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Short-term sPECAM-Fc treatment ameliorates EAE while chronic use hastens onset of symptoms

Emily K. Reinke^{1,3}, JangEun Lee^{1,2}, Alla Zozulya¹, Jozsef Karman^{1,2}, William A. Muller⁴, Matyas Sandor^{1,2}, and Zsuzsanna Fabry^{1,2,3}

1 Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison WI

2 Cellular and Molecular Pathology Training Program University of Wisconsin-Madison; Madison, WI

3 Neuroscience Training Program University of Wisconsin-Madison, Madison, WI

4 Department of Pathology, Weill Medical College of Cornell University, New York, NY 10021

Abstract

The homotypic cell adhesion molecule PECAM-1 is a major participant in the migration of leukocytes across endothelium. We examined the ability of a chimeric soluble sPECAM-1 fused to human IgG-Fc to impair leukocyte entry through the blood-brain barrier and reduce CNS autoimmunity. sPECAM-Fc impaired migration of lymphocytes across brain endothelial monolayers and diminished the severity of EAE, an experimental model of MS, when administered at the onset of symptoms. However, in mice transgenic for sPECAM-Fc, the chronically elevated levels of sPECAM-Fc hastened onset of EAE disease without significantly changing clinical score severity. Our data suggests that short-term treatment of diseases like MS with sPECAM-Fc has therapeutic potential.

Keywords

PECAM-1; EAE; sPECAM-Fc; MS; cellular migration

1. Introduction

Platelet endothelial cell adhesion molecule (PECAM)-1 (CD31) is a cell adhesion molecule found on leukocytes and vascular endothelium. Homophilic interactions between PECAM-1 on both leukocyte and endothelium is necessary for the migration of leukocytes through the vasculature from blood to tissue (Muller et al., 1993). In CNS autoimmunity, leukocytes migrate through the blood-brain barrier, a unique vascular structure, to enter the brain and

Corresponding Author: Zsuzsanna Fabry, University of Wisconsin-Madison, Department of Pathology and Laboratory Medicine, 6130 Medical Sciences Center, 1300 University Ave, Madison, WI 53706, Office: (608) 265-8716, Lab: (608) 262-0812, Fax: (608) 262-0846, zfabry@facstaff.wisc.edu

Emily K. Reinke, Alla Zozulya, JangEun Lee, University of Wisconsin-Madison, Department of Pathology and Laboratory Medicine, 6330 MSC, 1300 University Ave, Madison, WI 53706

Jozsef Karman, Current address: Harvard University, Brigham and Women's Hospital, Center for Neurologic Diseases, 77 Avenue Louis Pasteur, HIM 785, Boston, MA 02115

William A. Muller, Cornell University Weill Medical College, Dept of Pathol & Lab Med, 1300 York Ave C-312, New York, NY 10021
Matyas Sandor, University of Wisconsin-Madison, Department of Pathology and Laboratory Medicine, 5468 MSC Medical Sciences Center, 1300 University Ave, Madison, WI 53706

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spinal cord. Soluble (s)PECAM-1 has the potential to compete with and interfere with the PECAM-PECAM interactions between the brain microvascular endothelial cells and the entering leukocytes and may reduce the number of invading cells and improve clinical outcome.

Several *in vitro* and *in vivo* studies have examined the effects of PECAM-1 inhibition on cellular migration across endothelial cells or vessels. *In vitro*, sPECAM-1 and anti-PECAM-1 antibodies inhibit the passage of immune cells across layers of endothelial cells (Goldberger et al., 1994; Liao et al., 1995; Liao et al., 1997; Muller et al., 1993; Wong et al., 1995). *In vivo* in several species, treatment with sPECAM-1 blocks leukocyte entry into the peritoneum in response to thioglycolate (Bogen et al., 1994; Liao et al., 1999; Schenkel et al., 2004), lungs in response to immune complexes (Vaporciyan et al., 1993) and muscle in response to ischemia/reperfusion injury (Farooq et al., 2001; Gumina et al., 1996; Murohara et al., 1996). Additionally, T cell accumulation in the cerebral spinal fluid in response to infused antigen is impaired in the presence of exogenous sPECAM-Fc or anti-PECAM-1 antibody (Qing et al., 2001). These studies promote the hypothesis that sPECAM-1 may ameliorate MS pathology.

The role of PECAM-1 in experimental autoimmune encephalomyelitis (EAE) has partially been examined. The use of anti-PECAM antibody had no effect on EAE in rats, although this antibody was not shown to be effective at blocking leukocyte migration (Williams et al., 1996). Furthermore, PECAM-1 deficient animals had an earlier onset of EAE clinical signs compared to wild-type animals (Graesser et al., 2002). This effect was attributed to increased vascular permeability, particularly of the blood-brain barrier, in the PECAM-1 deficient mice. Similar results were seen in studies of PECAM-1 deficient mice in collagen induced arthritis models (Tada et al., 2003; Wong et al., 2005). Both of these studies were carried out in the C57Bl/6 strain of mice, which are unique in that they do not respond to PECAM blockade in several inflammatory models (Schenkel et al., 2004). However, these studies suggest that PECAM-1 interactions could play an important role in autoimmune responses.

