

COMPOSITIONS AND METHODS FOR BLOOD-BRAIN BARRIER DELIVERY

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Application No. 63/006,998, filed on April 8, 2020, and U.S. Provisional Application No. 63/036,020, filed on June 8, 2020, the disclosures of which are incorporated herein by reference in their entireties.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0002] This application contains a sequence listing, which is submitted electronically via EFS-Web as an ASCII formatted sequence listing with a file name "004852.158WO1-Sequence... Listing" and a creation date of March 30, 2021, and having a size of 1.3 MB. The sequence listing submitted via EFS-Web is part of the specification and is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention relates to a blood-brain barrier shuttle that binds to the transferrin receptor (TfR) and methods of using the same.

BACKGROUND OF THE INVENTION

[0004] While the blood-brain barrier (BBB) prevents harmful substances from entering the brain and is essential for brain homeostasis, it presents a formidable obstacle for efficiently delivering drugs to the brain. Large molecules, such as monoclonal antibodies and other biotherapeutics, have great therapeutic/diagnostic potential for treating/detecting pathology in the central nervous system (CNS). However, their route into the brain is prevented by the BBB. Previous studies have illustrated that only a very small percentage (approximately 0.1%) of an IgG injected in the bloodstream are able to penetrate the BBB into the CNS compartment (Felgenhauer, *Klin. Wschr.* 52: 1158-1164, 1974)). This will limit any pharmacological effect due to the low concentration within the CNS of the antibody.

[0005] Numerous approaches have been studied to improve the brain delivery of therapeutic monoclonal antibodies (mAbs), including the use of receptor-mediated transcytosis (RMT). RMT utilizes abundantly expressed receptors on the luminal side of the BBB for transport through brain endothelial cells. Previous efforts to generate a clinically feasible platform for delivery of therapeutic mAbs into the brain have been focused on antibody engineering to increase the efficiency of transcytosis, with gains made through observations on valency of binding, pH dependency and affinity (reviewed in Goulatis et al., 2017, *Curr Opin Struct Biol* 45: 109-115). However, translation into NHPs and the clinic has been limited by rapid peripheral clearance from target-mediated drug disposition (TMDD) and safety from acute reticulocyte depletion (Gadkar, 2016, *Eur J Pharm Biopharm.* 2016 Apr;101:53-61). Transferrin receptor (TfR), particularly TfR1, mediates the transport of iron-loaded transferrin (Tf) from blood to brain and the return of iron-depleted Tf to the blood (Kawabata, *Free Radical Biology & Medicine*, 133, 46–54, 2019). Anti-TfR1 monoclonal antibodies have been used to deliver drugs to the brain (Burkhart, et al. *Progress in neurobiology*, 181, 101665, 2019). However, safety liabilities and poor pharmacokinetics (PK) of anti-TfR1 monoclonal antibodies have hampered their clinical development as BBB carriers.

[0006] Therefore, there is a need for an anti-TfR monoclonal antibody or antigen binding fragment thereof that can be used to shuttle drugs into the brain efficiently with improved safety and PK.

SUMMARY OF THE INVENTION

[0007] The application relates to an optimized platform for brain delivery, accounting for not just brain concentration of a delivered agent, such as a therapeutic monoclonal antibody (mAb), but also therapeutically relevant characteristics of the mAb, including peripheral pharmacokinetics, safety and the pharmacodynamics of the mAb. The platform utilizes a TfR binding molecule, in particular, an antibody or antigen-binding fragment thereof that binds to transferrin receptor (TfR), preferably a human transferrin receptor 1 (huTfR1), wherein the TfR binding molecule has optimized transport function defined by the on-rate k_a and off-rate k_d values both at a neutral pH of 6.8 to 7.8, such as a physiological pH (e.g., 7.4), and at an acidic pH of 4.5 to 6.5, such as an acidic pH often found in endosomal compartments.

[0008] The inventors discovered, unexpectedly, the optimal values are not simply the fastest on-rate k_a values and the slowest off-rate k_d values as one might expect in typical antibody-target interactions. That is, for this system, one would not necessarily want to use a molecule that “binds” and associates with TfR at a relatively high rate and then dissociates from the TfR more slowly to have the longest life span of the antibody-target complex. Instead, in one embodiment, the optimized transport function of the TfR binders described herein preferably have k_a rates that are similar (e.g., within the same order of magnitude) at both physiologic pH (e.g., 7.4) and at lower pH (e.g., 6.5 or 6.0) but have faster off-rate k_d at a lower pH (e.g., pH 6.5 or 6.0) when compared to the k_d rates at physiological pH (e.g., 7.4).

[0009] In one general aspect, the application describes an anti-TfR antibody or antigen-binding fragment thereof for delivering a therapeutic or diagnostic agent to the brain of a subject in need thereof, wherein the anti-TfR antibody or antigen-binding fragment thereof binds to a transferrin receptor (TfR), preferably human TfR1, with a dissociation constant K_D of at least 1 nM, preferably 1 nM to 500 nM, at neutral pH and an off-rate constant k_d of at least 10^{-4} sec^{-1} , preferably 10^{-4} to 10^{-1} sec^{-1} , at an acidic pH, preferably pH 5.

[0010] In some embodiments, the anti-TfR antibody or antigen-binding fragment thereof of claim 1 has an off-rate constant k_d of 2×10^{-2} to $2 \times 10^{-4} \text{ sec}^{-1}$, preferably $2.0 \times 10^{-3} \text{ sec}^{-1}$ at a neutral pH.

[0011] In another embodiment, the optimized transport function of certain TfR binders described herein preferably have a k_a rate of at least 1.05×10^5 and a k_d rate of at least $2.0 \times 10^{-3} \text{ s}^{-1}$ or faster at physiologic acidic pH (e.g., 7.4). The aforementioned pH, K_D , k_a and k_d parameters reflect optimized transcytosis conditions only and in no way limit our findings that TfR-mediated transport, of certain molecules conjugated to certain TfR binders herein, may nonetheless occur outside of the preferred parameters described.

[0012] In one general aspect, the application relates to an antibody or antigen-binding fragment thereof for delivering an agent to the brain of a subject in need thereof, wherein the antibody or antigen-binding fragment thereof binds to transferrin receptor (TfR), preferably a human transferrin receptor 1 (huTfR1), comprising

- (1) a heavy chain variable region comprising heavy chain complementarity determining regions (HCDRs) HCDR1, HCDR2 and HCDR3, and a light chain variable region comprising light chain complementarity determining regions (LCDRs) LCDR1,

LCDR2 and LCDR3, wherein the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 have the amino acid sequences of:

- (i) SEQ ID NOs: 292, 293, 294, 295, 296, and 297, respectively;
- (ii) SEQ ID NOs: 279, 280, 281, 282, 283 and 284, respectively;
- (iii) SEQ ID NOs: 29, 30, 31, 32, 33 and 34, respectively;
- (iv) SEQ ID NOs: 57, 58, 59, 60, 61 and 62, respectively;
- (v) SEQ ID NOs: 85, 86, 87, 88, 89 and 90, respectively;
- (vi) SEQ ID NOs: 110, 111, 112, 113, 114 and 115, respectively;
- (vii) SEQ ID NOs: 135, 136, 137, 138, 139 and 140, respectively;
- (viii) SEQ ID NOs: 191, 192, 193, 194, 195 and 196, respectively;
- (ix) SEQ ID NOs: 244, 245, 246, 247, 248 and 249, respectively;
- (x) SEQ ID NOs: 263, 264, 265, 266, 267 and 268, respectively;
- (xi) SEQ ID NOs: 345, 346, 347, 348, 349 and 350, respectively;
- (xii) SEQ ID NOs: 355, 356, 357, 358, 359 and 360, respectively;
- (xiii) SEQ ID NOs: 365, 366, 367, 368, 369 and 370, respectively;
- (xiv) SEQ ID NOs: 375, 376, 377, 378, 379 and 380, respectively;
- (xv) SEQ ID NOs: 385, 386, 387, 388, 389 and 390, respectively;
- (xvi) SEQ ID NOs: 395, 396, 377, 398, 399 and 400, respectively;
- (xvii) SEQ ID NOs: 405, 406, 407, 408, 409 and 410, respectively;
- (xviii) SEQ ID NOs: 415, 416, 417, 418, 419 and 420, respectively;
- (xix) SEQ ID NOs: 425, 426, 427, 428, 429 and 430, respectively;
- (xx) SEQ ID NOs: 435, 436, 437, 438, 439 and 440, respectively;
- (xxi) SEQ ID NOs: 445, 446, 447, 448, 449 and 450, respectively;
- (xxii) SEQ ID NOs: 455, 456, 457, 458, 459 and 460, respectively;
- (xxiii) SEQ ID NOs: 465, 466, 467, 468, 469 and 470, respectively;
- (xxiv) SEQ ID NOs: 475, 476, 477, 478, 479 and 480, respectively;
- (xxv) SEQ ID NOs: 485, 486, 487, 488, 489 and 490, respectively;
- (xxvi) SEQ ID NOs: 495, 496, 497, 498, 499 and 500, respectively;
- (xxvii) SEQ ID NOs: 505, 506, 507, 508, 509 and 510, respectively;
- (xxviii) SEQ ID NOs: 515, 516, 517, 518, 519 and 520, respectively;
- (xxix) SEQ ID NOs: 525, 526, 527, 528, 529 and 530, respectively;

- (xxx) SEQ ID NOs: 535, 536, 537, 538, 539 and 540, respectively; or
- (xxxi) SEQ ID NOs: 545, 546, 547, 548, 549 and 550, respectively; or
- (2) a single variable domain on a heavy chain (VHH) comprising heavy chain complementarity determining regions (HCDRs) HCDR1, HCDR2 and HCDR3 having the amino acid sequences of:
 - (i) SEQ ID NOs: 7, 8 and 9, respectively;
 - (ii) SEQ ID NOs: 317, 318 and 319, respectively;
 - (iii) SEQ ID NOs: 324, 325 and 326, respectively;
 - (iv) SEQ ID NOs: 331, 332 and 333, respectively; or
 - (v) SEQ ID NOs: 338, 339 and 340, respectively.

