Supplementary Methods

Growth and Induction of scFv library.

The nonimmune human scFv library in EBY100 yeast (GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1 GAL) was grown at 30°C in SD-CAA (20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, 5.0 g/L casamino acids, 10.19 g/L Na₂HPO₄·7H₂O, 8.56 g/L NaH₂PO₄·H₂O) plus 50 µg/mL kanamycin for 24 hours (OD₆₀₀~10). Yeast at 10-fold excess of the library diversity (5x10⁹) were subsequently induced in 500 mL SG-CAA medium (same as SD-CAA except dextrose replaced by galactose) at 20 °C for 22 hours prior to panning against RBE4 monolayers.

Panning of scFv library against RBE4 cell monolayers.

The RBE4 rat brain endothelial cell line was used as the brain endothelial cell source as RBE4 cells have previously been demonstrated to display many attributes characteristic of the BBB in vivo. RBE4 cells exhibit a nontransformed phenotype, express typical endothelial markers, respond to astrocyte cues, and exhibit BBB-specific properties such as the expression and correct localization of the tight junction protein occludin. In addition, plasma membrane-localized transporters characteristic to brain endothelial cells including those that transport glucose (GLUT1), large neutral amino acids (LAT1), and iron (transferrin receptor), and those that function in active efflux at the BBB (p-glycoprotein, MDR1), are expressed by RBE4 cells. RBE4 cells were a kind gift from Dr. Françoise Roux and were maintained as described previously. RBE4 cells were seeded on collagen type I-coated (Sigma) 6-well plates at 25% confluency two days prior to panning. Induced yeast cells at 10-fold excess of the library size (5x10⁹ yeast) were washed twice with 0.01 M PBS, pH 7.4, supplemented with 1 mM CaCl₂, 0.5 mM Mg₂SO₄ and 0.1% bovine serum albumin (BSA) (Wash buffer) and the yeast mixture was added dropwise onto 100 cm² of RBE4 cell monolayer to ensure even distribution.
across the monolayer. The density of yeast (5 x 10⁷ yeast/cm²) is at the upper limit for panning in that the yeast completely coat the RBE4 monolayer. Panning at high density allows ~30% recovery of binding yeast while also providing appropriate oversampling of the library diversity. The monolayers were then incubated at 4°C for 2 hours to allow yeast-RBE4 cell contacting. The washing strategy was optimized to recover a model scFv that binds to RBE4 cells with nanomolar affinity. The resulting method involved washing the RBE4 layers with ice cold wash buffer by gently rocking the plate twenty-five times, rotating the plate five times (repeated twice), and rotating the plate ten times. The washing supernatant was removed after each step and replaced with fresh wash buffer. After the washing steps, 1 mL of wash buffer was added into each well and all cells were scraped off the plate and pooled together. The yeast/RBE4 cell mixture was resuspended in 5 mL kanamycin-supplemented SD-CAA and grown at 30°C overnight, followed by SG-CAA induction for 20 hours at 20°C. In parallel, a small fraction of the recovered cells were plated on SD-CAA agar plate to quantify the total number of recovered yeast cells after each round. Since the pool diversity was greatly reduced after round 1, the yeast panning density was lowered to 5x10⁶ yeast/cm², and the RBE4 area was reduced to 20 cm² for round 2 and 10 cm² for rounds 3-5. After round 2 of panning, the recovered yeast clones numbered 8.2 x 10⁴, and a parallel experiment with control yeast displaying an anti-fluorescein scFv (4-4-20) showed very little background using the same washing regimen indicating that the panning strategy was yielding primarily RBE4-binding yeast clones. To confirm that the yeast-RBE4 interactions were scFv-based, the scFv-encoding plasmids for several RBE4-binding yeast clones (7 from round 3, 12 from round 4) were recovered using the Zymoprep yeast miniprep kit (Zymo Research). The scFv-encoding plasmid was then retransformed into yeast surface display parent strain, EBY100, using the lithium acetate method and Trp+ transformants were selected. After RBE4 binding with the retransformed clones was confirmed, the
plasmids were sequenced with the Gal1-10 (5’-CAACAAAAAATTGTTAATACCT-3’)
and alpha terminator primers (5’-GTTACATCTACACTGTTGTTAT-3’) (UW-Madison
Biotechnology Center).

High-throughput analysis of recovered yeast clones.

