B-Cell Very Late Antigen-4 Deficiency Reduces Leukocyte Recruitment and Susceptibility to Central Nervous System Autoimmunity

Klaus Lehmann-Horn, MD,1,2 Sharon A. Sagan, BS,1,2 Claude C.A. Bernard, PhD,3 Raymond A. Sobel, MD,4 and Scott S. Zamvil, MD, PhD1,2

Natalizumab, a monoclonal antibody (mAb) directed against the human α4 (CD49d) subunit of the integrin very late antigen-4 (VLA-4), is a potent treatment for relapsing–remitting multiple sclerosis (RRMS).1 Studies of anti–VLA-4 treatment in experimental autoimmune encephalomyelitis (EAE), considered predominantly a T-cell–mediated disease, indicate that its effects on T cells,2-4 in particular Th1 cells,5 are responsible for the clinical benefit of natalizumab. The recent successful use of anti–CD20 B-cell–depleting agents in multiple sclerosis (MS) treatment trials6 has renewed appreciation for the role of B cells in MS pathogenesis and interest in evaluating their response to MS therapeutics. Although VLA-4 is more highly expressed on the surface of mature B cells than on T cells,7 less is known regarding the influence of anti–VLA-4 therapy on B cells than on T cells. One in vitro study suggested that engagement of VLA-4 on B cells with its endothelial ligand VCAM-1 is required for their migration across the blood–brain barrier (BBB).8 In this regard, natalizumab treatment of MS has been associated with elevation of B cells in peripheral blood9 and reduction in cerebrospinal fluid.10

Thus, given these observations and the recent increased appreciation for the role of B cells in MS and EAE,6,11–13 we questioned whether the clinical benefit of anti–VLA-4 therapy could also relate to its potential influence on B-cell trafficking into the CNS.

Materials and Methods

Mice

α4disc mice14 (referred to as α4flox/flox below) were kindly provided by Dr Thalia Papayannopoulou (University of Washington). CD19cre mice15 and wild-type C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All studies have been approved by the University of California, San Francisco Institutional Animal Care and Use Committee and were in accordance with the US Public Health Service’s Policy on Humane Care and Use of Laboratory Animals.

Antigen

Recombinant human (rh) myelin oligodendrocyte glycoprotein (MOG) was provided by Dr C. C. A. Bernard and was synthesized, purified, and refolded as previously reported.12

EAE Induction

EAE was induced in 8- to 12-week-old mice by immunization with 100μg rhMOG in complete Freund adjuvant containing 200μg Mycobacterium tuberculosis H37RA (DIFCO Laboratories, Detroit, MI) on day 0. Mice intraperitoneally (i.p.) received either 100ng (Fig 1) or 200ng (all other experiments) Bordetella pertussis toxin (List Biological Laboratories, Campbell, CA) on days 0 and 2. Mice were observed daily for clinical EAE.12

In Vivo Blockade of α4

Mice received 200μg of rat anti–α4 antibody (PS/2) or rat IgG2b isotype control (LTF-2) (both Bio X Cell, West Lebanon, NH) i.p. on days 4, 7, and 10 after immunization.

From the 1Department of Neurology and 2Program in Immunology, University of California, San Francisco, San Francisco, CA; 3Multiple Sclerosis Research Group, Australian Regenerative Medicine Institute, Monash University, Clayton, Australia; and 4Department of Pathology, Stanford University School of Medicine, Stanford, CA

Address correspondence to Dr Zamvil, Department of Neurology and Program in Immunology, 675 Nelson Rising Lane, Room 215A, San Francisco, CA 94158. E-mail: zamvil@ucsf.neuroimmunol.org

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Cell Isolation
Blood was collected via cardiac puncture. After erythrocyte lysis, leukocytes were washed. Spleen and CNS mononuclear cells were isolated after perfusion with phosphate-buffered saline (PBS).