We examined the therapeutic potential of a chimeric soluble PECAM-1 fused human IgG-Fc in EAE. We found that sPECAM-Fc was able to impair migration of lymphocytes across brain endothelium *in vitro* and was able to reduce the severity of clinical symptoms in SJL mice treated at the onset of disease. To examine the effect of continued sPECAM-Fc therapy, we examined EAE symptoms in transgenic mice secreting serum sPECAM-Fc. Interestingly, animals with long-term elevated levels of sPECAM-Fc experienced earlier onset of symptoms. Our data suggest that sPECAM-Fc may be an efficacious acute, but not extended, therapy for multiple sclerosis (MS).

2. Materials and Methods

2.1 Mice

SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice transgenic for a chimeric, soluble, murine PECAM-1 fused to the Fc region of human IgG₁ (Liao et al., 1999) were backcrossed six generations from the FVB/n to the SJL/J background. Expression of the sPECAM-Fc was monitored by serum ELISA using goat anti-human IgG-Fc γ fragment specific antibodies (Jackson ImmunoResearch, West Grove, PA) at Weill College of Medicine (New York, NY). Transgene expression in the colony was heterogeneous, producing offspring of varying levels of transgene expression. "High" expression of sPECAM-Fc in serum was defined as 9-12.5 μ g/ml. "Low" expression was defined as 2-9 μ g/ml. Mice used in EAE experiments were 7-13 week old females. The University of Wisconsin-Madison School of Medicine and Public Health Institutional Animal Care and Use Committee approved all experimental protocols.

2.2 Migration assay

Brain endothelial cells were harvested as described (Deli et al., 2003; Deli and Joo, 1996). In brief, brain cortexes of adult female SJL/J mice were isolated and processed. Brain tissue microvessels were plated on to μ m Transwells® (Corning, Acton, MA) precoated with fibronectin and collagen IV (Sigma-Aldrich) in 20% fetal bovine serum in DMEM supplemented with 2mM L-glutamine, 1ng/ml basic fibroblast growth factor (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, IN) and antibiotics. Cells were incubated in the presence of 4 μ g/ml puromycin (Sigma-Aldrich) for two days to eliminate contaminating pericytes. Two days prior to use, cell monolayers were cultured in serum-free DMEM-HAM's-F12 media containing L-glutamine and antibiotics and supplemented with 550 nM hydrocortisone (Sigma-Aldrich) (Weidenfeller et al., 2005). Transendothelial resistance of endothelial monolayers was measured prior to use to ensure integrity of the layer. Splenocytes were isolated according to as previously described (Fee et al., 2003; Qing et al., 2001; Qing et al., 2000; Fabry et al., 1993; Fabry et al., 1990) and incubated in a plastic dish for 30 min to deplete adherent cells. Non-adherent cells were washed and counted. Immediately prior to use, the chambers were washed and the media replaced with 2% FBS DMEM-HAM's-F12 media supplemented with hydrocortisone. 2×10^5 cells were put into the upper chambers with 20 μ g/ml sPECAM-Fc (Liao et al., 1999) or purified Fc fragment of human IgG (Bethyl Laboratories, Inc., Montgomery, TX). Cells were allowed to migrate at 37°C for four hours after which the abluminal face of the Transwell was gently rinsed into the lower chamber media. Cells were then collected and counted. Experiments were repeated two times using three or four separate migration chambers.

2.3 EAE induction and evaluation

Proteolipid protein (PLP₁₃₉₋₁₅₁) synthesized by Annovis (Ashton, PA) was dissolved at 1 mg/ml in sterile PBS and mixed by sonication in equal volumes with complete Freund's adjuvant (CFA), supplemented with Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) to 5 mg/ml. 100 μ l of the homogenized solution was injected subcutaneously between the shoulder blades of the mouse. Animals were coded and clinical scores were monitored on a blind manner daily beginning 5-7 days after EAE induction. Clinical scoring was as follows: 0, no motor defect; 1, tail weakness; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, complete fore limb and hind limb paralysis; 5, moribund or dead. Intermediate scores were assigned for intermediate symptoms.

