α4-integrin deficiency in B cells does not affect disease in a T-cell–mediated EAE disease model

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Abstract

Objective

The goal of this study was to investigate the role of CD 19$^+$ B cells within the brain and spinal cord during CNS autoimmunity in a peptide-induced, primarily T-cell–mediated experimental autoimmune encephalomyelitis (EAE) model of MS. We hypothesized that CD19$^+$ B cells outside the CNS drive inflammation in EAE.

Methods

We generated CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice. EAE was induced by active immunization with myelin oligodendrocyte glycoprotein peptide (MOG$_{p35-55}$). Multiparameter flow cytometry was used to phenotype leukocyte subsets in primary and secondary lymphoid organs and the CNS. Serum cytokine levels and Ig levels were assessed by bead array. B-cell adoptive transfer was used to determine the compartment-specific pathogenic role of antigen-specific and non–antigen-specific B cells.

Results

A genetic ablation of α4-integrin in CD19$^{+/-}$ B cells significantly reduced the number of CD19$^+$ B cells in the CNS but does not affect EAE disease activity in active MOG$_{p35-55}$-induced disease. The composition of B-cell subsets in the brain, primary lymphoid organs, and secondary lymphoid organs of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice was unchanged during MOG$_{p35-55}$-induced EAE. Adoptive transfer of purified CD19$^+$ B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice or C57BL/6 wild-type (WT) control mice immunized with recombinant rMOG$_{1-125}$ or ovalbumin$_{323-339}$ into MOG$_{p35-55}$-immunized CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice caused worse clinical EAE than was observed in MOG$_{p35-55}$-immunized C57BL/6 WT control mice that did not receive adoptively transferred CD19$^+$ B cells.

Conclusions

Observations made in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice in active MOG$_{p35-55}$-induced EAE suggest a compartment-specific pathogenic role of CD19$^+$ B cells mostly outside of the CNS that is not necessarily antigen specific.

Glossary

BM = bone marrow; **DC** = dendritic cell; **EAE** = experimental autoimmune encephalomyelitis; **GC** = germinal center; **IFN** = interferon gamma; **IL** = interleukin; **LN** = lymph node; **MGZ** = marginal zone; **MOG** = myelin oligodendrocyte glycoprotein; **OVA** = ovalbumin; **PB** = plasma blast; **PC** = plasma cell; **WT** = wild type.

Recent clinical trials with B-cell–depleting anti-CD20 therapeutic monoclonal antibodies illustrated a pathogenic role for B lymphocytes in MS.[1–4] Whether B-cell depletion outside of the CNS is sufficient to provide a detectable benefit in MS or whether a reduction in the number of B lymphocytes within the CNS compartment is required to diminish inflammation remains incompletely understood.

In 1992, it was determined that the binding of leukocytes to inflamed CNS venules was inhibited by antibodies against $\alpha 4$-integrin.[5] Natalizumab, a humanized recombinant monoclonal antibody, was the first approved $\alpha 4$-integrin antagonist for treatment of relapsing forms of MS.[6] Natalizumab is highly effective in decreasing the number of $CD19^+$ B cells in CSF.[7]

The goal of this study was to investigate the role of $\alpha 4$-integrin ablation in $CD19^+$ B cells in a peptide-induced, primarily T-cell–mediated experimental autoimmune encephalomyelitis (EAE) model and to identify compartment-specific contributions of B cells to disease initiation and perpetuation. A T-cell–mediated EAE model was chosen to reflect the role of $\alpha 4$-integrin in B cells in patients with MS as closely as possible. Genetically, MS is most strongly associated with human leukcoyte antigen-DRB1*15:01,[8,9] an association that implies a pathogenic involvement of an antigen-specific $CD4^+$ T cell in MS.

Flow cytometry was used to phenotype leukocyte subsets in lymphoid organs and the CNS. Serum cytokine levels and immunoglobulin (Ig) levels were assessed by ELISA. B-cell adoptive transfer was used to determine the compartment-specific pathogenic role of antigen-specific B cells.

Methods

Generation of CD19.Cre$^{+/-}$ $\alpha 4$-integrin–deficient mice

Because $\alpha 4$-integrin is an absolute requirement for normal organ development, $\alpha 4$-integrin–deficient $(\alpha^{-/-})$ mice are embryonic lethal.[10] Thus, it is not possible to conduct EAE experiments in animals that are completely devoid of $\alpha 4$-integrin. To examine how the deficiency of $\alpha 4$-integrin affects the migration of dendritic cells and B cells into the CNS and T-cell reactivation and retention in the CNS, we used cre-loxP–mediated recombination[11] to create B-cell lineage–specific $\alpha 4$-integrin gene knockout mice. Specifically, we crossed female mice that are homozygous for the $\alpha 4$-integrin–floxed allele $(\alpha 4^{f/f})$[12] with commercially available CD19.Cre$^+$ males for the ablation of $\alpha 4$-integrin in B cells. Insertion of *cre* disrupts the *CD19* coding sequence, leading to a CD19 deficiency and a concomitant reduction in germinal centers (GCs) in homozygous animals. Consequently, CD19.Cre$^{+/+}$ mice behave functionally very similarly to B-cell–deficient mice. CD19.Cre$^{+/+}$ mice on the C57BL/6 background were used to generate CD19.Cre$^{+/-}$ $\alpha 4$-integrin$^{fl/fl}$ mice that appear developmentally normal and fertile. C57BL/6 mice were purchased from (The Jackson Laboratories, Bar Harbor, MN). $\alpha 4$-integrin$^{fl/fl}$ mice were used as controls. Male and female mice were used for experiments. We observed no differences regarding disease scores, cellular composition, or any of the biochemical and cellular outcomes between the 2 sexes.

Peptides

Mouse myelin oligodendrocyte glycoprotein peptide (MOGp)$_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK) and ovalbumin (OVA)$_{323-339}$ (ISQAVHAAHAEINEAGR) were synthesized by solid-phase Fmoc chemistry by QCB, Inc. (Hopkinton, MA) and CS Bio (Menlo Park, CA). Recombinant rMOG$_{1-125}$ was as donation of Dr. Hans-Christian von Büdingen at the University of California, San Francisco (UCSF).