As described above, yeast are typically grown first in SD-CAA followed by SG-
CAA to promote scFv expression. However, this technique yielded comparatively low
levels of scFv surface expression level and lowered percentages of yeast displaying
scFv when a 96-well format was used. Therefore, the scFv display methodology was
optimized for 96-well plates, and it was found that simultaneous growth and induction in
SG-CAA allowed for scFv display having similar efficiency to that observed using the
traditional yeast display methods. Thus, for high throughput screening, yeast clones
were inoculated into 200 µL of SG-CAA (induced sample) and SD-CAA (control
uninduced sample) in a 96-well plate and incubated at 30°C for 24 hours. After
removing 160 µL of SD culture to ensure similar total yeast numbers as the parallel SG
culture, the 96-well plate of yeast was centrifuged, and the supernatant was carefully
removed. The yeast were then washed once with 150 µL wash buffer and resuspended
in 150 µL wash buffer. In parallel, RBE4 cells cultured to confluency in a 96-well plate
were washed once with ice-cold wash buffer. The yeast clones were then transferred
into corresponding wells containing RBE4 monolayers and incubated at 4°C for 2 hours.
After washing, light microscopy was used to assess the binding capacity of the scFv
yeast clones. After visual inspection, a yeast clone was defined as RBE4-binding if
induced yeast remained bound while uninduced yeast originating from the same clone
were washed away.

The scFv genes harbored by binding yeast clones were directly amplified by
whole yeast cell PCR. Briefly, a small amount of a fresh, uninduced yeast colony was
transferred into 30 µL 0.2% SDS, vortexed, frozen at –80 °C for 2 minutes and incubated at 95°C for 2 minutes (temperature shift repeated once). One microliter of the cell lysis solution was then used as a PCR reaction template with primers, PNL6 Forward (5'-G T A C G A G C T A A A A G T A C A G T G - 3') and PNL6 Reverse (5'-TAGATACCCATACGACGTTC-3'). Subsequently, 20 µL of PCR product was subjected to BstNI (New England Biolabs) restriction digest at 60°C for 14 hours. The digested products were resolved on a 3% agarose gel for unique scFv clone identification. The PCR product of each clone displaying a unique BstNI digestion pattern was sequenced with Rev Seq P2 (5'-CCGCCGAGCTATTACAAGTC-3') and For Seq P2 (5'-TCTGCAAGGCTAGTGGTGGTG-3') primers. The sequence was then analyzed by IgBLAST to identify the human germline origin (http://www.ncbi.nlm.nih.gov/igblast/).

**Yeast colony Northern blotting.**

Yeast colony Northern blotting was used to detect and presubtract class 1 and class 2 scFv from the yeast binding pool (Figure 1C, Panel 8). Reagents and instruments were prepared as in standard Northern blotting experiments to eliminate RNase contamination. Yeast clones were cultured on SD-CAA agar plates and the resulting colonies were transferred onto ethanol-sterilized nitrocellulose membranes. The colony-loaded membrane was then layered on top of SG-CAA agar plates, cell side facing up, and incubated at 30°C for 2 days to induce transcription of the scFv gene. To prepare the induced yeast colonies for Northern blotting, the nitrocellulose membranes were layered onto Whatman filter paper soaked with 10% SDS and incubated at 65°C for 30 minutes. The filters were then fixed by transferring to formaldehyde-soaked filter paper at 65°C for 30 minutes (3X SSC, 10% formaldehyde in ddH₂O). Air-dried membranes were subsequently baked for 2 hours at 80°C under vacuum. Oligonucleotide probes corresponding to Class 1 VHCDR2 and Class 2 VHCDR2 were
radiolabeled with a 10 residue $^{32}$P-dATP tail using the Starfire™ kit according to manufacturer's instructions (IDT), and their specific radioactivity was determined by scintillation counting. Being part of the germline V-region, the VHCDR2 regions exhibited 100% homology within class 1 and class 2, and were therefore amenable to hybridization-based subtraction. The membranes were blocked in prehybridization buffer (50% formamide, 5X Denhardt's solution, 5X SSPE, 1% SDS, 0.1% salmon sperm DNA) at 43°C for 2 hours, and then hybridized (prehybridization buffer with $8 \times 10^5$ cpm/ml of each probe) at 43°C overnight. After hybridization, the nitrocellulose membranes were washed as follows: 2X SSC, 0.1% SDS at room temperature for 8 minutes, 0.5X SSC, 0.1% SDS at room temperature for 8 minutes, 0.1X SSC, 0.1% SDS at room temperature for 8 minutes, 0.1X SSC, 1% SDS at 50°C for 30 minutes. The membranes were then exposed to ECL Hyperfilm (Amersham) at −80°C for 24 or 72 hours. Although VHCDR2 was used as the probe in these subtractive screens, the diversity of the recovered scFv clones can be readily expanded as desired via subtraction using any combination of CDR probes.