Flow Cytometric Analysis
Antimouse FcRIIB/FcRIIIA mAb (2.4G2; BD, Franklin Lakes, NJ) was used to avoid nonspecific staining. Aqua dead cell stain kit was used for live/dead cell separation and CountBright counting beads (both Molecular Probes, Eugene, OR) for absolute cell number quantification. Antibodies to mouse CD19 PerCP-Cy5.5 (eBio1D3), B220 (CD45R) APC-Cy7 (RA3–6B2), MHC-II (I-A/I-E) PE-Cy7 (M5/114.15.2), CD80 (B7-1) APC (16-10A1), CD4 APC-Cy7 (RM4–5), and CD11b PE-Cy7 (M1/70) were purchased from eBioscience (San Diego, CA). Antibodies to B220 (CD45R) FITC (RA3–6B2), CD45 APC (30-F11), and CD49d PE (9C10) were purchased from BD. An isotype- and fluorochrome-matched control antibody (IgG2a kappa PE; R35–95; BD) was used to assess nonspecific staining for CD49d. Analysis was performed on a BD LSRFortessa flow cytometer using FACSDiva software (BD).

Intracellular Cytokine Staining
Intracellular cytokine staining (ICS) was performed as described, using aqua dead cell stain kit (Molecular Probes) and antibodies to CD4 PE-Cy7 (RM4–5), IL-17A PerCP-Cy5.5 (eBio17B7), and IFN-γ APC (XMG1.2; all eBioscience).

Detection of Anti-MOG Antibodies
Anti-rhMOG immunoglobulin G (IgG) was measured with a noncommercial enzyme-linked immunosorbent assay as described, using rhMOG and horseradish peroxidase–labeled goat antimouse IgG (Thermo Scientific, Waltham, MA).

Immunohistochemistry
Mice were perfused with PBS followed by 10% formalin for fixation. Brains and spinal cords were dissected, paraffin-embedded, and sectioned. Immunohistochemical staining (CD3 and B220) and hematoxylin counterstaining were performed on representative sections. Meningeal and parenchymal B220+ T cells were counted by a blinded observer (R.A.S.).

Statistical Analysis
Mann–Whitney U test was used for clinical scores. All other statistical analysis was performed using an unpaired t test. A value of \( p \leq 0.05 \) was considered significant.

Results
In this investigation, EAE was induced by rhMOG, which requires the participation of both T and B cells. Administration of anti-\( \alpha \)4 mAb ameliorated EAE induced by rhMOG protein in C57BL/6 wild-type mice (see Fig 1). To investigate the role of VLA-4 expression on B cells in EAE pathogenesis, we created mice (CD19cre/\( \alpha \)4f/f) that selectively lack B-cell \( \alpha \)4 expression by crossing the CD19cre knockin, which selectively directs gene expression in B cells, but not in T cells or in other leukocytes, (and data not shown) onto the \( \alpha \)4f/f background. VLA-4 was not detected on B cells from naive CD19cre/\( \alpha \)4f/f mice, whereas CD4+ T cells expressed normal levels (Fig 2A). Similar results were observed in the blood and spleen of rhMOG-immunized mice (data not shown). In comparison to either CD19cre/\( \alpha \)4f/f or CD19cre control mice, CD19cre/\( \alpha \)4f/f mice exhibited a reduction in EAE susceptibility, which was most pronounced at the peak of disease (see Fig 2B). Similar results were observed in an independent experiment using \( \alpha \)4f/f mice as control group (data not shown).