Experimental autoimmune encephalomyelitis

To induce active EAE, experimental mice were immunized subcutaneously with myelin MOG$_{p35-55}$ (200 μg/100 μL/mouse), emulsified in an equal volume of complete Freund adjuvant containing 4 mg/mL H37Ra *Mycobacterium tuberculosis* (Difco, BD, Franklin Lakes, NJ) in each flank as described.[13]

For B-cell adoptive transfer, spleens of donor mice immunized with MOG$_{1-125}$ or OVA$_{323-339}$ were removed at day 12, and single-cell suspensions were prepared as previously described.[14] The Miltenyi kit 130-090-862 was used to purify a total of 10×10^6 $CD19^+$ donor B cells (Miltenyi Biotec, San Diego, CA). Briefly, highly pure resting B cells were isolated by magnetic labeling and depleted of CD43-expressing B cells (activated B cells, plasma cells [PCs], and $CD5^+$B-1a cells) and non–B cells. Purified cells were subsequently transferred IV into recipient CD19.cre$^{+/-}$ $\alpha 4$-integrin$^{fl/fl}$ mice that were then immediately immunized with MOG$_{p35-55}$. For all experiments, individual animals were observed and scored as described.[13]

Isolation of lymph node cells and splenocytes

Lymph node (LN) cells and splenocytes were isolated by pressing through a 70-μM nylon mesh cell strainer as described.[13]

Percoll PLUS density gradient

In all experiments in which tissue is referred to as CNS, and not specifically as brains or spinal cords, CNS leukocytes were isolated by Percoll PLUS (GE Healthcare Bio-Sciences, Pittsburgh, PA) gradient as previously described.[13]

Enzymatic CNS digestions

For some experiments, brains and spinal cords were dissociated enzymatically as described.[13]

Flow cytometry

To determine the absolute number of B cells and T cells in different compartments during EAE, multiparameter flow cytometry was used. Cells from bone marrows (BMs), spleens, LNs, brains, and spinal cords were brought into single-cell suspension as described.[13] Cells were stained for 30 minutes at 4°C with the following antibodies: CD45 PE-Cyannin-7 (30-F11; eBioscience, San Diego, CA) CD3 Alexa Flour 700 (17A2, eBioscience), CD19 PE-Texas Red (6D5, Invitrogen, Waltham, MA), CD19 PE (1D3 BD Bioscience, San Jose, CA), CD49d FITC (PS/2, Santa Cruz Biotechnology, Dallas, TX), CD4 APC (RM4-5, BD Bioscience), CD45R B220-APC (RA3-6B2, BioLegend, San Diego, CA), PE, PE Dazzle 594, PE-Texas Red, AF700, Pac Orange, PerCP, CD138 BV421(281-2, BioLegend), CD11b (APC, APC-Cy-7, PE, PerCP/Cy5.5, V450, B700 (M1/70, BioLegend), CD23 PE-Cy7 (B3B4, BioLegend), CD5 PE-Cy5 (53-7.3, BioLegend), CD1d PE (1B1, BioLegend), GL-7 FITC (GL7, BioLegend), and CD21/CD35 BV510 (7G6, BD Bioscience, San Jose, CA). Isotype-matched mAbs were used to set the gates. Next, 30,000–300,000 gated events were acquired on an FACSAria II flow cytometer (BD Biosciences), equipped with Diva acquisition software (BD Biosciences). FlowJo (BD Biosciences) software was also used for some data analysis.

Intracellular cytokine staining

Phorbol 12-myristate 13-acetate (500 ng), ionomycin (500 ng), and GolgiPlug (1 μL) (BD Biosciences) were added to 1×10^6 cells and incubated for 3 hours at 37°C. Cells were then washed in phosphate-buffered saline and stained following the extracellular staining protocol for CD45-Alexa Fluor 700. After extracellular staining, CD19$^+$ cells were purified as described and fixed using Fixation Buffer (BioLegend) for 15 minutes in the dark at room temperature. Cells were then washed with Permeabilization Buffer (BioLegend) twice and then stained using interferon gamma (IFNγ)-PE-Cy7 (BD Biosciences), interleukin (IL)-17A-FITC (BioLegend), tumor necrosis factorα-PE (eBioscience), and IL-6-APC (BioLegend). Cells were then washed, and acquired, and analyzed by flow cytometry.

ELISA

Serum samples were collected by submandibular bleeding at days 13, 19, and 29 during early active EAE, maximum EAE disease activity, and during chronic EAE. Quantitative ELISA for IL-17, IL-10, IL-4, IL-5, and IFNγ was performed using paired mAb specific for corresponding cytokines as per the manufacturer's recommendations (BD Biosciences or R&D Systems). IgM and IgG serum levels were also determined by ELISA. The results of ELISA assays are expressed as an average of triplicate wells ± SD. The EPOCH (BioTek, Winooski, VT) ELISA plate reader and software were used for data analysis (Molecular Devices Corporation, Sunnyvale, CA).

T-cell proliferation assay

To determine the capability of B cells to serve as APC to CD4$^+$ T cells, flow cytometric proliferation assays using the green fluorescent dye carboxyfluorescein succinimidyl ester or V450 (BD Biosciences) were performed according to published methods.[15] On day 15 after active induction of EAE, spleens and LNs were harvested and processed into single-cell suspension. Splenocytes and lymphocytes were stained with carboxyfluorescein succinimidyl ester and plated at 1×10^6 cells per well. The cells were then restimulated with 10 μg/mL of MOG$_{p35-55}$ and incubated at 37°C for 5 days. Con A at a concentration of 2 mg/mL and media were used as controls. On day 6, the cells were collected, blocked with Fc block antibody, stained with APC CD4 antibody, and acquired and analyzed by flow cytometry.

Histology

Following fixation in 10% buffered formalin, LNs were processed and embedded in paraffin blocks. Four-micrometer sections were cut, mounted on Fisherbrand Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA), and stained with hematoxylin & eosin (Fisher Scientific) or anti-CD19.

Statistical analysis

For parametric tests, data were assessed for normality using the Kolmogorov-Smirnov test. Normally distributed values were compared using the unpaired 2-sided Student t test. Correlations between continuous and categorical variables were assessed using the Mann-Whitney U-test. All statistical tests were 2 sided, and $p < 0.05$ indicated significance. All analyses were performed with Prism 7 (Graphpad, La Jolla, CA).

Standard protocol approvals and registrations

All experimental animals were maintained in a specific pathogen-free facility at the University of Texas (UT) Southwestern Medical Center. All protocols involving mice handling were approved by the UT Southwestern animal care facility.

Data availability

Data not shown will be shared by request.

