



Targeting endothelial CD146 attenuates neuroinflammation by limiting lymphocyte extravasation to the CNS

SUBJECT AREAS:
BLOOD-BRAIN BARRIER
MULTIPLE SCLEROSIS
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The ability to selectively block the entry of leukocytes into the central nervous system (CNS) without compromising the immune system is an attractive therapeutic approach for treating multiple sclerosis (MS). Using endothelial CD146-deficient mice as a MS model, we found that endothelial CD146 plays an active role in the CNS-directed extravasation of encephalitogenic T cells, including CD146⁺ T_{H1} and T_{H17} lymphocytes. Moreover, treating both active and passive MS models with the anti-CD146 antibody AA98 significantly decreased the infiltrated lymphocytes in the CNS and decreased neuroinflammation. Interestingly, the ability of AA98 to inhibit the migration of CD146⁺ lymphocytes was dependent on targeting endothelial CD146, but not lymphocytic CD146. These results suggest a key molecular target located on the blood-brain barrier endothelium that mediates the extravasation of inflammatory cells into the CNS. In addition, our data suggest that the AA98 is a promising candidate for treating MS and other CNS autoimmune diseases.

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system (CNS). MS is a progressive disorder characterized by perivascular leukocyte infiltration into the CNS, leading to inflammatory lesions^{1,2}. A key step in the pathogenesis of this neuroinflammatory disease is the attachment of blood-borne lymphocytes to the vasculature, followed by the diapedesis (transmigration) of immune cells across the blood-brain barrier (BBB)³. Impaired BBB function is associated with an increase in trafficking of leukocytes (T lymphocytes in particular) into the CNS, which can result in neural inflammation^{1,4}. The expression of adhesion molecules on the BBB is upregulated in response to pro-inflammatory factors—including TNF α and IFN γ —and facilitates the extravasation of leukocytes to the parenchyma of the CNS^{5,6}. For example, both intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on BBB endothelial cells mediate leukocyte transmigration during inflammation by interacting with LFA-1 and $\alpha 4\beta 1$ integrin (their respective ligands) on leukocytes^{7,8}. Other studies have shown that the interaction between endothelial CD166 and its ligands CD166 and CD6^{9,10} on leukocytes facilitates the transmigration of leukocytes across the junction barrier during the development of neuroinflammatory diseases¹¹. Thus, blocking lymphocyte-endothelial interactions is a promising therapeutic target for treating MS^{12,13}.

Most antibodies that have been studied for treating MS primarily target lymphocytes in order to block their infiltration or impair their function. One such example is natalizumab, a humanized monoclonal antibody that targets $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrin on lymphocytes and was approved in 2004 for treating MS symptoms¹⁴. Another antibody, alemtuzumab, which targets CD52 on mature B cells, T cells and natural killer cells, is also used for treating MS¹⁵. Although these antibodies have shown promise for ameliorating the symptoms of MS, they can have side effects that must be considered. Many reports have noted that the use of these antibodies can lead to immunosuppressive infections, and this has greatly limited their use in clinical settings^{16,17}. In addition, the weak efficacy of currently available immunosuppressive agents such as corticosteroids and interferon β —which are



also known to affect the host's immune system¹⁸—underscores the need to develop novel therapeutic strategies for treating MS. The ideal agent should specifically block lymphocyte transmigration and dampen neuroinflammation while sparing the host's protective immune response^{17,19}. Here, we generated a monoclonal antibody to selectively target the endothelial adhesion molecule CD146.

CD146 (also known as MCAM and Muc-18) is a member of the immunoglobulin superfamily that was originally identified as a melanoma marker and plays a role in tumor metastasis^{20,21}. We previously reported that CD146 is a novel endothelial biomarker that acts as a VEGFR2 co-receptor, playing a key role in tumor-related angiogenesis^{22,23}. Although initially considered to be an important molecule in tumor invasion and angiogenesis, CD146 is emerging as a key player in other pathological conditions, including rheumatoid arthritis²⁴, Crohn's disease²⁵ and vasculitis²⁶. Recently, Larochelle and colleagues reported that MCAM (i.e., CD146) can be used to identify encephalitogenic T cells during neuroinflammation²⁷. Their findings clarified the role that lymphocytic CD146 on T_H17 cells plays in the pathogenesis of experimental autoimmune encephalomyelitis (EAE); they also found that depleting CD146⁺ T cells reduced the disease symptoms in a transfer model of EAE. However, no study has fully elucidated the role of BBB endothelial CD146 in the pathogenesis of EAE or MS.

In this study, we investigated the role of BBB endothelial CD146 in lymphocyte extravasation and EAE pathogenesis using endothelial CD146 conditional knockout (CD146^{EC-KO}) mice to generate both active and passive models of EAE together with *in vitro* BBB models. We found that eliminating CD146 in endothelial cells significantly reduced both the severity of EAE and the infiltration of pathogenic T lymphocytes (including CD4⁺, CD8⁺, T_H1 and T_H17 cells) *in vivo* and *in vitro*. These data suggest that BBB endothelial CD146 plays a key role in the development of autoimmune diseases such as MS. Moreover, treating animals with passive or active EAE using the monoclonal anti-CD146 antibody AA98 had a clear beneficial effect on the disease symptoms. Furthermore, we found that the AA98 antibody inhibits the development of EAE by blocking lymphocyte recruitment selectively via endothelial CD146, but is independent of lymphocytic CD146. Taken together, our results suggest that the anti-CD146 antibody AA98 may be a promising therapeutic agent for treating MS by selectively targeting endothelial CD146 while sparing the host's immune response.

Results

Reduced EAE severity in endothelial CD146 knockout mice. Consistent with Larochelle et al.²⁷, we found higher levels of CD146 in BBB endothelial cells in active MS and EAE lesions compared with their respective controls (Supplementary Fig. 1a and b). Using flow cytometry, we confirmed this finding by measuring CD146 in BBB endothelial cells that were isolated from naïve mice and mice with EAE (Supplementary Fig. 1c). Together, these results suggest that endothelial CD146 may serve as a potential therapeutic target and may play an important role in the pathogenesis of MS and EAE. To examine the function of endothelial CD146 in EAE pathogenesis, we generated conditional knockout mice that lack endothelial CD146 expression (CD146^{EC-KO}; see Methods). Our first step was to use immunofluorescence to confirm that CD146 expression is selectively ablated in endothelial cells but is unaffected in other cells, including lymphocytes, the principal player in EAE pathogenesis (Supplementary Fig. 2a). Importantly, we also found no difference in either the total number of leukocytes or the percentage of T cells between CD146^{EC-KO} mice and wild type mice (Supplementary Fig. 2b and c). In addition, using flow cytometry, we confirmed that the expression of CD146 in both hematopoietic stem cells (i.e., CD117⁺CD146⁺ cells) and peripheral immune cells (including CD4⁺, CD8⁺, T_H1 and T_H17 cells) was not altered in the CD146^{EC-KO} mice (Supplementary Fig. 2d and e). These results

confirm that the CD146^{EC-KO} mouse lacks CD146 expression in their endothelial cells and that CD146 expression in their lymphocytes is intact.

Having verified our CD146^{EC-KO} mice, we next used these mice to actively induce experimental autoimmune encephalomyelitis (EAE) as a model for MS by subcutaneously injecting CD146^{EC-KO} and wild type (WT) mice with an emulsification of MOG(35–55)/complete Freund's adjuvant (to induce active EAE). The CD146^{EC-KO} and WT mice responded similarly in terms of both the timing of EAE onset and the disease incidence, for which both groups reached 100% (Fig. 1a). However, the clinical EAE scores were significantly lower in the CD146^{EC-KO} mice compared with wild type mice (Fig. 1b), indicating that the knockout mice were affected less severely by the disease. Consistent with this finding, the incidence of severe EAE (defined as a clinical score ≥ 3) was significantly lower in the CD146^{EC-KO} group during the course of EAE ($p < 0.05$) (Fig. 1c). Importantly, Luxol fast blue staining of spinal cord sections revealed fewer inflammatory lesions and smaller demyelinated areas in the CD146^{EC-KO} mice compared with wild type mice (Fig. 1d). Together, these data suggest that endothelial CD146 plays an important role in the severity of EAE.

Fewer lymphocytes infiltrate the CNS in CD146^{EC-KO} EAE mice.

