B cell VLA-4-deficiency reduces leukocyte recruitment and susceptibility to central nervous system autoimmunity

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

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, Victoria 3800, Australia, 4Department of Pathology, Stanford University School of Medicine, Stanford, CA

Corresponding author’s contact information:
Scott S. Zamvil, MD, PhD
Department of Neurology and Program in Immunology
675 Nelson Rising Lane, Room 215A
San Francisco, CA 94158
Tel: 415-502-7395
Fax: 415-502-7241
zamvil@ucsf.neuroimmunol.org

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Abstract

Natalizumab, which binds very late antigen-4 (VLA-4), is a potent therapy for multiple sclerosis (MS). Studies have focused primarily upon its capacity to interfere with T cell-migration into the central nervous system (CNS). B cells are important in MS pathogenesis and express high levels of VLA-4. Here, we report that the selective inhibition of VLA-4-expression on B cells impedes CNS accumulation of B cells, recruitment of Th17 cells and macrophages, and reduces susceptibility to experimental autoimmune encephalomyelitis (EAE). These results underscore the importance of B cell VLA-4-expression in pathogenesis of CNS autoimmunity and provide insight regarding mechanisms that may contribute to the benefit of natalizumab in MS, as well as candidate therapeutics that selectively target B cells.
Introduction

Natalizumab, a monoclonal antibody (mAb) directed against the human \( \alpha_4 \) (CD49d) subunit of the integrin very late antigen-4, is a potent treatment for relapsing-remitting multiple sclerosis (RRMS).\(^1\) Studies of anti-VLA-4-treatment in 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 MS treatment trials\(^6\) has renewed appreciation for the role of B cells in MS pathogenesis and interest in evaluating their response to MS therapeutics. While 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 blood\(^9\) and reduction in cerebrospinal fluid (CSF).\(^10\) 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

α4\textsuperscript{flox/flox} mice\textsuperscript{14} (referred to as α4\textsuperscript{f/f} below) were kindly provided by Dr. Thalia Papayannopoulou (University of Washington). CD19\textsuperscript{cre} mice\textsuperscript{15} and wild-type C57BL/6J mice were purchased from the Jackson Laboratory. All studies have been approved by the UCSF Institutional Animal Care and Use Committee and were in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Antigen

Recombinant human (rh) MOG was provided by Dr. C.C.A. Bernard and was synthesized, purified, and refolded as previously reported.\textsuperscript{12}

EAE induction

EAE was induced in 8-12 week-old mice by immunization with 100 µg rhMOG in Complete Freund’s Adjuvant (CFA) containing 200 µg Mycobacterium tuberculosis H37RA (DIFCO Laboratories) on day 0. Mice received either i.p. 100 ng (Fig 1) or 200 ng (all other experiments) Bordetella pertussis toxin (List Biological Laboratories) on days 0 and 2. Mice were observed daily for clinical EAE.\textsuperscript{12}

In vivo blockade of α4

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

Cell isolation

Blood was collected via cardiac puncture. After erythrocyte lysis, leukocytes were washed. Spleen and CNS mononuclear cells were isolated after perfusion with PBS.\textsuperscript{12}

Flow cytometric analysis

Anti-mouse FcRIIB/FcRIIIA mAb (2.4G2; BD) 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) 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. 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 LRSFortessa flow cytometer using FACSDiva software (BD).

**Intracellular cytokine staining (ICS)**
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 IgG was measured with a noncommercial ELISA as described, using rhMOG and HRP-labeled goat anti-mouse IgG (Thermo Scientific).

**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 was performed on representative sections. Meningeal and parenchymal B220+ B cells and CD3+ T cells were counted by a blinded observer (R.A. Sobel).

**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 ≤ 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-α4 mAb ameliorated EAE induced by rhMOG protein in C57BL/6 wild-type mice (Fig 1). In order to investigate the role of VLA-4-expression on B cells in EAE pathogenesis, we created mice (CD19cre/α4<sup>f/f</sup>) that selectively lack B cell α4-expression by crossing the CD19cre knock in, which selectively directs gene expression in B cells, but not in T cells or in other leukocytes, onto the α4<sup>f/f</sup> background (and data not shown). VLA-4 was not detected on B cells from naive CD19cre/α4<sup>f/f</sup> mice whereas CD4<sup>+</sup> 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/α4<sup>WT</sup> or CD19cre control mice, CD19cre/α4<sup>f/f</sup> mice exhibited a reduction in EAE susceptibility, which was most pronounced at the peak of disease (Fig 2B). Similar results were observed in an independent experiment using α4<sup>f/f</sup> 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/α4<sup>f/f</sup> mice with EAE, there were no evident differences in peripheral B cell-activation as measured by expression of CD80 or MHC II (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 (Fig 2D). Lastly, as immunization with rhMOG protein can elicit secretion of pathogenic (demyelinating) antibodies, we examined MOG-specific antibodies in control and CD19cre/α4<sup>f/f</sup> mice with rhMOG-induced EAE. No significant differences in anti-rhMOG IgG titers were observed (Fig 2E). By these measures, selective elimination of VLA-4-expression did not significantly influence 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⁺B220⁺ B cells in CD19cre/α4f/f mice (Fig 3A), findings that were confirmed by immunohistologic staining. Reduction of B220⁺ (CD45R⁺) B cells was most pronounced in the meninges, which contained the majority of CNS-infiltrating B cells in control mice with EAE (Fig 3B). There were no clear detectable differences in the histological appearances of microglia or astrocytes in lesions in CD19cre/α4f/f and control mice with EAE.