2.4 Acute treatment of SJL mice with EAE

EAE was induced in SJL mice, and 200 ng of Pertussis toxin (List Biological Laboratories, Inc. Campbell, CA) was administered intraperitoneally on the day of induction and two days later. Mice were injected with 10 μ g purified sPECAM-Fc or 10 μ g human Ig-Fc intraperitoneally in 10 μ l on the day of EAE symptom onset, then every other day for a total of 5 injections. The half-life of sPECAM-Fc is ~24 hrs (Liao et al., 1999). Statistical comparison of clinical scores included calculating the average clinical score for each mouse after onset and performing a Wilcoxon analysis (Fleming et al., 2005). p values were calculated using the statistical package R version 1.12 (<http://www.R-project.org>) (R-Development-Core-Team, 2005)

2.5 Treatment of transgenic and wildtype mice

4 μ g purified sPECAM-Fc, 4 μ g human IgG-Fc, or PBS was injected intravenously in 10 μ l into "high" sPECAM-Fc expressing, "low" sPECAM-Fc expressing, or wildtype mice, respectively; every other day from day 5-13. Average clinical score was calculated for each mouse for 15 days after onset and a Kruskal-Wallis analysis was performed (Fleming et al., 2005). Onset comparisons were performed using survival analysis

2.6 Histology

At 40 days post EAE induction, spinal cords were removed from the spinal column by liquid expulsion, fixed in 10% formalin and embedded in paraffin. 1 μ m sections were cut and stained with hematoxylin and eosin (H&E) or luxol fast blue (LFB) and examined with an Olympus BX40 microscope (Olympus America Inc., Melville, N.Y.) with an Olympus Qcolor 3 camera (Olympus America Inc., Melville, N.Y. Images were adjusted in Adobe Photoshop (Adobe Systems, Inc.)

3. Results

3.1 Migration of lymphocytes across brain endothelium *in vitro* in the presence of sPECAM-Fc

sPECAM-Fc is capable of blocking migration of monocytes and neutrophils across human umbilical vein endothelial cells (Liao et al., 1997), and anti-PECAM antibody is capable of reducing T cell migration across human brain microvessel endothelial cells (Wong et al., 1999). We investigated whether sPECAM-Fc would also block migration of lymphocytes across a primary murine brain endothelial layer. Isolated murine SJL/J brain microvessels were grown to confluence on μ m Transwell® membranes and hydrocortisone was used to induce blood-brain barrier-like characteristics (Weidenfeller et al., 2005). Splenocytes harvested from a naïve SJL/J were enriched for lymphocytes by plastic adherence. Purity of lymphocytes reached approximately 90%. 2×10^5 cells were applied to the luminal face of the endothelial monolayer in media with either purified sPECAM-Fc or human IgG-Fc. Cells were allowed to migrate for four hours, then collected and counted. In the presence of sPECAM-Fc, 2.4 times fewer lymphocytes migrated across the endothelial cells into the lower chamber. This supports previous findings and established that *in vitro*, sPECAM-Fc blocks lymphocyte migration across brain endothelium.

3.2 Treatment of EAE with sPECAM-Fc

In order to analyze the effect of sPECAM-Fc in an autoimmune disease of the CNS *in vivo*, we tested the effect of injected sPECAM-Fc in EAE. We induced EAE in SJL/J mice using PLP₁₃₉₋₁₅₁ peptide and injected 10 μ g sPECAM-Fc or human IgG-Fc intraperitoneally on the day when clinical symptoms were detected and then every other day for a total of five injections throughout of the disease. As we demonstrate in figures 2B and C, the proportion of mice without clinical symptoms or with mild EAE (clinical score 0.5-2) was higher in the sPECAM-Fc treated groups. This also corresponded with the lower proportion of mice experiencing severe EAE (clinical score higher than 2) in this treated group. Although the initial peak of disease was similar in the sPECAM-Fc and control Fc injected groups, improvement of clinical signs in the sPECAM-Fc treated group was evident in the relapse phase of the disease, after the injections had ended (Figure 2A). These data indicate that the inhibition by sPECAM-Fc is capable of providing a lasting clinical improvement of ongoing EAE disease *in vivo*.

3.3 EAE in transgenic mice with elevated levels of sPECAM-Fc

In the above experiments, soluble PECAM levels *in vivo* were acutely increased by injection of sPECAM-Fc. To test whether sPECAM-Fc would be more effective with extended sPECAM-Fc exposure, we utilized transgenic mice expressing serum sPECAM-Fc (Liao et al., 1999) bred onto the SJL/J background. These mice allowed us to avoid complications from an accumulation of potentially blocking antibodies to the exogenous human portion of the protein chimera that might develop with many injections in a wildtype animal and kept stress to the animals at a minimum. To avoid possible complications from parental strain influences, wildtype littermates were used as controls. Mice from the colony were divided into three groups based on serum level of sPECAM-Fc. High sPECAM-Fc expressing mice were defined as

having serum levels of sPECAM-Fc ranging from 9-12.5 μ g/ml, and low expressors defined as having levels ranging from 2-9 μ g/ml. Wildtype mice had no detectable sPECAM-Fc. Initial experiments with low sPECAM-Fc expressing and wildtype mice indicated that sPECAM-Fc at a concentration in serum between 2-9 μ g/ml is not sufficient to significantly alter the disease course from that seen in wildtype mice (Figure 3).