Results

The percentage of CD49d$^+$ CD19$^+$ B cells is diminished in primary and secondary lymphoid organs in naive CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice

To characterize CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice, the expression of α4-integrin (CD49d) was determined by multiparameter flow cytometry. The percentage of CD49d$^+$ CD19$^+$ B cells in the BM, LNs, spleens, and Peyer patches is significantly diminished in naive CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice compared with C57BL/6 wild-type (WT) control mice (figure 1A). Cre recombination efficiency is incomplete[16] and determined mostly by the nucleotide sequence in the spacer region of the lox site. Also, there is a negative correlation between the Cre/lox recombination efficiency and the length of DNA between the 2 lox sites. As

stated above, other investigators found a deletion efficiency between 75% and 80% in BM-derived pre–B cells and approximately 90% in splenic B cells.[17]
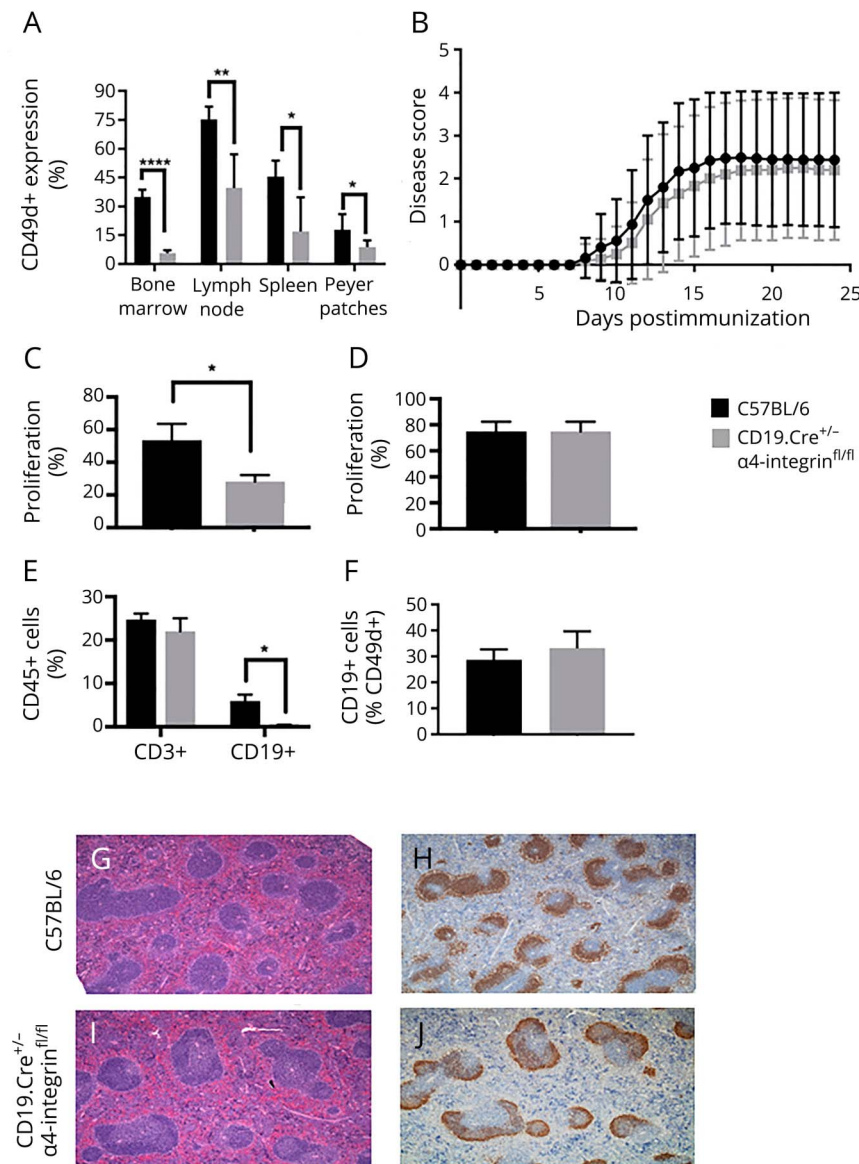
CD19.Cre^{+/−} α4-integrin^{fl/fl} mice immunized with MOG[p35-55] display a regular clinical EAE course

To determine the effect of α4-integrin expression of B cells in a T-cell–mediated active model of EAE, CD19.Cre^{+/−} α4-integrin^{fl/fl} mice and C57BL/6 control mice were immunized with MOG[p35-55]. No significant differences regarding the EAE disease incidence, severity, or phenotype were observed between the mouse strains (figure 1B and table). α4-integrin^{fl/fl} mice were also used as controls and showed similar disease activity (data not shown).

α4-integrin expression on splenic B cells affects antigen-specific CD4^{+} T-cell proliferation

It was previously demonstrated that α4-integrin can participate in costimulation of CD4^{+} T cells.[18,19] Yet other investigators showed that anti-CD49d plus anti-CD3/anti-CD28–coated polystyrene beads induced significantly greater T-cell proliferation than anti-CD3/anti-CD28 polystyrene beads alone.[20] To test the effect of α4-integrin ablation on the capability of CD19^{+} B cells to present antigen to antigen-reactive CD4^{+} T cells, flow cytometric proliferation assays were performed. CD4^{+} T-cell recall responses to MOG[p35-55] in spleens were significantly diminished in CD19.Cre^{+/−} α4-integrin^{fl/fl} mice (figure 1C). However, in LNs, we did not observe any difference in CD4^{+} T-cell proliferation between CD19.Cre^{+/−} α4-integrin^{fl/fl} mice and C57BL/6 WT control mice (figure 1D).

Figure 1 CD19.Cre^{+/−} α4-integrin^{fl/fl} mice are fully susceptible to actively MOGp[35-55]-induced experimental autoimmune encephalomyelitis

(A) The percentage of CD49d^{+} CD19^{+} B cells in the bone marrow, lymph nodes, spleens, and Peyer patches is significantly diminished in naive CD19.Cre^{+/−} α4-integrin^{fl/fl} mice compared with C57BL/6 control mice. Cells were immunophenotyped by multiparameter flow cytometry. (B) CD19.Cre^{+/−} α4-integrin^{fl/fl} mice behave similar to C57BL/6 wild-type (WT) mice regarding the disease incidence, onset, and severity of active experimental autoimmune encephalomyelitis (EAE). α4-integrin^{fl/fl} mice were used as controls and showed similar disease activity (data not shown). Active EAE was induced in CD19.Cre^{+/−} α4-integrin^{fl/fl} and C57BL/6 age-matched control mice by subcutaneous immunizations with MOGP[35-55] in incomplete Freund adjuvant (IFA) containing 4 mg/mL mycobacteria. Mice received intraperitoneal injection of 200 μL of pertussis toxin (Ptx) at 200 ng/mL on days 0 and 2. Mice were observed daily, and EAE severity was scored using a 5-point scale. CD4^{+} T-cell recall responses to MOGp[35-55] in (C) spleens were significantly diminished in CD19.Cre^{+/−} α4-integrin^{fl/fl} mice, but (D) indistinguishable to those in C57BL/6 WT mice in lymph nodes. Cell proliferation was determined by a flow cytometric proliferation assay using the green fluorescent dye CFSE or V450. (E) At maximum disease activity (days 13–15), the percentage of CD3^{+} T cells in the CNS was similar in CD19.Cre^{+/−} α4-integrin^{fl/fl} and C57BL/6 WT mice. In contrast, the percentage of CD19^{+} B cells in the CNS was significantly diminished in CD19.Cre^{+/−} α4-integrin^{fl/fl} mice. (F) The percentage of α4-integrin–positive (CD49d^{+}) CD19^{+} B cells in the CNS during maximum EAE disease activity was comparable between both mouse strains. Lymphocytes were immunophenotyped by multiparameter flow cytometry. The number, appearance, and architecture of germinal centers in lymph nodes of (G and H) C57BL/6 WT control mice were comparable to those in (I and J) CD19.Cre^{+/−} α4-integrin^{fl/fl} mice. Panels G and I were stained with hematoxylin & eosin, and panels H and J were stained with anti-CD19. Magnification for G–J is ×4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. CFSE = carboxyfluorescein succinimidyl ester.

