CD8 T Cell-Initiated Blood–Brain Barrier Disruption Is Independent of Neutrophil Support

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CD8 T Cell-Initiated Blood–Brain Barrier Disruption Is Independent of Neutrophil Support

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Blood–brain barrier (BBB) disruption is a common feature of numerous neurologic disorders. A fundamental question in these diseases is the extent inflammatory immune cells contribute to CNS vascular permeability. We have previously shown that CD8 T cells play a critical role in initiating BBB disruption in the peptide-induced fatal syndrome model developed by our laboratory. However, myelomonocytic cells such as neutrophils have also been implicated in promoting CNS vascular permeability and functional deficit in murine models of neuroinflammatory disease. For this reason, we evaluated neutrophil depletion in a murine model of CD8 T cell-initiated BBB disruption by employing traditionally used anti-granulocyte receptor-1 mAb RB6-8C5 and Ly-6G–specific mAb 1A8. We report that CNS-infiltrating antiviral CD8 T cells express high levels of granulocyte receptor-1 protein and are depleted by treatment with RB6-8C5. Mice treated with RB6-8C5, but not 1A8, display: 1) intact BBB tight junction proteins; 2) reduced CNS vascular permeability visible by gadolinium-enhanced T1-weighted magnetic resonance imaging; and 3) preservation of motor function. These studies demonstrate that traditional methods of neutrophil depletion with RB6-8C5 are broadly immune abating. Our data also provide evidence that CD8 T cells initiate disruption of BBB tight junction proteins and CNS vascular permeability in the absence of neutrophil support. The Journal of Immunology, 2012, 189: 000–000.
Compass monocytes and neutrophils were perceived to be the critical cell type promoting vascular permeability, associated with CD8 T cell-dependent morbidity. This conclusion was based on the results of neutrophil depletion with anti-granulocyte receptor-1 (GR-1) mAb RB6-8C5, a widely used method to evaluate the role of neutrophils in several disease processes, including cancer (23, 24). Using this approach, it was proposed that CD8 T cells may primarily serve to attract other effector populations such as neutrophils to induce BBB disruption (13). However, RB6-8C5 has been shown to be nonspecific for neutrophils, as it binds to both Ly-6G on neutrophils and Ly-6C on lymphocytes and monocytes (25, 26). RB6-8C5 has also been shown to bind to F4/80 macrophages/monocytes, plasmacytoid dendritic cells, and CD8 T cells in a model of HSV type I infection (27). Consequently, it is possible that this Ab is also ablating activated CD8 T cells, which we have previously shown to be critical for initiating BBB disruption (6, 15, 20, 28). We have also demonstrated that depletion of Ag-specific CD8 T cells confers protection (15). Therefore, due to the lack of specificity of the broadly used mAb RB6-8C5, it is essential to reassess the capacity of neutrophils to contribute to BBB disruption in models of neurologic diseases. In this study, we employed both anti-GR-1 mAb RB6-8C5 and anti-Ly-6G mAb IA8 to deplete neutrophils in vivo (25–27, 29). We investigated the impact of these neutrophil-depletion strategies on brain-infiltrating CD8 T cell populations, BBB tight junction protein alterations, CNS vascular permeability, and functional deficit using the PIFS model system.

Materials and Methods

Animals

C57BL/6 male mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 wk of age. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and Mayo Clinic.

Induction of CNS vascular permeability using the PIFS model

CNS vascular permeability was induced as previously described (15, 20, 28). Briefly, all mice were intracranially infected with 2 \times 10^8 PFU Daniel’s strain of TMEV. Seven days later, during the peak of CD8 T cell expansion in the brain, mice were i.v. administered 0.1 mg VP21-130 (FHAGSLVVFM) peptide or mock control E7 (RAHYNVITF) peptide (GenScript) (15).

Neutrophil-depletion strategies

Mice were administrated 250 µg anti–GR-1 mAb RB6-8C5 (#BE0075; BioXCell), 500 µg anti–Ly-6G mAb IA8 (#BE0075-1; BioXCell), or 500 µg normal rat serum (NRS) (#89531; Jackson ImmunoResearch Laboratories) on days 5–7 post-TMEV infection.