Because EAE is characterized by a high infiltration of T lymphocytes—particularly pathogenic T_H1 and T_H17 cells—into inflammatory sites^{28–31}, we measured the number of immune cells that infiltrated the CNS in CD146^{EC-KO} and WT mice with EAE. Compared to wild type mice, the CD146^{EC-KO} mice had significantly fewer infiltrated cells in the EAE lesions in terms of both the total number of cells and the proportion of CD45^{high}CD11b⁻ lymphocytes ($p < 0.05$) (Fig. 2a). Furthermore, fewer CD4⁺ T cells, CD8⁺ T cells and T_H1 (i.e., CD4⁺IFN γ ⁺) and T_H17 (i.e., CD4⁺IL-17⁺) cells infiltrated the EAE lesions in the CD146^{EC-KO} EAE mice (Fig. 2b). Because CD146⁺ lymphocytes are known to play an important role in inflammatory diseases, including MS²⁷, we next measured the CD146⁺ lymphocytes that infiltrated the CNS in the CD146^{EC-KO} and WT mice with EAE. We found that the number of CD146⁺ lymphocytes—including CD4⁺CD146⁺, CD8⁺CD146⁺, CD146⁺ T_H1 and CD146⁺ T_H17 cells—was significantly reduced in the CD146^{EC-KO} mice (Fig. 2c and d), suggesting that endothelial CD146 plays an important role in the preferential migration of inflammatory cells across the BBB. Interestingly, we found that these differences between the CD146^{EC-KO} and wild type mice occurred within the CNS, but not in other organs, including the spleen, lymph nodes and peripheral blood (Fig. 2c and d), suggesting that BBB endothelial CD146 plays an important role in recruiting inflammatory cells into the CNS.

In vivo CNS-directed recruitment of pathogenic lymphocytes is impaired in a passive EAE model.

Because we observed a reduction in the infiltration of lymphocytes into the CNS in the CD146^{EC-KO} mice with active EAE, we next examined the role of endothelial CD146 in lymphocyte transmigration by performing an *in vivo* experiment using a passive EAE model³² in which the transferred encephalitogenic T cells were previously re-activated *in vitro*. On post-transfer day 9, we harvested the total population of lymphocytes that had infiltrated the CNS and analyzed the cells by FACS. Consistent with our active EAE data, fewer lymphocytes infiltrated the CNS in the CD146^{EC-KO} mice with passive EAE compared with the wild type mice. Specifically, fewer CD45^{high}CD11b⁻ lymphocytes (Fig. 3a) and lower proportions of CD4⁺, T_H1, and T_H17 cells (Fig. 3a and b) and CD146⁺ lymphocytes (Fig. 3c) infiltrated the CNS in the CD146^{EC-KO} mice. These data suggest that BBB endothelial CD146 may actively facilitate the infiltration of pathogenic lymphocytes into the CNS, thereby promoting EAE pathogenesis.

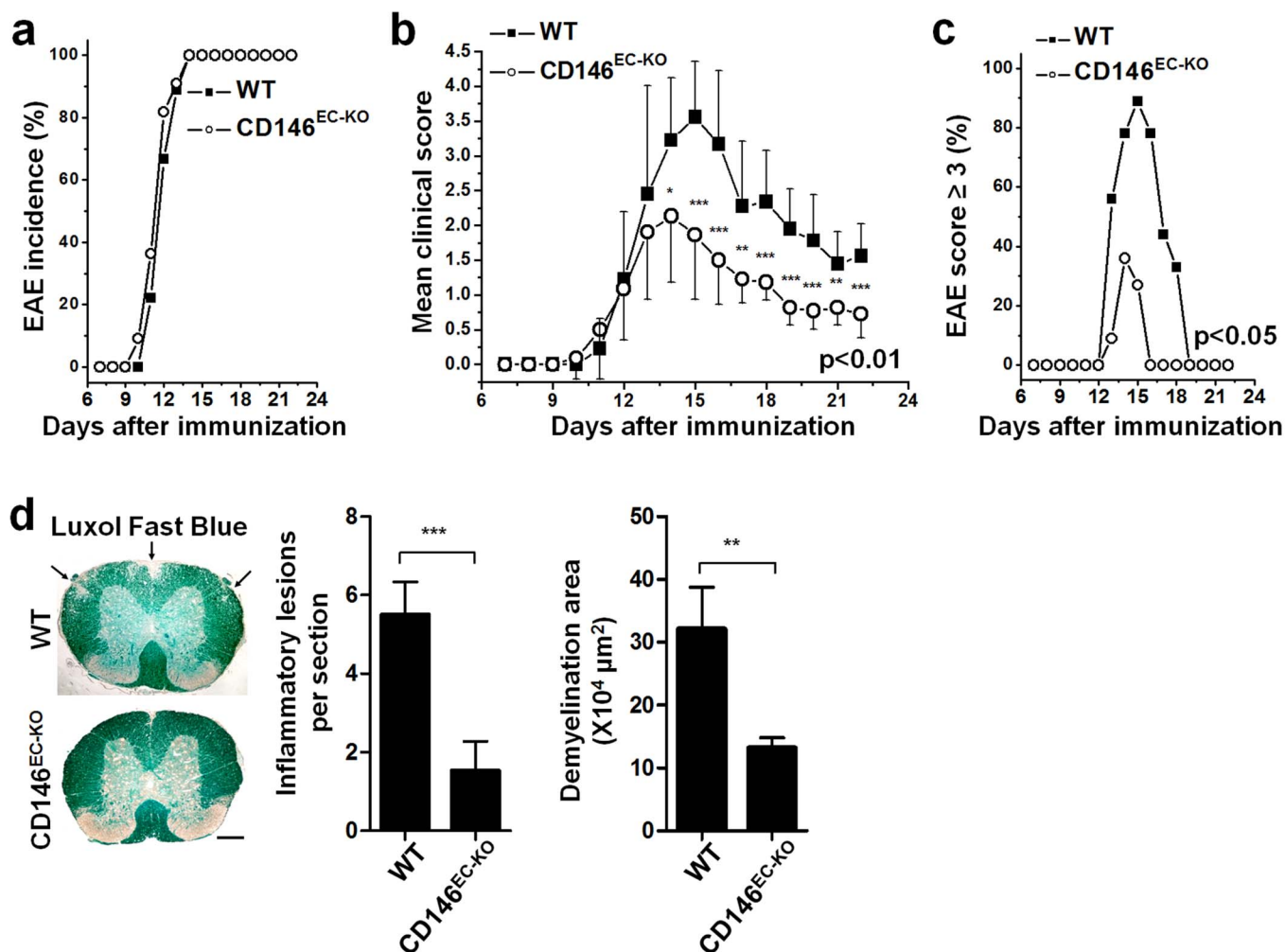


Figure 1 | CD146^{EC-KO} mice have less severe EAE than wild type mice. (a) Wild type (WT) and CD146^{EC-KO} mice (n = 11 mice per group) have a similar time of onset and incidence of EAE following immunization. (b) CD146^{EC-KO} mice had lower mean clinical scores than WT mice (n = 11 mice per group). (c) Fewer CD146^{EC-KO} mice had severe EAE (a clinical score ≥ 3) compared to the WT group ($p < 0.01$). (d) Luxol fast blue staining of spinal cord sections from CD146^{EC-KO} and WT mice with EAE, showing inflammatory lesions (arrows) and demyelinated areas (n = 5 mice/group, 15 lesions/mouse). Scale bar, 200 μm .

Endothelial CD146 is required for lymphocyte transmigration in an *in vitro* BBB model. To directly test whether BBB endothelial CD146 promotes MS pathogenesis by mediating the infiltration of lymphocytes into the CNS, we used an *in vitro* BBB model to measure the transmigration of the human brain endothelial cell line hCMEC/D3 (BBBEC)³³. We first established that like ICAM-1 and VCAM-1, CD146 was also upregulated in BBBECs following incubation with the inflammatory cytokine TNF α (Supplementary Fig. 3); these factors are elevated in EAE and MS lesions and promote the transmigration of lymphocytes^{34,35}. We next isolated human peripheral blood lymphocytes and performed a cell transmigration assay. We found that pretreating the BBBECs with TNF α led to a higher number of transmigrated lymphocytes. Importantly, pretreating the endothelial cells with the monoclonal anti-CD146 antibody AA98 significantly blocked the transmigration of lymphocytes, including CD4⁺ and CD8⁺ T cells, T_H1 and T_H17 cells, as well as CD146⁺ T cells; importantly, the antibody blocked transmigration regardless of whether the BBBECs were pretreated with TNF α (Fig. 4a and b). Furthermore, knocking down CD146 in BBBECs using siRNA (see Supplementary Fig. 4) significantly reduced the transmigration of T lymphocytes, whereas restoring endothelial CD146 expression restored transmigration (Fig. 4c and d). These results confirm that endothelial CD146 plays a functional role in the transmigration of lymphocytes across the BBB.

