8A). This was not seen in SLO of animals treated with anti-BLyS mAb, anti-APRIL mAb, or a single dose of anti-CD20 mAb (studies 1 and 3; Supplemental Fig. 4B, 4C).

In the spleen of placebo-treated EAE animals, stimulation with all three mitogens increased the percentage of CD3⁺CD83⁺,

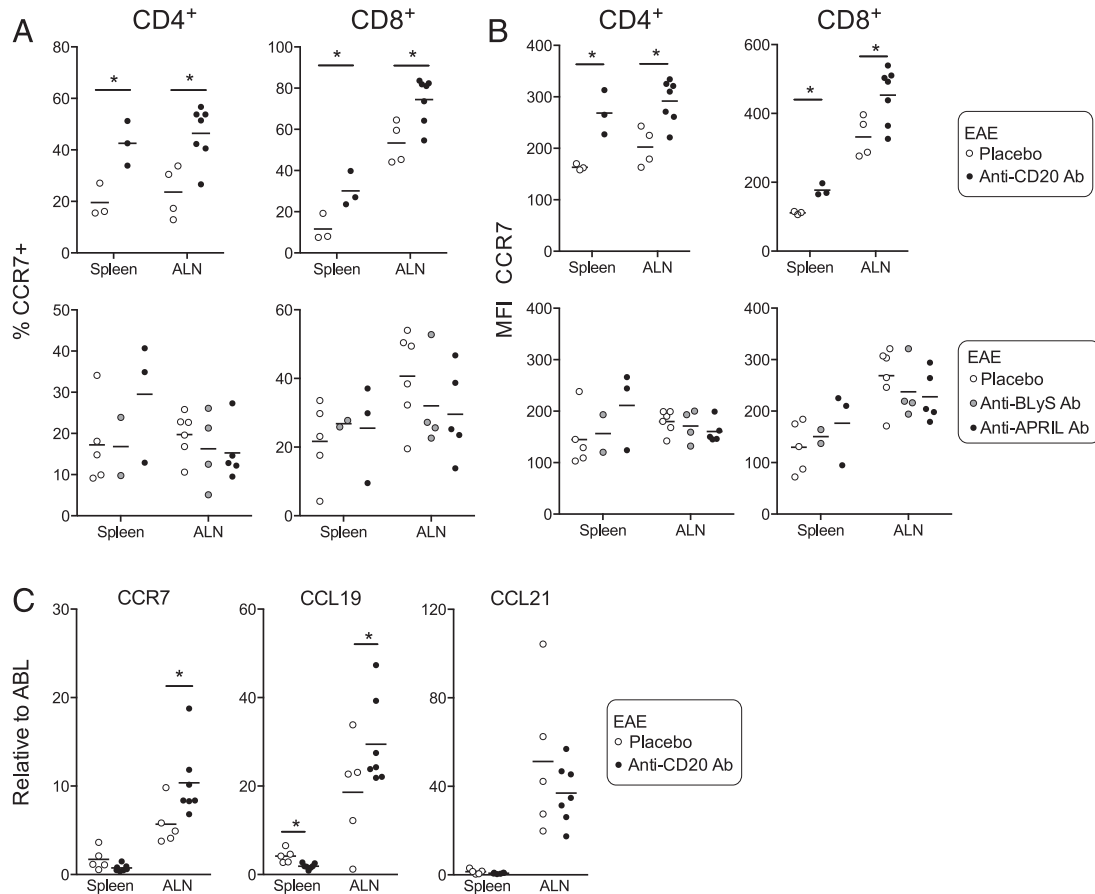
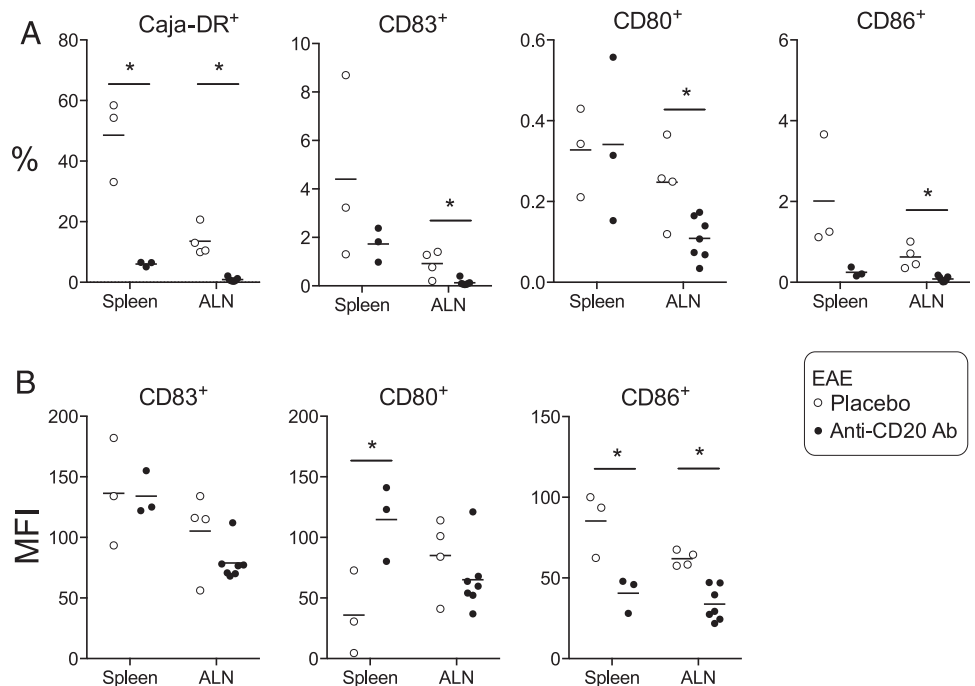


