Hyaluronan Anchored to Activated CD44 on Central Nervous System Vascular Endothelial Cells Promotes Lymphocyte Extravasation in Experimental Autoimmune Encephalomyelitis*§

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Background: Multiple sclerosis (MS) is a demyelinating disease involving lymphocyte infiltration into the central nervous system (CNS).

Results: The glycosaminoglycan hyaluronan (HA), anchored to brain blood vessels via the CD44 receptor, facilitates lymphocyte binding to vessels and CNS infiltration.

Conclusion: HA-CD44 interactions on brain endothelial cells facilitate the initiation of inflammatory demyelinating disease.

Significance: Findings elucidate mechanisms promoting lymphocyte rolling in inflammatory CNS diseases.

The extravasation of lymphocytes across central nervous system (CNS) vascular endothelium is a key step in inflammatory demyelinating diseases including multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). The glycosaminoglycan hyaluronan (HA) and its receptor, CD44, have been implicated in this process but their precise roles are unclear. We find that CD44<sup>−/−</sup> mice have a delayed onset of EAE compared with wild type animals. Using an in vitro lymphocyte rolling assay, we find that fewer slow rolling (<1 μm/s) wild type (WT) activated lymphocytes interact with CD44<sup>−/−</sup> brain vascular endothelial cells (ECs) than with WT ECs. We also find that CD44<sup>−/−</sup> ECs fail to anchor HA to their surfaces, and that slow rolling lymphocyte interactions with WT ECs are inhibited when the ECs are treated with a pegylated form of the PH20 hyaluronidase (PEG-PH20). Subcutaneous injection of PEG-PH20 delays the onset of EAE symptoms by ~1 day and transiently ameliorates symptoms for 2 days following disease onset. These improved symptoms correspond histologically to degrada-tion of HA in the lumen of CNS blood vessels, decreased demyelination, and impaired CD4<sup>+</sup> T-cell extravasation. Collectively these data suggest that HA tethered to CD44 on CNS ECs is critical for the extravasation of activated T cells into the CNS providing new insight into the mechanisms promoting inflammatory demyelinating disease.

Multiple sclerosis (MS)<sup>2</sup> is a central nervous system (CNS) disorder characterized by the extravasation of pathogenic lymphocytes into the brain and spinal cord. Lymphocyte recruitment into the CNS during MS attacks results in inflammation, demyelination, and axonopathy, leading to neurological disability (1, 2). Experimental autoimmune encephalomyelitis (EAE) is an animal model of inflammatory demyelinating disease that recapitulates many of the pathological and clinical traits of MS. The molecular events that contribute to lymphocyte extravasation in MS and EAE include interactions between adhesion molecules on the surface of both activated lymphocytes and CNS vascular endothelial cells (ECs). These interactions lead to intracellular signaling events that enhance cell-cell adhesion and promote the crossing of lymphocytes across CNS vascular endothelium (3). In most tissues, this process is initiated by L-selectin on lymphocytes and P- and E-selectins on ECs binding to their transmembrane glycoprotein ligands (4). These transient interactions result in lymphocyte rolling along the endothelial cell surface, enabling signaling that induces expression and activation of integrins that mediate firm adhesion (5). However, the involvement of selectins in EAE and MS pathogenesis is a contentious issue. Although there is evidence that P- and E-selectins are expressed by superficial blood vessels of the brain and that they can mediate rolling (6–8), evidence from both antibody blocking experiments and experiments with knock-out and transgenic mouse models suggest selectins and their ligands are not essential for the development of EAE (9, 10). These findings indicate the involvement of other adhesive molecules in lymphocyte rolling on vessels within the CNS.

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<sup>2</sup>The abbreviations used are: MS, multiple sclerosis; EC, endothelial cell; EAE, experimental autoimmune encephalomyelitis; HA, hyaluronan; HABP, biotinylated HA-binding protein; TLR, Toll-like receptor; MOG, myelin oligodendrocyte glycoprotein; qRT, quantitative reverse transcription.
CD44 is a single-pass transmembrane glycoprotein widely expressed on a number of cell types including lymphocytes and ECs (11–14). CD44 functions as a receptor for hyaluronan (HA), a glycosaminoglycan that is synthesized in a wide range of sizes up to $\sim 10 \times 10^6$ Da. HA is composed of repetitive disaccharide units of N-acetyl-d-glucosamine and d-glucuronic acid. It is synthesized at the inner plasma membrane by one of three HA synthases (HAS1–3) that extrude HA into the extracellular matrix of many cell types including ECs (15, 16). Once extruded, HA can be tethered to the surface of ECs by HA synthases or CD44 (17). CD44 and HA have been implicated in regulating cell-cell adhesion, proliferation, migration, and differentiation (18).

CD44 expression and HA synthesis are increased in response to proinflammatory signals and numerous studies have implicated CD44 and HA in lymphocyte-endothelial cell interactions as well as the regulation of inflammatory responses (19). Proinflammatory stimulation of lymphocytes and ECs facilitates post-translation modification of CD44 inducing HA binding activity (17–19). Disruption of HA-CD44 interactions by anti-CD44 antibodies is sufficient to impair activated T-cell adhesion to endothelial cells in vitro (20). Additionally, CD44-HA interactions are required for superantigen-stimulated T-cells to efficiently home to sites of inflammation in the peritoneal cavity (21).

In the context of inflammatory CNS disease, blocking antibodies against CD44 delay EAE onset and decrease disease severity coincident with fewer lymphocytes present in the CNS (22–24). Similarly, one report found that EAE induced in CD44$^{-/-}$ mice is significantly attenuated (25). However, in contrast to the studies utilizing CD44 blocking antibodies, this study attributed the decrease in EAE disease severity to a phenotypic shift in the activated lymphocyte population through an HA-independent mechanism (25). It is unclear, therefore, whether the contribution of CD44 to EAE and MS disease progression is linked to lymphocyte extravasation or alterations in lymphocyte phenotypes. The requirement for HA in EAE onset and progression is also not clear.