Because we observed that in addition to CD146, several adhesion molecules—including ICAM-1 and VCAM-1—are upregulated in BBB endothelial cells following induction with inflammatory factors, we tested whether treating cells with anti-CD146 in combination with another antibody would inhibit the transmigration of lymphocytes even further. Accordingly, we performed a transmigration assay with the AA98 and anti-ICAM-1 antibodies, as targeting endothelial ICAM-1 has been shown to block the transmigration of T lymphocytes³⁶. When applied separately, these two antibodies blocked lymphocytes migration to a similar degree. However, their combined application led to a much higher block of lymphocyte migration, particularly with respect to CD4⁺ and T_H17 cells (Fig. 4e and f). These results suggest that using the anti-CD146 together with an antibody against another adhesion molecule has an additive effect in blocking the transmigration of leukocytes into the CNS.

Because targeting endothelial CD146 with the AA98 antibody reduced the migration of both CD146⁺ and CD146⁻ lymphocytes across the BBB, we next examined whether targeting lymphocytic CD146 with AA98 affects the migration of CD146⁺ lymphocytes. Accordingly, we isolated human CD146⁺ T cells from total peripheral lymphocytes using flow cytometry sorting and pre-incubated these cells with the AA98 antibody for one hour prior to measuring transmigration. Interestingly, we found that pretreating lymphocytes

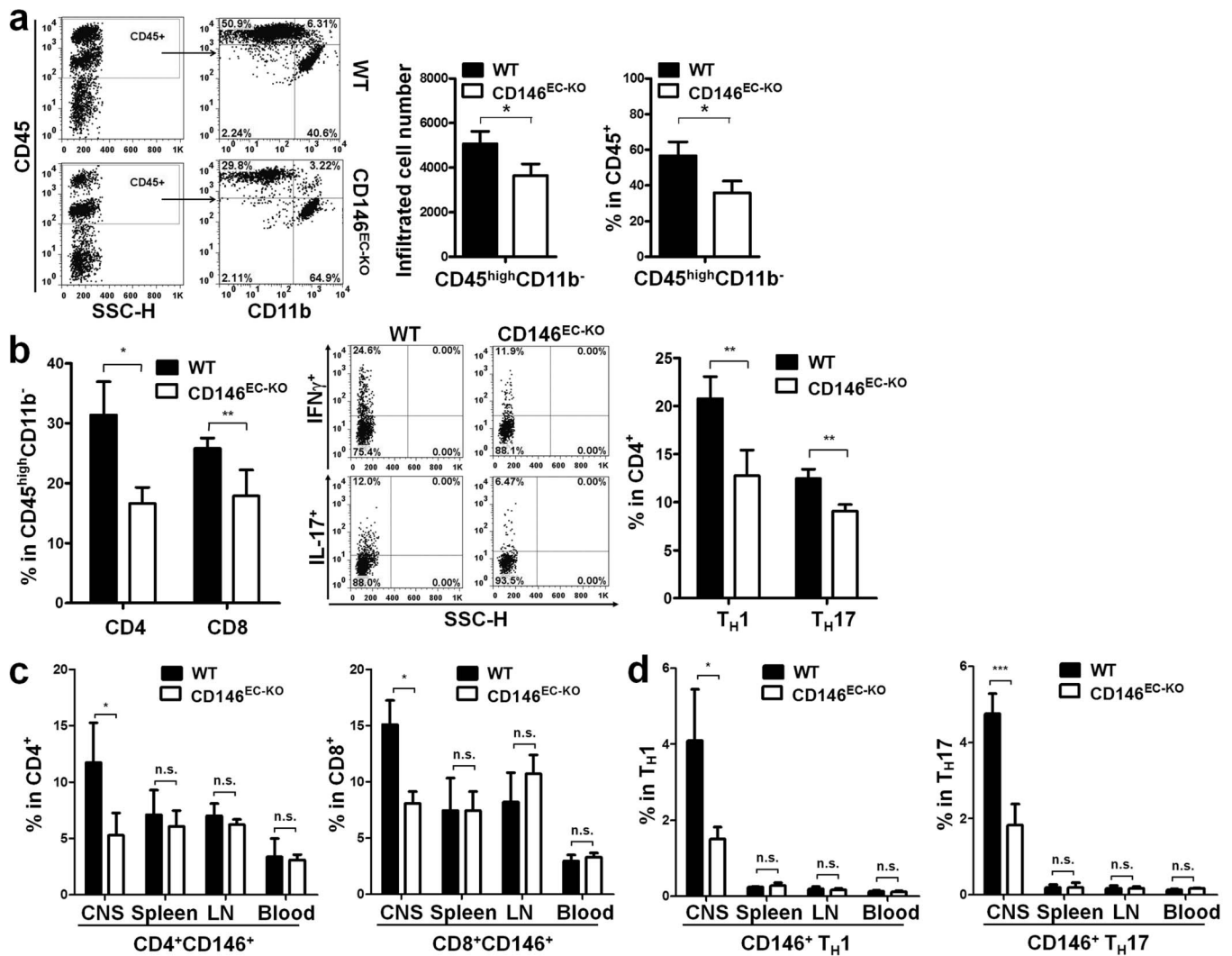


Figure 2 | Fewer lymphocytes infiltrate the CNS in CD146^{EC-KO} mice. (a) Flow cytometry analysis revealed a reduction in the number and percentage of infiltrated leukocytes (CD45^{high}CD11b⁻) in the CNS of CD146^{EC-KO} mice compared to WT mice with EAE (n = 5 mice/group). (b) Flow cytometry analysis of the proportion of CD4⁺ and CD8⁺ cells in CD45^{high}CD11b⁻ cells and T_H1 and T_H17 lymphocytes in CD4⁺ cells in the CD146^{EC-KO} and WT mice with EAE (n = 5 mice/group). (c and d) Flow cytometry analysis of the percentage of CD146⁺ cells in the CD4⁺ and CD8⁺ cells (c), and T_H1 and T_H17 (d) cells in the CNS of CD146^{EC-KO} and WT mice with EAE (n = 5 mice/group). The data represent three independent experiments. *, p < 0.05, **, p < 0.01, ***, p < 0.001, n.s., not significant.

with the AA98 antibody had no effect on the number of CD146⁺ cells that transmigrated across the barrier (Fig. 4g). These data suggest that the AA98 antibody selectively targets endothelial CD146—but not lymphocytic CD146—as the mechanism for inhibiting the transmigration of lymphocytes. These results are also consistent with a recent report that CD146⁺ T_H17 cells enter the CNS via a heterophilic interaction with laminin-411 rather than via a homophilic interaction with endothelial CD146³⁷.

Targeting CD146 with the AA98 antibody suppresses the development of EAE. Because endothelial CD146 plays an important role in EAE pathogenesis, and because targeting lymphocytic CD146 with the AA98 antibody did not affect the *in vitro* transmigration of CD146⁺ lymphocytes, we next examined the therapeutic value of the AA98 antibody in treating EAE. We first initiated active EAE in C57BL6/J mice; on day 7 (prior to the onset of EAE symptoms), we administered either the AA98 antibody or the control mIgG. The time course of the disease onset and the incidence of EAE were similar between the two groups (Fig. 5a); however, the AA98-treated mice had a significant reduction in terms of both the

mean clinical score and EAE severity (Fig. 5b and c). Luxol fast blue staining revealed fewer inflammatory lesions and a reduction in the demyelination area in the AA98-treated mice (Fig. 5d). Analyzing the infiltrated lymphocytes by flow cytometry confirmed that treatment with the AA98 antibody blocked the entry of lymphocytes into the CNS, including CD4⁺, CD8⁺ and CD146⁺ cells, as well as T_H1 and T_H17 pathogenic lymphocytes (Fig. 5e and f).

Because MS is generally a chronic progressive disease, we next tested the therapeutic effect of administering the AA98 antibody after EAE has developed. We induced active and passive EAE in C57BL6/J mice and then administered the AA98 antibody or the control mIgG after the mice became symptomatic (i.e., with a mean clinical score > 1). We found that the mean clinical scores in the mice with active and passive EAE were significantly reduced in the mice that received the AA98 antibody therapy (p < 0.001) (Fig. 5g and h). Moreover, the AA98-treated group entered remission one day earlier than the control mIgG-treated group (Fig. 5g and h). Importantly, the severity of EAE was significantly reduced in the AA98-treated mice compared to the mIgG-treated mice (p < 0.01) (Fig. 5i and j). In