To increase the likelihood of protection, we supplemented the serum level of sPECAM-Fc in high producing mice to raise it closer to the level found previously to be effective in blocking transmigration after thioglycollate challenge (Laio et al., 1999). Every other day from day 5-13, mice received an intravenous injection of 4 μ g sPECAM-Fc into high expressors, 4 μ g of human IgG-Fc into low expressors, or PBS into wildtype. The mice were monitored for clinical symptoms until day 40. Onset of EAE clinical symptoms was on average 2-3 days earlier in the high expressing mice treated with additional sPECAM-Fc (Figure 4), indicating that in contrast to the results with acute treatment after disease onset, chronically elevated levels of sPECAM-Fc were not protective. However, the severity of disease post-onset was unaffected by the presence of increased sPECAM-Fc. On average, within the first fifteen days after onset, all three groups experienced similar numbers of days with severe symptoms (clinical scores of two or above); mild symptoms (clinical scores less than two, but more than zero); or without discernable disability. This indicates that chronically high levels of sPECAM-Fc were no longer an effective inhibitor of clinical symptoms in EAE.

4. Discussion

Multiple lines of *in vitro* and *in vivo* evidence suggest that blocking PECAM-PECAM interactions between leukocytes and endothelium either through anti-PECAM-1 antibodies or through the use of recombinant sPECAM-1 constructs diminishes migration of leukocytes across endothelium (Liao et al., 1995; Liao et al., 1997; Muller et al., 1993; Wakelin et al., 1996; Wong et al., 1999). We investigated the hypothesis that blocking PECAM-PECAM interactions could improve clinical outcome in EAE.

We first addressed whether our sPECAM-Fc would block lymphocyte migration across brain endothelial cells *in vitro* using a Transwell® assay. We determined that passage of murine lymphocytes across murine brain endothelium was diminished in the presence of sPECAM-Fc, similar to PECAM blocking experiments using monocytes and neutrophils on human umbilical vein endothelial cells (Liao et al., 1995; Liao et al., 1997) and T cells on human brain endothelium (Wong et al., 1999). Thus, we had additional support for the hypothesis that sPECAM-Fc might prevent mice from developing EAE or at least diminish the severity of the disease by impairing cell entry into the CNS.

We then investigated whether the blocking effect of sPECAM-Fc seen *in vitro* would be sufficient to change the course of infiltration and destruction seen in CNS autoimmunity using EAE in SJL mice. We treated animals with a regimen of five injections of sPECAM-Fc over nine days beginning at the onset of symptoms. We found that sPECAM-Fc was effective *in vivo* and capable of reducing the severity of clinical disease, even after the treatment was no longer being administered. This data shows that acutely administered sPECAM-Fc is a novel treatment with potential as a therapeutic agent for MS.

Evaluation of sPECAM-Fc as a possible long-term therapy was evaluated using sPECAM-Fc transgenic mice. Unexpectedly, sPECAM-Fc did not show a protective effect in the transgenic animals, but instead allowed an earlier onset of disease onset without changing the disease severity. Earlier onset of EAE without an overall increase in disease severity was also described in the PECAM-1 deficient strain (Graesser et al., 2002). In their paper, the authors conclude that the accelerated onset of EAE in the absence of PECAM-1 is due to brain microvessel

hyperpermeability in response to inflammatory stimuli. In previous studies, sPECAM-Fc transgenic mice had reduced migration into the peritoneum in response to acute inflammatory challenge (Liao et al., 1999), indicating that sPECAM-Fc transgenicity does not lead to vascular hyperpermeability. Our data suggest, therefore, that long-term sPECAM-Fc exposure can contribute to the onset of the inflammatory process.

Interestingly, a number of pro-inflammatory effects have been shown when cross-linked anti-PECAM antibodies were used to stimulate PECAM-1 outside-in signaling. For example, when macrophages or neutrophils were treated with anti-PECAM antibodies an increased CD11b/CD18 activity (Berman and Muller, 1995; Elias et al., 1998) and pro-matrix metalloproteinase-9 production were observed (Nelissen et al., 2002), in parallel with elevated superoxide and TNF- α (Elias et al., 1998). Activation of naïve T cells can also be potentiated using anti-PECAM-1 antibodies (Elias et al., 1998). We measured T cell activation markers in sPECAM-Fc transgenic and wildtype mice (data not shown). Brains and spleens from EAE mice were harvested 40 days after EAE induction. Expression of IFN- γ and high LFA-1 were measured as indicators of activation. The proportion of LFA-1 high, CD4+ cells in the spleen was unchanged among groups. Similarly, in brain we did not observe changes in the PLP₁₃₉₋₁₅₁-induced IFN- γ + CD4+ populations in the transgenic mice (data not shown). It seems unlikely then, that sPECAM-Fc has an effect on T cell activation once the disease has been initiated. We cannot exclude that with extended exposure, sPECAM-Fc may be signaling through PECAM-1 on leukocytes to contribute to initial inflammatory responses in mice chronically producing sPECAM-Fc *in vivo*.