**ScFv secretion and purification.**

Open reading frames for scFv were isolated from the PCR products used for BstNI typing by *NheI-HindIII* restriction digest and were shuttled to an scFv yeast secretion vector (pRS316-GAL4-4-20) that has been used extensively for scFv secretion$^{12}$. The resultant pRS316-GALscFv plasmids were then transformed into YVH10, a yeast strain overexpressing protein disulfide isomerase. Yeast harboring scFv secretion vector were grown in minimal SD medium (2% dextrose, 0.67% yeast nitrogen base) supplemented with 2X SCAA amino acid (190 mg/L Arg, 108 mg/L Met, 52 mg/L Tyr, 290 mg/L Ile, 440 mg/L Lys, 200 mg/L Phe, 1260 mg/L Glu, 400 mg/L Asp, 480 mg/L Val, 220 mg/L Thr, 130 mg/L Gly, 20 mg/L tryptophan lacking leucine and uracil) at 30 °C for 72 hours.
Subsequently, scFv secretion was induced at 20°C for 72 hours in SG-SCAA (dextrose substituted by galactose) with 1 mg/ml BSA as a nonspecific carrier. For experiments requiring purified scFv, Ni-NTA columns (Qiagen) were used to purify the six histidine-tagged scFv from 50 mL or 1 L batches as described previously. For experiments requiring purified scFv, Ni-NTA columns (Qiagen) were used to purify the six histidine-tagged scFv from 50 mL or 1 L batches as described previously.

The size, purity, and secretion yields of scFv were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with a 4% stacking and 12.5% separating gel followed by Coomassie blue staining. Protein concentrations were estimated by comparison to a series of carbonic anhydrase standards (31 kDa) and by BCA protein assay (Pierce). In parallel, the SDS-PAGE resolved proteins were also blotted onto a nitrocellulose membrane (BioRad) for Western blotting. The nitrocellulose membrane was blocked at 4°C overnight in TBST solution (8 g/L NaCl, and 0.1% Tween-20, buffered to pH 7.6 with 20 mM Tris) supplemented with 5% nonfat milk and probed with 1 µg/mL 9E10 anti-c-myc antibody (Covance) followed by an anti-mouse IgG horse radish peroxidase conjugate (Sigma). Detection was performed using enhanced chemiluminescence and multiple time point exposures to ECL hyperfilm (Amersham) were evaluated by NIH ImageJ software for quantification.

**Affinity determination.**

RBE4 cells were labeled at various scFvA concentrations at 4°C, and the bound scFv detected by anti-c-myc (9E10) antibody labeling followed by anti-mouse IgG AlexaFluor 555. Fluorescence intensity was monitored by FACSCalibur flow cytometer and used to quantitate fractional bound ligand. The scFvA binding data was fit to an equilibrium binding model to determine the monovalent affinity dissociation constant (K_d). The cell-labeling assay was not sensitive enough to produce a binding curve using monomeric scFvD, so scFvD was predimerized with anti-epitope tag antibody, 9E10, to provide the requisite avidity for the ligand binding measurements. ScFv D dimer-labeled
cells were then probed with anti-mouse IgG AlexaFluor 555 conjugate and assessed by flow cytometry. The resulting data were fit to an equilibrium binding model to derive an apparent affinity (avidity). For antigen density experiments, living RBE4 cells were labeled at 4°C with 62.5 nM of pre-dimerized scFvA, scFvD, 4-4-20 scFv, OX26 monoclonal antibody, or IgG2a isotype control. A uniform secondary antibody (anti-mouse IgG AlexaFluor 555 conjugate) was used for each sample to facilitate quantitative comparisons of labeling intensity. These antibody labeling concentrations were adequate for saturation binding of the cell surface antigens, and the cell surface labeling was quantitatively assessed by flow cytometry.