[0013] In certain embodiments, the application relates to an anti-TfR VHH fragment comprising an amino acid sequence having at least 80%, such as at least 85%, 90%, 95% or 100%, sequence identity to SEQ ID NO: 6, 316, 323, 330, or 337.

[0014] In other embodiments, the application relates to an anti-TfR single-chain variable fragment (scFv) comprising a heavy chain variable region covalently linked to a light chain variable region via a linker, preferably, the linker has the amino acid sequence of SEQ ID NO: 314. More preferably, the scFv comprises an amino acid sequence having at least 80%, such as at least 85%, 90%, 95% or 100%, sequence identity to the amino acid sequences of SEQ ID NO: 278, 291, 28, 56, 84, 109, 134, 162, 190, 218, 243, 262, 344, 354, 364, 374, 384, 394, 404, 414, 424, 434, 444, 454, 464, 474, 484, 494, 504, 514, 524, 534 or 544.

[0015] Another aspect of the application relates to a conjugate comprising an anti-TfR antibody or antigen-binding fragment thereof of the application coupled to a therapeutic or diagnostic agent, such as a neurological disorder drug or an agent for detecting a neurological disorder. Preferably, the therapeutic or diagnostic agent is a second antibody or an antigen binding fragment thereof that binds to a brain target.

[0016] In certain embodiments, the application relates to a fusion construct comprising an anti-TfR antibody or antigen-binding fragment thereof of the application covalently linked to a second antibody or an antigen binding fragment thereof that binds to a brain target, such as a brain target selected from the group consisting of beta-secretase 1 (BACE1), amyloid beta (A β), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), Tau, apolipoprotein E4 (ApoE4), alpha-synuclein, CD20, huntingtin, prion protein

(PrP), leucine rich repeat kinase 2 (LRRK2), parkin, presenilin 1, presenilin 2, gamma secretase, death receptor 6 (DR6), amyloid precursor protein (APP), p75 neurotrophin receptor (p75NTR), and caspase 6.

[0017] In certain embodiments, a fusion construct of the application comprises a second antibody or antigen binding fragment thereof that binds to Tau and comprises HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 having the amino acid sequences of SEQ ID NOs: 554 to 559, respectively. Preferably, the second antibody is a monoclonal antibody comprising a heavy chain having the amino acid sequence of SEQ ID NO: 310 and a light chain having the amino acid sequence of SEQ ID NO: 311.

[0018] In one embodiment, a fusion construct of the application comprises an anti-TfR antibody or antigen-binding fragment thereof, preferably an anti-huTfR1 VHH or scFv fragment, of the application covalently linked, via a linker, to the carboxyl terminus of only one of the two heavy chains of a second antibody or antigen binding fragment thereof that binds to a brain target. Preferably, the linker has the amino acid sequence of SEQ ID NO: 312 or SEQ ID NO: 313.

[0019] In certain embodiments, each of the two heavy chains of the second antibody or antigen binding fragment thereof comprises a modified constant heavy chain 3 (CH3) domain as compared to a wild-type CH3 domain to facilitate the formation of a heterodimer between the two heavy chains. Any mutation that facilitates the formation of a heterodimer between the two heavy chains can be used. Preferably, the modified CH3 domain of the first heavy chain comprises amino acid modifications at positions T350, L351, F405, and Y407, and the modified CH3 domain of the second heavy chain comprises amino acid modifications at positions T350, T366, K392 and T394. Preferably, the amino acid modification at position T350 is T350V, T350I, T350L or T350M; the amino acid modification at position L351 is L351Y; the amino acid modification at position F405 is F405A, F405V, F405T or F405S; the amino acid modification at position Y407 is Y407V, Y407A or Y407I; the amino acid modification at position T366 is T366L, T366I, T366V or T366M, the amino acid modification at position K392 is K392F, K392L or K392M, and the amino acid modification at position T394 is T394W. More preferably, the modified heterodimeric CH3 domain of the first heavy chain comprises mutations T350V, L351Y, F405A and Y407V, and the modified heterodimeric CH3 domain of the second heavy chain comprises mutations T350V, T366L, K392L and T394W. The numbering of amino

acid residues in the antibody throughout the specification is performed according to the EU index as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991), unless otherwise explicitly stated.

[0020] In certain embodiments, the fragment crystallizable region (Fc region) of the second antibody or antigen binding fragment thereof contains substitutions that alter (increase or diminish), preferably eliminate, effector function, such as antibody dependent cellular cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC). Preferably, the Fc region of the second antibody or antigen binding fragment thereof comprises one or more amino acid modifications that decrease or abolish the binding of the second antibody or antigen binding fragment thereof to Fc gamma receptors (FcγR) and avoid effector function mediated toxicity. For example, the Fc region of the second antibody or antigen binding fragment thereof can comprise one or more amino acid modifications at positions L234, L235, D270, N297, E318, K320, K322, P331, and P329, such as one, two or three mutations of L234A, L235A and P331S, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat.

[0021] In certain embodiments, the Fc region of the second antibody or antigen binding fragment thereof contains substitutions that alter (increase or diminish), preferably increase, the binding of the second antibody or antigen binding fragment thereof to neonatal Fc receptor (FcRn). Preferably the one or more mutations enhance the binding at an acidic pH, more preferably the Fc has the M252Y/S254T/T256E (YTE) mutations, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat.

[0022] In certain embodiments, a fusion construct of the application comprises:

- (1) a first heavy chain having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 301, 304, 307, 285, 288, 298, 10, 13, 16, 19, 22, 25, 35, 38, 41, 44, 47, 50, 53, 63, 66, 69, 72, 75, 78, 81, 91, 94, 97, 100, 103, 106, 116, 119, 122, 125, 128, 131, 141, 144, 147, 150, 153, 156, 159, 169, 172, 175, 178, 181, 184, 187, 197, 200, 203, 206, 209, 212, 215, 225, 228, 231, 234, 237, 240, 250, 252, 256, 259, 269, 272, 275, 320, 327, 334, 341, 351, 361, 371, 381, 391, 401, 411, 421, 431, 441, 451, 461 and 471;

- (2) two light chains each independently having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of 302, 305, 308, 286, 289, 299, 11, 14, 17, 20, 23, 26, 36, 39, 42, 45, 48, 51, 54, 64, 67, 70, 73, 76, 79, 82, 92, 95, 98, 101, 104, 107, 117, 120, 123, 126, 129, 132, 142, 145, 148, 151, 154, 157, 160, 170, 173, 176, 179, 182, 185, 188, 198, 201, 204, 207, 210, 213, 216, 226, 229, 232, 235, 238, 241, 251, 253, 257, 260, 270, 273, 276, 321, 328, 335, 342, 352, 362, 372, 382, 392, 402, 412, 422, 432, 442, 452, 462 and 472, respectively; and
- (3) a second heavy chain having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of 303, 306, 309, 287, 290, 300, 12, 15, 18, 21, 24, 27, 37, 40, 43, 46, 49, 52, 55, 65, 68, 71, 74, 77, 80, 83, 93, 96, 99, 102, 105, 108, 118, 121, 124, 127, 130, 133, 143, 146, 149, 152, 155, 158, 161, 171, 174, 177, 180, 183, 186, 189, 199, 202, 205, 208, 211, 214, 217, 227, 230, 233, 236, 239, 242, 252, 254, 258, 261, 271, 274, 277, 322, 329, 336, 343, 353, 363, 373, 383, 393, 403, 413, 423, 433, 443, 453, 463 and 473, respectively.

[0023] Another general aspect of the application relates to an isolated nucleic acid encoding the antibody or antigen-binding fragment, a conjugate, or a fusion construct of the application. Also provided is a vector comprising the isolated nucleic acid of the application, a host cell comprising the nucleic acid or the vector of the application.

[0024] Another general aspect of the application relates to a method of producing the antibody or antigen-binding fragment, a conjugate, or a fusion construct of the application. The method comprises culturing a cell comprising a nucleic acid of the application under conditions to produce the antibody or antigen-binding fragment, the conjugate or the fusion construct, and recovering the antibody or antigen-binding fragment, the conjugate or the fusion construct from the cell or cell culture.

[0025] Further provided is a pharmaceutical composition comprising a conjugate or a fusion construct of the application and a pharmaceutically acceptable carrier.