To elucidate the role of CD44 and HA in lymphocyte-EC interactions during EAE pathogenesis, we utilized CD44$^{-/-}$ mice and a pegylated form of recombinant human PH20 (PEG-PH20) to degrade HA in the lumen of CNS blood vessels. We find that HA is tethered by CD44 to the luminal surface of TNFα stimulated ECs isolated from the brain and that slow rolling lymphocyte interactions are disrupted on ECs lacking CD44. In contrast, CD3/CD28-stimulated CD44$^{-/-}$ lymphocytes interact normally with wild type brain ECs. Removal of HA from ECs with PEG-PH20 treatment also results in impaired lymphocyte rolling. In vivo PEG-PH20 treatment delays the onset of EAE and reduces the number of T-cell infiltrates early in disease. These data indicate that HA tethered to CD44 on ECs promotes lymphocyte rolling during EAE pathogenesis.

**EXPERIMENTAL PROCEDURES**

**Induction of EAE**—EAE was induced using mouse myelin oligodendrocyte glycoprotein, peptides 35–55 (MOG$_{35-55}$), synthesized artificially by Peptides International. MOG$_{35-55}$ was combined with complete Freund’s adjuvant containing heat-inactivated mycobacterium tuberculosis as previously described (26).

**EAE Scoring**—Beginning the day following EAE induction, an experimenter, blinded to genotype or treatment condition, assigned a clinical disease score daily until days 13 or 21. The following clinical disease scoring scale was used: 0, no symptoms; 1, tail weakness (completely flaccid); 2, hindlimb weakness (animal can be easily flipped radially onto its back when grasped at base of tail); 3, animal walks with hind limbs splayed outwards; 4, one hindlimb partially or substantially paralyzed; 5, both hindlimbs completely paralyzed, no spastic movement; 6, moribund (animal is euthanized immediately). Increments of 0.5 were used for disease severity between the indicated scores.

**Hyaluronidase Administration in Mice**—PEG-PH20 was provided by Halyozyme Therapeutics Inc. An aliquot of a PEG-PH20 stock solution was prepared in advance each day that injections were to take place. Aliquots were diluted in PBS and passed through a 0.22-μm low protein binding syringe filter to sterilize the solution. Mice were randomly assigned to two groups to receive injections every other day of either 50 μl of subcutaneous sterile PBS (vehicle control) or 50 μl of subcutaneous PEG-PH20 (50 units/kg) into hind flanks. Injections continued until the experiment was terminated on days 13 or 21 postinduction of EAE or 6 days after beginning the injections in the case of naïve animals.

**Splenocyte Culture and Isolation**—Splenocytes from WT C57BL/6 mice were cultured in T75 flasks coated with anti-CD3 and anti-CD28 (eBioscience) antibodies for 72 h to induce T-cell-specific activation and clonal expansion as previously described (27). Cultures were harvested using a Lympholyte® (Sigma) gradient according to the manufacturer’s protocol. The lymphocyte layer was removed using a sterile Pasteur pipette and pelleted by centrifugation. The resulting pellet was washed and suspended in RPMI medium supplemented with 1% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate at a concentration of $1 \times 10^7$ splenocytes/ml. Cultures were maintained in a humidified 5% CO$_2$, 95% air atmosphere at 37°C.

**Murine Primary Brain Endothelial Cell Culture**—Primary brain endothelial cells were isolated and grown as previously described (28). Briefly, forebrains from 8-week-old WT or CD44$^{-/-}$ mice were isolated, minced, then digested with 1 mg/ml of collagenase CLS2 (Worthington Biochemical) in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 25 mM l-glutamine, and suspended in RPMI supplemented with 1% FBS, and 2 mM sodium pyruvate at a concentration of $1 \times 10^7$ cells/ml. Cultures were maintained in a humidified 5% CO$_2$, 95% air atmosphere at 37°C.
HA Anchored to CD44 on ECs Mediates Lymphocyte Extravasation

Interactions between lymphocytes and brain ECs were visualized in real-time by phase-contrast digital video microscopy. A single field of view (×10; 0.55 mm²) was monitored during each trial. The number of total interacting cells and the average rolling velocity of each interacting cell were analyzed for each experiment. Interacting lymphocytes were denominated as those that interacted with the EC monolayer for at least 1 s. Average rolling speed was determined using the particle tracking features of the Stallion imaging software. Criteria were set with the automated path.

CD44 Exon-specific RT-PCR Analysis—Total RNA was isolated from WT and CD44−/− EC cultures and CD3/CD28-stimulated lymphocytes using TRIzol reagent (Invitrogen). cDNAs were synthesized using MultiScribe™ reverse transcriptase (Applied Biosystems) according to the manufacturer’s protocol and a CD44 primer from the 3′ nonvariant portion of mouse CD44 (exon 19, 5′-tag gca cta cac ccc aat ctt ca-3′). cDNA products were amplified using Phusion® Hot Start II DNA Polymerase (Finnzymes) and a primer from the 5′ nonvariant region of mouse CD44 (exon 1, 5′-tcc ctc cgt ttc aac gga tgg cag g-3′) and another primer straddling two nonvariant 3′ exons (exon 16–17, 5′-ggt tgc cac tgt agt gtc ca-3′). The PCR was performed using a Mastercycler thermocycler (Eppendorf) with the following protocol: 40 cycles of 10 s at 98 °C, 30 s at 64 °C, and 1.5 min at 72 °C, followed by incubation at 72 °C for 5 min. This reaction was stopped at the end of the 25th cycle and 2 μl was removed for CD44 variant-specific nested primer analysis. The thermocycler protocol was subsequently re-started at the 25th cycle and the reaction was continued to completion.