Rotarod

Mice were placed on the Rotamex-5 rotarod apparatus (Columbus Instruments) and covered with Vectashield medium (H-1000; Vector Laboratories). Slides were incubated with the following primary Abs at a concentration of 1:250 overnight at room temperature: mouse anti–claudin-5 (Alcon SA, Lausanne, Switzerland), Alexa Fluor 532 goat anti-mouse IgG (A-11001; Invitrogen) and Alexa Fluor 647 goat anti-rabbit IgG (A-21244; Invitrogen). Slides were then rinsed five times in PBS followed by Hoechst staining at a concentration of 1:500 in PBS for 5 min. Slides were then rinsed five times in PBS, dried, and covered with Vectashield medium (H-1000; Vector Laboratories). Images were acquired using a Leica DM 2500 confocal microscope (Leica Microsystems) equipped with a 63× oil immersion objective (numerical aperture 1.30). All images were collected at room temperature using Type F immersion liquid (Leica Microsystems) and analyzed using LAS AF stimulator AF 6000 acquisition software (Leica Microsystems).

MRI acquisition

A Bruker Avance II 7 Tesla vertical bore small animal MRI system (Bruker Biospin) was used for image acquisition to evaluate CNS vascular permeability. Image acquisition was performed as previously described (6, 30). Briefly, inhalation anesthesia was induced and maintained via 3 to 4% isoflurane. Respiratory rate was monitored during the acquisition sessions using an MRI compatible vital sign monitoring system (Model 1030; SA Instruments, Stony Brook, NY). Mice were given an i.p. injection of gadolinium using weight-based dosing of 100 mg/kg, and after a standard delay of 15 min, a volume acquisition T1-weighted spin echo sequence was used (repetition time = 150 ms, echo time = 8 ms, field of view: 32 mm × 19.2 mm × 19.2 mm, matrix: 160 × 96 × 96; number of averages = 1) to obtain T1-weighted images.

Image analysis

Three-dimensional (3D) volumetry was performed as previously described (31–33). Briefly, the 3D volume of vascular permeability was quantified using Analyze 10 software (Biomedical Imaging Resource, Mayo Clinic).Brains from the gadolinium-enhanced T1-weighted images were extracted using the 3D volume extractor tool. The 3D region of interest tool was used to define areas of gadolinium leakage using semi-automated methods, including a combination of thresholding and seed-growing tools. The identified volume of gadolinium enhancement was saved as a 3D object designating a subvolume of the brain. The 3D sampling tool was then used to calculate the actual volume of this object, which corresponds with the volume of gadolinium leakage in the studied brain. To generate figures, 3D object rendering using the volume-rendering tool was performed to visualize Ly-6G+ cells.

Conflcut microscopy

Frozen fresh coronal slices from FITC-albumin injected mice were cut on a cryostat and placed onto positively charged slides. Slides were washed twice with PBS and then fixed in 3% paraformaldehyde for 15 min. Slides were then rinsed three times in PBS, followed by a 1-h incubation in 5% normal goat serum plus 0.5% IgEgal CA-630 (I5021; Sigma-Aldrich) in PBS. Slides were incubated with the following primary Abs at a concentration of 1:250 overnight at room temperature: mouse anti–claudin-5 (35-2500; Invitrogen) and rabbit anti-occludin (71-1500; Invitrogen). Slides were then rinsed five times in PBS. The following secondary Abs were then added at a concentration of 1:250 for 1 h at room temperature: Alexa Fluor 532 goat anti-mouse IgG (H+L) (A-11002; Invitrogen) and Alexa Fluor 647 goat anti-rabbit IgG (H+L) (A-21244; Invitrogen). Slides were then rinsed five times in PBS followed by Hoechst staining at a concentration of 1:500 in PBS for 5 min. Slides were then rinsed five times in PBS, dried, and covered with Vectashield medium (H-1000; Vector Laboratories). Images were acquired using a Leica DM 2500 confocal microscope (Leica Microsystems) equipped with a 63× oil immersion objective (numerical aperture 1.30). All images were collected at room temperature using Type F immersion liquid (Leica Microsystems) and analyzed using LAS AF stimulator AF 6000 acquisition software (Leica Microsystems).
**Statistical analysis**