FIGURE 6. T cells retained within the SLO highly express CCR7. **(A)** The percentage of CCR7⁺ cells was determined within the CD4⁺ and CD8⁺ T cell populations. **(B)** The mean fluorescence intensity (MFI) of CCR7 is shown. **(C)** The mRNA expression levels of CCR7, CCL19, and CCL20 were determined in spleen and ALN of placebo-treated and anti-CD20 mAb-treated EAE animals by qPCR and related to the household gene Abelson (ABL). Statistical significance is indicated by an asterisk: **p* < 0.05.

CD3⁻CD80⁺, and CD3⁻CD86⁺ cells. In contrast, splenocytes of animals treated with a repetitive dose of anti-CD20 mAb hardly responded to stimulation by upregulation of their costimulatory

markers (study 2; Fig. 8A). In the ALN of placebo-treated EAE animals, the percentages of CD3⁻CD83⁺ and CD3⁻CD86⁺ cells were increased after Con A and PHA stimulation, whereas the

FIGURE 7. The immunostimulatory environment is reduced by B cell depletion. MNCs from spleen and ALN of study 2 were stained for CD3, MHC class II (Caja-DR), the costimulatory molecules CD80 and CD86, and the DC maturation marker CD83. Cells were gated on CD3⁻ cells within the living lymphocyte population. Shown are the percentages of cells within the live lymphocyte population **(A)** and the mean fluorescence intensity (MFI) **(B)**. Statistical significance is indicated by an asterisk: **p* < 0.05.