The 2 μl from the above reaction was amplified using the same reagents and protocols listed above with the exception that variant exons 6 (5′-tgg ttt gac aac gga tgg cag g-3′), 7 (5′-cca cca cca cca tcc aag tca aa-3′), and 10 (5′-tct tcc ccc aga tac aac ttc tt-3′) specific nested 5′ primers were added. All reaction products were analyzed by electrohoresis in 1.5% agarose and visualized by ethidium bromide staining.

SYBR Green I Real-time RT-PCR Analysis (qRT-PCR)—Total RNA from EC cultures was obtained as above and single-stranded cDNAs were synthesized using the ImProm-II Reverse Transcriptase synthesis kit (Promega Corporation) according to the manufacturer’s protocol. The primer sets used were designed using Primer Express® software version 3.0 (Applied Biosystems) and synthesized by Integrated DNA Technologies. The primer sequences were: HAS1 forward, 5′-ggc tact gat cat cct-3′ and reverse, 5′-cca gga gct cag gat ctt-3′; HAS2 forward, 5′-aa ggg acc tgt tga gac aga a-3′ and reverse, 5′-cct ttatt gta tga tgc aa-3′; HAS3 forward, 5′-ggc cag tgc ctt tcc aaa-3′ and reverse, 5′-tgc cac cca gca cct-3′. The 18 S ribosomal RNA was used as a normalizing unit for each reaction. Primer sets were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit; Applied Biosystems). The qPCR assays were carried out with Platinum SYBR Green qPCR Supermix-UDG (Invitrogen) in a 7500 Fast TaqMan Instrument (Applied Biosystems) using a default thermocycling program. Assays were performed in triplicate. The normalized expression of the target gene with respect to 18 S was computed using the ΔΔCt method in Microsoft Excel.

HA Quantification—HA was quantified from culture medium supernatant or cellular lysates of WT and CD44−/− ECs cultured to confluence in 35-mm dishes. Cell lysates were obtained by incubation of EC monolayers in 200 μl of buffer containing 20 mm Tris-HCl, 150 mm NaCl, 1 mm EDTA, and 1% Triton X-100. Samples were applied to an enzyme-linked immunosorbent assay (ELISA)-based assay (Echelon Biosciences) according to the manufacturer’s instructions. Medium supernatant samples were diluted 1:4 and cell lysates 1:2 to a final volume of 350 μl in the kit diluent buffer. Triplicate 100-μl fractions were transferred into the ELISA plate. At the end of the assay, absorbances were read at 450 nm on a 96-well plate reader (Molecular Devices).

Immunohistochemistry—At appropriate EAE time points, mice were euthanized using isoflurane (Novaplus) and perfused transcardially with heparin saline followed by 4% paraformaldehyde. Lumbar spinal cords were removed, freeze-embedded, and serially sectioned at a thickness of 10 μm on a cryostat (Leica) and placed on glass slides. Sections were washed 3 × 5 min with PBS and blocked in PBS with 5% BSA with 0.05% Triton X-100.
HA Anchored to CD44 on ECs Mediates Lymphocyte Extravasation

FIGURE 1. EAE disease onset is delayed in CD44−/− animals. Disease was induced by inoculation of female WT (A) or CD44−/− (B) C57/B6/S129 background mice with MOG35–55 peptide emulsified in CFA. Disease symptoms manifest on or near day 10 postinoculation in WT animals but 3–8 days later in CD44−/−. $\Psi$ represents animals being euthanized due to severe disease.

Triton X-100 (blocking buffer) for 1 h at room temperature. Sections were then incubated with primary antibodies against CD4 (1:300, BD Pharmingen), CD31 (1:50, Ab cam), neurofilament-L (1:1000, Millipore), and/or HAS1 (1:25, Santa Cruz Biotechnology) in PBS overnight at 4 °C. Biotinylated HA-binding protein (bHABP, 1:250, Calbiochem) was used in place of a primary antibody to visualize HA. Negative controls included omitting the primary antibody. Sections were washed 3 × 5 min with PBS the next day and then incubated with the appropriate secondary antibody (goat anti-rat Alexa 488 and goat anti-rabbit Alexa 633, 1:1000, Molecular Probes) or Cy3 streptavidin (1:1000, Jackson Labs) in place of secondary to visualize HA for 2 h at room temperature. Sections were washed in PBS, and then incubated in FluoroMyelin (1:300, Invitrogen) for 20 min at room temperature to visualize myelin and Hoechst 33342 (1:5000, Invitrogen) for 10 min at room temperature to label nuclei. Sections were washed in PBS and mounted with Prolong Gold mounting media (Invitrogen), then imaged using a Zeiss Axioskop 40 fluorescence microscope (Zeiss) or an inverted Leica SP5 AOB5 spectral confocal system (Leica).

Immunocytochemistry—EC cultures on coverslips were fixed in 4% paraformaldehyde in PBS at room temperature for 15 min, rinsed with PBS, and treated for 1 h with blocking buffer (see above). Cultures were incubated overnight at 4 °C with the primary antibody diluted in PBS. Cells were stained with antibodies against CD44 (IM7 hybridoma, 1:40, ATCC), CD31 (see above), and glial fibrillary acidic protein (GFAP, 1:500, Dako). bHABP (see above) was used to visualize HA. Subsequently, cells were rinsed 3 × 5 min in PBS and incubated with the relevant secondary antibodies or Cy3 streptavidin as above for 2 h at room temperature. Cultures were rinsed 3 × 5 min in PBS and incubated in Hoechst for 10 min, mounted, and examined as above.