Mean and SE values were calculated using SigmaStat software (SYSTAT Software). GraphPad Prism Software (GraphPad) was used to construct bar graphs with SE bars. A Student t test was performed using SigmaStat (SYSTAT Software) to evaluate the extent CD8 T cells bind to GR-1+ or Ly-6G+ protein and also to assess the efficacy of neutrophil depletion. A Mann–Whitney rank sum test was performed using SigmaStat (SYSTAT Software) to evaluate total numbers of GR-1+ or Ly-6G+ cells. An ANOVA followed by a test for pairwise multiple comparisons (Tukey’s test or Dunn’s method) using SigmaStat (SYSTAT Software) was employed to determine significance among the three treatment groups with regards to ablation of CD8 T cells, quantification of the 3D volume of CNS vascular permeability, and rotarod scores.

**Results**

*The anti–GR-1 mAb RB6-8C5 binds to CD8+ cells*

CNS-infiltrating lymphocytes isolated from TMEV-infected C57BL/6 mice that received VP2_{121–130} peptide to induce PIFS (n = 8) were stained with Ab to GR-1, Ly-6G, CD45, CD8, and D^{b}:VP2_{121–130} tetramer. We found that the vast majority (94.9%) of brain-infiltrating CD8 T cells also stained positive for GR-1 protein when compared with the percentage of Ly-6G\(^+\)CD8\(^+\) cells (p < 0.001) (Fig. 1A–D). Additionally, we found that CNS-infiltrating D\(^{b}:\)VP2_{121–130} epitope-specific CD8\(^+\) cells isolated from C57BL/6 mice induced to undergo PIFS (n = 8) had significantly higher GR-1 protein

![Figure 1](http://www.jimmunol.org/)
expression (95.7% ± 0.314) when compared with CD8+ cells isolated from the spleen of uninfected (10.4% ± 0.819) (n = 3; p < 0.001) or intracranially TMEV-infected (25.4 ± 3.901%) (n = 6; p < 0.001) C57BL/6 mice. These results demonstrate that peripheral CD8 T cells do not express GR-1 protein at the level of activated CNS-infiltrating antiviral CD8 T cells (Fig. 1E). Percentages are depicted as mean ± SEM. In addition, we performed a magnetic Ly-6G+ sort (see Materials and Methods) to visualize brain-infiltrating Ly-6G+ cells and determined these cells have morphological features characteristic of neutrophils (i.e., segmented nuclei) (Fig. 1F). This observation is in accordance with previously published reports that anti-Ly-6G mAb 1A8 is highly specific for neutrophils (25–27, 29). Furthermore, we evaluated GR-1 positivity on other brain-infiltrating cells and found that the vast majority of CD3+, CD11c+, and Mac-1+ cells also stained positive for GR-1 protein. Approximately half of CD4+ cells and a quarter of NK1.1+ cells were also positive for GR-1 protein (n = 4) (Fig. 1G–K).

Efficacy of mAb RB6-8C5 and 1A8 neutrophil-depletion strategies during acute TMEV infection