Accumulation of sPECAM-1 in blood is a characteristic of diseases effecting endothelium. An increase of serum levels of sPECAM-1 is well documented in MS (Losy et al., 1999; Jimenez et al., 2005; Minagar et al., 2001; Kuenz et al., 2005.), although studies have not been able to link genetic changes in PECAM-1 with MS (Sciacca et al., 2000; Nelissen et al. 2000.) In addition to MS, increased levels of sPECAM-1 are detected in serum after acute myocardial infarction (Serebruany et al., 1999b; Soeki et al., 2003), congestive heart failure (Serebruany et al., 1999a), stroke (Zaremba and Losy, 2002), and umbilical placental vascular disease (Wang et al., 2002). This indicates that elevated sPECAM-1 is naturally associated with disease states. It is possible that chronic elevation of sPECAM-1 can be an inflammatory mediator and not merely a marker of chronic inflammation, although the mechanisms for this remain to be identified.

Our data together suggest sPECAM-1 acts both to enhance and curb inflammatory processes. sPECAM-Fc is a new and exciting possible treatment for MS during acute attack, but should not be used chronically or in periods of remission as it may also have long-term proinflammatory effects.

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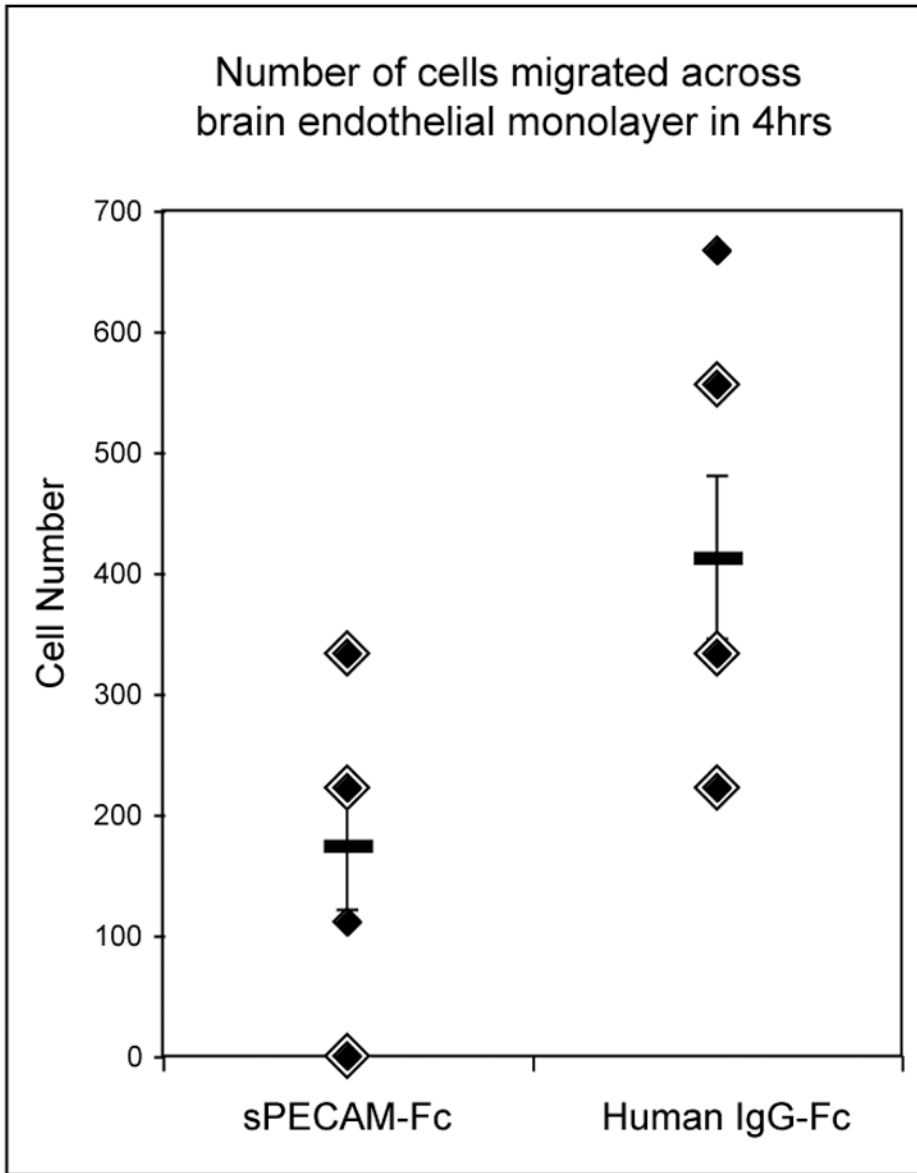


Figure 1. sPECAM-Fc blocks migration of lymphocytes across an *in vitro* murine brain endothelial cell monolayer. Lymphocytes were incubated for four hours in QCM™ 24-well Invasion Assay chambers with endothelial cell monolayers in the presence of 20 μ g/ml sPECAM-Fc or human IgG-Fc. Migrated cells were collected, washed, and counted as described in Material and Methods. In the presence of sPECAM-Fc, on average, 2.4 times fewer cells migrated across the monolayers. Error bars represent the standard deviation. $p < 0.02$ Double size diamonds indicate a second measurement of the same magnitude.