Stereology—Following immunohistochemical labeling, 12 sections of the lumbar spinal cord were analyzed from each animal (n = 8 animals per group). Digital photomicroscopic images were captured on a Zeiss Axiovert 200M (Zeiss) fluorescence microscope interfaced with a Marianas Digital Microscopy Work station (Intelligent Imaging Innovation Inc.). Montages of the entire lumbar cord were created and analyzed using SlideBookTM software. Using FluoroMyelin labeling, a mask delimiting the spinal cord white matter was created for each section and a grid was generated within the mask area for analysis. The grid consisted of 10-μm boxes spaced 50 μm apart in the xy axis with each box representing a 2500-μm square area. Based on FluoroMyelin labeling, boxes that fell entirely within the lesion areas were counted and divided by the total number of boxes within the grid and multiplied by 100 to determine a percent lesion area.

Infiltrating T-cell counts were performed by setting a threshold value for CD4 immunolabeling that resolved single cells. Subsequently, a mask was applied within the CD4 channel to record each cell’s position in the section. The software was then used to overlay the CD4 mask onto the mask generated for the lesion analysis and to record the number of CD4+ cells within the white matter in each section.

Statistical Analysis—Differences between treatment groups in HA ELISA and parallel plate assays were analyzed by a Student’s t test. Differences in mean EAE disease score between groups were analyzed by a repeated measures analysis of variance. Statistical significance was defined as p < 0.05 for all analyses.

RESULTS

EAE Onset Is Delayed in CD44−/− Mice—Inhibition of CD44 activity using neutralizing antibodies (22–24) or deletion of the CD44 gene (25) are reported to attenuate EAE onset and progression. To confirm that genetic ablation of CD44 is sufficient to ameliorate EAE symptoms, we tested how EAE progresses in CD44−/− mice that develop severe disease. Clinical disease signs were observed over a 25-day period in 8-week-old female WT and CD44−/− mice on a C57/B6;129 background, actively immunized with MOG35–55. Disease was evident beginning at day 10 postinoculation in WT animals with peak scores occurring by day 14 (Fig. 1A). Consistent with the findings of Guan and co-workers (25), CD44−/− mice manifested disease with a delayed onset, no earlier than day 14 postinoculation and as late as day 18 with an average peak disease on day 16 (Fig. 1B). However, in contrast to the previous study (25), we found that CD44−/− mice developed similar levels of disability. For both
groups, scores remained elevated to at least a score of 2.5 after 20 days postinoculation. These data demonstrate that CD44 contributes to the initiation of EAE onset but is not necessary for disease progression.

CD44 on Brain ECs but Not Lymphocytes Is Critical for Lymphocyte Recruitment and Rolling—Previous studies utilizing CD44 neutralizing antibodies suggest that CD44 contributes to EAE onset by promoting lymphocyte extravasation (22–24). A second study suggests that CD44 promotes EAE pathogenesis by regulating lymphocyte phenotypes (25). The exact role of CD44 in EAE pathogenesis remains unclear.

We used an in vitro parallel plate assay to determine whether CD44 on the lymphocytes and/or the EC cells affects adhesion and rolling during early stages of lymphocyte extravasation. We examined WT and CD44−/− lymphocyte adhesion and rolling on WT and CD44−/− brain ECs. Fewer WT lymphocytes (44.6% less, *p < 0.005) interacted with CD44−/− ECs (Fig. 2, C and D) compared with WT EC controls (Fig. 2A). The presence of CD44 on the lymphocytes, however, was not critical. Thus there was no difference in the number of interacting cells between WT and CD44−/− lymphocytes when superfused across WT ECs (Fig. 2, B and D).

Using particle-tracking techniques, the average rolling speed of all interacting cells was determined. Bins for slow (0–1 μm/s), medium (1–5 μm/s), and fast (>5 μm/s) rolling cells are plotted against the mean number of interacting cells for each group (Fig. 2D). Within the slow rolling population, significantly fewer (*, p < 0.05) interacting cells were observed when WT lymphocytes were superfused across CD44−/− ECs, but not when CD44−/− lymphocytes were superfused across WT ECs (Fig. 2D). Meanwhile, the number of medium and fast rolling interacting lymphocytes in the experimental groups was not significantly different from controls. These results indicate that CD44 on CNS ECs, but not on lymphocytes, contributes to the capture and slowing of activated lymphocytes along the endothelial surface.

Standard CD44 Tethers HA to the Surface of Brain ECs—Inflammatory mediators trigger HA synthesis and surface expression of CD44 by ECs (19). CD44 binds HA as it is extruded through the cell membrane by HA synthases and can tether it to the cell surface (17). We therefore tested if HA is similarly anchored to CD44 on the surface of brain ECs. Brain EC cultures from WT (Fig. 3, A and B) and CD44−/− (Fig. 3, C and D) mice were isolated, stimulated with TNFα, and assayed for expression of CD31, HA, and CD44. Both WT and CD44−/− cultures expressed high levels of the tight junctional marker CD31 as expected (Fig. 3, A, C and D, and supplemental Fig. S1B). WT cultures also displayed high levels of membrane CD44 that co-localized with areas of HA labeling (Fig. 3B, arrows). In contrast, CD44 and HA were undetectable in CD44−/− brain EC cultures (Fig. 3D).
HA Anchored to CD44 on ECs Mediates Lymphocyte Extravasation