Although VLA-4 is recognized primarily for its role in endothelial transmigration of leukocytes, the interaction of VLA-4 with its receptor, VCAM-1, can also mediate costimulation. Therefore, selective elimination of B-cell VLA-4 expression could conceivably alter EAE susceptibility via its influence on peripheral immune responses and/or CNS B-cell migration. First, in comparison between control and CD19cre/\( \alpha \)4f/f mice with EAE, there were no evident differences in peripheral B-cell activation as measured by expression of CD80 or MHC II (see Fig 2C). Second, there were no significant differences in peripheral numbers of Th1 and Th17 cells, indicating that selective elimination of B-cell VLA-4 expression did not alter in vivo antigen presentation by B cells or other antigen-presenting cells (see Fig 2D). Lastly, as...
FIGURE 2: Selective α4-deficiency on B cells reduces disease severity in recombinant human myelin oligodendrocyte glycoprotein (rhMOG)-induced experimental autoimmune encephalomyelitis (EAE), whereas peripheral B-cell properties appear to be unchanged. (A) α4 (CD49d) surface expression on CD19+ B220+ B cells and CD4+ T cells in the peripheral blood of naïve CD19cre/α4f/f or control mice assessed by flow cytometry. An isotype control antibody (ab) was used to define the negative population, and a healthy wild-type (WT) mouse served as a positive control. Histograms show 1 representative mouse per group (n = 4 mice/group). (B) CD19cre/α4f/f (n = 9 mice) and CD19cre/α4f/WT control mice (n = 7 mice) were immunized with 100μg rhMOG (upper panel). CD19cre/α4f/f (n = 17 mice) and CD19cre control mice (n = 10 mice) were immunized similarly (lower panel). In the lower panel, cumulative data from 2 independent experiments are shown. Similar results were obtained in another independent experiment with an α4f/f control group (not shown). Data shown represent mean disease score ± standard error of the mean (SEM). **p ≤ 0.01; Mann–Whitney U test; differences were significant (p ≤ 0.05) for days 14 to 35, except day 28, in the upper panel and for days 12 to 16 in the lower panel. (C) Surface activation markers CD80 (B7-1) and MHC II were assessed on B cells (gated on viable CD19+ B220+ cells) by flow cytometry in the spleen at peak of disease (day 14 postimmunization). Similar results were obtained in the blood, at a later time point (31 days postimmunization), and for CD86 (B7-2; not shown). Flow cytometry plots show 1 representative mouse per group. Bar graphs represent mean ± SEM of n = 3 mice/group. ns = not significant; t test. (D) Th1 (IFN-γ) and Th17 (IL-17A) T-cell differentiation is measured by intracellular cytokine staining in the spleen 31 days postimmunization. Flow cytometry plots show 1 representative mouse per group (gated on viable CD4+ T cells). Bar graphs represent mean ± SEM of n = 3 mice/group. ns = not significant; t test. Similar results were obtained at peak of disease (14 days postimmunization; not shown). (E) Serum immunoglobulin G (IgG) antibodies against rhMOG were detected by enzyme-linked immunosorbent assay. The optical density (OD) at 450nm is indicated. Serum was obtained on day 31 postimmunization with 100μg rhMOG in CD19cre/α4f/f or control mice (n = 3 mice/group) and diluted 1:9,000 before analysis. ns = not significant; t test. Control mice were CD19cre in all experiments, except in B, upper panel, where they were CD19cre/α4f/WT. FS, forward scatter.
immunization with rhMOG protein can elicit secretion of pathogenic (demyelinating) antibodies,17 we examined MOG-specific antibodies in control and CD19cre/\textsuperscript{a4f/f} mice with rhMOG-induced EAE. No significant differences in anti-rhMOG IgG titers were observed (see Fig 2E). By these measures, selective elimination of VLA-4 expression did not significantly influence proinflammatory peripheral B-cell function.

Our observation that selective B-cell VLA-4 deficiency reduced EAE susceptibility, but did not impact peripheral immune responses, indicates its primary effect may be attributed to its influence on B-cell migration. Flow cytometric analysis revealed a significant reduction in absolute numbers of CNS-infiltrating CD19\textsuperscript{+}B220\textsuperscript{+} B cells in CD19cre/\textsuperscript{a4f/f} mice (Fig 3A), findings that were confirmed by immunohistological staining. Reduction of B220\textsuperscript{+} (CD45R\textsuperscript{+}) B cells was most pronounced in the meninges, which contained the majority of CNS-infiltrating B cells in control mice with EAE (see Fig 3B). There were no clearly detectable differences in the histological appearances of microglia or astrocytes in lesions in CD19cre/\textsuperscript{a4f/f} and control mice with EAE.