[0026] Another general aspect of the application relates to a method of treating or detecting a neurological disorder in a subject in need thereof, comprising administering to the subject an effective amount of an anti-TfR antibody or antigen binding fragment thereof, a conjugate or a

fusion construct, or a pharmaceutical composition of the application. Preferably, the neurological disorder is selected from the group consisting of neurodegenerative diseases (such as Lewy body disease, postpoliomyelitis syndrome, Shy-Draeger syndrome, olivopontocerebellar atrophy, Parkinson's disease, multiple system atrophy, striatonigral degeneration, spinocerebellar ataxia, spinal muscular atrophy), tauopathies (such as Alzheimer disease and supranuclear palsy), prion diseases (such as bovine spongiform encephalopathy, scrapie, Creutzfeldt-Jakob syndrome, kuru, Gerstmann-Straussler-Scheinker disease, chronic wasting disease, and fatal familial insomnia), bulbar palsy, motor neuron disease, and nervous system heterodegenerative disorders (such as Canavan disease, Huntington's disease, neuronal ceroid-lipofuscinosis, Alexander's disease, Tourette's syndrome, Menkes kinky hair syndrome, Cockayne syndrome, Halervorden-Spatz syndrome, lafora disease, Rett syndrome, hepatolenticular degeneration, Lesch-Nyhan syndrome, and Unverricht-Lundborg syndrome), dementia (such as Pick's disease, and spinocerebellar ataxia), and cancer of the CNS and/or brain (such as brain metastases resulting from cancer elsewhere in the body).

[0027] Preferably, the antibody or antigen binding fragment thereof, the conjugate, the fusion construct or the pharmaceutical composition of the application is administered intravenously.

[0028] Also described is a method of delivering a therapeutic or diagnostic agent to the brain of a subject in need thereof, comprising administering to the subject a conjugate comprising the therapeutic or diagnostic agent coupled to an anti-TfR antibody or antigen-binding fragment thereof of the application. Preferably, the therapeutic or diagnostic agent is a second antibody or an antigen binding fragment thereof that binds to a brain target. More preferably, the administration of the therapeutic or diagnostic agent coupled to an anti-TfR antibody or antigen-binding fragment thereof of the application to the brain of a subject results in reduced Fc-mediated effector function and/or does not induce rapid reticulocyte depletion, as compared to the administration of the therapeutic or diagnostic agent not coupled to the anti-TfR antibody or antigen-binding fragment thereof.

[0029] Yet another general aspect of the invention relates to a method of inducing antibody dependent phagocytosis (ADP) without stimulating secretion of a pro-inflammatory cytokine in a subject in need thereof, comprising administering to the subject a complex comprising a therapeutic antibody or antigen binding fragment thereof coupled to, preferably covalently conjugated to, an antigen-binding fragment thereof according to an embodiment of the invention,

wherein the therapeutic antibody or antigen binding fragment thereof does not have effector function, for example, the therapeutic antibody or antigen binding fragment thereof comprises one or more amino acid modifications at positions L234, L235, D270, N297, E318, K320, K322, P331, and P329, such as one, two or three mutations of L234A, L235A and P331S, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat. Preferably, the therapeutic antibody or antigen binding fragment thereof binds specifically to tau aggregates.

[0030] Other aspects, features and advantages of the invention will be apparent from the following disclosure, including the detailed description of the invention and its preferred embodiments and the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

[0031] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise embodiments shown in the drawings.

[0032] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0033] FIG. 1 is an illustration of tripod mAb format (also referred to as a TTP mAb) used for the brain delivery platform.

[0034] FIG. 2 is an image showing internalization of tripod mAbs in human brain endothelial cells. Tripod mAbs are stained red, nucleus is blue, and actin green.

[0035] FIG. 3 is a graph showing pH dependent binding, which was assessed by comparing off-rates at pH 7.4 to off-rate as pH was reduced to 6.5 and 6.0. Tripod mAbs were scored positive if the off-rate was faster as the pH decreased.

[0036] FIG. 4 is an image showing internalization of the tripod mAb BBBB383, in human brain endothelial cells. Tripod mAbs are stained red, nucleus is blue, and actin green.

[0037] FIG. 5A-FIG. 5B are graphs showing plasma (FIG. 5A) and brain (FIG. 5B) PK of BBBB383 and BBBB426. Brain shuttle containing anti-BACE mAbs, BBBB383 and BBBB426

were compared with BBBB456 (anti-BACE mAb without the brain shuttle). Symbols represent the average of 4 mice (at 4 and 24 hours) or 5 mice (at 72 hours).

[0038] FIG. 6 is a graph showing A β 1-40 concentrations in brain following treatment with BBBB383 and BBBB426. Brain shuttle containing anti-BACE mAbs, BBBB383 and BBBB426, were compared with BBBB456 (anti-BACE mAb without the brain shuttle). Symbols represent the average of 4 mice (at 4 and 24 hours) or 5 mice (at 72 hours).

[0039] FIG. 7A-FIG. 7B are graphs showing plasma (FIG. 7A) and brain (FIG. 7B) PK of brain shuttle anti-BACE mAbs. Brain shuttle containing anti-BACE mAbs were compared with BBBB456 (anti-BACE mAb without the brain shuttle, solid diamond with dotted line). Each symbol represents the average of two mice per timepoint.

[0040] FIG. 8 is a graph showing A β 1-40 concentrations in brain following treatment with brain shuttle mAbs. Brain shuttle containing anti-BACE mAbs, were compared with BBBB456 (anti-BACE mAb without the brain shuttle, solid diamond with dotted line). Dose dependent decrease in AB levels was observed for all brain shuttles except BBBB983. Each symbol represents the average of two mice per timepoint.

[0041] FIG. 9 is an image showing internalization of tripod mAb BBB-00489 in human brain endothelial cells. Tripod mAbs are stained red and actin green.

[0042] FIG. 10 are graphs showing brain pharmacokinetics in cynomolgus monkey. Cynomolgus monkeys were dosed 10mg/kg intravenously with three TTP mAbs, BBBB1134 and BBBB1136 (left) and BBBB1133 (right), and compared to control mAb, PT1B844. Brain exposure measured 72 hours following dosing (n= 3 cynomolgus monkeys/mAb). Brain concentration was determined for the mAbs across a variety of areas and averaged across animals. Each symbol represents a region of the brain.

[0043] FIG. 11 is a graph showing brain concentration of a brain shuttle-containing mAb as compared to the non-brain shuttle control in different regions. Individual points represent each animal (n=3).

[0044] FIG. 12 is a graph showing plasma concentration of mAbs dosed *i.v.* at 4, 24 and 72 hours. All brain shuttle mAbs had faster clearance than non-brain shuttle mAbs. Individual points represent each animal (n=3).

[0045] FIG. 13 is a graph showing reticulocyte depletion observed during the cyno study for BBBB1134 but not the other mAbs, confirming the impact of Fc function on TfR binding mAbs and reticulocyte depletion.

[0046] FIG. 14A-FIG. 14C: Brain pharmacokinetics and pharmacodynamics of tripod mAbs in huTfR knock-in mice, human TfR knock-in mice were dosed 10mg/kg intravenously with a panel of tripod mAbs (BBBBx) compared with one control mAb, and brain exposure was assessed at 24 hours:

FIG. 14A: a range of enhanced exposure was observed from no enhancement (BBBB974, open square) to 10.5x (BBBB978, open triangle) (n= 2 mice, symbols represent each individual animal with the bar representing the mean and error bars standard deviation).

FIG. 14B: the tripod mAb off-rates correlated well with brain exposure, with an off-rate that was neither too fast nor too slow observed to be optimal.

FIG. 14C: brain pharmacodynamics of the mAb, anti-BACE antagonist mAb, were assessed and a strong PK/PD relationship was observed in the brain for all tripod mAbs, except BBBB983. BBBB983 had enhanced brain exposure (5.5x) but similar concentration of A β ₁₋₄₀ as the control mAb (each triangle represents an individual). It is hypothesized that the slow-neutral off-rate is preventing diffusion in the brain to the target.

[0047] FIG. 15 is a graph showing mAb mediated uptake into microglial phagosomes. All brain shuttle mAbs promoted more efficient uptake into phagosomes than the non-brain shuttle mAb, PT1B844. Within the brain shuttle mAbs those with full effector function (BBBB1131, 1134 and 1046) were more efficient than those without effector function.

[0048] FIG. 16 is a graph showing mAb mediated uptake into macrophage phagosomes. All brain shuttle mAbs promoted more efficient uptake into phagosomes than the non-brain shuttle mAb, B21M-IgG1.

[0049] FIG. 17A-FIG. 17F: Brain pharmacokinetics in cynomolgus monkey demonstrate enhanced brain delivery of therapeutic mAb.

FIG. 17A: cynomolgus monkeys were dosed 10 mg/kg intravenously with two tripod mAbs, BBBB1134 and BBBB1136, and one control mAb, PT1B844. Brain exposure measured 72 hours following dosing (n= 3 cynomolgus monkeys/mAb. Symbols represent each individual animal with the bar representing the mean and error bars

standard deviation). Enhanced brain exposure was observed for both brain shuttle mAbs across all regions of the brain assessed.

FIG. 17B: a 7x and 11x enhancement in brain concentration was observed for BBBB1134 and BBBB1136, respectively, compared with the control mAb.