FIGURE 3. CD44 anchors HA to the surface of CNS endothelial cells. A and B, WT EC cultures express high levels of CD31 (white) in addition to high levels of CD44 (green) and HA (red). Areas of CD44-HA colocalization are indicated (B, arrows). C and D, CD44−/− EC cultures also express high levels of CD31, but CD44 and HA were undetectable. All cultures were stimulated with TNFα (5 ng/ml) for 4 h. Unstimulated WT cultures expressed little CD44 or HA (A and B, inset). Scale bar is 10 μm. All images are 6-μm z-projections. E, qRT analysis of HAS mRNA in WT and CD44−/− EC cultures show similar levels of induction when stimulated with TNFα for 4 h. F, significantly more HA was found in the supernatant of CD44−/− EC cultures compared with WT ECs in response to a 4-h TNFα stimulation. Conversely, significantly less HA was found in the cell lysis fraction of CD44−/− ECs compared with WT ECs. *p < 0.005, t test compared with WT ECs.

with and without TNFα stimulation for CD44 variant 6, 7, and 10 mRNA expression. CD3/CD28-stimulated lymphocytes, which transiently express CD44 variant 6 were used as a positive control (29). Using CD44-specific primers corresponding to the 5′ and 3′ nonvariant exons, we determined that WT ECs, regardless of TNFα stimulation, express predominantly standard CD44 (supplemental Fig. S1C). Stimulated lymphocytes express predominately standard CD44, but a weaker band corresponding to CD44 variant 6 at the predicted size of 906 bp is present (supplemental Fig. S1C, red arrow). These results were confirmed using nested CD44 variant 6, 7, and 10 specific primers to re-amplify EC and lymphocyte CD44 nonvariant exon PCR products. A band corresponding to the predicted size product for CD44 variant 6 but not 7 and 10 was observed in lymphocytes and no predicted variant bands were observed in ECs (data not shown). These data indicate that standard CD44 is the predominant form of CD44 expressed by CNS ECs.

Our finding that HA is absent from the surface of CD44−/− ECs indicates that either CD44 anchors HA to brain EC cells or that HA synthesis is impaired in brain ECs lacking CD44. We examined HAS1–3 mRNA expression from WT and CD44−/− EC cultures via qRT-PCR. In WT cultures, mRNA expression of HAS1 increased ~4-fold, HAS2 was unchanged, and HAS3 decreased ~0.5-fold in response to TNFα stimulation (Fig. 3E). The same trend of mRNA expression for all the HAS genes was observed in CD44−/− cultures following TNFα stimulation (Fig. 3E). Furthermore, an ELISA was used to measure the amount of HA produced by WT and CD44−/− EC cultures in response to TNFα stimulation. CD44−/− cultures contained significantly more HA in the culture supernatant and less on the cell surface than WT controls (Fig. 3F, *p < 0.05). All together, these findings indicate that loss of CD44 does not affect HA synthesis by brain vascular ECs and that standard CD44 is required to anchor HA to the surface of these cells.

Degrading HA at the Surface of Brain ECs Impairs Lymphocyte Recruitment and Rolling—Given that CD44 anchors HA to brain vascular ECs, we tested if HA on the surface of brain ECs is required for lymphocyte recruitment and rolling. WT and CD44−/− brain EC cultures were treated with a pegylated form of a hyaluronidase that functions at neutral pH (PEG-PH20) or vehicle prior to analysis in the parallel plate assay. PEG-PH20 treatment resulted in a loss of HABP staining, indicating that HA had been effectively degraded in these cultures (data not shown). Compared with vehicle controls, there was a significant decrease in the number of lymphocytes interacting with WT ECs pre-treated with PEG-PH20 (47.5%, *p < 0.05; Fig. 4A). PEG-PH20 did not further decrease lymphocyte binding to CD44−/− ECs (Fig. 4A, NS). Similar to our findings with CD44−/− brain ECs (Fig. 2C), PEG-PH20 treatment of WT ECs significantly (*, p < 0.05) reduced the number of slow rolling cells to a similar extent as observed in both the vehicle and PEG-PH20-treated CD44−/− ECs (Fig. 4B). No significant differences were observed between groups at the medium and fast rolling speeds. Taken together, these results demonstrate that HA tethered to CD44 on the surface of brain ECs is involved in the recruitment and rolling of lymphocytes.
Subcutaneous Treatment with PEG-PH20 Delays the Onset of EAE but Does Not Ameliorate Later Disease—Because degradation of HA by PEG-PH20 leads to decreased lymphocyte binding and rolling on brain ECs in vitro, we hypothesized that degradation of HA by PEG-PH20 would ameliorate the onset and severity of EAE in vivo. To test the ability of PEG-PH20 to remove HA from the luminal surface of CNS vessels, naive animals were subcutaneously injected every other day with PEG-PH20 or vehicle for 6 days. Spinal tissue was harvested and labeled for CD31 and HA (supplemental Fig. S2). Vehicle-treated vessels displayed a thin band of HABP label along the luminal surface (supplemental Fig. S2, A–C), whereas PEG-PH20-treated vessels were devoid of HABP within the vessel lumen (supplemental Fig. S2, D–F). These results demonstrate that subcutaneous PEG-PH20 administration removes HA from the lumen of CNS blood vessels.

Mice with EAE were injected with either vehicle (PBS) or PEG-PH20 every other day beginning 7 days post-MOG inoculation. Similar to our observations in CD44−/− mice, clinical disease signs of EAE were delayed in animals treated with PEG-PH20 compared with animals treated with vehicle (Fig. 5). Mean clinical disease scores were also significantly lower on both days 11 and 12 in PEG-PH20-treated animals as compared with controls (*, p < 0.05; Fig. 5). Clinical scores of PEG-PH20-treated animals lagged behind vehicle-injected animals until day 19 postinoculation. At later times in disease progression, PEG-PH20-treated animals demonstrated more severe symptoms, starting at day 19 and became significant by day 21 (*, p < 0.05; Fig. 5).