Table Disease incidence, mean maximum disease score, and mean day of active MOG$_{p35-55}$-induced EAE disease onset in CD19$^{+/-}$ α4-integrin mice and C57BL/6 control mice

Group	Incidence	Mean maximum disease score	Mean day of disease onset
C57BL/6	72/80	2.89	10.57 ± 5.18
CD19$^{+/-}$ α4-integrin$^{fl/fl}$	62/81	2.45	9.26 ± 6.07

Abbreviations: EAE = experimental autoimmune encephalomyelitis; MOG = myelin oligodendrocyte glycoprotein.
Results of 15 experiments are shown.

In both mouse strains, T-cell proliferation was substantial. Our observations appear to differ from those of other investigators, who showed that B cells do not present MOG$_{p35-55}$ to T cells.[21] The Methods section of that article states that "…B cells were magnetically activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany)-separated from lymph nodes or spleens." Thus, a differential capability of B cells isolated from either LNs or spleens on MOG peptide presentation may not have been fully tested.

The number of B cells in the CNS during active MOG$_{p35-55}$-induced EAE is diminished in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice

To examine the effect of α4-integrin deletion in CD19$^+$ B cells on the ability of B lymphocytes to enter the CNS, cells were isolated from brains and spinal cords by Percoll gradient and analyzed by multiparameter flow cytometry. As MOG$_{p35-55}$-induced EAE is a T-cell–mediated form of EAE, the number of CD3$^+$ T cells was also assessed by the same method. At maximum disease activity (days 13–15), the percentage of CD3$^+$ T cells in the CNS was similar in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57Bl/6 WT mice (figure 1E). In contrast, the percentage of CD19$^+$ B cells in the CNS was significantly reduced in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice (figure 1E). These observations suggest that antigen-specific B cells within the CNS play a minor role in initiating and perpetuating EAE, given that these mice showed the same disease susceptibility and disease course as C57Bl/6 WT mice. Immunohistochemical studies to determine a differential anatomic distribution of B cells in the CNS between CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57Bl/6 WT mice were not performed, as the absolute number of B cells in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice was very low.

CD19$^+$ B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice use CD49d to gain access to the CNS during active MOG$_{p35-55}$-induced EAE

To determine whether CD19$^+$ B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice that gain access to the CNS during active EAE use α4-integrin, the percentage of α4-integrin–positive (CD49d$^+$) CD19$^+$ B cells was determined by multiparameter flow cytometry and found to be comparable between CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57Bl/6 WT controls (figure 1F).

The number and architecture of GCs in LNs of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice are normal

CD19.Cre mice express cre under the transcriptional control of the B lineage–restricted CD19 gene.[17] Insertion of *cre* disrupts the *CD19* coding sequence, leading to a CD19 deficiency and a concomitant reduction in GCs in CD19.Cre$^{+/+}$ mice. At day 15 during the acute phase of MOG$_{p35-55}$-induced EAE, the number and architecture of GCs in LNs of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT control mice were comparable by hematoxylin & eosin (H&E) staining (figure 1, G and H) and anti-CD19 staining (figure 1, I and J).

B cells of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice have normal cytokine profiles during active MOG$_{p35-55}$-induced EAE

To test whether the absence of α4-integrin in CD19$^+$ B cells affects their differentiation during inflammatory conditions, experimental animals were killed at day 13 during the acute phase of MOG$_{p35-55}$-induced EAE, and B cells in disease-relevant compartments were immunophenotyped by multiparameter flow cytometry and intracellular cytokine staining. Compared with B cells from C57BL/6 WT control mice, the percentage of CD19$^+$ B cells expressing IFNγ, IL-6, IL-10, or IL-17 in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice was similar in the BM (figure 2A), spleen (figure 2B), LNs (figure 2C), and brain (figure 2D) of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice.

Outside the CNS, B cells of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice express normal maturation and activation markers during active MOG$_{p35-55}$-induced EAE

As stated above, α4-integrin is considered a costimulatory molecule through which CD19$^+$ B cells are capable of interacting with CD4$^+$ T cells. To further elucidate the effect of α4-integrin deficiency on CD19$^+$ B cells on their maturation and activation in secondary lymphoid organs, experimental animals were killed at day 13 during the acute phase of MOG$_{p35-55}$-induced EAE, and B cells in spleens and LNs were immunophenotyped by multiparameter flow cytometry. The percentages of CD19$^+$ B220$^+$ B cells expressing IgD, IgM, IgG, and major histocompatibility complex (MHC) II in LNs (figure 2E) and spleen (figure 2F) were comparable in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT control mice.

CNS B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice do not upregulate activation markers during active MOG$_{p35-55}$-induced EAE

The absolute number of CD19$^+$ B cells is diminished in the CNS of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice compared with C57BL/6 WT control mice during acute MOG$_{p35-55}$-induced EAE (figure 1E). To evaluate the activation state of these cells, CNS B cells were isolated and assessed by multiparameter flow cytometry during active EAE at day 13 after active induction or EAE with MOG$_{p35-55}$. The surface expression of MHC II was similar in CNS-derived B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ and C57BL/6 WT controls