FIG. 17C: the plasma exposure over 72 hours demonstrated target-mediated drug disposition for the tripod mAbs with accelerated clearance observed compared with the control mAb. The tripod mAbs differ in their binding affinity for FcRn, with BBBB1136 containing the high binding affinity “YTE” mutations; BBBB1136 (triangle) had approximately 2x enhanced plasma concentration at 72 hours compared with BBBB1134 (open square).

FIG. 17D: in vitro ADCC activity of the tripod mAbs (BBBB1134 and BBBB1136) compared with the positive control, BBBB175 high affinity anti-TfR binding IgG1 mAb, and negative control, CNTO3930, an IgG1 mAb that does not bind the target cells. BBBB1134, IgG1 mAb, potentiates robust ADCC of target cells with both human and cyno PBMCs. BBBB1136, an effector function silent IgG1 mAb, was observed to have no ADCC activity.

FIG. 17E: SPR binding data of BBBB1134 and BBBB1136 for the complement component 1q (C1q). BBBB1134 binds C1q while BBBB1136 does not.

FIG. 17F: Reticulocyte depletion observed in the cynomolgus monkey PK study.

Reticulocyte loss 2 days following dosing was not observed for the control mAb or BBBB1136, while robust depletion was observed following treatment with BBBB1134 (symbols represent the individual animals, bars the average and error bars the standard deviation).

[0050] FIG. 18A-FIG. 18D: Brain and Serum pharmacokinetics of repeat dosing and dose response of BBBB1133 in cynomolgus monkey:

FIG 18A: Cynomolgus monkeys were dosed intravenously with either 2mg/kg, 10mg/kg or 30mg/kg with BBBB1133 and brain exposure assessed at 1, 7 or 15 days following (n=3 monkeys/mAb and timepoint. Symbols represent the average brain concentration and error bars the standard deviation). Linear brain PK was observed between 2 and 10mg/kg but nonlinear PK observed between 10 and 30 mg/kg, suggesting that 30mg/kg is a saturating dose for the TfR.

FIG. 18B: Serum concentration of BBBB1133 was measured throughout the study (1, 6 hours post-dosing and on days 1, 2, 4, 10 and 14). Linear pharmacokinetics was observed at all three doses. A $T_{1/2} = 6$ days was determined for BBBB1133 in serum.

FIG. 18C: Cynomolgus monkeys were dosed intravenously weekly for three weeks with BBBB1133 at either 2mg/kg, 10mg/kg or 30mg/kg. Brain exposure assessed at 1, 7, 15, or 21 days following dosing (n=3 monkeys/mAb and timepoint. Symbols represent the average brain concentration and error bars the standard deviation). Linear brain PK was observed between 2 and 10mg/kg but nonlinear PK observed between 10 and 30 mg/kg, suggesting that 30mg/kg is a saturating dose for the TfR. Evidence for accumulation was observed at the 30mg/kg dose.

FIG. 18D: Serum concentration of BBBB1133 was measured throughout the study (1, 6 hours post first dose and on days 1, 2, 4, 10, 14, 14.02, 14.25, 15, 16, 18 and 21). Linear pharmacokinetics was observed at all three doses with no evidence for PK tolerance with repeat dosing.

[0051] FIG. 19A-FIG. 19C: Non-classical, non-FcγR mediated ADP promotes the efficient phagocytosis of Tau aggregates in human microglia:

FIG. 19A: To assess the potential of the effector function impaired IgG1 tripod mAbs, BBBB1133 and BBBB1136, to promote uptake of Tau aggregates in microglia, human iPSC derived microglia were incubated with mAbs and biotinylated phospho-tau oligomers labeled with streptavidin Alexa Flour 488 (AF488). At 4 hours post incubation, cells were washed, fixed, permeabilized, stained and imaged using confocal microscopy. Cells containing tau aggregates that co-localized with Lamp-1 stained lysosomes were quantitated. BBBB1133 and BBBB1136 promoted more efficient uptake and lysosomal trafficking than the anti-Tau WT IgG1 mAb, PT1B844.

FIG. 19B: Uptake of Tau oligomers can be blocked with excess of soluble TfR ECD but is not impacted by addition of soluble Fc, demonstrating the uptake occurs through TfR.

FIG. 19C: Human iPSc-derived microglia were incubated with Alexa Fluor 488-labeled phosphoTau peptide (green) in the presence of PT1B844 or BBBB1133 for 4 hours. After fixation, cells were stained with antibodies against Clathrin, EEA1, Rab17 or Lamp1, and detected with Alexa Fluor 647-secondary antibodies (red). Cells were imaged using a Perkin Elmer Opera Phenix, 60x magnification, confocal mode. Representative cell

images at the 2 μm plane are shown. Scale bar = 10 μm . Arrows point at the colocalization area detailed in insets. Third column for each phosphoTau-antibody treatment is the merged result of the other two columns. Cells were also stained and imaged with DAPI to detect nuclei and hcs Cellmask orange to detect cytoplasm (not shown).

[0052] FIG. 20A-FIG. 20E: Non-classical, non-Fc γ R mediated ADP promotes the efficient phagocytosis of Tau aggregate derived from human AD patient brains in human macrophages and microglia:

FIG. 20A: Human monocyte-derived macrophages were incubated with Tau aggregates and BBBB1133 (open square) and the control anti-Tau mAb, PT1B844 (circle). The amount of pTau remaining in the culture supernatant was quantified with time. Similar degradation of pTau was observed up 8 hours, at which point the PT1B844-mediated ADP stalls while the BBBB1133-mediated ADP continues to promote degradation.

FIG. 20B: A similar trend was observed using human iPSC-derived microglia, with BBBB1133 (open square) potentiating more robust degradation of pTau with time compared with PT1B844. The mechanism of BBBB1133-mediated pTau degradation was demonstrated to occur through the TfR by blocking degradation using excess amount of soluble TfR ECD.

FIG. 20C-FIG. 20E: Supernatants from the microglia experiment were assess for cytokine concentrations. PT1B844-mediated pTau ADP simulated the release of proinflammatory cytokines, TFN α (FIG. 20C), IL6 (FIG. 20D) and IL1 β (FIG. 20E), while BBBB1133 did not simulate similar release.

[0053] FIG. 21: Co-injection of PHFs with the indicated tau antibodies reduced the induction of tau pathology:

FIG. 21A: Partial dependency of the model on Fc-dependent activity is demonstrated by the statistically significant differences in neutralization of Tau by the mouse IgG2a.

FIG. 21B: Both anti-Tau mAbs neutralized Tau seeding compared with the isotype control. No statistical difference was observed between the mAb and the TTP mAb, with the TTP mAb having slightly improved neutralization compared with the mAb, demonstrating that non-classical ADP mechanism is functional *in vivo*.

DETAILED DESCRIPTION OF THE INVENTION

[0054] Various publications, articles and patents are cited or described in the background and throughout the specification; each of these references is herein incorporated by reference in its entirety. Discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is for the purpose of providing context for the present invention. Such discussion is not an admission that any or all of these matters form part of the prior art with respect to any inventions disclosed or claimed.

[0055] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention pertains. Otherwise, certain terms used herein have the meanings as set in the specification. All patents, published patent applications, and publications cited herein are incorporated by reference as if set forth fully herein. It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise.

[0056] Unless otherwise stated, any numerical value, such as a concentration or a concentration range described herein, are to be understood as being modified in all instances by the term “about.” Thus, a numerical value typically includes $\pm 10\%$ of the recited value. For example, a dosage of 10 mg includes 9 mg to 11 mg. As used herein, the use of a numerical range expressly includes all possible subranges, all individual numerical values within that range, including integers within such ranges and fractions of the values unless the context clearly indicates otherwise.

[0057] As used herein, the conjunctive term “and/or” between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by “and/or,” a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term “and/or” as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term “and/or.”

[0058] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise,” and variations such as “comprises” and “comprising,” will be

understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or “including” or sometimes when used herein with the term “having.”

[0059] When used herein “consisting of” excludes any element, step, or ingredient not specified in the claim element. When used herein, “consisting essentially of” does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. Any of the aforementioned terms of “comprising,” “containing,” “including,” and “having,” whenever used herein in the context of an aspect or embodiment of the invention can be replaced with the term “consisting of” or “consisting essentially of” to vary scopes of the disclosure.

[0060] The term “antibody” herein is used in the broadest sense and specifically includes full-length monoclonal antibodies, polyclonal antibodies, and, unless otherwise stated or contradicted by context, antigen-binding fragments, antibody variants, and multispecific molecules thereof, so long as they exhibit the desired biological activity. Generally, a full-length antibody is a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarily determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. General principles of antibody molecule structure and various techniques relevant to the production of antibodies are provided in, e.g., Harlow and Lane, *ANTIBODIES: A LABORATORY MANUAL*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., (1988).

[0061] Depending on the amino acid sequence of the constant domain of their heavy chains, full length antibodies can be assigned to different “classes”. There are five major classes of full-

length antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into “subclasses” (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0062] An “antibody” can also be a single variable domain on a heavy chain (VHH) antibody, also referred to as a heavy chain only antibody (HcAb), which are devoid of light chains and can be naturally produced by camelids or sharks. The antigen binding portion of the HcAb is comprised of a VHH fragment.