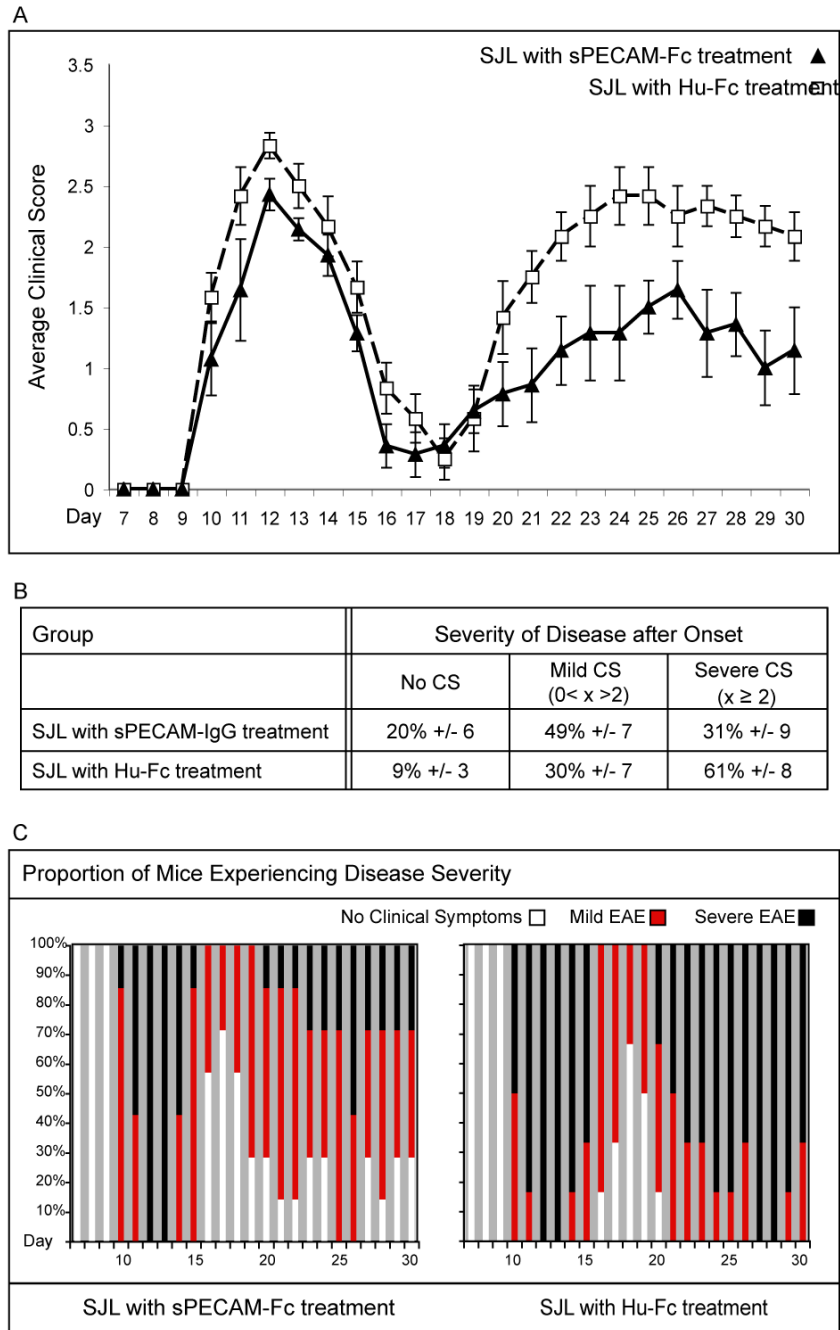
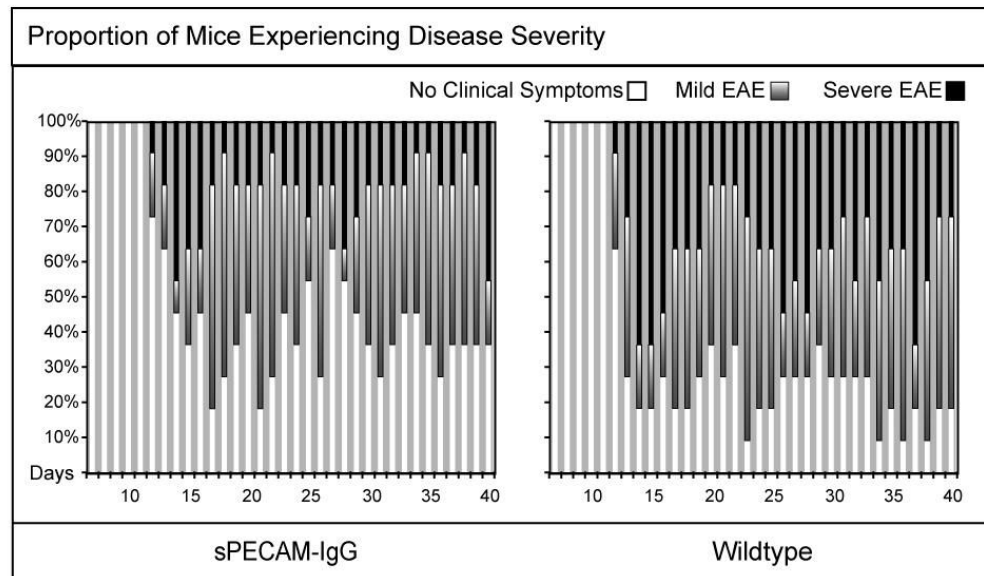