Figure 2 CD19$^+$ B cells in the CNS of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice are phenotypically similar to wild-type (WT) B cells during myelin oligodendrocyte glycoprotein peptide (MOG$_{p35-55}$)-induced experimental autoimmune encephalomyelitis
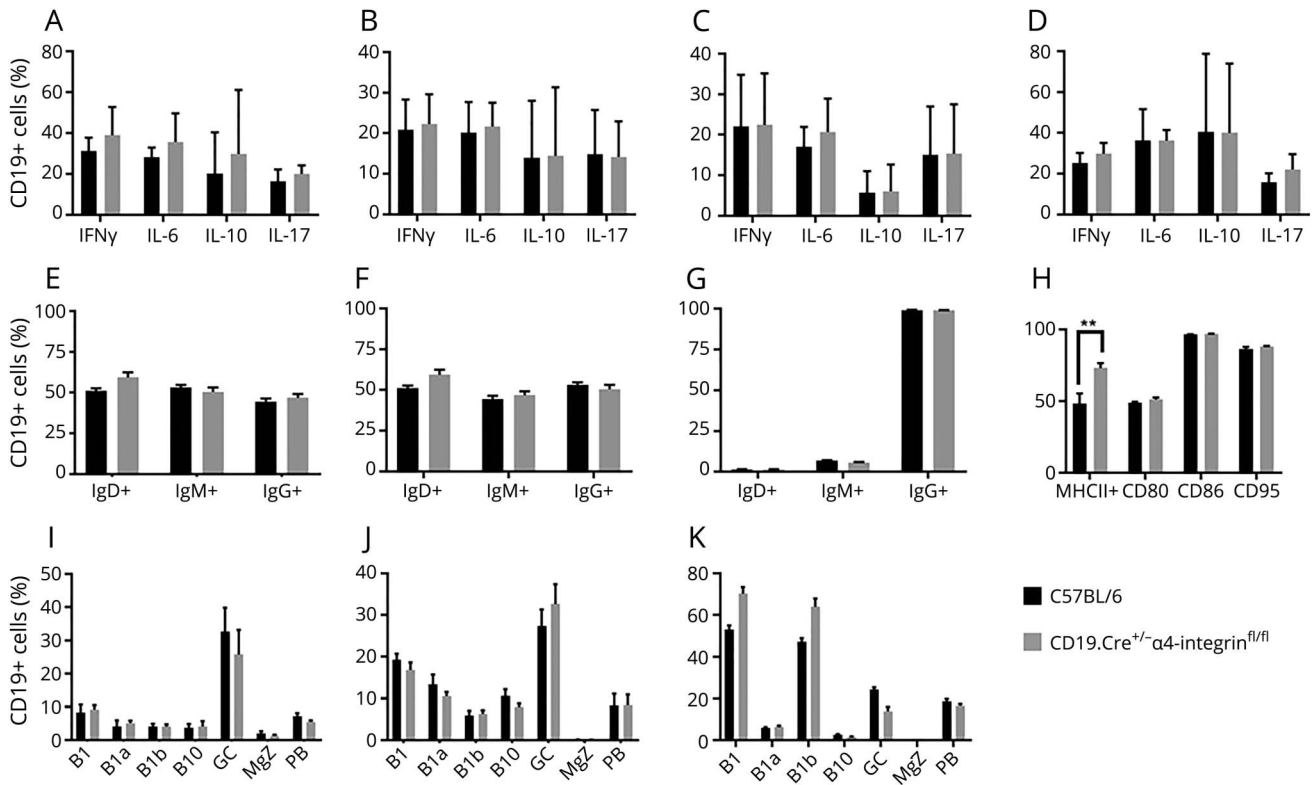
Experimental animals were killed at day 13 during the acute phase of MOGp$_{35-55}$-induced experimental autoimmune encephalomyelitis (EAE), and B cells in disease-relevant compartments were immunophenotyped by multiparameter flow cytometry and intracellular cytokine staining. Compared with B cells from C57BL/6 WT control mice, the percentage of CD19$^+$ B cells expressing interferon gamma (IFNγ), interleukin (IL)-6, IL-10, or IL-17 in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice was similar in the (A) bone marrow (BM), (B) spleen, (C) lymph node (LNs), and (D) brain of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice. The percentages of CD19$^+$ B220$^+$ B cells expressing IgD, IgM, IgG, and MHC II in (E) LNs and (F) spleen were comparable in both mouse strains. (G) In the CNS, the surface expression of MHC II was similar in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and controls (H) In the brain, the expression of the costimulator molecules CD80 and CD86 and the first apoptosis signal receptor CD95 was indistinguishable between CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT controls. The percentage of CD11b$^+$CD23$^-$B220^{lo} B1 B cells, CD11b$^+$CD23$^-$B220$^{lo/-}$CD5$^+$ B1a B cells, CD11b$^+$CD23$^-$B220$^{lo/-}$CD5$^-$ B1b B cells, B220$^+$CD5$^+$CD1d^{hi} B10 B cells, CD19$^+$CD23$^+$CD5$^-$ naive follicular B cells (FB), B220$^+$CD138$^-$CD95$^+$GL7$^+$ germinal center (GC) B cells, B220$^+$CD1d$^+$CD5$^-$CD23$^-$CD21$^+$ marginal zone (MGZ) B cells, CD138$^+$B220$^{low/-}$ plasma blast (PB), and B220$^-$CD138$^+$ plasma cells (PC) was similar in both mouse strains at (I) day 13 during early active EAE, (J) day 19 during maximum EAE disease activity, and (K) day 29 during chronic EAE. Lymphocytes were immunophenotyped by multiparameter flow cytometry. A gating strategy used to identify some of the B-cell subsets is shown in Figure 3. **$p < 0.01$.

(figure 2G). Also, the expression of the costimulator molecules CD80 and CD86 and the first apoptosis signal receptor CD95 was comparable in brain-derived B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT controls (figure 2H).

The composition of B-cell subsets in the brain of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice is unchanged during MOG$_{p35-55}$-induced EAE

To determine whether the deletion of α4-integrin on CD19$^+$ B cells affects B-cell subsets differentially in their capability to enter the CNS, the percentage of innate and adaptive B-lymphocyte subsets, including CD11b$^+$CD23$^-$B220$^-$ B1 B cells, CD11b$^+$CD23$^-$B220$^{lo/-}$CD5$^-$ B1b B cells, CD11b$^+$CD23$^-$B220l$^{o/-}$CD5$^+$ B1a B cells, CD19$^+$CD5$^+$CD1d^{hi} B10 B cells, CD19$^+$CD23$^+$CD5$^-$ naive follicular B cells, B220$^+$CD138$^-$CD95$^+$GL7$^+$ GCs B cells, B220$^+$CD1d$^+$CD5$^-$CD23$^-$CD21$^+$ marginal zone

(MGZ) B cells, CD19$^{low/-}$CD138$^+$B220$^{low/-}$ plasma blast (PB), and CD19$^-$CD138$^+$ PCs, was assessed by multiparameter flow cytometry during acute MOG$_{p35-55}$-induced EAE at day 13 during early active EAE (figure 2I), day 19 during maximum EAE disease activity (figure 2J), and day 29 during chronic EAE (figure 2K) after active induction or EAE with MOG$_{p35-55}$ and was found to be similar in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT controls at all time points. A gating strategy used to identify some of the B-cell subsets is shown in figure 3.