[0063] The term “recombinant antibody”, as used herein, refers to an antibody (e.g. a chimeric, humanized, or human antibody or antigen-binding fragment thereof) that is expressed by a recombinant host cell comprising nucleic acid encoding the antibody. Examples of “host cells” for producing recombinant antibodies include: (1) mammalian cells, for example, Chinese Hamster Ovary (CHO), COS, myeloma cells (including YO and NSO cells), baby hamster kidney (BHK), Hela and Vero cells; (2) insect cells, for example, sf9, sf21 and Tn5; (3) plant cells, for example plants belonging to the genus *Nicotiana* (e.g. *Nicotiana tabacum*); (4) yeast cells, for example, those belonging to the genus *Saccharomyces* (e.g. *Saccharomyces cerevisiae*) or the genus *Aspergillus* (e.g. *Aspergillus niger*); (5) bacterial cells, for example *Escherichia coli* cells or *Bacillus subtilis* cells, etc.

[0064] An “antigen-binding fragment” of an antibody is a molecule that comprises a portion of a full-length antibody which is capable of detectably binding to the antigen, typically comprising one or more portions of at least the VH region. Antigen-binding fragments include multivalent molecules comprising one, two, three, or more antigen-binding portions of an antibody, and single-chain constructs wherein the VL and VH regions, or selected portions thereof, are joined by synthetic linkers or by recombinant methods to form a functional, antigen-binding molecule. Antigen-binding fragments can also be a single-domain antibody (sdAb), also known as a nanobody, which is an antibody fragment consisting of a single monomeric variable antibody domain (VHH). While some antigen-binding fragments of an antibody can be obtained by actual fragmentation of a larger antibody molecule (e.g., enzymatic cleavage), most are typically produced by recombinant techniques. The antibodies of the invention can be prepared as full-length antibodies or antigen-binding fragments thereof. Examples of antigen-binding fragments

include Fab, Fab', F(ab)₂, F(ab')₂, F(ab)₃, Fv (typically the VL and VH domains of a single arm of an antibody), single-chain Fv (scFv, see e.g., Bird et al., *Science* 1988; 242:423-426; and Huston et al. *PNAS* 1988; 85:5879-5883), dsFv, Fd (typically the VH and CH1 domain), and dAb (typically a VH domain) fragments; VH, VL, VHH, and V-NAR domains; monovalent molecules comprising a single VH and a single VL chain; minibodies, diabodies, triabodies, tetrabodies, and kappa bodies (see, e.g., Ill et al., *Protein Eng* 1997; 10:949-57); camel IgG; IgNAR; as well as one or more isolated CDRs or a functional paratope, where the isolated CDRs or antigen-binding residues or polypeptides can be associated or linked together so as to form a functional antibody fragment. Various types of antibody fragments have been described or reviewed in, e.g., Holliger and Hudson, *Nat Biotechnol* 2005; 23:1126-1136; WO2005040219, and published U.S. Patent Applications 20050238646 and 20020161201. Antibody fragments can be obtained using conventional recombinant or protein engineering techniques, and the fragments can be screened for antigen-binding or other function in the same manner as are intact antibodies.

[0065] Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of full-length antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods*, 24:107-117 (1992); and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter et al., *Bio/Technology*, 10:163-167 (1992)). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. In other embodiments, the antibody of choice is a single-chain Fv fragment (scFv). See WO 1993/16185; U.S. Pat. No. 5,571,894; and U.S. Pat. No. 5,587,458. The antibody fragment may also be a "linear antibody", e.g., as described in U.S. Pat. No. 5,641,870, for example. Such linear antibody fragments can be monospecific or bispecific.

[0066] The term "antibody derivative" as used herein refers to a molecule comprising a full-length antibody or an antigen-binding fragment thereof, wherein one or more amino acids are chemically modified or substituted. Chemical modifications that can be used in antibody derivative includes, e.g., alkylation, PEGylation, acylation, ester formation or amide formation or the like, e.g., for linking the antibody to a second molecule. Exemplary modifications include

PEGylation (e.g., cysteine- PEGylation), biotinylation, radiolabeling, and conjugation with a second agent (such as a cytotoxic agent).

[0067] Antibodies herein include “amino acid sequence variants” with altered antigen-binding or biological activity. Examples of such amino acid alterations include antibodies with enhanced affinity for antigen (e.g. “affinity matured” antibodies), and antibodies with altered Fc region, if present, e.g. with altered (increased or diminished) antibody dependent cellular cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) (see, for example, WO 00/42072, Presta, L. and WO 99/51642, Iduosogie et al); and/or increased or diminished serum half-life (see, for example, WO00/42072, Presta, L.).

[0068] A “multispecific molecule” comprises an antibody, or an antigen-binding fragment thereof, which is associated with or linked to at least one other functional molecule (e.g. another peptide or protein such as another antibody or ligand for a receptor) thereby forming a molecule that binds to at least two different binding sites or target molecules. Exemplary multispecific molecules include bi-specific antibodies and antibodies linked to soluble receptor fragments or ligands.

[0069] The term “human antibody”, as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from (i.e., are identical or essentially identical to) human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is “derived from” human germline immunoglobulin sequences. The human antibodies of the invention can include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term “human antibody”, as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0070] A “humanized” antibody is a human/non-human chimeric antibody that contains a minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or non-human primate having the desired specificity, affinity, and capacity. In some instances, FR residues of the human

immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR residues are those of a human immunoglobulin sequence. The humanized antibody can optionally also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, e.g., Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992), WO 92/02190, US Patent Application 20060073137, and U.S. Pat. Nos. 6,750,325, 6,632,927, 6,639,055, 6,548,640, 6,407,213, 6,180,370, 6,054,297, 5,929,212, 5,895,205, 5,886,152, 5,877,293, 5,869,619, 5,821,337, 5,821,123, 5,770,196, 5,777,085, 5,766,886, 5,714,350, 5,693,762, 5,693,761, 5,530,101, 5,585,089, and 5,225,539.

[0071] The term “hypervariable region” when used herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region generally comprises amino acid residues from a “complementarity-determining region” or “CDR” (residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light-chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy-chain variable domain; (Kabat et al. (1991) *Sequences of Proteins of Immunological Interest*, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and/or those residues from a “hypervariable loop” (residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light-chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy-chain variable domain; Chothia and Lesk, *J. Mol. Biol.* 1987; 196:901-917). Typically, the numbering of amino acid residues in this region is performed by the method described in Kabat et al., *supra*. Phrases such as “Kabat position”, “variable domain residue numbering as in Kabat” and “according to Kabat” herein refer to this numbering system for heavy chain variable domains or light chain variable domains. Using the Kabat numbering system, the actual linear amino acid sequence of a peptide can contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or CDR of the variable domain. For example, a heavy chain variable domain can include a single amino acid insert (residue 52a according to Kabat) after residue 52 of CDR H2 and inserted residues (e.g.

residues 82a, 82b, and 82c, etc. according to Kabat) after heavy chain FR residue 82. The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a “standard” Kabat numbered sequence.

[0072] “Framework region” or “FR” residues are those VH or VL residues other than the CDRs as herein defined.

[0073] An “epitope” or “binding site” is an area or region on an antigen to which an antigen-binding peptide (such as an antibody) specifically binds. A protein epitope can comprise amino acid residues directly involved in the binding (also called the immunodominant component of the epitope) and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked by the specifically antigen binding peptide (in other words, the amino acid residue is within the “solvent-excluded surface” and/or “footprint” of the specifically antigen binding peptide).

[0074] A “paratope” is an area or region of an antigen-binding portion of an antibody that specifically binds an antigen. Unless otherwise stated or clearly contradicted by context, a paratope can comprise amino acid residues directly involved in epitope binding, several of which are typically in CDRs, and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked by the specifically bound antigen (in other words, the amino acid residue is within the “solvent-excluded surface” and/or “footprint” of the specifically bound antigen).

[0075] An “antibody that binds to the same epitope” as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more.

[0076] An “isolated” antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For a review of methods for assessment of antibody purity, see, e.g., Flatman et al, J. Chromatogr. B 848:79-87 (2007).

[0077] The term “administering” with respect to the methods of the invention, means a method for therapeutically or prophylactically preventing, treating or ameliorating a syndrome, disorder

or disease as described herein by using a conjugate of the invention or a form, composition or medicament thereof. Such methods include administering an effective amount of said antibody, antigen-binding fragment thereof, or conjugate, or a form, composition or medicament thereof at different times during the course of a therapy or concurrently in a combination form. The methods of the invention are to be understood as embracing all known therapeutic treatment regimens.

[0078] The ability of a target antibody to “block” the binding of a target molecule to a natural target ligand, means that the antibody, in an assay using soluble or cell-surface associated target and ligand molecules, can detectably reduce the binding of a target molecule to the ligand in a dose-dependent fashion, where the target molecule detectably binds to the ligand in the absence of the antibody.

[0079] The “blood-brain barrier” or “BBB” refers a physiological barrier between the peripheral circulation and the brain and spinal cord which is formed by tight junctions within the brain capillary endothelial plasma membranes, creating a tight barrier that restricts the transport of molecules into the brain. The BBB can restrict the transport of even very small molecules such as urea (60 Daltons) into the brain. Examples of the BBB include the BBB within the brain, the blood-spinal cord barrier within the spinal cord, and the blood-retinal barrier within the retina, all of which are contiguous capillary barriers within the CNS. The BBB also encompasses the blood-CSF barrier (choroid plexus) where the barrier is comprised of ependymal cells rather than capillary endothelial cells.