With the observation that GR-1 protein was highly expressed on CNS-infiltrating CD8 T cells, we next assessed the efficacy of our neutrophil-depletion strategies using anti–GR-1 mAb RB6-8C5 and anti–Ly-6G mAb 1A8 during acute TMEV infection. We performed flow cytometric analysis of lymphocytes isolated from the brains of C57BL/6 mice i.p. administered NRS (n = 6), anti–GR-1 mAb RB6-8C5 (n = 6), or anti–Ly-6G mAb 1A8 (n = 6). Isolated CNS-infiltrating lymphocytes were then stained with Ab to GR-1, Ly-6G, CD45, CD8, and D2-VP2121–130 tetramer. We determined that i.p. administered mAb RB6-8C5 significantly depleted GR-1+ cells as a percentage of CD45 hi cells (Fig. 2B) (p < 0.001), and mAb 1A8 significantly depleted Ly-6G+ cells as a percentage of CD45 hi cells (Fig. 2F) (p = 0.008) when compared with the levels of these cells in mice treated with NRS (Fig. 2A, 2D). We also analyzed absolute numbers of GR-1+ or Ly-6G+ cells per 100,000 events and found that treatment with mAb RB6-8C5 significantly depleted GR-1+ cells (Fig. 2G) (p = 0.004) and mAb 1A8 significantly depleted Ly-6G+ cells (Fig. 2H) (p = 0.002) when compared with treatment with NRS. Additionally, we used IgG2b as an isotype control for mAb RB6-8C5 and IgG2a as an isotype control for mAb 1A8. Treatment with IgG2b (n = 3) did not significantly affect the percentage of GR-1+ cells when compared with treatment with NRS (p = 0.935). Similarly, treatment with IgG2a (n = 3) also did not significantly affect the percentage of Ly-6G+ cells when compared with treatment with NRS (p = 0.400) (data not shown). Additionally, we determined that RB6-8C5 is broadly immune ablating, in that we observed significant reduction of CD45+ cells as a percentage of whole brain cells when compared with positive controls treated with NRS (p = 0.009) (Fig. 2I).

FIGURE 2. Efficacy of mAb RB6-8C5 and 1A8 neutrophil-depletion strategies during acute TMEV infection. Histograms showing the efficacy of neutrophil-depletion strategies in representative 7-d TMEV-infected C57BL/6 mice (n = 6/group) using NRS (A, D), RB6-8C5 (B, E), or 1A8 (C, F). Neutrophil-depletion Abs were administered on days 5–7 post-TMEV infection. The CD45 hi population was gated and analyzed for the percentages of either GR-1+ or Ly-6G+ cells. (B) RB6-8C5 significantly depleted GR-1+ cells (p < 0.001 by Student t test), and (F) 1A8 significantly depleted Ly-6G+ cells (p < 0.001 by Student t test). (G) RB6-8C5 also significantly depleted the total number of GR-1+ cells per 100,000 events (p = 0.004 by Mann–Whitney rank sum test), and (H) 1A8 significantly depleted the total number of Ly-6G+ cells per 100,000 events (p = 0.002 by Mann–Whitney rank sum test). (I) RB6-8C5 also caused a significant reduction in CD45+ cells as a percentage of whole brain cells when compared with positive controls treated with NRS (p = 0.009 by Student t test). Error bars indicate SEM. Shown in (A)–(F) are the results of a representative animal in one experiment of two conducted.
The anti-GR-1 mAb RB6-8C5 depletes CNS-infiltrating CD8\(^+\) cells in mice administered VP\(_{212-130}\) peptide to induce PIFS. The above experiments prompted the question of whether treatment with RB6-8C5 would also cause ablation of virus-specific CD8 T cells in addition to neutrophils. To address this, isolated CNS-infiltrating lymphocytes from C57BL/6 mice treated with NRS (\(n=6\)), anti-GR-1 mAb RB6-8C5 (\(n=6\)), or anti-Ly-6G mAb 1A8 (\(n=6\)) were stained with Ab to GR-1, Ly-6G, CD45, CD8, and D\(^b\):VP\(_{212-130}\) tetramer. Fig. 3A–D illustrates that treatment with RB6-8C5 resulted in a significant reduction in CNS-infiltrating CD8 T cells as a percentage of CD45\(^+\) cells when compared with treatment with NRS (\(p<0.05\)) or 1A8 (\(p<0.05\)). There was no significant difference between treatment with NRS and treatment with 1A8 (\(p=0.386\)). (E) Treatment with RB6-8C5 also significantly reduced the total number of CD8\(^+\) cells when compared with treatment with NRS (\(p<0.05\)). Significance between groups was determined by an ANOVA followed by Tukey’s multiple comparison test. Error bars indicate SEM. Shown are the results of one experiment representative of two.