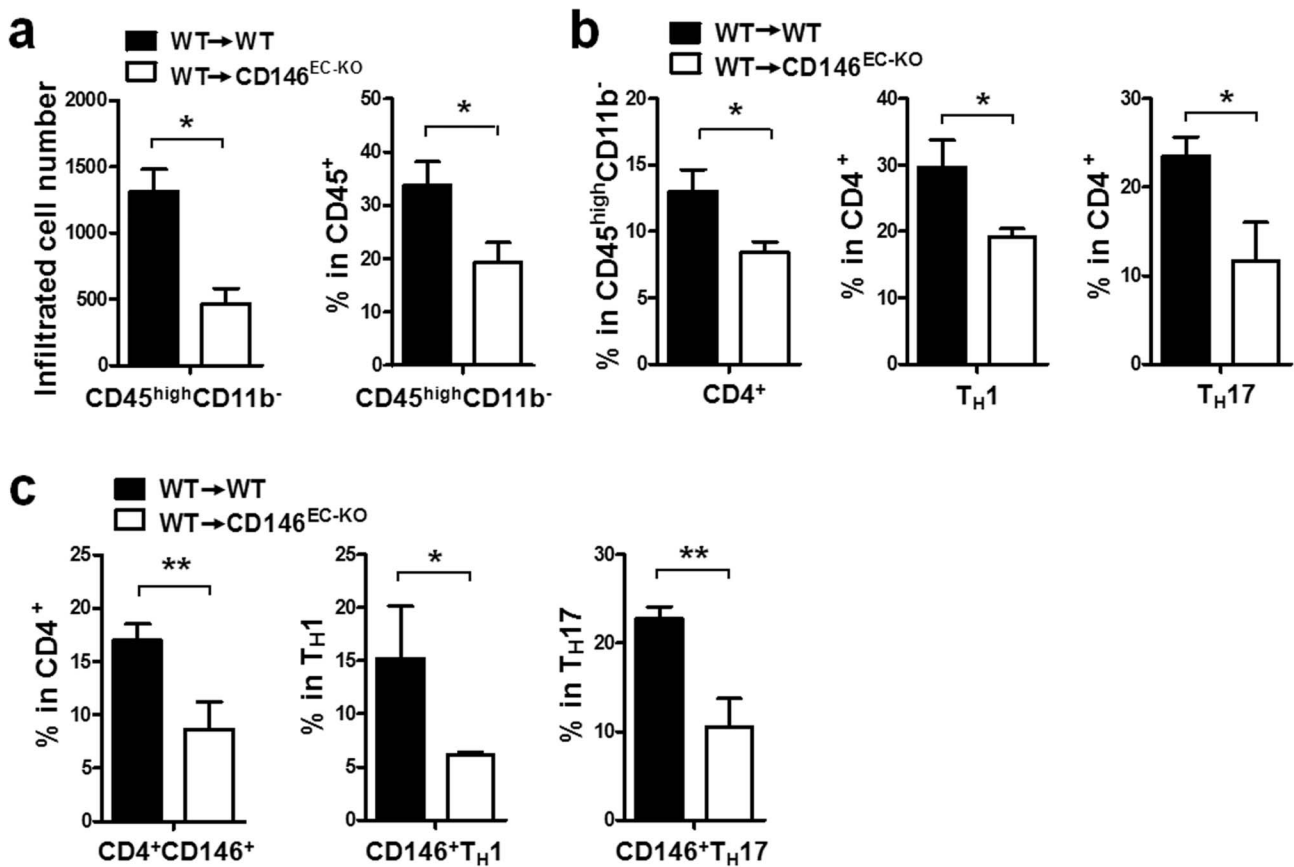


Figure 3 | Fewer lymphocytes infiltrate the CNS in CD146^{EC-KO} mice than in WT mice following the induction of passive EAE. Flow cytometry analysis of CD45^{high}CD11b⁻ lymphocytes (a), CD4⁺ T_H1 and T_H17 lymphocytes (b) and CD146⁺ T cells (c) from the CNS of CD146^{EC-KO} and WT mice with passive EAE. The data represent three independent experiments; 5 mice/group. *, $p < 0.05$, **, $p < 0.01$.

addition, the number of inflammatory lesions and the size of the demyelinated areas in the CNS lesions were significantly decreased in the AA98-treated animals (Fig. 5k). Lastly, fewer T cells—including CD4⁺, CD8⁺, T_H1, T_H17, CD146⁺ T_H1 and CD146⁺ T_H17 cells—infiltrated the CNS in the AA98-treated mice (Fig. 5l and m). Together, these data suggest that the AA98 antibody exerts a therapeutic effect by drastically suppressing the accumulation of pathogenic T lymphocyte at the sites of inflammation, thereby ameliorating the disease.

The AA98 antibody does not affect lymphocyte proliferation or activation. We demonstrated that our monoclonal anti-CD146 antibody AA98 has a therapeutic effect on EAE pathogenesis (and by extension, MS), and we obtained mechanistic data suggesting that the inhibitory effect of the AA98 antibody is achieved by selectively targeting endothelial CD146, thereby inhibiting the migration of lymphocytes into the CNS. However, although targeting lymphocytic CD146 did not affect the migration of CD146⁺ lymphocytes, whether targeting lymphocytes with the AA98 antibody will affect the proliferation and/or function of lymphocyte remains unclear. To investigate the specific effect of the AA98 antibody on lymphocyte migration in further detail, we performed several *in vitro* and *in vivo* experiments. First, we examined the effect of the AA98 antibody on MOG(35–55)- and phytohemagglutinin-P (PHA-P)-induced lymphocyte proliferation *in vitro*. Interestingly, the AA98 antibody did not affect either the proliferation or the production of cytokines (IFN γ and IL17) by lymphocytes that were stimulated with MOG(35–55) or PHA-P (Fig. 6a and b). Moreover, the AA98 antibody did not affect the proliferation of CD146⁺ lymphocytes *in vitro* (Fig. 6c). Second, we

examined whether the AA98 antibody perturbed the immune response of mice with EAE. We found that treating mice with the AA98 antibody had no effect on either the number of lymphocytes or the proportions of various lymphocyte sub-populations (including CD146⁺ lymphocytes) within the peripheral immune system (Fig. 6d and e); the antibody also had no effect on IFN γ or IL-17 expression in CD4⁺ T cells within the CNS lesions (Fig. 6f). In addition, the AA98 antibody had no effect on the expression of the cytokines IFN γ , IL-17 and Granzyme B in CD146⁺ lymphocytes (Fig. 6g). These findings suggest that unlike other antibodies that block lymphocyte migration and perturb the normal immune response, the AA98 antibody selectively targets endothelial CD146 and blocks lymphocyte migration without compromising the immune system.

Discussion

As a marker for vascular endothelial cells, CD146 has been found in many inflammatory diseases, including rheumatoid arthritis²⁴, Crohn's disease²⁵ and vasculitis²⁶. Although a recent report found that CD146 is upregulated in BBB endothelial cells in CNS lesions of MS patients, neither this recent report nor any other study has investigated the role of BBB endothelial CD146 on EAE or MS pathogenesis²⁷. Here, we provide detailed evidence to support the hypothesis that BBB endothelial CD146 is an important adhesion receptor that is actively involved in the transmigration of lymphocytes across the BBB and promotes the formation of CNS lesions in MS and EAE. Using endothelial CD146-specific knockout mice and the monoclonal anti-CD146 AA98 antibody, we found that selectively deleting CD146 expression in vascular endothelial cells or blocking endothelial CD146 with the AA98 antibody causes a significant reduction in the infiltration of pathogenic lymphocytes into the CNS, including