[0080] A “blood-brain barrier receptor” (abbreviated “R/BBB” herein) is an extracellular membrane-linked receptor protein expressed on brain endothelial cells which is capable of transporting molecules across the BBB or be used to transport exogenous administered molecules. Examples of R/BBB include, but are not limited to, transferrin receptor (TfR), insulin receptor, insulin-like growth factor receptor (IGF-R), low density lipoprotein receptors including without limitation low density lipoprotein receptor-related protein 1 (LRP1) and low density lipoprotein receptor-related protein 8 (LRP8), and heparin-binding epidermal growth factor-like growth factor (HB-EGF). An exemplary R/BBB herein is transferrin receptor (TfR).

[0081] The “central nervous system” or “CNS” refers to the complex of nerve tissues that control bodily function, and includes the brain and spinal cord.

[0082] A “conjugate” as used herein refer to a protein covalently linked to one or more heterologous molecule(s), including but not limited to a therapeutic peptide or protein, an antibody, a label, or a neurological disorder drug.

[0083] As used herein the term “coupled” refers to the joining or connection of two or more objects together. When referring to chemical or biological compounds, coupled can refer to a covalent connection between the two or more chemical or biological compounds. By way of a non-limiting example, an antibody of the invention can be coupled with a peptide of interest to form an antibody coupled peptide. An antibody coupled peptide can be formed through specific chemical reactions designed to conjugate the antibody to the peptide. In certain embodiments, an antibody of the invention can be covalently coupled with a peptide of the invention through a linker. The linker can, for example, be first covalently connected to the antibody or the peptide, then covalently connected to the peptide or the antibody.

[0084] An “effective amount” or “therapeutically effective amount” of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[0085] A “linker” as used herein refers to a chemical linker or a single chain peptide linker that covalently connects two different entities. A linker can be used to connect any two of an antibody or a fragment thereof, a blood brain barrier shuttle, a fusion protein and a conjugate of the present invention. The linker can connect, for example, the VH and VL in scFv, or the monoclonal antibody or antigen-binding fragment thereof with a therapeutic molecule, such as a second antibody. In some embodiment, if the monovalent binding entity comprises a scFv directed to TfR, preferably huTfR1, and the therapeutic molecule comprises an antibody directed to a CNS target, such as Tau, then the linker can connect the scFv to the antibody directed to Tau. Single chain peptide linkers, comprised of from 1 to 25 amino acids, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids, joined by peptide bonds, can be used. In certain embodiments, the amino acids are selected from the twenty naturally occurring amino acids. In certain other embodiments, one or more of the amino acids are selected from glycine, alanine, proline, asparagine, glutamine and lysine. Chemical linkers, such as a hydrocarbon linker, a polyethylene glycol (PEG) linker, a polypropylene glycol (PPG) linker, a polysaccharide linker, a polyester linker, a hybrid linker consisting of PEG and an embedded heterocycle, and a hydrocarbon chain can also be used.

[0086] A “neurological disorder” as used herein refers to a disease or disorder which affects the CNS and/or which has an etiology in the CNS. Exemplary CNS diseases or disorders include, but are not limited to, neuropathy, amyloidosis, cancer, an ocular disease or disorder, viral or microbial infection, inflammation, ischemia, neurodegenerative disease, seizure, behavioral disorders, and a lysosomal storage disease. For the purposes of this application, the CNS will be understood to include the eye, which is normally sequestered from the rest of the body by the blood-retina barrier. Specific examples of neurological disorders include, but are not limited to, neurodegenerative diseases (including, but not limited to, Lewy body disease, postpoliomyelitis syndrome, Shy-Draeger syndrome, olivopontocerebellar atrophy, Parkinson's disease, multiple system atrophy, striatonigral degeneration, spinocerebellar ataxia, spinal muscular atrophy), tauopathies (including, but not limited to, Alzheimer disease and supranuclear palsy), prion diseases (including, but not limited to, bovine spongiform encephalopathy, scrapie, Creutzfeldt-Jakob syndrome, kuru, Gerstmann-Straussler-Scheinker disease, chronic wasting disease, and fatal familial insomnia), bulbar palsy, motor neuron disease, and nervous system heterodegenerative disorders (including, but not limited to, Canavan disease, Huntington's disease, neuronal ceroid-lipofuscinosis, Alexander's disease, Tourette's syndrome, Menkes kinky hair syndrome, Cockayne syndrome, Halervorden-Spatz syndrome, lafora disease, Rett syndrome, hepatolenticular degeneration, Lesch-Nyhan syndrome, and Unverricht-Lundborg syndrome), dementia (including, but not limited to, Pick's disease, and spinocerebellar ataxia), cancer (e.g. of the CNS and/or brain, including brain metastases resulting from cancer elsewhere in the body).

[0087] A “neurological disorder drug” is a drug or therapeutic agent useful in treating or ameliorating the effects of one or more neurological disorder(s). Neurological disorder drugs of the invention include, but are not limited to, small molecule compounds, antibodies, peptides, proteins, natural ligands of one or more CNS target(s), modified versions of natural ligands of one or more CNS target(s), aptamers, inhibitory nucleic acids (i.e., small inhibitory RNAs (siRNA) and short hairpin RNAs (shRNA)), ribozymes, or active fragments of any of the foregoing. Exemplary neurological disorder drugs of the invention are described herein and include, but are not limited to: antibodies, aptamers, proteins, peptides, inhibitory nucleic acids and small molecules and active fragments of any of the foregoing that either are themselves or specifically recognize and/or act upon (i.e., inhibit, activate, or detect) a CNS antigen or target

molecule such as, but not limited to, amyloid precursor protein or portions thereof, amyloid beta, beta-secretase, gamma-secretase, tau, alpha-synuclein, parkin, huntingtin, DR6, presenilin, ApoE, glioma or other CNS cancer markers, and neurotrophins. Non-limiting examples of neurological disorder drugs and the corresponding disorders they may be used to treat: Brain-derived neurotrophic factor (BDNF), Chronic brain injury (Neurogenesis), Fibroblast growth factor 2 (FGF-2), Anti-Epidermal Growth Factor Receptor Brain cancer, (EGFR)-antibody, Glial cell-line derived neural factor Parkinson's disease, (GDNF), Brain-derived neurotrophic factor (BDNF) Amyotrophic lateral sclerosis, depression, Lysosomal enzyme Lysosomal storage disorders of the brain, Ciliary neurotrophic factor (CNTF) Amyotrophic lateral sclerosis, Neuregulin-1 Schizophrenia, Anti-HER2 antibody (e.g. trastuzumab) Brain metastasis from HER2-positive cancer.

[0088] The term “pharmaceutical formulation” refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

[0089] As used herein, “pharmaceutically acceptable carrier or diluent” means any substance suitable for use in administering to an individual. For example, a pharmaceutically acceptable carrier can be a sterile aqueous solution, such as phosphate buffer saline (PBS) or water-for-injection.

[0090] As used herein, “pharmaceutically acceptable salts” means physiologically and pharmaceutically acceptable salts of compounds, such as oligomeric compounds or oligonucleotides, i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0091] Pharmaceutically acceptable acidic/anionic salts for use in the invention include, and are not limited to acetate, benzenesulfonate, benzoate, bicarbonate, bitartrate, bromide, calcium edetate, camsylate, carbonate, chloride, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, fumarate, glyceplate, gluconate, glutamate, glycolylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, malate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, mucate, napsylate, nitrate, pamoate, pantothenate, phosphate/diphosphate, polygalacturonate, salicylate, stearate, subacetate, succinate, sulfate, tannate, tartrate, teoclate, tosylate and

triethiodide. Organic or inorganic acids also include, and are not limited to, hydriodic, perchloric, sulfuric, phosphoric, propionic, glycolic, methanesulfonic, hydroxyethanesulfonic, oxalic, 2-naphthalenesulfonic, p-toluenesulfonic, cyclohexanesulfamic, saccharinic or trifluoroacetic acid. Pharmaceutically acceptable basic/cationic salts include, and are not limited to aluminum, 2-amino-2-hydroxymethyl-propane-1,3-diol (also known as tris(hydroxymethyl)aminomethane, tromethane or "TRIS"), ammonia, benzathine, t-butylamine, calcium, chlorprocaine, choline, cyclohexylamine, diethanolamine, ethylenediamine, lithium, L-lysine, magnesium, meglumine, N-methyl-D-glucamine, piperidine, potassium, procaine, quinine, sodium, triethanolamine, or zinc.

[0092] "Polypeptide" or "protein" means a molecule that comprises at least two amino acid residues linked by a peptide bond to form a polypeptide. Small polypeptides of less than 50 amino acids may be referred to as "peptides".