The composition of B-cell subsets in primary and secondary lymphoid organs of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice is similar to those of WT control mice during MOG$_{p35-55}$-induced EAE

To assess the effect of α4-integrin ablation on CD19$^+$ B cells on B-cell phenotypes in lymphoid organs, innate and adaptive B-lymphocyte subsets were characterized by flow cytometry.

We were unable to detect a change in composition of $CD19^+CD11b^+CD23^-B220^-$ B1 B cells, $CD19^+CD11b^+CD23^-B220^-CD5^+$ B1a B cells, $CD19^+CD11b^+CD23^-B220^-CD5^-$ B1b B cells, $CD19^+CD5^+CD1d^{hi}$ B10 B cells, $CD19^+B220^+CD1d^+CD5^-CD23^-CD21^+$ MGZ B cells, and $CD19^{low/-}CD138^+B220^{low/-}$ PB CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT controls at day 13 during early active EAE in the BM (figure 4A), LNs (Figure 4B), and spleen (figure 4C).

Serum soluble inflammatory markers are unchanged in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice during different stages of MOG$_{p35-55}$-induced EAE

Deletion of α4-integrin on $CD19^+$ B cells diminishes the ability of $CD19^+$ B cells to enter the CNS during acute MOG$_{p35-55}$-induced EAE (figure 1E). It is conceivable that inflammatory B cells accumulate in peripheral blood, altering the inflammatory milieu in that compartment. This was observed in patients with MS treated with natalizumab.[22] We found that the serum expression of the cytokines IL-17A (figure 5A), IL-10 (Figure 5B), IL-4 (Figure 5C), IL-5 (figure 5D), and IFNγ (figure 5E), as measured by ELISA (ELISA) during acute and chronic MOG$_{p35-55}$-induced EAE on days 13, 19, and 29, was comparable between CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT control mice.

Serum Ig levels are unchanged in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice during different stages of MOG$_{p35-55}$-induced EAE

To further investigate whether the sequestration of $CD19^+$ B cells through deletion of α4-integrin affects their activation, proliferation, and antibody secretion, IgM and IgG levels in serum were determined by ELISA. Serum levels of IgM (figure 5F) and IgG (figure 5G) as measured by ELISA were also similar between CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT control mice on days 13, 19, and 29 of MOG$_{p35-55}$-induced EAE. Given that the absolute levels of IgM and IgG were similar between the mouse strains, and that other investigators previously showed that B cells from mice on the C57BL/6 background do not readily recognize MOG$_{p35-55}$,[21] we did not test the serum levels of MOGp$_{35-55}$-specific IgM or IgG.

Figure 3 Immunophenotyping of murine B cells
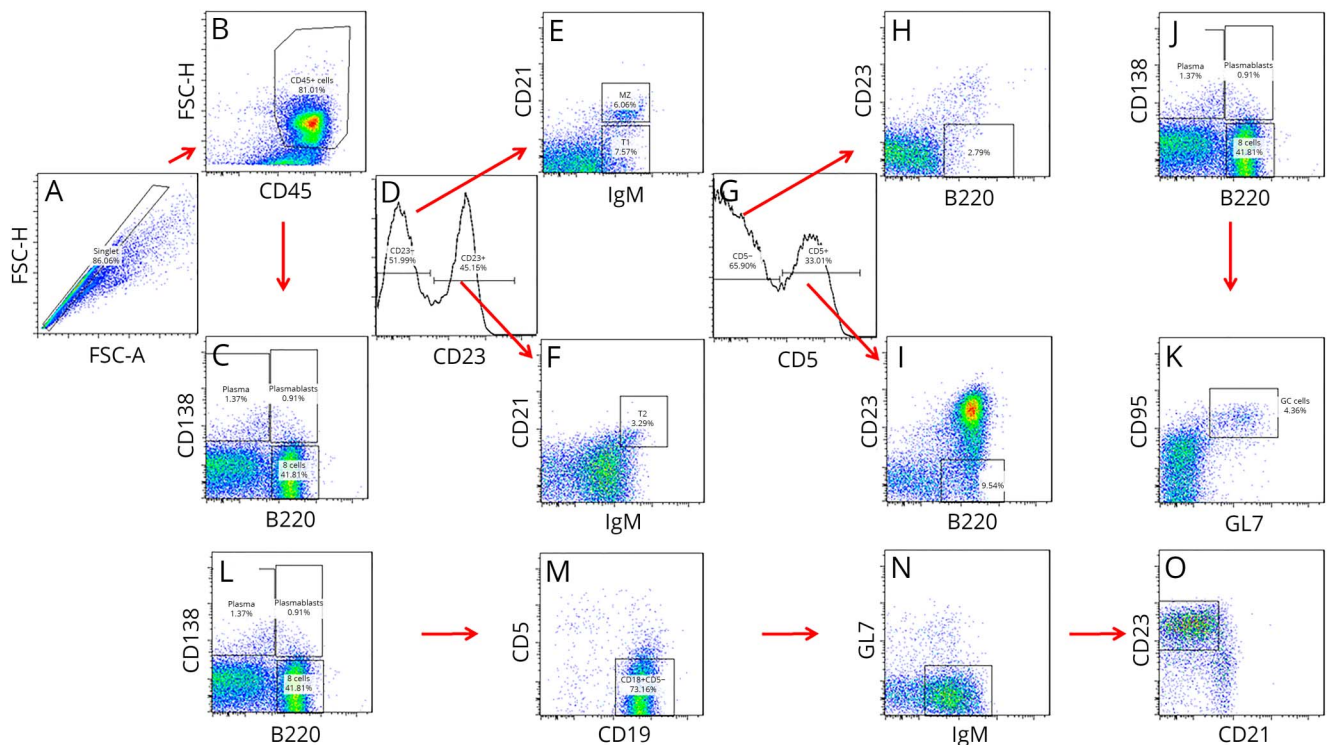
B-lymphocyte subsets were immunophenotyped by multiparameter flow cytometry. A gating strategy that was used to identify some of the B-cell subpopulations is shown. (A) First, cellular area, height, and width measurements were obtained in a channel with linear scale to gate on singlets and exclude doublets. (B) Next, gates were set to include $CD45^+$ leukocytes and exclude FSC^{low} cell debris out. (C) CD138 and B220 were used to gate on plasmablasts ($CD138^+B220^{low/-}$), plasma cells ($CD138^+B220^-$), and B cells ($B220^+$). Panels D–F show the gating for (E) $B220^+CD1d^+CD5^-CD23^-CD21^+IgM^+$ marginal zone B cells, (E) $B220^+CD1d^+CD5^-CD23^-CD21^-IgM^+$ transitional 1 (T1) B cells, and (F) $B220^+CD1d^+CD5^-CD23^+CD21^+IgM^+$ T2 B cells. Panels G–I show gating for B1 B-cell populations, and the identification of (H) $CD11b^+CD23^-B220^{lo/-}CD5^-$ B1b B cells and (I) $CD11b^+CD23^-B220^{lo/-}CD5^+$ B1a B cells. Panels J and K show the identification of $B220^+CD138^-CD95^+GL7^+$ germinal center B cells. Panels L–O illustrate the gating for $B220^+CD19^+CD5^-GL7^-CD23^+CD21^{lo/int}$ naive follicular B cells. FSC = forward side scatter.