The paucity of CNS B cells in CD19cre/\textsuperscript{a4f/f} mice was also associated with a decrease in the total number of CNS-infiltrating hematopoietic cells (CD45\textsuperscript{hi}), CD45\textsuperscript{hi}CD11b\textsuperscript{+}macrophages, and CD45\textsuperscript{lo}CD11b\textsuperscript{+}CD4\textsuperscript{+} T cells (see Fig 3C–E). Similarly, meningeal CD3\textsuperscript{+} T cells were reduced in CD19cre/\textsuperscript{a4f/f} mice, as measured by immunohistochemistry (see Fig 3B). Moreover, the frequency of Th17 but not Th1 cells was significantly reduced in the CNS of CD19cre/\textsuperscript{a4f/f} mice with EAE (see Fig 3F). In summary, our results indicate that conditional B-cell VLA-4 deficiency inhibited CNS B-cell accumulation, reduced recruitment of other effector leukocytes, and suppressed development of EAE.

Discussion

Since 1992, when it was reported that anti–VLA-4 antibody treatment could prevent EAE,4 providing the foundation for advancing development of natalizumab in MS,1 mechanistic studies of anti–VLA-4 therapy have focused primarily on its influence on T cells. However, B-cell VLA-4 expression promotes B-cell trafficking across the BBB,6,18 and clinical studies suggest this process may be inhibited by natalizumab.9,10 Clinical investigations demonstrating pronounced beneficial effects of anti-CD20 B-cell–depleting mAbs in the treatment of RRMS have renewed interest in understanding the cellular and humoral contribution of B cells in MS pathogenesis and response to therapy.18 To assess the influence of VLA-4 expression on B cells in CNS autoimmune disease, we created mice that were deficient in VLA-4 on B cells only and studied these mice in an EAE model that requires the participation of B cells.12 Mice containing VLA-4–deficient B cells demonstrated a modest but significant and reproducible reduction in EAE susceptibility, which was associated with a marked decrease in CNS B cells as well as significantly fewer CNS proinflammatory Th17 cells and macrophages. In contrast, selective B-cell VLA-4 deficiency did not alter peripheral B-cell activation, modulate peripheral T-cell function, or reduce anti-MOG antibodies. Thus, our results indicate that the reduction in both clinical EAE and other CNS leukocytes was due to the selective block in accumulation of B cells within the CNS, and not an effect on peripheral immunity. Similarly, reduction in VLA-4–dependent CNS B-cell recruitment may contribute to the benefit of natalizumab therapy in MS.

The proinflammatory Th17 subset was described in 2006, 2 years after the initial approval of natalizumab. Of interest, 1 recent study demonstrated that anti–VLA-4 prevented EAE induced by myelin-specific Th1, but not Th17 cells.5 VLA-4–deficient Th17 cells induced an atypical form of EAE, which was attributed to their ability to enter the brain through use of the integrin LFA-1 (\alphaL\beta2 integrin) instead of VLA-4. Our results, demonstrating that selective B-cell VLA-4 deficiency was associated with a reduction in CNS Th17 cells, are complementary to the study demonstrating that anti–VLA-4 directly inhibited CNS accumulation of Th1 cells.5 Anti–VLA-4 mAb may also indirectly block accumulation of proinflammatory Th17 cells through binding of VLA-4 on B cells that may enter the CNS in a VLA-4–dependent manner.

Although effective in MS, natalizumab has exacerbated neuromyelitis optica (NMO).19 There may be several contributing factors. First, NMO is a humoral autoimmune disease that is associated with aquaporin-4 (AQP4)-specific antibodies, which are generated primarily outside of the CNS. We observed that B-cell VLA-4 deficiency did not influence peripheral humoral responses. Second, neutrophils are abundant in NMO lesions20 and are thought to contribute to NMO pathogenesis. Th17 cells can promote CNS neutrophil accumulation,21 and AQP4-specific T cells in NMO exhibit Th17 polarization.22 Third, as resting human neutrophils do not express VLA-4,23 CNS migration of this leukocyte subset may be unopposed by natalizumab. Thus, one can speculate that natalizumab treatment, which may be less effective in Th17-mediated CNS disease,5 could facilitate CNS neutrophil accumulation in NMO.