At 8 days postinoculation, lumbar spinal tissues from control and PEG-PH20-treated animals contained no histological evidence of CD4+ cell extravasation within the spinal cord (data not shown). By day 13 postinoculation, demyelinated lesions in vehicle-treated mice, visualized by staining with FluoroMyelin, contained numerous CD4+ cells (Fig. 6, A and B). In addition to having lower clinical scores, there was a significantly less demyelination (69.1% less than controls; p < 0.005; Fig. 6, A and C) and fewer infiltrating CD4+ cells (2.3-fold fewer cells; p < 0.005; Fig. 6, B and D) in the spinal cords of PEG-PH20-treated mice.
To address the possibility that delayed disease onset in PEG-PH20-treated animals is related to reduced axonopathy, spinal cords from naive, day 13 control and PEG-PH20-treated animals were labeled with neurofilament-L. Representative, high magnification images of similar sized lesion areas are illustrated in supplemental Fig. S3. PEG-PH20-treated animals had fewer dysmorphic axons than controls (supplemental Fig. S3, F and E, red arrows). Additionally, more denuded axons were evident in PEG-PH20-treated lesions than controls (supplemental Fig. S3, H and I, white arrows).

Stereologic analysis of spinal cords from mice with EAE 21 days postinoculation revealed that PEG-PH20 treatment continued to significantly reduce the degree of demyelination albeit to a lesser extent than at day 13 (24.4% smaller lesion volume than controls; Fig. 7, A versus C and E). However, consistent with the elevated disease scores at this time point, these animals demonstrated significantly increased numbers of infiltrating T-cells (1.9-fold more than controls; Fig. 7, C versus D and F). Additionally, the number of neurofilament-L positive axons proximal to and within areas of demyelination was visually indistinguishable between treatment groups (supplemental Fig. S3, J and K).

Overall, these data suggest that PEG-PH20 treatment effectively delays disease onset by impairing T-cell extravasation resulting in less CNS inflammation, demyelination, and axonopathy early in disease. However, prolonged administration of PEG-PH20 increases CNS inflammation resulting in more severe clinical disease and negates/diminishes, respectively, its benefit to axon survival/demyelination observed in early disease.
HA Is Removed from CNS Blood Vessels at EAE Day 13 by PEG-PH20 but Is Re-expressed at EAE Day 21—To confirm that PEG-PH20 treatment leads to chronic reductions in HA in the lumen of CNS vessels, lumbar spinal tissue from animals with EAE was harvested from PEG-PH20-treated and vehicle-treated mice at 13 days postinoculation and labeled for ECs, T-cells, and HA (Fig. 8, A–F). A representative image of a blood vessel from a vehicle-treated animal shows HA labeling within the EC lumen adjacent to CD31/H11001 tight junctions (Fig. 8A). Additionally, CD4/H11001 cells were often observed (18/32 vessels) co-localizing with HA within the lumen of the vessel (Fig. 8B and C, arrow and arrowhead). CD4+ cells are also observed in the perivascular space co-localized with high levels of HA, as is typical of active EAE lesions (Fig. 8B and C, curved arrows). In contrast, HA is undetectable within the luminal area of blood vessels from PEG-PH20-treated animals (Fig. 8D). No CD4+ cells are observed within the lumen of PEG-PH20-treated vessels (0/26 vessels), however, some CD4+ cells are present in HA-rich regions within the perivascular area (Fig. 8, E and F, asterisks).

A possible explanation for increased T-cell infiltration into the CNS of mice with EAE following prolonged PEG-PH20 treatment is that HA is re-expressed in the lumen of CNS vessels at later times of disease progression. To address this possibility, CNS vessels from day 21 EAE animals treated with vehicle or PEG-PH20 were examined for HA expression. We find that HA is present along the lumen surface (arrows) in both vehicle- and PEG-PH20-treated EAE day 21 vessels (Fig. 9, A and D), but that the intensity of staining in both cases is significantly less than is observed in vessels from day 13 vehicle-treated EAE animals. Consistent with the low intensity HA staining, few vessels in vehicle-treated day 21 EAE mice contained CD4+ cells interacting with the luminal surface (Fig. 9B and C, 2/34 vessels). However, despite the relatively weak HA label, vessels from PEG-PH20-treated day 21 mice often had CD4+ cells interacting with the luminal surface (14/27 vessels, Fig. 9E and F, block arrow and arrowheads).

Given that CNS ECs increase expression of HAS1 mRNA in response to proinflammatory stimulation (Fig. 3E), we reasoned that HAS1 expression may be elevated in EAE day 21 PEG-PH20-treated vessels relative to PBS EAE controls. We examined HAS1 expression in EAE day 21 vessels via immunohistochemistry and found HAS1 expression was higher in PH20-treated vessels relative to PBS controls (Fig. 10, D versus A). Additionally, HA was observed in the luminal area of vehicle- and PEG-PH20-treated vessels (Fig. 10, B and E), but only co-localized with HAS1 labeling in PEG-PH20-treated vessels suggesting the HA in those vessels may be newly synthesized (Fig. 10E, white arrows).

These results are consistent with the hypothesis that PEG-PH20 degrades HA from the vessel lumen in early disease, impairing T-cell extravasation. However, HA is re-expressed within the lumen of PEG-PH20-treated vessels at EAE day 21 due to increased HAS1 expression. HA re-expression may therefore account for the elevated disease score and the increased number of T-cells observed within the spinal cord.
DISCUSSION

We have demonstrated that HA and the standard form of CD44 on the luminal surface of CNS ECs contribute significantly to the initial capture and subsequent rolling of encephalitogenic lymphocytes in an inflammatory demyelinating disease model. We found that both CD44^+/H11002^/H11002^ animals and animals treated with a hyaluronidase experience delayed onset of EAE that could be accounted for by reduced numbers of encephalitogenic lymphocytes in demyelinating lesions. Our *in vitro* data indicate that CD44 expressed by stimulated brain ECs, but not by stimulated lymphocytes, contributes to lymphocyte rolling on ECs, and that this effect depends on HA being anchored to CD44 on the ECs. Although this finding is novel in regard to CNS disease, a role for endothelial HA tethered to CD44 in lymphocyte extravasation has also been observed in a model of arthritis (30). Together, these data support the idea that HA anchored to the CNS endothelial surface is a critical mediator of encephalitogenic immune cell trafficking in multiple organs and may represent a therapeutic target to treat inflammatory disease.