Neutrophil depletion with mAb 1A8 does not preserve BBB tight junctions in CD8 T cell-initiated BBB disruption

We have previously shown that CD8 T cells initiate BBB tight junction protein alterations in microvessels and CNS vascular permeability in the PIFS model of CD8 T cell-initiated BBB disruption (28). We therefore evaluated the effect of neutrophils and CD8 T cells on vascular integrity and the tight junction architecture in animals induced to undergo PIFS through administration of VP\(_{212-130}\) peptide. Administration of mock human papillomavirus virus E7 peptide was used as a negative control, as this peptide does not induce PIFS (6, 15, 20, 28) (Fig. 4A).

Treatment with anti-GR-1 mAb RB6-8C5 (Fig. 4C) preserved BBB tight junction proteins and vascular integrity similar to that observed in negative controls. However, treatment with NRS (Fig. 4B) or neutrophil-ablating Ly-6G mAb 1A8 (Fig. 4D) resulted in loss of the BBB tight junction proteins claudin-5 and occludin in areas of vascular permeability. Through extensive analysis of numerous areas of vascular permeability, we found that leakage of FITC-albumin coincided with disruption of tight junction proteins in 15 out of 15 areas for mice treated with NRS and 12 out of 12 areas for mice treated with 1A8. This correlation is consistent with...
BBB tight junction protein disruption colocalizing with vascular permeability. Therefore, CD8 T cell-initiated disruption of BBB tight junction proteins and ensuing CNS vascular permeability are not dependent on the contribution of neutrophils.

**Neutrophil depletion with mAb 1A8 does not preserve vascular integrity as measured by 3D volumetric analysis of gadolinium enhancement visible on T1-weighted MRI**

Our analysis using confocal microscopy demonstrated that CD8 T cell-initiated vascular permeability colocalized with BBB tight junction protein degradation. To quantitatively determine the full volume of CNS vascular leakage in animals undergoing CD8 T cell-initiated BBB disruption, we employed volumetric analysis of 3D gadolinium-enhanced MRI. Because neutrophils have been strongly implicated in promoting CNS vascular permeability (13, 34, 35), we sought to determine the extent neutrophils were contributing to CD8 T cell-initiated BBB disruption. To accomplish this, we conducted gadolinium-enhanced T1-weighted MRI to measure vascular permeability following neutrophil-depletion strategies using anti–GR-1 mAb RB6-8C5 and Ly-6G–specific mAb 1A8 24 h postinduction of PIFS with administration of VP2121–130 peptide. The extent gadolinium leaked into the CNS was quantified using 3D volumetric analysis with Analyze 10 software developed by Mayo Clinic’s Biomedical Imaging Resource (36, 37). Quantification of the 3D volume of gadolinium leakage from vasculature (Fig. 5I) revealed that treatment with RB6-8C5, which we have also shown to deplete CD8 T cells (Fig. 3B, 3D), was effective at reducing CNS vascular permeability (Fig. 5C, 5G) when compared with NRS (Fig. 5B, 5F) \( p < 0.05 \). In contrast, highly neutrophil-specific depletion with mAb 1A8 was not effective at reducing 3D gadolinium leakage (Fig. 5D, 5H). As an additional control, we evaluated the effect of treatment with isotype-matched Ab on vascular integrity. Treatment with IgG2b (isotype control for RB6-8C5) and IgG2a (isotype control for 1A8) did not significantly affect the extent of CNS vascular permeability when compared with treatment with NRS \( (p = 0.368) \) (data not shown). Therefore, anti–GR-1 treatment with mAb RB6-8C5 is highly effective at protecting from CNS vascular permeability in the PIFS model. Meanwhile, specific ablation of neutrophils with mAb 1A8 did not reduce CNS vascular permeability in this study.