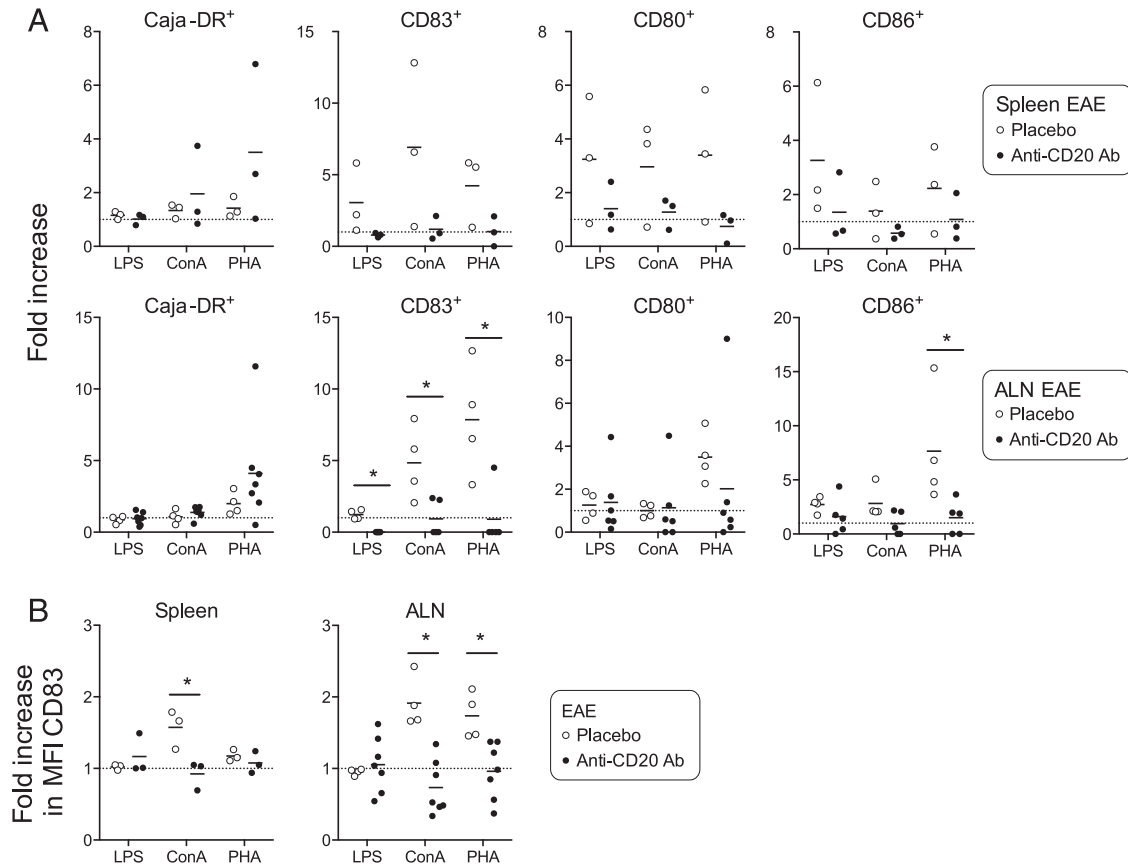


FIGURE 8. APCs from B cell-depleted SLO are less responsive to mitogenic stimulation. **(A)** MNCs isolated from the spleen and ALN of placebo-treated and anti-CD20 mAb-treated EAE animals (study 2) were stimulated with LPS, Con A, or PHA for 48 h. The expression of Caja-DR, CD80, CD86, and CD83 was determined by flow cytometry. Cells were gated on CD3⁺ cells within the live lymphocyte population. Shown is the fold increase of stimulated cells compared with nonstimulated cells. **(B)** Depicted is the fold increase in the mean fluorescence intensity for CD83 after stimulation. The mean fluorescence intensity (MFI) of CD80 and CD86 was not changed by stimulation. Statistical significance is indicated by an asterisk: **p* < 0.05.

percentage of CD3⁺CD80⁺ cells was only increased by PHA. Similar to the spleen, ALN cells of anti-CD20 mAb-treated animals responded hardly to any of the three stimuli (study 2; Fig. 8A). The expression levels per cell of CD80 and CD86 were not changed by stimulation, neither in placebo-treated nor in anti-CD20 mAb-treated EAE animals (study 2, data not shown). In placebo-treated animals, stimulation by Con A or PHA led to a higher expression of CD83, suggesting induction of DC maturation (study 2; Fig. 8B). In anti-CD20 mAb-treated animals, expression of CD83 could not be induced, suggesting that B cell depletion affects the maturation of DC upon mitogenic stimulation (study 2; Fig. 8B).

In contrast with placebo-treated animals, in animals treated with a single dose of anti-CD20 mAb, the percentage of CD3⁺CD83⁺ and CD3⁺CD86⁺ cells could not be increased by mitogenic stimulations (study 1; Supplemental Fig. 4B). Upon stimulation, the percentage of CD3⁺CD80⁺ cells was similarly increased in anti-CD20 mAb and placebo-treated animals (Supplemental Fig. 4B). Treatment with anti-BLyS mAb or anti-APRIL mAb did not affect the percentage or the expression level of CD80, CD83, or CD86 induced by mitogenic stimulation (study 3; Supplemental Fig. 4C). Taken together, the expression of APC markers required for full T cell activation is profoundly reduced after anti-CD20 mAb treatment.

Discussion

The primary objective of this study was to find a mechanistic explanation for the remarkable clinical effect of anti-CD20 mAbs in the marmoset EAE model and in clinical applications. For our

studies, we used the well-validated rhMOG/CFA-induced EAE model. As therapeutic anti-CD20 mAb, we used HuMab 7D8, which is a clonal variant of ofatumumab binding the same epitope (32, 33). The remarkably robust effect of the anti-CD20 mAb on clinical and pathological aspects of the model has been reported previously (16, 17). In this article, we report that B cell depletion induced several dramatic changes in SLO, in particular, in the expression of markers engaged in survival and homing of T cells.

The space left by depleted B cells seems to be replenished by T cells. The ubiquitous high expression of CCR7 indicates that T cells are retained within the SLO and not released into the circulation. T cells within SLO constitutively express CCR7, which is normally downregulated after Ag stimulation (34). In the poorly immunostimulatory milieu of the B cell-depleted SLO, T cells not only express high levels of CCR7, but also of CD127, the receptor for IL-7. Similar to CCR7, CD127 is downregulated upon antigenic activation. CD127 is crucial for T cell survival during steady-state conditions (35). The higher level of CD127 was confined to the CD8⁺ T cell population and not observed in CD4⁺ T cells. This may be due to the higher sensitivity of CD8⁺ T cells for a low level of IL-7 leading to upregulation of CD127, as previously suggested by others (35, 36).