Figure 2. SJL mice treated with sPECAM-Fc at symptom onset have improved clinical symptom severity. **A.** Daily mean clinical score for each group is plotted. Error bars represent the standard error of the mean. $p = 0.02$, $n = 7$ PECAM-IgG treated, 6 human IgG-Fc, combined from 2 independent experiments. **B.** The average percentage of time after onset that mice in each group experienced no clinical symptoms, mild EAE with clinical symptoms greater than zero and less than 2, and severe EAE with clinical symptoms of 2 and above is shown. **C.** Graphical representation shows the proportion of mice in the group on each day experiencing no onset or no clinical symptoms (white portion of bar), mild EAE (red portion of bar), or severe EAE (black portion of bar).

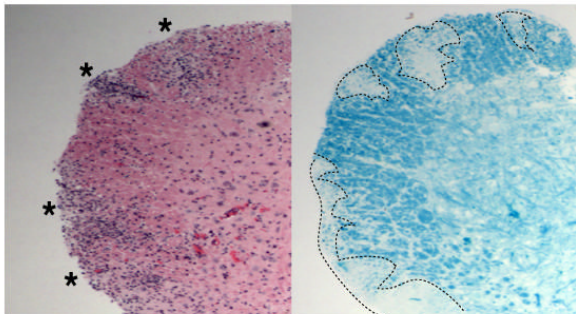
A

Group	Day of Onset						Severity of Disease after Onset		
	Average	0-15	16-20	21-26	27-40	>40	No CS	Mild CS (0 < x < 2)	Severe CS (x ≥ 2)
sPECAM-IgG	14 +/- 0.7	7/11	2/11	0/11	0/11	2/11	19% +/- 7	30% +/- 7	51% +/- 5
Wildtype	15.5 +/- 2.1	9/11	0/11	1/11	1/11	0/11	9% +/- 7	43% +/- 8	47% +/- 6

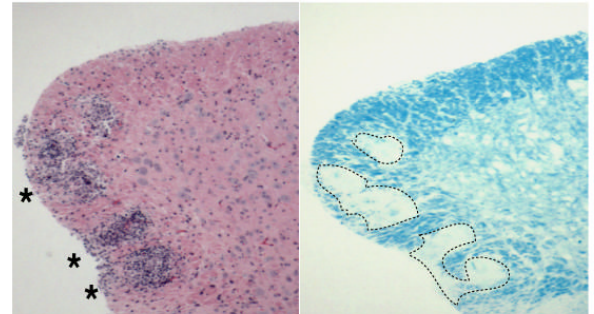
B



C



D

**Figure 3.**

sPECAM-Fc mice with 2-9 μ g/ml in serum do not have different onset or severity of EAE symptoms than wildtype mice. A. Shown is average day onset, plus or minus standard error of the mean, and the number of mice per group to experience onset within the given time frames. The average percentage of time during the fifteen days after onset that mice in each group experienced no clinical symptoms, mild EAE with clinical symptoms greater than zero and less than 2, and severe EAE with clinical symptoms of 2 and above is shown. B. Graphical representation shows the proportion of mice in the group on each day experiencing no onset or no clinical symptoms (white portion of bar), mild EAE (gray portion of bar), or severe EAE (black portion of bar). The protection afforded by the low levels of sPECAM-Fc did not reach

statistical significance. (C) Wildtype and (D) sPECAM-Fc mice. Spinal cords were removed 40 days post EAE induction. Paraffin-embedded sections were stained with H&E or LFB to detect infiltrating mononuclear cells and demyelinated areas, respectively. Both wildtype and sPECAM-Fc transgenic mice showed visible signs of infiltration and demyelination in the spinal cord.

A

Group	Day of Onset						Severity of Disease after Onset		
	Average	0-15	16-20	21-26	27-40	>40	No CS	Mild CS (0 < x < 2)	Severe CS (x ≥ 2)
High sPECAM-IgG 9-12.5 µg/ml w/ PECAM	15.1 +/- 1.0	6/10	4/10	0/10	0/10	0/10	3% +/- 2	39% +/- 9	58% +/- 11
Low sPECAM-IgG 2-9 µg/ml w/ Fc	17.3 +/- 1.8	6/14	3/14	2/14	1/14	2/14	13% +/- 7	41% +/- 9	46% +/- 11
Wildtype w/ PBS	18.3 +/- 1.9	6/14	2/14	3/14	1/14	2/14	5% +/- 2	41% +/- 9	54% +/- 10