**Neutrophil depletion with mAb 1A8 does not preserve functional ability in the PIFS model**

In the above experiments, we determined that anti–GR-1 mAb RB6-8C5 causes ablation of CD8 T cells in addition to neutrophils. In contrast, Ly-6G–specific mAb 1A8 is more selective for neutrophils. We next assessed the effect of these treatments on functional deficit by assessing mice on the Rotamex rotarod (Columbus Instruments) before and 24 h postinduction of PIFS. Seven-day TMEV-infected animals were administered VP2121–130 peptide to initiate BBB disruption, CNS vascular permeability, and functional deficit (6, 15, 20, 28). As depicted in Fig. 6, we determined that treatment with mAb RB6-8C5 significantly preserved motor function when compared with mice treated with NRS or neutrophil-depleting mAb 1A8 \( (p < 0.05) \). These results indicate that functional deficit is dependent on the presence of GR-1+ cells, including CD8 T cells. Meanwhile, consistent with the above data demonstrating a negligible effect of mAb 1A8 on BBB disruption, neutrophil depletion did not affect functional deficit in animals undergoing PIFS.

**Discussion**

Depletion of neutrophils with anti–GR-1 mAb RB6-8C5 is a widely used method to evaluate the role of neutrophils in several disease processes (13, 23, 24, 34, 38–43). However, this study demonstrates that the use of mAb RB6-8C5 is highly nonspecific. Importantly, activated CNS-infiltrating CD8 T cells express high levels of GR-1 and are depleted by treatment with mAb RB6-8C5. For this reason, previous reports implying a neutrophil subset being required for immune-mediated BBB disruption may require further evaluation before such a conclusion can be drawn (13, 34, 35). Depletion of neutrophils with mAb 1A8, which is highly specific for the neutrophil subset, did not affect CD8 T cell-initiated BBB disruption in these studies.

The extent inflammatory immune cells contribute to CNS vascular permeability in immune-mediated neurologic diseases, including multiple sclerosis, AHLE, stroke, dengue hemorrhagic fever, and cerebral malaria, remains largely unknown (1–7). Insult to the CNS results in homing of inflammatory immune cells. However, although these cells are capable of entering the CNS, the BBB remains relatively impermeable to other blood-derived products. This may be due to the route immune cells use to gain access to the CNS. Rather than opening tight junctions between cerebral endothelial cells of the vasculature, immune cells enter...
Shown are the results of one experiment representative of two. Followed by Tukey's multiple comparison test. Error bars indicate SEM. Significant difference between treatment with NRS and treatment with 1A8 preserved motor function when compared with treatment with NRS (n = 8; p < 0.05) and treatment with IAB (n = 10; p < 0.05). There was no significant difference between treatment with NRS and treatment with IAB (p = 0.853). Significance between groups was determined by an ANOVA followed by Tukey’s multiple comparison test. Error bars indicate SEM. Shown are the results of one experiment representative of two.

the CNS via postcapillary venules (44–46). Current models propose a role for inflammatory immune cells in opening tight junctions, resulting in increased permeability of blood-derived products (3, 47). Immune cells may also mediate the release of certain chemokines and cytokines, including VEGF, IFN-γ, TNF-α, IL-1β, and IL-6, which may also contribute to increased vascular permeability (8, 20, 22, 48–53).

Studies performed in the various experimental model systems have proposed mechanisms for immune-mediated BBB disruption. The readily inducible PIFS model of CNS vascular permeability developed by our laboratory has enabled us to analyze the kinetics of immune-mediated BBB disruption. We have previously shown that this disruption is initiated by CD8 T cells and requires perforin expression (6, 15, 20, 28). Additionally, a decrease in the BBB tight junction protein occludin has been associated with CNS vascular permeability in the PIFS model (28), the EAE model (8), and the LPS injection model of CNS vascular permeability (10, 54). Our laboratory has also demonstrated that a blockade of VEGF preserves the BBB tight junction protein occludin, reduces CNS vascular permeability, and promotes survival (20). In situ mRNA analysis revealed that neurons are the major cellular source of VEGF expression (20). In parallel studies, we also determined that CD8 T cells actively engage Theiler's virus-infected neurons (55). Based on the results of these studies, we propose a direct hypothesis for BBB disruption in which CD8 T cells engage neurons to promote upregulation of VEGF, resulting in disruption of the tight junction architecture and ensuing CNS vascular permeability. Our alternative hypothesis is that CD8 T cells may engage a different CNS cell type to promote vascular permeability through a mechanism that is dependent or independent of neuronally expressed VEGF. Both the direct and indirect hypotheses require perforin expression.