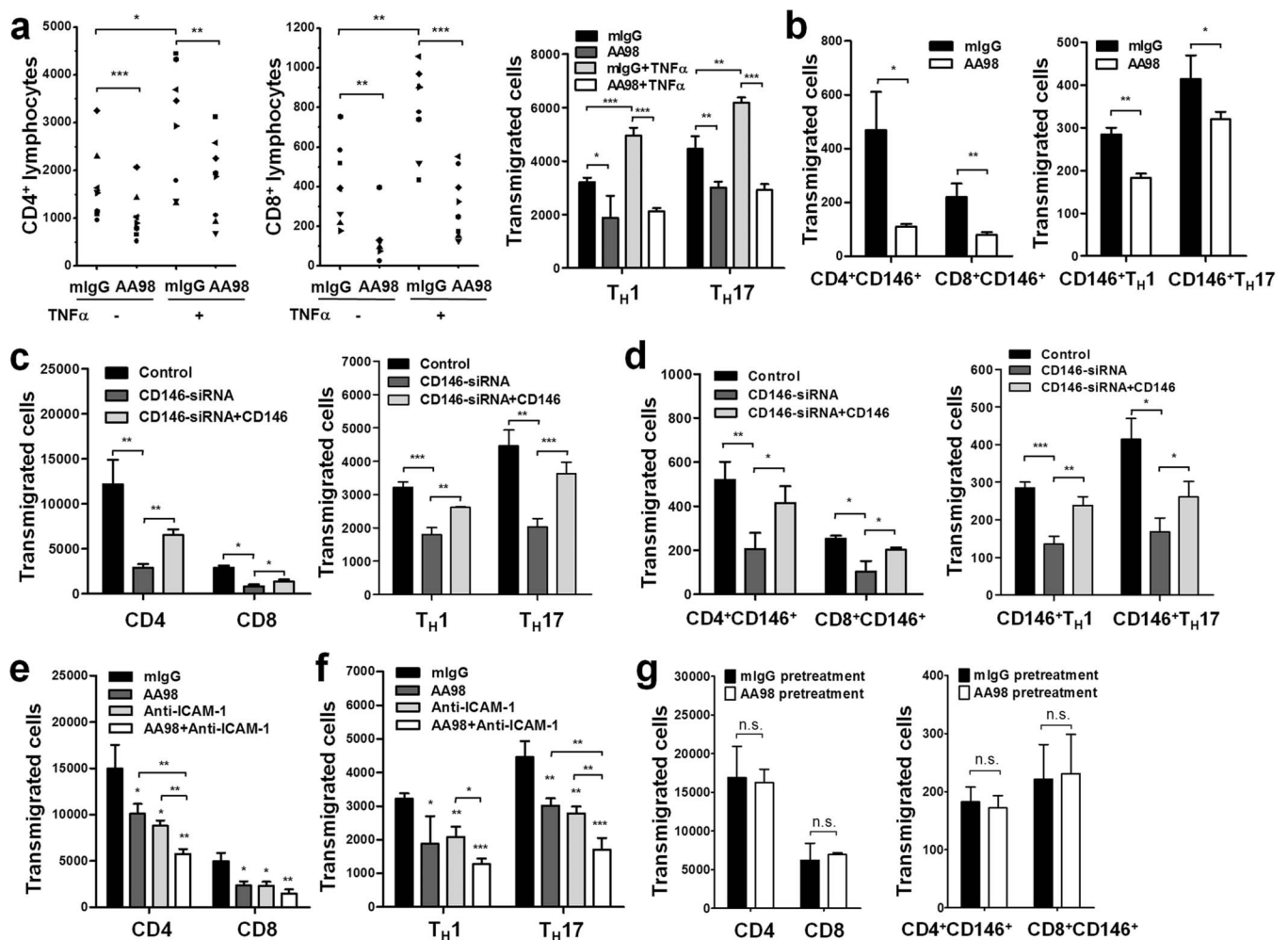


Figure 4 | Endothelial CD146 is necessary for the transmigration of lymphocytes in an *in vitro* BBB model. (a) Flow cytometry analysis of T lymphocyte ($CD4^+$, $CD8^+$, T_H1 and T_H17 cells) transmigration across BBBECS that were treated with or without $TNF\alpha$ (50 ng/ml) in the presence of the AA98 antibody or control mlgG (50 μ g/ml). Each data point represents the mean of each experiment with each donor ($n = 8$ donors). (b) The AA98 antibody blocked the migration of $CD146^+$ T cells across the BBBECS monolayer. (c, d) Flow cytometry analysis of the T lymphocytes that transmigrated across BBBECS transfected with CD146 siRNA with or without rescuing CD146 expression. (e, f) Flow cytometry analysis of the lymphocytes that transmigrated across BBBECS in the presence of either the control mlgG or the AA98 antibody (50 μ g/ml) and/or anti-ICAM-1 (10 μ g/ml). (g) Pre-treatment of lymphocytes ($CD146^-$ and $CD146^+$) with the AA98 antibody did not affect their transmigration. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$, n.s., not significant ((a), paired Student's *t*-test; (b–g), unpaired Student's *t*-test). The data represent eight (a) or three (b–g) independent experiments.

$CD4^+$, $CD8^+$, $CD146^+$, T_H1 and T_H17 cells, thereby reducing the severity of MOG(35–55)-induced EAE without affecting lymphocyte activation or proliferation. *In vitro* and *in vivo* transmigration models confirmed that endothelial CD146 is essential for the transmigration of lymphocytes into the CNS. Our findings provide new insights into the molecular mechanisms by which leukocytes home into CNS-specific lesions and provide a novel therapeutic antibody for treating debilitating CNS autoimmune diseases such as MS.

A key finding in this study is that targeting endothelial CD146 with the AA98 antibody inhibits the transmigration of lymphocytes across the CNS endothelium without affecting normal lymphocyte activation or proliferation. This selectivity is critical to this antibody's potential as a therapeutic agent, as many immunosuppressive agents for treating MS therapy—including corticosteroids and interferon β —have limited usefulness due to their generalized immunosuppressive effects; even the commonly used antibody natalizumab has been reported to cause melanoma and progressive multifocal leukoencephalopathy (PML, a lethal viral CNS disease) as a consequence of the antibody's immunosuppressive effects^{17,19}. Therefore, developing a therapy for treating MS that is both effective and safe is essential.

CD146 is expressed primarily by endothelial cells as well as by a small fraction of lymphocytes. Here, we found that the anti-CD146 antibody AA98 is superior to natalizumab, as it specifically targets endothelial CD146 while sparing lymphocytic CD146, thereby inhibiting the infiltration of pathogenic lymphocytes into the CNS without affecting normal lymphocyte proliferation or activation. Therefore, we suggest that the monoclonal anti-CD146 antibody AA98 may be a safe and effective therapeutic agent for treating MS treatment.

The entry of pathogenic T cells into the CNS is a critical step in the pathogenesis of MS and EAE. The BBB prevents peripheral immune cells from randomly accessing the CNS and maintains immune homeostasis. Pro-inflammatory factors can activate BBB endothelial cells and compromise the integrity of the BBB by upregulating adhesion molecules such as integrins and selectins. A recent report showed that regional neural activation of the spinal cord at the fifth lumbar level in mice can open “gates” in specific blood vessels in which the chemokine CCL20 was upregulated, thereby allowing immune cells—in particular, T_H17 cells—to enter the CNS and cause paralytic disease³⁸. In MS and EAE lesions, T_H1 cells and other immune cells have also been detected at the sites of inflammation^{39,40}.