**ScFv-RBE4 immunocytochemistry.**

Predimerization of scFv via the c-myc epitope tag by the 9E10 antibody was used as a method to provide bivalency which is often an important component for promoting cellular internalization of scFv. To this end, the RBE4-binding scFv was first incubated with 9E10 to form artificial dimers. Equal volumes of purified scFv (diluted to 8 µg/mL for scFvA or 32 µg/mL for scFvD and 4-4-20 using 40% goat serum in PBS supplemented with 1 mM CaCl₂, 0.5 mM MgSO₄) and 10 µg/mL 9E10 were mixed and incubated at room temperature for 1 hour to form artificial dimer. RBE4 cells at about 90% confluency were washed 3x with wash buffer. RBE4 cells were then incubated with scFv artificial dimer or OX26 monoclonal antibody (10 µg/mL) (Serotec) at 4°C for 30 minutes and then switched to 37° for another 30 minutes. An anti-mouse IgG secondary antibody conjugated with AlexaFlour555 (Molecular Probes) was applied for 30 minutes at 4°C to label cell surface-bound scFv. The cells were then permeabilized with 0.5% saponin (SigmaAldrich) diluted in wash buffer at 4°C for 5 minutes, and subsequently labeled with an anti-mouse IgG antibody conjugated with AlexaFluor488 (Molecular Probes) for 30 minutes at 4°C to detect internalized scFv. Labeled cells were then fixed.
with 4% paraformaldehyde and examined using a fluorescence microscope (Olympus IX70).

**Yeast display immunoprecipitation.**

ScFv-displaying yeast cells selected from the human scFv library were directly used to immunoprecipitate the cognate plasma membrane antigens. Yeast cells displaying anti-hen egg lysozyme (D1.3) scFv were used as a negative control. As a positive control, an anti-transferrin receptor OX26 scFv yeast display plasmid was created by excising OX26 scFv open reading frame from pRS316-GALOX26 as an NheI-XhoI fragment and ligating into pCT-LWHI. Yeast clones were grown and induced in 50 mL cultures as described above. Induced yeast were collected by centrifugation, washed and fixed with 3% vol/vol formalin in PBS. RBE4 plasma membrane proteins were biotinylated using 0.5 mg/mL Sulfo-NHS-LC-Biotin (Pierce). To prepare RBE4 cell lysate, approximately 5×10^6 biotinylated RBE4 cells were lysed using a 1% (w/v) n-octyl-β-D-glucopyranoside (scFvA, B, C and J Sigma) or 0.1% (w/v) Triton X-100 (scFvD, I, J, and OX26, Sigma) detergent solution in PBS, supplemented with a protease inhibitor cocktail (Calbiochem). For immunoprecipitation, 400 µg of cell lysate protein was mixed with approximately 10^8 yeast cells and incubated overnight at 4°C. Elution of immunoprecipitated product was performed by resuspending yeast cells in 30 µL of 0.5% SDS in 0.4 M Tris (pH 6.8) for 15 minutes. The eluates were separated with SDS-PAGE (8% separating gel) with or without reducing agent (DTT) present, and blotted onto a nitrocellulose membrane (BioRad). Western blotting was subsequently performed with an anti-biotin monoclonal antibody (0.5 µg/mL, clone BTN.4, Labvision), OX26 monoclonal antibody (5 µg/mL, Serotec), or anti-insulin receptor β-subunit monoclonal antibody (1 µg/mL, clone CT-3, Labvision) as described above. Neither scFvA nor scFvD were active in Western blotting format with immunoprecipitated
products or with cell lysates, likely a result of selections being performed under native conditions with living cells.

**Immunohistochemical labeling of rat brain sections by scFv A.**

Brain tissue sections were prepared from the brain of an adult male Sprague Dawley rat. The brain was snap-frozen with tissue freezing medium (Triangle Biomedical Sciences) using a liquid nitrogen bath, and 7 µm coronal sections were cut from the frozen brain. The brain sections were blocked with 40% goat serum and 0.2% TritonX-100 in PBSCM at room temperature for 30 minutes. Purified scFv A or 4-4-20 was diluted with 40% goat serum and incubated with an equal volume of 10 µg/mL 9E10 for 1 hour at room temperature to form artificial dimer. Brain sections were then incubated with scFv A artificial dimer at 4 °C for 1 hour. A secondary labeling solution consisting of phycoerythrin-conjugated anti-mouse IgG and FITC conjugated *Griffonia simplicifolia* lectin (GSA-FITC 10 µg/ml, Sigma) was applied for 30 minutes at 4 °C. After washing, the brain sections were immediately fixed with 4% paraformaldehyde for 10 minutes on ice and examined by fluorescence microscopy.