The finding that CD44 expressed by activated lymphocytes was dispensable for lymphocyte-EC interactions implies that lymphocytes possess an alternative mechanism for HA binding. One candidate HA-binding protein is the receptor for hyaluronan-mediated motility, which is expressed by developing CD3^+^ thymocytes, and is reported to be largely absent from mature lymphocytes (31–33). Like CD44, the receptor for hyaluronan-mediated motility can be expressed at the cell surface where it can bind and tether HA and is capable of signaling through Src kinase to influence cytoskeletal rearrangement (34). If receptor for hyaluronan-mediated motility expression was retained beyond thymic development in CD44^-/-^ lymphocytes, it could account for the lack of phenotype observed in our flow model. Another candidate is the HA receptor for endocytosis. It can be expressed on the cell surface, but it predominately localizes within sinusoidal endothelia and its expression by lymphocytes has not been reported (35, 36). Interestingly, antibody blockage of HA binding to HA receptor for endocytosis impairs tumor cell metastasis, a process likened to active lymphocyte extravasation (37, 38). HA receptor for endocytosis is also capable of initiating intracellular signaling via MAP kinases (39). If a redundant or compensatory mechanism for HA binding on activated lymphocytes exists, it may function beyond the initial capture and rolling of lymphocytes to influence cellular rearrangement in preparation for extravasation.

**FIGURE 8.** Subcutaneously injected PEG-PH20 degrades and removes HA from CNS blood vessels of day 13 EAE animals. Representative images of CNS blood vessels (CD31, white) from EAE animals treated with PBS (vehicle, A–C) or PEG-PH20 (D and E) are shown. In vehicle-treated EAE vessels, HA (red) is present in the vessel lumen (A, arrows) and frequently co-localizes with CD4^+^ (green) T-cells (B, block arrows, 18/32 vessels). The orthogonal view (C) demonstrates a CD4^+^ T-cell contacting HA tethered to the endothelial lumen proximal to a CD31 positive tight junction (arrowhead). In PEG-PH20-treated vessels, HA is absent from the vessel lumen (D). CD4^+^ T-cells were never visualized contacting the vessel lumen (E and F, 0/26 vessels). CD4^+^ cells were only found in the immediate perivascular area (E, asterisks) and not widespread throughout the demyelinating lesion as in vehicle-treated animals (B, curved arrows). Scale bar is 25 μm. Images are 8-μm z-projections.
PEG-PH20 treatment of mice with EAE results in the degradation and removal of HA from the luminal surface of spinal cord vessels after 6 days of treatment (EAE day 13). However, after 14 days of PEG-PH20 treatment (EAE day 21), HAS1 protein expression was elevated in the vessel lumen relative to PBS controls and HA was re-expressed in the same area. Coincident with HA re-expression, the number of CD4⁺/H11001 infiltrates within the spinal cord of PEG-PH20-treated animals was elevated relative to controls. The mechanism underlying this reversal in disease progression is unknown. It is possible that degradation products of the injected enzyme may be signaling back onto ECs to enhance HAS1 expression. A likely candidate receptor is Toll-like receptor (TLR) 4, which induces NF-κB-dependent signaling in response to HA fragments resulting in proinflammatory cytokine production (40). Indeed, cytokines such as TNFα have been shown to increase HAS expression and HA synthesis on human ECs resulting in enhanced monocyte adhesion (41).

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Additionally, HA signaling through TLR4 on ECs increases the expression of other molecules involved in immune cell extravasation. For example, in dermal ECs, TLR4 signal induced by HA fragments increases expression of the cell adhesion molecule ICAM-1 and the chemokine MIP-2, which are involved in the recruitment of neutrophils (42). CD44/HA interactions mediate neutrophil and lymphocyte adhesion and migration across endothelium (43). Additionally, siRNA inhibition of endogenous hyaluronidases in fibroblasts subjected to collagen-induced arthritis decreases TLR4 expression, NF-κB activation, and IL-1β production elicited by TNFα stimulation (44).

An alternative explanation for enhanced T-cell infiltration in PEG-PH20-treated EAE animals at day 21 may be that the injected enzyme is being inactivated by the endogenous humoral response. Both nonhuman primates and mice have been shown to generate anti-PH20-specific antibodies against injected recombinant PH20 protein (45, 46). Interestingly, co-injection of recombinant protein with an adjuvant results in higher anti-PH20 titers. Because our subcutaneous PEG-PH20 injections were made coincident with adjuvant injections, the humoral response against PEG-PH20 may have been enhanced.