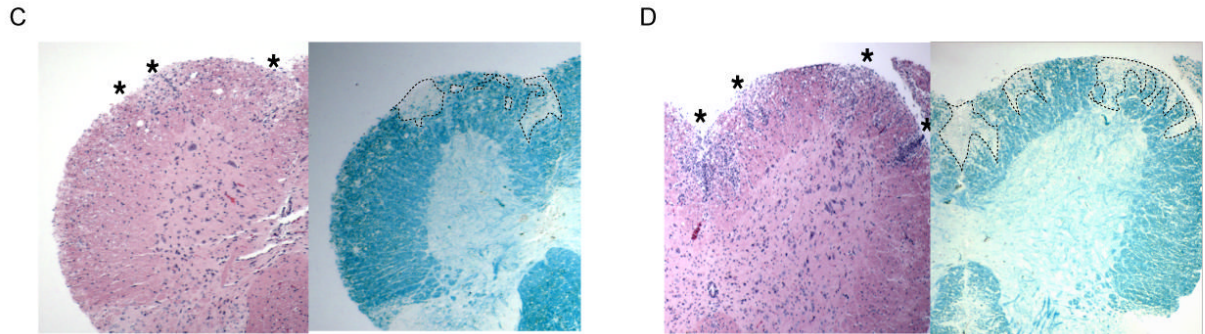
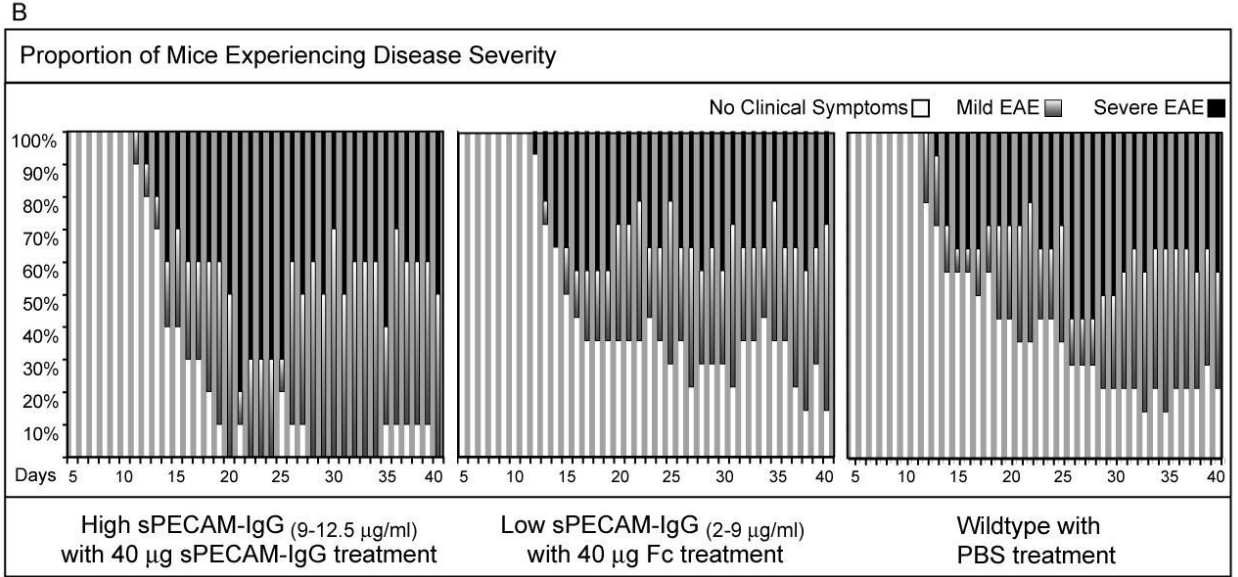


Figure 4. sPECAM-Fc treatment of high sPECAM-Fc expressing mice increases disease onset but not disease severity as compared to wildtype mice. High sPECAM-Fc expressors, low sPECAM-Fc expressors, and wildtype mice were treated 5,7,9,11,13 days after induction of EAE. High expressors received 4µg of purified sPECAM-Fc; low expressors received 4µg of human IgG-Fc; wildtype mice received PBS alone. A. Shown is average day onset, plus or minus standard error of the mean, and the number of mice per group to experience onset within the given time frames for all three groups. For wildtype to high comparison, $p=0.06$, for all other comparisons, $p > 0.1$. The average percentage of time during the fifteen days after onset that mice in each group experienced no clinical symptoms, mild EAE with clinical symptoms greater than zero

and less than 2, and severe EAE with clinical symptoms of 2 and above is shown. B. Graphical representation shows the proportion of mice in the group on each day experiencing no onset or no clinical symptoms (white portion of bar), mild EAE (red portion of bar), or severe EAE (black portion of bar). (C) Low sPECAM-Fc expressor and (D) high sPECAM-Fc expressor. Spinal cords were removed 40 days post EAE induction. Paraffin-embedded sections were stained with H&E or LFB to detect infiltrating mononuclear cells and demyelinated areas, respectively. Both low and high expressors showed visible signs of infiltration and demyelination in the spinal cord.