[0093] The phrases "sequence identity" or "percent (%) sequence identity" or "% identity" or "% identical to" when used with reference to an amino acid sequence describe the number of matches ("hits") of identical amino acids of two or more aligned amino acid sequences as compared to the number of amino acid residues making up the overall length of the amino acid sequences. In other terms, using an alignment, for two or more sequences the percentage of amino acid residues that are the same (e.g. 90%, 91%, 92%, 93%, 94%, 95%, 97%, 98%, 99%, or 100% identity over the full-length of the amino acid sequences) may be determined, when the sequences are compared and aligned for maximum correspondence as measured using a sequence comparison algorithm as known in the art, or when manually aligned and visually inspected. The sequences which are compared to determine sequence identity may thus differ by substitution(s), addition(s) or deletion(s) of amino acids. Suitable programs for aligning protein sequences are known to the skilled person. The percentage sequence identity of protein sequences can, for example, be determined with programs such as CLUSTALW, Clustal Omega, FASTA or BLAST, e.g. using the NCBI BLAST algorithm (Altschul SF, et al (1997), *Nucleic Acids Res.* 25:3389-3402).

[0094] The term "substantially identical" in the context of two amino acid sequences means that the sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least about 50 percent sequence identity. Typically sequences that

are substantially identical will exhibit at least about 60, at least about 70, at least about 80, at least about 90, at least about 95, at least about 98, or at least about 99 percent sequence identity.

[0095] “Specific binding” or “specifically binds” or “binds” refer to antibody binding to an antigen or an epitope within the antigen with greater affinity than for other antigens. Typically, the antibody binds to the antigen or the epitope within the antigen with a dissociation constant (K_D) of about 1×10^{-8} M or less, for example about 1×10^{-9} M or less, about 1×10^{-10} M or less, about 1×10^{-11} M or less, or about 1×10^{-12} M or less, typically with a K_D that is at least one hundred fold less than its K_D for binding to a non-specific antigen (e.g., BSA, casein). K_D is the equilibrium dissociation constant, a ratio of k_{off}/k_{on} , between the antibody and its antigen. K_D and affinity are inversely related. The “on-rate” (k_{on}) is a constant used to characterize how quickly the antibody binds to its target. The “off-rate” (k_{off}) is a constant used to characterize how quickly an antibody dissociates from its target. The dissociation constant K_D can be measured using standard procedures. For example, the K_D of an antibody can be determined by using surface plasmon resonance, such as by using a biosensor system, e.g., a Biacore® system, or by using bio-layer interferometry technology, such as an Octet RED96 system. The smaller the value of the K_D of an antibody, the higher affinity that the antibody binds to a target antigen. Antibodies that specifically bind to the antigen or the epitope within the antigen can, however, have cross-reactivity to other related antigens, for example to the same antigen from other species (homologs), such as human or monkey, for example *Macaca fascicularis* (cynomolgus, cyno), *Pan troglodytes* (chimpanzee, chimp) or *Callithrix jacchus* (common marmoset, marmoset). While a monospecific antibody specifically binds one antigen or one epitope, a bispecific antibody specifically binds two distinct antigens or two distinct epitopes.

[0096] The term “subject” as used herein refers to a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human. When the subject is human, they can also be referred to as a “patient”.

[0097] The term “transferrin receptor” or “TfR,” as used herein, refers to a cell surface receptor necessary for cellular iron uptake by the process of receptor-mediated endocytosis. carrier protein for transferrin. A TfR is involved in iron uptake in vertebrates and is regulated in response to intracellular iron concentration. It imports iron by internalizing the transferrin-iron

complex through receptor-mediated endocytosis. Two transferrin receptors in humans, transferrin receptor 1 and transferrin receptor 2, have been characterized. Both these receptors are transmembrane glycoproteins. TfR1 is a high affinity ubiquitously expressed receptor. TfR2 binds to transferrin with a 25-30-fold lower affinity than TfR1. The expression of TfR2 is restricted to certain cell types and is unaffected by intracellular iron concentrations. In one embodiment, the TfR is a human TfR comprising the amino acid sequence as in Schneider et al. Nature 311: 675-678 (1984), for example. It can have a molecular weight of about 180,000 Dalton, having two subunits each of apparent molecular weight of about 90,000 Dalton. Preferably, the TfR is a human TfR1.

[0098] A “target antigen” or “brain target,” as used herein, refers to an antigen and/or molecule expressed in the CNS, including the brain, which can be targeted with an antibody or small molecule. Examples of such antigens and/or molecules include, without limitation: beta-secretase 1 (BACE1), amyloid beta (Abeta), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), Tau, apolipoprotein E4 (ApoE4), alpha-synuclein, CD20, huntingtin, prion protein (PrP), leucine rich repeat kinase 2 (LRRK2), parkin, presenilin 1, presenilin 2, gamma secretase, death receptor 6 (DR6), amyloid precursor protein (APP), p75 neurotrophin receptor (p75NTR), and caspase 6. In some embodiments, the target antigen is BACE1. In some embodiments, the target antigen is Tau.

[0099] As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

[00100] Antibodies or immunoglobulins can be assigned to five major classes, namely IgA, IgD, IgE, IgG and IgM, depending on the heavy chain constant domain amino acid sequence. IgG is the most stable of the five types of immunoglobulins, having a serum half-life in humans of about 23 days. IgA and IgG are further sub-classified as the isotypes IgA₁, IgA₂, IgG₁, IgG₂,

IgG₃ and IgG₄. Each of the four IgG subclasses has different biological functions known as effector functions. These effector functions are generally mediated through interaction with the Fc receptor (FcγR) and/or by binding C1q and fixing complement. Binding to FcγR can lead to antibody dependent cell mediated cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC), whereas binding to complement factors can lead to complement mediated cell lysis or complement-dependent cytotoxicity (CDC). An anti-TfR antibody of the invention, or a therapeutic or diagnostic antibody to be conjugated or fused to the anti-TfR antibody can have no or minimal effector function, but retains its ability to bind FcRn, the binding of which can be a primary means by which antibodies have an extended *in vivo* half-life.

[00101] Binding of FcγR or complement (e.g., C1q) to an antibody is caused by defined protein-protein interactions at the so-called Fc part binding site. Such Fc part binding sites are known in the art. Such Fc part binding sites include, e.g., characterized by the amino acids L234, L235, D270, N297, E318, K320, K322, P331, and P329 (numbering according to EU index of Kabat). In some embodiment, an anti-TfR antibody of the invention, or a therapeutic or diagnostic antibody to be conjugated or fused to the anti-TfR antibody contains one or more substitutions in one or more Fc part binding sites to eliminate the effector function. For example, an anti-TfR antibody of the invention, or a therapeutic or diagnostic antibody to be conjugated or fused to the anti-TfR antibody can contain a Fc region containing one or more of the following substitutions: substitution of proline for glutamate at residue 233, alanine or valine for phenylalanine at residue 234 and alanine or glutamate for leucine at residue 235 (EU numbering, Kabat, E. A. et al. (1991) Sequences of Proteins of Immunological Interest, 5th Ed. U.S. Dept. of Health and Human Services, Bethesda, Md., NIH Publication no. 91-3242). Preferably, the antibody of interest contains one, two or three mutations of L234A, L235A and P331S (EU numbering, Kabat).

[00102] Antibodies of subclass IgG1, IgG2, and IgG3 usually show complement activation including C1q and C3 binding, whereas IgG4 does not activate the complement system and does not bind C1q and/or C3. Human IgG4 Fc region has reduced ability to bind FcγR and complement factors compared to other IgG sub-types. Preferably, an anti-TfR antibody of the invention, or a therapeutic or diagnostic antibody to be conjugated or fused to the anti-TfR antibody comprises a Fc region derived from human IgG4 Fc region. More preferably, the Fc region contains human IgG4 Fc region having substitutions that eliminate effector function. For

example, removing the N-linked glycosylation site in the IgG4 Fc region by substituting Ala for Asn at residue 297 (EU numbering) is another way to ensure that residual effector activity is eliminated.

Anti-TfR Antibodies and antigen binding fragments thereof

[00103] In one general aspect, the application relates to an antibody or an antigen binding fragment thereof that binds to a primate TfR, such as a human TfR or a monkey TfR, and the antibody or an antigen binding fragment thereof is optimized for delivering an agent to the brain of a subject in need thereof. The relationship between the binding affinity of an anti-TfR antibody to the TfR and transcytosis efficiency has been described previously as improved transcytosis with decreased affinity for TfR (Yu, Zhang et al. 2011, *Sci Transl Med* 3(84): 84ra44). The inventors of the present invention surprisingly discovered a more nuanced relationship between affinity and transcytosis efficiency than what has been previously described, with influence from both on- and off-rates impacting brain concentration. In particular, a neutral off-rate that is neither too fast nor too slow is required for optimal brain PK and PD of an agent (such as an mAb) to be efficiently delivered by the anti-TfR antibody or antigen binding fragment thereof.