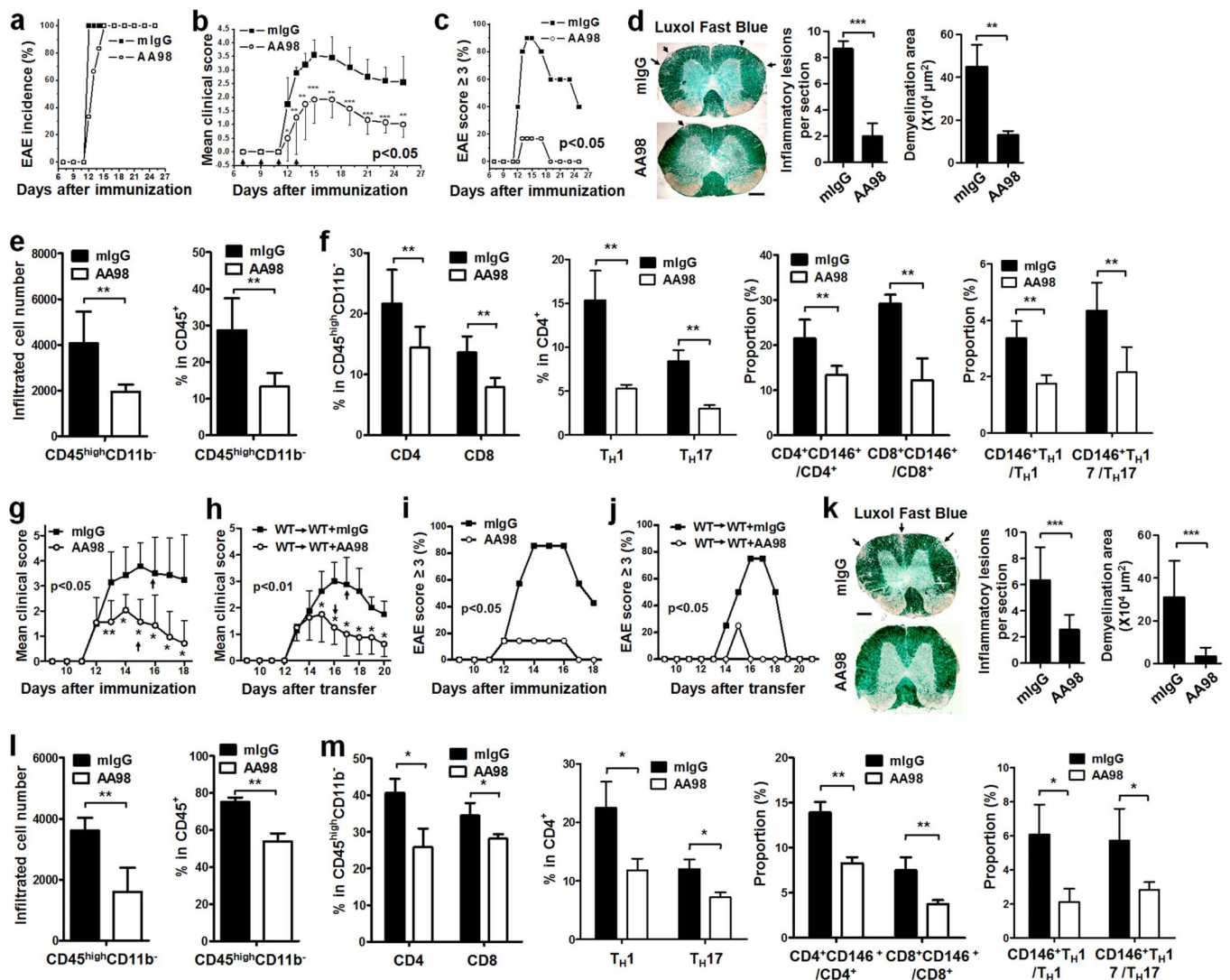


Figure 5 | Treating mice with the AA98 antibody reduces both the severity of EAE and the infiltration of lymphocytes into the CNS. (a) Mice treated with either the AA98 antibody or the control mIgG have a similar time of onset and incidence of EAE following immunization. (b and c) Mice that received preventive treatment with the AA98 antibody had lower mean clinical scores and less EAE severity (defined as a clinical score ≥ 3) than control-treated mice ($n = 11$ mice/group). (d) Luxol fast blue staining of spinal cord sections from control mIgG-treated and AA98-treated mice with EAE ($n = 5$ mice/group, 15 lesions/mouse). Scale bar, 200 μm . (e) Flow cytometry analysis of the number and percentage of infiltrated leukocytes ($\text{CD45}^{\text{high}}\text{CD11b}^-$) isolated from the CNS of mIgG-treated and AA98-treated mice ($n = 5$ mice/group). (f) Flow cytometry analysis of the proportion of CD4^+ and CD8^+ cell (in $\text{CD45}^{\text{high}}\text{CD11b}^-$ cells), $\text{T}_{\text{H}1}$ and $\text{T}_{\text{H}17}$ cells (in CD4^+ T cells) and CD146^+ T lymphocytes from mIgG-treated and AA98-treated mice ($n = 5$ mice/group). (g–j) Mean clinical scores (g and h) and percentage of mice with severe EAE (clinical score ≥ 3) (i and j) for mIgG-treated and AA98-treated mice with active EAE (g and i; $n = 7$ mice/group) or passive EAE (h and j; $n = 5$ mice/group). The arrows in panels g and h indicate the onset of remission, reflected by a significant decrease in the mean clinical score. (k) Luxol fast blue staining and analysis of the demyelinating lesions and area in mIgG-treated and AA98-treated mice with EAE ($n = 7$ mice/group). Scale bar, 200 μm . (l and m) Flow cytometry analysis of $\text{CD45}^{\text{high}}\text{CD11b}^-$ cells (l) as well as CD4^+ and CD8^+ cell (in $\text{CD45}^{\text{high}}\text{CD11b}^-$ cells), $\text{T}_{\text{H}1}$ and $\text{T}_{\text{H}17}$ cells (in CD4^+ T cells) and CD146^+ T cells (m) in mIgG-treated and AA98-treated mice with EAE ($n = 5$ mice/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

This finding may be explained by different entry sites for different cell types; nevertheless, the BBB is the final common site of entry to the CNS, and blocking the interaction between BBB endothelial cells and pathogenic cells is therefore an effective strategy for treating MS. Here, we show that BBB endothelial CD146 mediates the extravasation of pathogenic lymphocytes—including $\text{T}_{\text{H}1}$ and $\text{T}_{\text{H}17}$ cells—to the CNS both *in vivo* and *in vitro*. Targeting endothelial CD146 using the specific anti-CD146 antibody AA98 significantly reduced EAE severity by inhibiting the infiltration of pathogenic T cells into the CNS. Although we have identified the role of endothelial CD146 in recruiting pathogenic T cells during EAE pathogenesis, questions remain that warrant further study. For example, 1) Does CD146

participate in the permeability of the BBB; 2) Is CD146 involved in the migration of myeloid cells (e.g., macrophages) into the CNS; and 3) Does CD146 affect the activation of local CNS glial cells in EAE pathogenesis?

In our study, neither genetically deleting CD146 expression nor preventively treating wild type mice with the AA98 antibody altered the timing of EAE onset or its incidence, suggesting that the protective effect of targeting endothelial CD146 is likely to occur at the level of disease progression rather than at the initial influx of leukocytes. Numerous studies have shown that the accumulation of pathogenic T cells in the CNS does not occur rapidly. An anatomical study of the human CNS found that a few leukocytes initially enter via the

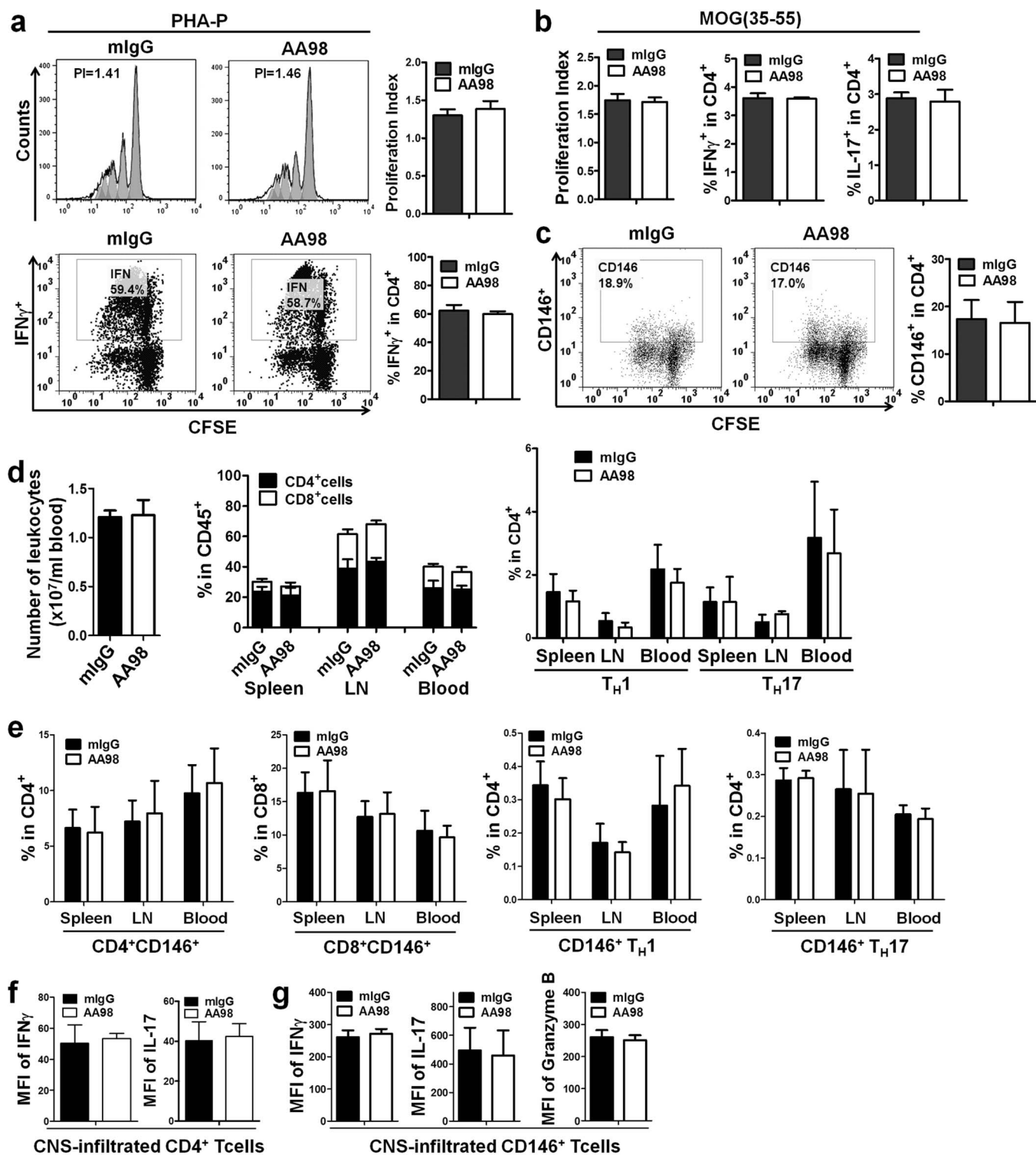


Figure 6 | The anti-CD146 antibody AA98 does not affect lymphocyte proliferation or activation *in vitro* or *in vivo*. (a and b) AA98 treatment did not affect either PHA-P-induced (5 μ g/ml) (a) or MOG(35–55)-induced (50 μ g/ml) (b) lymphocyte proliferation *in vitro* or the *in vitro* production of IFN γ or IL-17. (c) AA98 treatment did not affect the *in vitro* proliferation of CD146 $^+$ cells. (d) AA98 treatment did not affect the total number of leukocytes or the percentages of CD4 $^+$ and CD8 $^+$ T $_H$ 1 and T $_H$ 17 cells in the spleen, lymph nodes or peripheral blood of mice with EAE. (e) The percentage of CD146 $^+$ T cells in the spleen, lymph nodes and peripheral blood of EAE mice was not affected by treatment with the AA98 antibody. (f) The mean fluorescence intensity (MFI) of IFN γ and IL-17 expression in CD4 $^+$ T lymphocytes in the CNS of EAE mice was not affected by treatment with AA98 antibody. (g) AA98 treatment did not affect the MFI of IFN γ , IL-17 or Granzyme B in CD146 $^+$ T lymphocytes isolated from the CNS of mice with EAE. The data represent three independent experiments. PHA-P, phytohemagglutinin-P; LN, lymph nodes; MOG, myelin oligodendrocyte glycoprotein.