Using in vitro and in vivo approaches, we determined that HA anchored to standard CD44 on CNS endothelial cells contributes to the adhesion and subsequent extravasation of lymphocytes. However, ~50% of activated lymphocytes are still capable of slow rolling in our blood flow model and PEG-PH20-treated animals still develop EAE. The question remains what
other cell adhesion molecules contribute to the capture and rolling of encephalitogenic T-cells? Endothelial P-selectin and its T-cell ligand, PSGL-1, have been implicated in the development of EAE but this is a contentious issue. Several studies, using P-selectin \(^{-/-}\) animals and blocking antibodies downplay the importance of these molecules in EAE development (9, 10), whereas others contend they are critical mediators of this process (6, 7). A recent study claims that P-selectin facilitates the ability of T-cell \(^{4}\) integrin to induce firm adhesion of T-cells to CNS endothelium, but is not itself critical for the induction of EAE (8). These conclusions stem from the finding that P-selectin \(^{-/-}\) animals have delayed EAE onset but eventually develop severe disease. Additionally, if blocking antibodies to P-selectin and \(^{4}\) integrin are co-injected, animals have delayed onset and quicker recovery than \(^{4}\) integrin antibodies alone. The authors of this study speculate that P-selectin facilitates firm adhesion by mediating capture and rolling of T-cells to CNS endothelium, but is not itself critical for the induction of EAE (8). These conclusions stem from the finding that P-selectin \(^{-/-}\) animals have delayed EAE onset but eventually develop severe disease. Additionally, if blocking antibodies to P-selectin and \(^{4}\) integrin are co-injected, animals have delayed onset and quicker recovery than \(^{4}\) integrin antibodies alone. The authors of this study speculate that P-selectin facilitates firm adhesion by mediating capture and rolling of T-cells to the endothelial surface but that \(^{4}\) integrin mediates encephalitogenic T-cell extravasation into CNS tissue. These results mirror closely our own findings with regard to EAE in CD44 \(^{-/-}\) animals and suggest that HA tethered to CD44 on endothelial cells may be functioning to capture T-cells and facilitate signaling to induce high affinity \(^{4}\) integrin expression in inflammatory disease. Due to the complex nature of T-cell capture, rolling, and adherence to the CNS endothelium, a potential therapeutic strategy may include the blockage of a combination of CAM molecules so far discussed. Further experiments may be required to reveal additional mechanisms involved in this process.

Axonal degeneration is responsible for chronic disability in MS and EAE (50). We attempted to quantify neurofilament-L labeling but due to the extensive and highly variable axonal damage were unable to produce reliable counts. We

![FIGURE 10. CNS blood vessels of PEG-PH20-treated EAE animals express more HAS1 21 days postinoculation than PBS controls.](image)

Representative images of CNS blood vessels (CD31, white) from EAE animals treated with PBS (vehicle, A–C) or PEG-PH20 (D and E) are shown. In PBS-treated EAE vessels, HAS1 (green) and HA (red) are present in the vessel lumen (A and B), but no obvious co-localization is observed (B and C). HAS1 staining in PEG-PH20-treated vessels (D) is brighter than in PBS controls (A) and areas of co-localization with HA are observed (E, block arrows, and F). Co-localization may indicate that HA within this vessel is newly synthesized. Scale bar is 25 µm. Images are 6-µm z-projections.
determined that the quality and number of axons in and around lesions was increased with PEG-PH20 treatment on day 13 post-inoculation. However, the causative relationship between PEG-PH20 treatment and reduced axonal damage is unclear due to fewer CD4+ cells in the spinal cord at this time point. A recent EAE study demonstrates foci of infiltrating immune cells correlate with locally impaired axonal transport and markers of axonal damage at disease onset (51). It is likely the increase in spared axons is related to lower levels of inflammation within demyelinating lesions and not directly attributable to the activity of PEG-PH20. This idea is supported by the fact that the quality and number of axons in and around lesions was indistinguishable between treatment groups at 21 days postinoculation. These findings are reminiscent of other studies inhibiting immune cell infiltration where axonopathy continues despite decreased CNS inflammation (52).

Our experiments showed that both CD44+/− and PEG-PH20-treated mice eventually became symptomatic and attained disease scores equivalent to controls. A recent study (25) reported a delay in onset of disease, which the authors did not discuss further, coupled with prolonged attenuation of symptoms when EAE was induced in CD44+/− animals. The authors conclude that signaling through CD44 in encephalitogenic CD4+ T-cells results in epigenetic changes that drive differentiation of the cell toward a Th1/Th17 proinflammatory phenotype. Although such phenotypic shifts may contribute to EAE progression, results from our group and others (22–24) indicate that CD44 also plays a significant role in the initiation of T-cell rolling on ECs and, therefore, the extravasation of encephalitogenic lymphocytes into the CNS.

It is possible that the difference between our studies is related to differences in the severity of EAE that is being induced. Guan et al. (25) reported a maximal mean disease score of ~3.25 in WT controls and ~1.15 for CD44−/−, whereas our animals reached a maximal mean score of 4.33 for controls and 4.40 for CD44−/−. Recently it was shown that the monosaccharide N-acetylglucosamine, a component of HA, effectively inhibited T-cell activation and ameliorated MOG35–55 induced C57BL/6 EAE. However, treatment is clinically ineffective if EAE is induced in 2D2 T-cell receptor transgenic mice, which display more robust disease (53). The severity of EAE disease is known to vary depending on the population of cellular infiltrates. Other studies have shown that adoptive transfer of myelin reactive CD8+ T-cells induces more severe disease than active disease induction alone (54, 55). Although we did not directly assay CD8+ cells, our ability to induce more severe disease implies a greater contribution of the CD8+ phenotype to disease pathogenicity in our model. As such, the contribution of deficient CD44 signaling to CD4+ encephalitogenic T-cell phenotype described by Guan et al. (25) may be masked in our findings. Because myelin reactive CD8+ cells are more prevalent within MS lesions than CD4+ cells (56), our findings may better reflect the potential therapeutic outcomes of manipulating CD44 or HA.

In conclusion we have demonstrated that standard CD44 on CNS ECs but not activated lymphocytes contributes to lymphocyte rolling on the endothelial surface. These findings expand the knowledge of mechanisms promoting inflammatory demyelinating CNS disease and suggest that HA anchored to EC CD44 represents a therapeutic target to reduce immune cell infiltration into the brain. Future studies aimed at testing whether transient HA degradation during early stage disease might have the potential to enhance other therapeutic agents that limit MS attacks will reveal the potential of targeting HA as a means of limiting disease severity.

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