Lund and Randall (37) suggested that a break in tolerance in immune-mediated diseases leads to a feed-forward loop between B cells and effector T cells with autoimmunity as a consequence. This feed-forward loop may be disrupted by B cell depletion leading to an increase in Tregs. As a consequence, repopulated B cells may adopt a regulatory function. Indeed, Treg numbers

were increased in humans with lupus nephritis or SLE treated with rituximab (38, 39) and in a diabetic mouse model (40). However, this increase was not observed in EAE models because Treg numbers in EAE mice did not change by rituximab administration before or after disease induction (22). Remarkably, treatment with rituximab was found to reduce the frequency of Tregs in SLO and CNS in another EAE mouse model (41). Consistent with the data reported by Matsushita et al. (22) the expression of Treg markers (Foxp3, CTLA4, TGF- β , and IL-10) was not changed in B cell-depleted EAE marmosets. Our data suggest that the success of B cell depletion is caused by reduced activation of effector T cells and their retention within SLO rather than by the induction of Tregs.

To assess which of the observed changes are relevant for the therapeutic effect of the anti-CD20 mAb, we have included two additional treatment groups, one treated with anti-BLyS mAb and one treated with anti-APRIL mAb. In these monkeys, B cells were (partially) depleted from blood and SLO, but this was not associated with a robust clinical effect. The phenotypical changes detected in the anti-CD20 mAb and anti-BLyS mAb are summarized in Table II. The most striking effects of the clinically effective anti-CD20 mAb that was not observed in marmosets treated with the moderately effective anti-BLyS mAb were in the APC compartment and the expression of CCR7 and CD127 on T cells. A reasonable explanation for this phenomenon may be that an essential trigger for the intra-SLO activation of the pathogenic T cells is depleted by the anti-CD20 mAb, but not by anti-BLyS mAb. According to our recently reported observations, this may be the lymphocryptovirus (LCV)-infected B cell. We found that LCV⁺ B cells were more profoundly depleted by anti-CD20 mAb than with anti-BLyS mAb or anti-APRIL mAb (42). LCVs are a family of γ -herpesviruses that infect B cells. Best known is the human representative EBV, which is considered an important infectious risk factor for MS (43). Consistent with the strong association of EBV with MS, the important pathogenic role of B cells, and supported by results obtained in the marmoset EAE model, we have postulated that the LCV/EBV-infected B cell has an essential role in the presentation of autoantigen to core pathogenic T cells (44). The potent stimulation of adaptive and innate immune functions by LCV/EBV has been well established (45), and several of these features could be reproduced in marmosets or rhesus monkeys injected with LCV-infected autologous B lymphoblastoid cells (42, 46).

The essential contribution of the LCV-infected B cell may also underlie the observation that the SLOs from anti-CD20 mAb-treated monkeys have a low immunostimulatory capacity and fail to upregulate CD80/CD86 after mitogenic stimulation. It has been shown that deoxyuridine triphosphate nucleotidohydrolase produced by EBV can stimulate the innate immune response. Binding of EBV-derived deoxyuridine triphosphate nucleotidohydrolase to TLR2 induces NF- κ B activation and production of proinflammatory cytokines by innate immune cells (47, 48). Furthermore, latent membrane protein 1 of EBV induces CD83 expression via NF- κ B signaling (49). Thus, depletion of LCV/EBV⁺ B cells removes these stimuli of innate immune reactions and may lead to the reduced DC activation that we observed in the anti-CD20 mAb-treated group.

In conclusion, we postulate that one of the mechanisms underlying the remarkable clinical effect of B cell depletion in relapsing-remitting MS is that B cells are indispensable for the egress of T cells from SLO. B cell depletion prevents the full activation of T cells (16). Either directly, by disrupting the essential contribution of B cells in the activation of pathogenic T cells, but also indirectly because B cell depletion causes a reduction of DC maturation and activation. The lack of T cell ac-

tivation may lead to a constitutive expression of CCR7, and thereby to retention of CCR7⁺ T cells within the SLO.

Acknowledgments

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Disclosures

GlaxoSmithKline (GSK) funded the in vivo studies from which materials used for the reported analyses had been obtained. P.P.T. is currently an employee of GSK, but he was not during the episodes in which the in vivo studies were performed. GSK has had no influence on the analyses and conclusions documented in this report.

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