Possibly the most important observation in our study is that selective B-cell VLA-4 deficiency was associated with reduced CNS recruitment of other effector cells. This finding may be particularly relevant to therapies that selectively target B cells. In this regard, despite knowledge that B cells constitute a minority of
Selective B-cell α4 deficiency impairs B-cell migration into the central nervous system (CNS) in recombinant human myelin oligodendrocyte glycoprotein (rhMOG)-induced experimental autoimmune encephalomyelitis (EAE) and reduces CNS recruitment of CD11b⁺ macrophages and T cells. CD19cre/α4f/f or control mice (CD19cre) were immunized with 100 μg rhMOG.

(A) Frequency and absolute numbers of CNS-infiltrating CD19⁺B220⁺ B cells at peak of disease (day 14) in CD19cre/α4f/f or control mice. Flow cytometry plots show 1 representative mouse (gated on viable CD45hiCD11b⁻ lymphocytes). Similar results were obtained at a later time point (31 days postimmunization; not shown). n = 3 mice/group. (B) Leptomeningeal infiltrates in the brains of CD19cre/α4f/f (upper picture panels) and control (lower picture panels) mice with EAE 31 days postimmunization. CD19cre/α4f/f and control mice have abundant CD3⁺ T cells (left panels); B220⁺ B cells are present in the control mouse but not the CD19cre/α4f/f mouse (right panels). Immunohistochemistry was done with hematoxylin counterstain. Scale bar = 20 μm. Bar graphs show mean number of B220⁺ B cells (upper graph panels) and CD3⁺ T cells (lower graph panels) in the meninges (left panels) or parenchyma (right panels) of n = 3–4 mice/group. (C) Nonresident CD45⁺ cells (gated on viable cells), (D) CD11b⁺ macrophages (gated on viable CD45⁺ cells), and (E) CD4⁺ T cells (gated on viable CD45⁺CD11b⁻ cells) in the CNS at peak of disease (day 14 postimmunization). n = 3 mice/group. (F) Th1 (IFN-γ secreting) and Th17 (IL-17A secreting) T-cell differentiation is measured by intracellular cytokine staining in the CNS 31 days postimmunization. Flow cytometry plots show 1 representative mouse per group (gated on viable CD4⁺ T cells). n = 6 mice/group (pooled from 2 independent experiments). Similar results were obtained at peak of disease (14 days postimmunization; not shown). All data shown in bar graphs are mean frequency (%) or total number (#) ± standard error of the mean. *p ≤ 0.05, **p ≤ 0.01; ns = not significant; t test.
infiltrating cells in characteristic lesions of RRMS,24 B-cell depletion in RRMS results in a marked reduction in gadolinium-enhancing CNS lesions.6 Anti-CD20 B-cell depletion in EAE significantly reduces meningeal B cells.11 Similarly, the observation that selective B-cell VLA-4 deficiency not only inhibited accumulation of meningeal B cells, but also reduced CNS recruitment of other leukocytes, may provide an additional mechanism for reduction in CNS inflammatory lesions by therapies that eliminate B cells. Our study, which highlights the importance of VLA-4 on B cells in the pathogenesis of CNS autoimmunity, should promote investigations to further understand how CNS B cells influence cellular immune responses in situ.

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Authorship
K.L.-H. designed research, performed the experiments, analyzed data, and wrote the paper. S.A.S. performed the experiments, analyzed data, and discussed the results at all stages. C.C.A.B. provided reagents. R.A.S. performed the neuropathologic analysis. S.S.Z. designed research, wrote the paper, and supervised the study.

Potential Conflicts of Interest
S.S.Z.: consultancy, honoraria, Biogen Idec, EMD Serono, Genzyme, Novartis, Questcor, Roche, Teva Pharmaceuticals; data safety monitoring board, Lilly, BioMS, Teva, Opexa Therapeutics.

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