choroid plexus and then progress to the cerebral ventricle⁴¹. These newly entered cells then activate local cells, resulting in the secretion of pro-inflammatory factors and the activation of vascular endothelial cells within the CNS. This activation then facilitates the secondary influx of leukocytes into the CNS parenchyma and promotes further inflammation. Interestingly, CD146 expression is absent at the choroid plexus³⁷. Based on these findings and the observation that BBB endothelial CD146 can be upregulated by inflammatory factors, we speculate that endothelial CD146 promotes the secondary influx of lymphocytes without affecting the initial entry of leukocytes via the choroid plexus. This may explain our finding in EAE that targeting endothelial CD146 attenuated the absolute severity of EAE but did not affect the initial onset of the disease. This hypothesis suggests that targeting endothelial CD146 with the AA98 antibody would likely not compromise the routine immunosurveillance that occurs at the initial intrusion of virus into the CNS, which would be a benefit compared with natalizumab, which blocks the interaction that occurs between $\alpha 4$ integrin and VCAM-1 at the choroid plexus and plays a role in the penetration of memory T cells into the non-inflamed CNS^{42,43}.

Because neither genetically deleting CD146 expression nor treating animals with the AA98 antibody completely prevents EAE from developing, but rather ameliorates its severity, we postulate that endothelial CD146 may promote the progression of EAE to its more severe stages via the preferential enrichment of pathogenic cells by the upregulation of CD146 itself or in combination with other adhesion molecules, thereby providing additional ligands for lymphocytes to adhere and transmigrate. This hypothesis suggests that a multi-level therapeutic strategy is needed for treating MS and other CNS autoimmune diseases. Our *in vitro* transmigration experiments revealed that the combination of the AA98 and anti-ICAM antibodies had a partially additive effect on the transmigration of lymphocytes, particularly CD4⁺ and T_H17 cells. This finding indicates that combining the AA98 antibody with other leukocyte-blocking antibodies may be a promising therapeutic strategy for ameliorating the symptoms of MS and related neuroinflammatory diseases.

Since CD146 was first identified as a marker for activated T cells⁴⁴, CD146⁺ T cells have been found in many inflamed tissues, including skin hypersensitivity lesions and the synovial fluid of rheumatoid arthritis patients⁴⁵. Moreover, CD146⁺ leukocytes, including effector memory lymphocytes and T_H17 cells, have been reported to produce high levels of pro-inflammatory factors and have an increased ability to adhere to the endothelium, suggesting a possible advantage in their extravasation to sites of inflammation^{46–49}. Waldert and colleagues first identified the role of CD146⁺ T_H17 cells in MS⁴⁶. A recent study further reported that CD146 identifies a population of encephalitogenic T cells and promotes the recruitment of these cells into the CNS²⁷. Therefore, blocking the entry of these cells into the CNS is important for treating MS. Interestingly, our study reveals that although endothelial CD146 promotes the transmigration of all sub-populations of T cells (including CD4⁺, CD8⁺, T_H1 and T_H17 cells), it plays a key role in the accumulation of CD146⁺ T cells. We found a reduction in the number of CD146⁺ lymphocyte in the CNS of CD146^{EC-KO} and AA98-treated mice, including CD4⁺ (e.g., T_H1 and T_H17 cells) and CD8⁺ lymphocytes. Moreover, the similar numbers of CD146⁺ cells in the spleen, lymph nodes and blood among all groups of mice (i.e., CD146^{EC-KO}, AA98-treated and control mice) together with a decrease in their numbers within the CNS of CD146^{EC-KO} and AA98-treated mice support the selective inhibition of CD146⁺ cell entry into the CNS in CD146^{EC-KO} and AA98-treated mice rather than their depletion and/or inactivation in the periphery.

Previous studies have reported that CD146 homophilic interactions mediate the rolling and adhesion of natural killer (NK) cells on endothelial cells⁴⁸. However, a recent study found that CD146⁺ T_H17 cells enter the CNS via a heterophilic interaction with laminin-411 in the basement membrane of the choroid plexus³⁷. In our study,

pretreating T lymphocytes with the anti-CD146 antibody AA98 did not affect the transmigration of CD146⁺ lymphocytes, suggesting that CD146 homophilic interactions do not play a role in this process and that other ligand(s) expressed on lymphocytes interact with endothelial CD146 to promote lymphocyte transmigration. Therefore, identifying these ligand(s) will be a priority in future research.

In this study, we generated CD146^{EC-KO} mice using the Tg (Tek-cre) system. Tie-2 (Tek) is expressed by a small fraction of myeloid cells—including tumor-infiltrated macrophages and some monocytes—but not lymphocytes or granulocytes^{50,51}; therefore, using the Tg(Tek-cre) system should not have affected the development or function of CD146⁺ lymphocytes. Indeed, using flow cytometry and immunofluorescence, we found that the expression of CD146 in pathogenic T cells—the primary effective encephalitogenic player in EAE—was not affected in the CD146^{EC-KO} mice (see Supplementary Fig. 2). Although we cannot exclude a possible effect of a few Tie-2⁺ monocytes on EAE development, our *in vitro* and *in vivo* transmigration assays support our statement that endothelial CD146 is required for the transmigration of pathogenic T cells.

Our findings reveal an important role for BBB endothelial CD146 in the transmigration of pathogenic lymphocytes and suggest that targeting endothelial CD146 is a promising approach for effectively treating MS. Moreover, because of its improved safety, the monoclonal anti-CD146 antibody AA98 is an attractive alternative to other antibody-based therapies for treating MS and other neuroinflammatory diseases.

Methods

Antibodies and reagents. The three murine anti-CD146 monoclonal antibodies (AA98, AA1, and AA4, each of which targets a distinct epitope in the extracellular domain of CD146) were generated in our laboratory^{22,52}. AA1, AA4 and AA98 recognize domain 1, domain 4 and domain 4–5 in the extracellular domain, respectively. AA1 and AA98 were used for the flow cytometry experiments, and AA4 was used for the immunohistochemistry (paraffin-embedded) experiments. The labeled antibodies (AA98-PE, AA1-APC and AA1-PE) were generated by Tianjin Sungene Biotech Co., Ltd.

The following additional antibodies were used in this study: anti-mouse CD31-APC (Tianjin Sungene Biotech Co., Ltd.), anti-human CD31 (Cymbus Biotechnology), anti-ICAM-1-PE, anti-VCAM-1-PE, anti-human ICAM-1 (clone eBioKAT-1); anti-CD3-PerCP-eFluor 710, anti-CD8-PE, anti-IL-17A-PE, anti-IFN γ -PerCP-eFluor 710, anti-mouse CD3-PerCP-eFluor 710, anti-CD8-PE, CD45-FITC, CD11b-PerCP-eFluor 710, IL-17A-APC, IFN γ -PE (eBioscience); an isotype-matched control mIgG (Sigma-Aldrich); and horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare). The inflammatory human cytokines TNF α , IFN γ , IL-17A, IL-2, IL-12, IL-23, TGF- β and IL-1 β , as well as the anti-human CD3, CD28, IL-4, anti-IL-4, and anti-IFN γ antibodies were purchased from Peprotech. Phytohemagglutinin-P (PHA-P), brefeldin-A (BFA), phorbol myristate acetate (PMA), ionomycin, pertussis toxin (PTX) and complete Freund's adjuvant were purchased from Sigma-Aldrich. MOG(35–55) (MEVGVYRSPFRRVHLYRNGK) was synthesized by Sangon Biotech Co., Ltd. *Mycobacterium tuberculosis* was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. The Luxol fast-blue staining kit was purchased from GENMED (GMS80019.1, GENMED, Shanghai). Ficoll and Percoll lymphocyte separation media were purchased from Tianjin Haoyang Chemical Company, Ltd.

Mice and EAE models. All animal experiments were performed in compliance with the guidelines for the care and use of laboratory animals and were approved by the institutional biomedical research ethics committee of the Institute of Biophysics, Chinese Academy of Sciences.