The paucity of CNS B cells in CD19cre/α4f/f mice was also associated with a decrease in the total number of CNS-infiltrating hematopoietic cells (CD45hi), CD45hiCD11b⁺ macrophages and CD45hiCD11b⁻CD4⁺ T cells (Fig 3C-E). Similarly, meningeal CD3⁺ T cells were reduced in CD19cre/α4f/f mice, as measured by immunohistochemistry (Fig 3B). Moreover, the frequency of Th17, but not Th1, cells was significantly reduced in the CNS of CD19cre/α4f/f mice with EAE (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, providing the foundation for advancing development of natalizumab in MS, 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, and clinical studies suggest this process may be inhibited by natalizumab. Clinical investigations demonstrating pronounced beneficial effects of anti-CD20 B cell-depleting mAbs in treatment of RRMS have renewed interest for understanding cellular and humoral contribution of B cells in MS pathogenesis and response to therapy. In order 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. 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, two years after the initial approval of natalizumab. Of interest, one recent study demonstrated that anti-VLA-4 prevented EAE induced by myelin-specific Th1, but not Th17 cells. 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 (αLβ2 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. 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.
While effective in MS, why has natalizumab exacerbated neuromyelitis optica (NMO)? There may be several contributing factors. First, NMO is a humoral autoimmune disease that is associated with aquaporin-4-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 lesions and are thought to contribute to NMO pathogenesis. Th17 cells can promote CNS neutrophil accumulation and AQP4-specific T cells in NMO exhibit Th17 polarization. Third, as human neutrophils do not express VLA-4, 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, 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 infiltrating cells in characteristic lesions of RRMS, B cell-depletion in RRMS results in a marked reduction in gadolinium-enhancing CNS lesions. Anti-CD20 B cell-depletion in EAE significantly reduces meningeal B cells. 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 pathogenesis of CNS autoimmunity, should promote investigations to further understand how CNS B cells influence cellular immune responses in situ.
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Author contributions

K. Lehmann-Horn designed research, performed the experiments, analyzed data, and wrote the paper. S.A. Sagan performed the experiments, analyzed data, and discussed the results at all stages. C.C.A. Bernard provided reagents. R.A. Sobel performed the neuropathologic analysis. S.S. Zamvil designed research, analyzed data, wrote the paper, and supervised the study.

Potential Conflicts of Interest

K.L.-H., S.A.S., C.C.A.B. and R.A.S. have nothing to disclose. S.S.Z. has served as a consultant and received honoraria from Biogen-Idec, EMD-Serono, Genzyme, Novartis, Questcor, Roche, and Teva Pharmaceuticals, Inc., and has served or serves on Data Safety Monitoring Boards for Lilly, BioMS, Teva and Opexa Therapeutics.
References

Figure Legends

**Figure 1. α4-blocking antibodies prevent rhMOG-induced EAE in C57BL/6 mice.**

EAE was induced in wild-type C57BL/6 mice by immunization with 100 µg rhMOG. On days 4, 7 and 10 after immunization, 200 µg of anti-α4 antibody (n = 8 mice) or IgG2b isotype control antibody (n = 7 mice) were injected i.p. Data shown is mean ±SEM. *, p ≤ 0.05 for days 15-22; Mann-Whitney U test.

**Figure 2. Selective α4-deficiency on B cells reduces disease severity in rhMOG-induced EAE while 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/α4⁺f/f or control mice assessed by flow cytometry. An isotype control antibody was used to define the negative population and a healthy wild-type mouse served as a positive control. Histograms show one representative mouse per group (n = 4 mice/group). B) CD19cre/α4⁺f/f (n = 9 mice) and CD19cre/α4⁻WT control mice (n = 7 mice) were immunized with 100 µg rhMOG (upper panel). CD19cre/α4⁺f/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 is shown. Similar results were obtained in another independent experiment with an α4⁻WT control group (not shown). Data shown represent mean disease score ±SEM. ***, p ≤ 0.01; Mann-Whitney U test; differences were significant (p≤0.05) for days 14-35, except day 28, in the upper panel and for days 12-16 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 post immunization with 100 µg rhMOG) in CD19cre/α4⁺f/f or control mice. Similar results were obtained in another independent experiment (not shown). Data shown represent mean ±SEM. ***, p ≤ 0.01; Mann-Whitney U test; differences were significant (p≤0.05) for days 14-35.
obtained in the blood, at a later time point (31 days post immunization) and for CD86 (B7-2) (not shown). Flow cytometry plots show one 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 (ICS) in the spleen 31 days post immunization. Flow cytometry plots show one 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 post immunization; not shown). E) Serum IgG antibodies against rhMOG were detected by ELISA. The optical density (OD) at 450 nm is indicated. Serum was obtained on day 31 post immunization with 100 µg rhMOG in CD19cre/α4ff 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/α4ffWT.

Figure 3. Selective B cell α4-deficiency impairs B cell-migration into the CNS in rhMOG-induced EAE and reduces CNS recruitment of CD11b+ macrophages and T cells.

CD19cre/α4ff 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/α4ff or control mice. Flow cytometry plots show one representative mouse (gated on viable CD45hiCD11b– lymphocytes). Similar results were obtained at a later time point (31 days post immunization; not shown). n = 3 mice/group. B) Leptomeningeal infiltrates in the brains of CD19cre/α4ff (upper picture panels) and control (lower picture panels) mice with EAE 31 days post immunization. CD19cre/α4ff and control mice have abundant CD3+ T cells (left panels); B220+ B cells are present in the control mouse but not the CD19cre/α4ff mouse (right panels). Immunohistochemistry
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