Figure 4 CD19$^+$ B cells in primary and secondary lymphoid organs of CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice are phenotypically similar to wild-type B cells during MOG$_{p35-55}$-induced experimental autoimmune encephalomyelitis
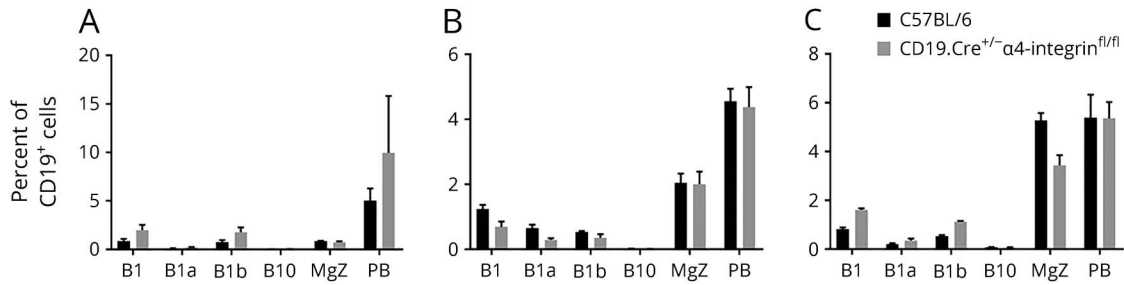
(A) In the bone marrow, (B) lymph nodes, and (C) spleen, the percentage of CD19$^+$CD11b$^+$CD23$^-$B220$^-$ B1 B cells, CD19$^+$CD11b$^+$CD23$^-$B220$^-$CD5$^+$ B1a B cells, CD19$^+$CD11b$^+$CD23$^-$B220$^-$CD5$^-$ B1b B cells, CD19$^+$CD5$^+$CD1d^{hi} B10 B cells, CD19$^+$B220$^+$CD1d$^+$CD5$^-$CD23$^-$CD21$^+$ marginal zone (MGZ) B cells, and CD19$^{low/-}$CD138$^+$B220$^{low/-}$ plasma blast (PB) was similar in CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT controls at day 13 during early active EAE. Lymphocytes were immunophenotyped by multiparameter flow cytometry.

Activated peripheral B cells contribute to disease severity in MOG$_{p35-55}$-induced EAE

To determine the compartment-specific pathogenic role of B cells in the induction and perpetuation of the EAE animal model, CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice were immunized with MOG$_{p35-55}$. As demonstrated, B cells in these mice have a diminished capacity to enter the CNS (figure 1E). Adoptive transfer of purified CD19$^+$ B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 WT mice immunized with rMOG$_{1-125}$ or the control antigen OVA323-339 worsened the clinical course of EAE in recipient mice immunized with MOG$_{p35-55}$ (figure 5H), although these changes were not significant.

Discussion

There is accumulating evidence to suggest that B cells and myelin-specific antibodies play crucial roles in the pathogenesis of MS. Whether B-cell depletion mediates its beneficial

Figure 5 Activated B cells outside of the CNS drive disease activity in myelin oligodendrocyte glycoprotein peptide (MOG$_{p35-55}$)-induced experimental autoimmune encephalomyelitis (EAE) in an antigen no-specific manner
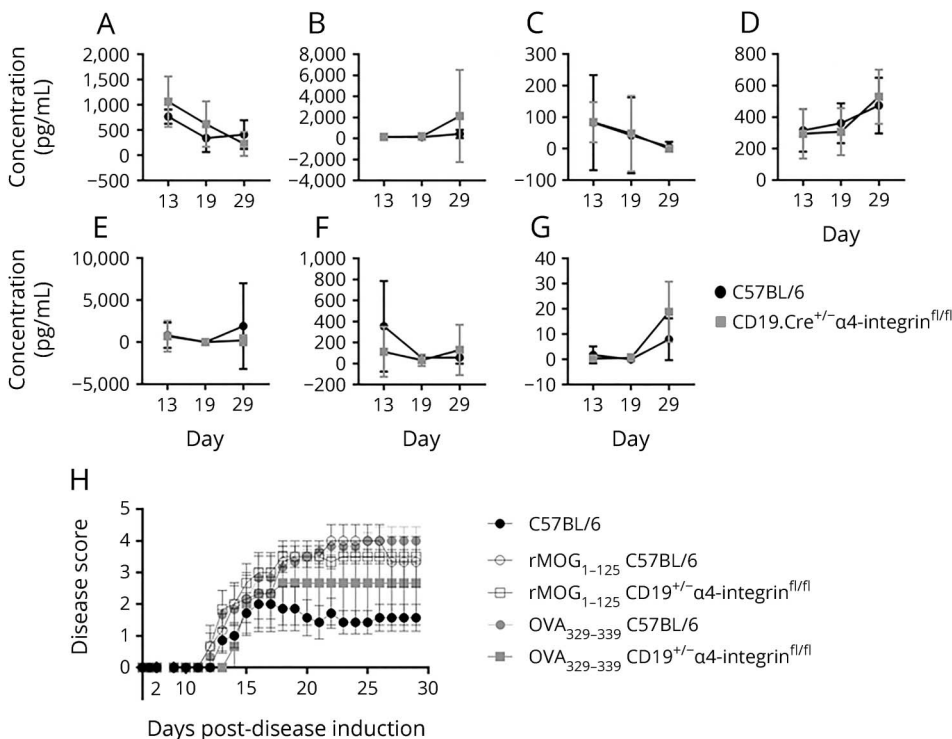
The serum expression of the cytokines (A) IL-17A, (B) IL-10, (C) IL-4, (D) IL-5, and (E) IFNγ as measured by ELISA during acute and chronic MOGp$_{35-55}$-induced EAE on days 13, 19, and 29 was comparable between CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice and C57BL/6 wild-type (WT) control mice. Serum levels of (F) IgM and (G) IgG measured by ELSA at the same time points were also similar between mouse strains. (H) Adoptive transfer of purified CD19$^+$ B cells from CD19.Cre$^{+/-}$ α4-integrin$^{fl/fl}$ mice or C57BL/6 WT control mice immunized with recombinant (r)MOG$_{1-125}$ or ovalbumin (OVA)$_{323-339}$ into CD19$^{+/-}$ α4-integrin$^{fl/fl}$ mice that were then immediately immunized with MOG$_{p35-55}$ caused worse clinical EAE than was observed in MOG$_{p35-55}$-immunized C57BL/6 WT control mice ("C57BL/6") that did not receive adoptively transferred CD19$^+$ B cells.

effects in patients with MS through B cells in the periphery, in the CNS, or both is currently not completely understood.