Wild type C57BL/6J and CD146^{EC-KO} (CD146 conditional knockout) mice were used in this study. Tek^{+/+}CD146^{flxed/flxed} mice (obtained from the Animal Model Research Center at Nanjing University) were backcrossed onto a C57BL/6J background a minimum of nine generations. Tek^{cre/+}CD146^{flxed/flxed} mice were generated using a Cre/loxP recombination system. In brief, Tek^{cre/+}CD146^{+/+} mice (obtained from Jackson Laboratories) were crossed with Tek^{+/+}CD146^{flxed/flxed} mice. The F1 Tek^{cre/+}CD146^{flxed/+} genotype was backcrossed with Tek^{+/+}CD146^{flxed/flxed} mice to obtain Tek^{cre/+}CD146^{flxed/flxed} mice, which we call CD146^{EC-KO} mice. Tek^{+/+}CD146^{flxed/flxed} mice (which we call WT mice here) were used as controls in the EAE disease induction experiments. All genotypes were confirmed by PCR analysis. CD146 deficiency in the vascular endothelial cells was confirmed by immunohistochemistry and immunofluorescence.

To induce the active EAE model⁵³, CD146^{EC-KO} mice and WT mice (on the C57BL/6J background) were injected subcutaneously with 250 μ g MOG(35–55) in complete



Freund's adjuvant mixed with *Mycobacterium tuberculosis* (4 mg/ml). Pertussis toxin (200 ng) was injected intraperitoneally on days 0 and 2. Female C57BL/6J mice were then injected intraperitoneally on days 7, 9, 11, and 13 with the anti-CD146 mAb AA98 antibody or the mouse control mIgG (200 µg per mouse). For the EAE treatment experiments, the antibody was injected on post-immunization days 12 (the day of EAE onset), 13, 14 and 15. The following clinical grades were assigned to each mouse as described previously¹¹: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 2, mild hind-limb paresis; 3, severe hind-limb paresis; 4, hind-limb paralysis; 5, hind limbs paralyzed and partial forelimb weakness.

For the lymphocyte infiltration assay, we isolated lymphocytes (by density gradient centrifugation using Ficoll or Percoll medium according to the manufacturer's instructions) from the peripheral blood, lymph nodes, spleen and spinal cord on post-immunization days 16 or 17, which corresponds to the day after peak disease severity. For calculating the lesion area, the spinal cords were collected on post-immunization day 18 and either frozen at -20°C or embedded in paraffin.

Isolated lymphocytes were stained with anti-CD45, anti-CD11b, anti-CD4, anti-IFN γ or anti-IL-17, and anti-CD146, and analyzed by flow cytometry. T_{H1} cells were defined as CD45⁺CD4⁺/IFN γ ⁺ cells, and T_{H17} cells were defined as CD45⁺CD4⁺IL-17⁺ cells.

For intracellular cytokine staining, PMA (1 ng/ml) and ionomycin (0.5 µg/ml) were added to the cells in 96 well-culture plates, and BFA (1 : 1000) was added 2 h later. After culturing for an additional four hours, the cells were harvested, fixed, permeabilized and stained using antibodies specific for IFN γ and IL-17A.

Passive EAE model. As described previously³², cells from the lymph nodes and spleens of MOG(35–55)-immunized mice were stimulated with 50 µg/ml MOG(35–55) for 72 hours. T cell blasts were then transferred by intravenous injection to host mice (irradiated wild type and CD146^{EC-KO} mice). Pertussis toxin (200 ng) was injected intraperitoneally on the day of transfer and on post-transfer day 2. For the *in vivo* lymphocyte transmigration assay, leukocytes were isolated on post-transfer day 9. For therapeutic treatment, the AA98 or mIgG antibody (200 µg per mouse) was injected intraperitoneally on days 12, 13, 14, and 15. Leukocytes were isolated from the peripheral blood (in red blood cell lysis buffer) and spinal cord (using 30% Percoll) of CD146^{EC-KO} mice and wild type (WT) littermates as well as antibody-treated C57BL/6J mice on post-immunization day 17 and stained with anti-CD45, anti-CD11b, anti-CD4, anti-IFN γ or anti-IL-17, and anti-CD146; these stained leukocytes were then analyzed by flow cytometry.

Luxol fast blue staining. Sequential mouse spinal cord sections were stained with Luxol fast blue and hematoxylin and eosin to identify areas of demyelination, and the number of parenchymal inflammatory lesions per µm² of spinal cord section in each treatment group was counted. The area of demyelination in each treatment group was measured using the ImageJ software program.

BBBEC cells and transfection. The immortalized human brain capillary endothelial cell line hCMEC/D3 (here called "BBBEC") was obtained from INSERM, France. The generation of this cell line was described previously³³. For all experiments, BBBECs were used between passage 25 and passage 35. The cells were cultured in EBM-2 medium supplemented with growth factors, hydrocortisone, ascorbate, 2.5% fetal calf serum (FCS), and antibiotics (Gibco Life Technologies Inc., UK) in a 5% CO₂ humidified atmosphere at 37°C. Lipofectamine 2000 (Invitrogen, San Diego, CA)-mediated siRNA transfection was performed according to the manufacturer's instructions. The p3XFLAG-CMV-14-CD146 construct was transfected in order to rescue CD146 expression. Synthetic siRNA duplexes for targeting CD146 and GFP (as a control) were synthesized by Invitrogen using previously reported sequences⁵⁴.

In vitro human T_{H1} and T_{H17} cell differentiation. Human T_{H1} and T_{H17} cells were differentiated *in vitro* as previously described^{55,56}. In brief, peripheral blood was obtained from healthy donors, and the human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat using Ficoll-hypaque density centrifugation. The PBMCs were cultured in 1640 medium supplemented with 10% FCS and antibiotics, and the cells were coated with anti-CD3 and anti-CD28 (2 µg/ml each). For T_{H1} differentiation, IL-12 and anti-IL-4 (10 µg/ml each) were added to the cultures; for T_{H17} differentiation, TGF- β (1 ng/ml), IL-23 and IL-1 β (50 ng/ml, each), and anti-IL-4 and anti-IFN γ (10 µg/ml each) were added to the cultures. After culturing for 4–5 days, human IL-2 (20 ng/ml) was added, and the cells were cultured for an additional two days. The differentiated T_{H1} and T_{H17} cells were then used for the transmigration experiments.

In vitro BBB transmigration model. The transmigration of human PBMCs or the induced T_{H1} or T_{H17} cells was assayed using a Transwell system equipped with a 3-µm pore filter (Corning Costar) as reported previously³⁷. BBBECs (10⁴ cells) were grown in the upper chamber in the presence or absence of TNF α for 24 h. The control mIgG or the anti-CD146 mAb AA98 (50 µg/ml) was added 1 h before lymphocyte migration. Equivalent numbers of induced T_{H1} or T_{H17} cells or purified lymphocytes (10⁵ cells) from human donors (n = 8) were added gently to the top chamber on the BBBEC cell layers and then left for 12 h. The cells that transmigrated to the bottom chamber were collected and stained for human CD146 (for the transmigrated T_{H1} and T_{H17} cells) or a combination of CD3, CD8 and CD146 (for the transmigrated purified lymphocytes); the stained cells were then analyzed by flow cytometry. CD4⁺ T cells were defined as CD3⁺CD8⁻ cells. Each experiment using purified lymphocytes

from each donor was performed in triplicate, and the means of these three experiments were used for the statistical analyses.

In vitro lymphocyte proliferation. Splenocytes were isolated from MOG(35–55)-immunized C57BL/6J mice and labeled with the vital dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), then re-stimulated with MOG(35–55) (50 µg/ml) or phytohemagglutinin-P (PHA-P) (5 µg/ml) for seven days in the presence of the anti-CD146 antibody AA98 or the control mIgG (50 µg/ml). The cells were stained with antibodies specific for mouse CD3, CD8 or the intracellular cytokines IFN γ and IL-17 and then subjected to FACS analysis.

Statistical analysis. All experiments were performed independently at least three times. The summary results are expressed as the mean \pm SEM (standard error of the mean). Statistical analyses were performed using the GraphPad Prism software program. The paired sign test or Student's *t*-test was used to compare the experimental and control groups. Differences with a *p*-value < 0.05 were considered to be statistically significant.

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Author contributions

Y.L., S.X. and H.D. designed and performed the experiments, analyzed the data and wrote the paper. F.L. obtained the clinical samples and analyzed the data. I.G. and Z.Q. analyzed the data and wrote the paper. Y.Z. performed the immunohistochemistry experiments. D.L., Q.Z. and K.F. provided expertise and performed the animal experiments. J.F. and D.Y. contributed to the experimental design and data analysis. P.-O.C., I.R. and B.W. provided the human BBECs and suggestions regarding the manuscript. X.Y. designed the project, directed the experiments, analyzed the data and wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

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