Other experimental model systems, including LCMV infection, have proposed that monocytic cells and neutrophils are the critical blood-derived cell types promoting BBB disruption. These studies showed that treatment with anti-GR-1 mAb RB6-8C5 extended survival and preserved vascular integrity (13). It was therefore concluded that rather than directly initiating CNS vascular permeability, CD8 T cells may serve to attract other effector populations such as neutrophils to induce damage (13). However, as shown in the results of this study, as well as in previously published results, treatment with anti-GR-1 mAb RB6-8C5 does not specifically deplete neutrophils (25–27, 29). Importantly, this method results in wide ablation of large numbers of activated CD8 T cells in addition to neutrophils, resulting in preservation of the BBB tight junction proteins and reduced CNS vascular permeability. Meanwhile, treatment with the more neutrophil-specific mAb IAB preserved CNS-infiltrating D9:VP2121–130 epitope-specific CD8 T-cell populations and resulted in disruption of BBB tight junction proteins, extensive CNS vascular permeability, and functional deficit. The difference in the effects of these two mAb treatments requires identification of the immune cell types depleted. We have evaluated GR-1 positivity on other brain-infiltrating cells and found that the vast majority of CD3+ T cells, CD11c+ dendritic cells, and Mac-1+ cells are positive for GR-1 protein. Approximately half of CD4+ T cells and a quarter of NK1.1+ cells are also positive for GR-1 protein. It is therefore likely that a proportion of these GR-1+ cells were also depleted by anti-GR-1 treatment with mAb RB6-8C5. Nevertheless, CD8 T cells are highly attenuated by anti-GR-1 treatment with mAb RB6-8C5 and therefore continue to support our central hypothesis that CD8 T-cell infiltration into the CNS is required for initiation of BBB disruption. The finding that virus-specific CD8 T cells correlate with immunopathology resulting from viral infection is in accordance with other previously published work using the LCMV model (11, 12, 14) and consistent with clinical observations in which CD8 T cells are associated with vascular permeability in human disease (56–59).

The results obtained in this study highlight the importance of CD8 T cells in promoting vascular permeability and warrants the re-evaluation of the literature pertaining to the extent neutrophils are required for BBB disruption (13, 34, 35). It remains possible that CD8 T cells could engage multiple CNS cell types to promote CNS vascular permeability, and this is currently under investigation in our laboratory. Nevertheless, the data put forward in this study offer new insight into the extent CD8 T cells contribute to disruption of the BBB in the absence of neutrophil support. Defining the specific immune cell types responsible for BBB disruption is essential to develop novel therapeutic approaches for neurologic diseases. Therefore, targeting CD8 T cells may hold promise as a therapeutic approach to ameliorate pathology associated with BBB disruption. Another approach may involve targeting VEGF and VEGF-related signaling pathways. VEGF has been associated with increased vascular permeability in both the PIFS model (20) and EAE model (8). Efforts are underway to evaluate the potential of this approach, as we have previously demonstrated that inhibition of neuropilin-1, a coreceptor for VEGF, caused preservation of occludin protein levels and a reduction in vascular permeability (20). Nevertheless, future research to define additional mechanisms by which immune cells contribute to BBB disruption will continue to aid in the development of potential therapies to treat immune-mediated neurologic disorders characterized by extensive CNS vascular permeability.

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Disclosures
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References
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