Other investigators investigated the role of α4-integrin deficiency in CD19^{+} B cells in EAE.[23] These investigators showed that B-cell very late activation antigen-4–deficient mice developed more severe clinical disease than control mice after active induction of EAE with MOG$_{p35-55}$ and that that this clinical effect is due to the requirement of regulatory B cells (Bregs) for α4-integrin to enter the CNS. Bregs were defined as CD19^{+}IL-10^{+} or CD1d^{hi}CD5^{+} B-cell subsets. Another group of investigators also tested CD19Cre Itga4^{fl/fl} mice in MOG$_{p35-55}$-induced active EAE.[24] CD19Cre Itga4^{fl/fl} mice developed more severe EAE than control mice, and these investigators demonstrated a critical role for α4-integrin on the generation of Bregs in peripheral immune organs.

Our own results diverge from these reports in that we could not detect a difference in active EAE disease severity between CD19.Cre^{+/−} α4-integrin^{fl/fl} mice and C57BL/6 WT control mice. These experiments were conducted 15 times (table), and we are confident in our findings. Differences in EAE disease severity and phenotype can be affected by conditions under which mouse colonies are maintained, as well as other factors that may affect the overall inflammatory milieu of experimental conditions. This may explain the differences in results. It is also conceivable that different mice were used by the other investigators for their experiments. The investigators of the 2 previous studies state that they used CD19.Creα4^{fl/fl} mice[23] or that they used CD19Cre Itga4^{fl/fl} mice.[25] It is not stated whether mice were heterozygous or homozygous for CD19.cre or a mixture of both. As stated in the Methods section, CD19.Cre^{+/+} mice behave functionally very similarly to B-cell–deficient mice, which develop more severe EAE when immunized with MOG$_{p35-55}$.[26]

We found one potentially meaningful biological difference between CD19.Cre^{+/−} α4-integrin^{fl/fl} mice and C57BL/6 control mice. There was decreased MOG$_{p35-55}$-specific CD4^{+} T-cell proliferation in spleens, which is an expected result, given that α4-integrin can participate in costimulation of CD4^{+} T cells.[18,19]

The observation that CD19^{+} B cells of CD19.Cre^{+/−} α4-integrin^{fl/fl} mice did deplete α4-integrin and that they were significantly less capable of entering the CNS after mice were immunized with MOG$_{p35-55}$ suggests 3 interpretations: (1) Antigen-activated B cells contribute to disease burden in EAE in a seemingly dose-dependent manner, (2) they do not absolutely require recognition of a CNS autoantigen or a mimic thereof to do so, and (3) they do not require full α4-integrin–mediated access to the CNS to exacerbate disease. The first interpretation is supported by work of other investigators who have previously demonstrated that B-cell–deficient C57BL/6 mice are resistant to active rMOG-induced EAE[26] and that the administration of a B-cell–depleting monoclonal antibody prevents or reverses established rMOG-induced EAE in C57BL/6 mice.[21] Weber and colleagues[21]

further showed that B-cell depletion results in a reduction of MOG$_{p35-55}$-specific Th1 and Th17 cells. Our own data suggest that the differentiation of MOG$_{p35-55}$-specific T cells to become encephalitogenic does not absolutely require the presentation of autoantigen, but is likely driven by other B-cell factors, including soluble inflammatory mediators. The third interpretation of this experiment is supported by our demonstration that B cells from CD19.Cre^{+/−} α4-integrin^{fl/fl} mice are significantly impaired in their ability to enter the CNS (figure 1E). Finally, it should be emphasized that our findings do not conclusively rule out a role of antigen-specific B cells in the CNS on the course of CNS autoimmunity, as threshold effects may be difficult to detect with the EAE experiments that were conducted.

Our data suggest that the beneficial effects of anti-CD20 therapies in patients with MS are mediated through their effects on peripheral B cells. We also conclude that benefits observed in patients with MS during anti-α4-integrin therapy are likely not predominantly due to its effect of suppressing the migration of B cells into the CNS. The interpretation of our findings should be limited to early forms of MS, as the EAE model that was used and the observation period that was used do not provide insight into late or progressive forms of CNS autoimmunity. Magliozzi et al[27] were the first investigators to show that some patients with secondary-progressive MS develop lymphoid tissue in the meninges that resemble B-cell follicles in secondary lymphoid organs. B cells in these structures may be susceptible to anti-CD20 therapies with monoclonal antibodies or small molecules, and it is conceivable that their depletion may benefit patients afflicted with that MS phenotype.

Appendix Authors

Name	Location	Role	Contribution
Rehana Z. Hussain, MSc	University of Texas Southwestern Medical Center, Dallas, TX	Author	Designed and conceptualized the study; analyzed the data; and drafted the manuscript for intellectual content
Petra C. Cravens, PhD	University of Texas Southwestern Medical Center, Dallas, TX	Author	Designed and conceptualized the study; analyzed the data; and drafted the manuscript for intellectual content
William A. Miller-Little, BA	University of Texas Southwestern Medical Center, Dallas, TX	Author	Acquired the data; analyzed the data; and revised the manuscript
Richard Doelger, MSc	University of Texas Southwestern Medical Center, Dallas, TX	Author	Acquired the data; analyzed the data; and revised the manuscript
Valerie Granados, PhD	University of Texas Southwestern Medical Center, Dallas, TX	Author	Acquired the data and analyzed the data
Emily Herndon, MD	University of Texas Southwestern Medical Center, Dallas, TX	Author	Interpreted the data and revised the manuscript for intellectual content
Darin T. Okuda, MD	University of Texas Southwestern Medical Center, Dallas, TX	Author	Interpreted the data and revised the manuscript for intellectual content
Todd N. Eagar, PhD	Houston Methodist Hospital, Houston, TX	Author	Designed and conceptualized the study; analyzed the data; and drafted the manuscript for intellectual content
Olaf Stüve, MD, PhD	University of Texas Southwestern Medical Center, Dallas, TX	Author	Designed and conceptualized the study; analyzed the data; and drafted the manuscript for intellectual content

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