[00104] Preferably, an anti-TfR antibody or antigen binding fragment thereof of the application is pH-sensitive, e.g., it has different binding affinities to TfR at different pHs. For example, an anti-TfR antibody of the application can bind to cell surface TfR at a neutral pH, such as physiological pH (e.g., pH 7.4), with high affinity, but upon internalization into an endosomal compartment, dissociates from TfR at an acidic pH, such as the relatively lower pH (pH 5.0-6.0). Affinity is a measure of the strength of binding between two moieties, e.g., an antibody and an antigen. Affinity can be expressed in several ways. One way is in terms of the dissociation constant (K_D) of the interaction. K_D can be measured by routine methods, include equilibrium dialysis or by directly measuring the rates of antigen-antibody dissociation and association, the k_{off} (k_d or k_{dis}) and k_{on} (or k_a) rates, respectively (see e.g., *Nature*, 1993 361:186-87). The ratio of k_{off}/k_{on} cancels all parameters not related to affinity, and is equal to the dissociation constant K_D (see, generally, Davies et al., *Annual Rev Biochem*, 1990 59:439-473). Thus, a smaller K_D means a higher affinity. Another expression of affinity is K_a , which is the inverse of K_D , or k_{on}/k_{off} . Thus, a higher K_a means a higher affinity. For example, an antibody or antigen binding fragment thereof for use in a composition and/or method of the application can

be an antibody or fragment thereof that binds to a TfR with a K_D of 1 nanomolar (nM, 10^{-9} M) or more at a neutral pH (e.g., pH 6.8-7.8), such as a physiological pH (e.g., pH 7.4), and dissociates from TfR with a k_{dis} of 10^{-4} sec $^{-1}$ or more at an acidic pH (e.g., pH 4.5-6.0), such as pH 5.0).

[00105] Accordingly, a general aspect of the application relates to an anti-TfR antibody or antigen-binding fragment thereof for delivering an agent to the brain of a subject in need thereof, wherein the anti-TfR antibody or antigen-binding fragment thereof binds to a transferrin receptor (TfR), preferably human TfR1, with a dissociation constant K_D of at least 1 nM, preferably 1 nM to 500 nM, at neutral pH and an off-rate constant k_a of at least 10^{-4} sec $^{-1}$, preferably 10^{-4} to 10^{-1} sec $^{-1}$, at an acidic pH, preferably pH 5.

[00106] In one embodiment, the anti-TfR antibody or antigen-binding fragment thereof of the application has an off-rate constant k_a of 2×10^{-2} to 2×10^{-4} sec $^{-1}$, such as 2×10^{-2} , 1×10^{-2} , 9×10^{-3} , 8×10^{-3} , 7×10^{-3} , 6×10^{-3} , 5×10^{-3} , 4×10^{-3} , 3×10^{-3} , 2×10^{-3} , 1×10^{-3} , 9×10^{-4} , 8×10^{-4} , 7×10^{-4} , 6×10^{-4} , 5×10^{-4} , 4×10^{-4} , 3×10^{-4} , 2×10^{-4} sec $^{-1}$, or any value in between, at the neutral pH.

[00107] In certain embodiments, the antibody or antigen binding fragment thereof that binds to human TfR is a single variable domain on a heavy chain (VHH) antibody comprising heavy chain complementarity determining regions (HCDRs) HCDR1, HCDR2 and HCDR3 having the amino acid sequences of:

- (i) SEQ ID NOs: 7, 8 and 9, respectively;
- (ii) SEQ ID NOs: 317, 318 and 319, respectively;
- (iii) SEQ ID NOs: 324, 325 and 326, respectively;
- (iv) SEQ ID NOs: 331, 332 and 333, respectively; or
- (v) SEQ ID NOs: 338, 339 and 340, respectively.

[00108] Preferably, it is a VHH fragment comprising an amino acid sequence having at least 80%, such as at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to SEQ ID NO: 6, 316, 323, 330, or 337.

[00109] In other embodiments, the antibody or antigen binding fragment thereof that binds to human TfR comprises a heavy chain variable region comprising heavy chain complementarity determining regions (HCDRs) HCDR1, HCDR2 and HCDR3, and a light chain variable region comprising light chain complementarity determining regions (LCDRs) LCDR1, LCDR2 and LCDR3, wherein the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 have the amino acid sequences of:

- (i) SEQ ID NOs: 292, 293, 294, 295, 296, and 297, respectively;
- (ii) SEQ ID NOs: 279, 280, 281, 282, 283 and 284, respectively;
- (iii) SEQ ID NOs: 29, 30, 31, 32, 33 and 34, respectively;
- (iv) SEQ ID NOs: 57, 58, 59, 60, 61 and 62, respectively;
- (v) SEQ ID NOs: 85, 86, 87, 88, 89 and 90, respectively;
- (vi) SEQ ID NOs: 110, 111, 112, 113, 114 and 115, respectively;
- (vii) SEQ ID NOs: 135, 136, 137, 138, 139 and 140, respectively;
- (viii) SEQ ID NOs: 191, 192, 193, 194, 195 and 196, respectively;
- (ix) SEQ ID NOs: 244, 245, 246, 247, 248 and 249, respectively;
- (x) SEQ ID NOs: 263, 264, 265, 266, 267 and 268, respectively;
- (xi) SEQ ID NOs: 345, 346, 347, 348, 349 and 350, respectively;
- (xii) SEQ ID NOs: 355, 356, 357, 358, 359 and 360, respectively;
- (xiii) SEQ ID NOs: 365, 366, 367, 368, 369 and 370, respectively;
- (xiv) SEQ ID NOs: 375, 376, 377, 378, 379 and 380, respectively;
- (xv) SEQ ID NOs: 385, 386, 387, 388, 389 and 390, respectively;
- (xvi) SEQ ID NOs: 395, 396, 397, 398, 399 and 400, respectively;
- (xvii) SEQ ID NOs: 405, 406, 407, 408, 409 and 410, respectively;
- (xviii) SEQ ID NOs: 415, 416, 417, 418, 419 and 420, respectively;
- (xix) SEQ ID NOs: 425, 426, 427, 428, 429 and 430, respectively;
- (xx) SEQ ID NOs: 435, 436, 437, 438, 439 and 440, respectively;
- (xxi) SEQ ID NOs: 445, 446, 447, 448, 449 and 450, respectively;
- (xxii) SEQ ID NOs: 455, 456, 457, 458, 459 and 460, respectively;
- (xxiii) SEQ ID NOs: 465, 466, 467, 468, 469 and 470, respectively;
- (xxiv) SEQ ID NOs: 475, 476, 477, 478, 479 and 480, respectively;
- (xxv) SEQ ID NOs: 485, 486, 487, 488, 489 and 490, respectively;
- (xxvi) SEQ ID NOs: 495, 496, 497, 498, 499 and 500, respectively;
- (xxvii) SEQ ID NOs: 505, 506, 507, 508, 509 and 510, respectively;
- (xxviii) SEQ ID NOs: 515, 516, 517, 518, 519 and 520, respectively;
- (xxix) SEQ ID NOs: 525, 526, 527, 528, 529 and 530, respectively;
- (xxx) SEQ ID NOs: 535, 536, 537, 538, 539 and 540, respectively; or
- (xxx) SEQ ID NOs: 545, 546, 547, 548, 549 and 550, respectively.

[00110] In other embodiments, an antibody or antigen binding fragment thereof of the application competes with an antibody or antigen binding fragment exemplified herein. The binding site of an antibody or antigen can be determined by known methods such as ELISA, Western blot, etc. In certain embodiments, such a competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by an exemplified antibody or antigen binding fragment thereof. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris, G. E., (ed.), "Epitope Mapping Protocols," In: Methods in Molecular Biology, Vol. 66, Humana Press, Totowa, N.J. (1996). An "antibody that binds to the same epitope" as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more.

[00111] Preferably, the antibody or antigen-binding fragment thereof is single-chain variable fragment (scFv) comprising the heavy chain variable region (Hv) covalently linked to the light chain variable region (Lv) via a flexible linker. The scFv can retain the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. In a scFv, the order of the domains can be either Hv-linker- Lv, or Lv-linker- Hv. The linker can be designed *de novo* or derived from known protein structure to provide a compatible length and conformational in bridging the variable domains of a scFv without serious steric interference. The linker can have 10 to about 25 amino acids in length. Preferably, the linker is a peptide linker spanning about 3.5 nm (35 Å) between the carboxy terminus of the variable domain and the amino terminus of the other domain without affecting the ability of the domains to fold and form an intact antigen-binding site (Huston et al., *Methods in Enzymology*, vol. 203, pp. 46–88, 1991, which is incorporated herein by reference in its entirety). The linker preferably comprises a hydrophilic sequence in order to avoid intercalation of the peptide within or between the variable domains throughout the protein folding (Argos, *Journal of Molecular Biology*, vol. 211, no. 4, pp. 943–958, 1990). For example, the linker can comprise Gly and Ser residues and/or together with the charged residues such as Glu, Thr and Lys interspersed to enhance the solubility. In one embodiment, the linker has the amino acid sequence of SEQ ID NO: 314 (GTEGKSSGSGSESKST). Any other suitable linker can also be used in view of the present disclosure.

[00112] In some embodiments, the scFv comprises an amino acid sequence having at least 80%, such as at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%, sequence identity to the amino acid sequences of SEQ ID NO: 278, 291, 28, 56, 84, 109, 134, 162, 190, 218, 243, 262, 344, 354, 364, 374, 384, 394, 404, 414, 424, 434, 444, 454, 464, 474, 484, 494, 504, 514, 524, 534 or 544.

[00113] In a preferred embodiment, an antibody or antigen binding fragment thereof that binds to Tfr, preferably human Tfr1, does not contain a free cysteine.

[00114] An anti-Tfr antibody or antigen-binding fragment thereof (such as a VHH or scFv fragment) can be produced using suitable methods in the art in view of the present disclosure. For example, a VHH or scFv fragment can be recombinantly produced by growing a recombinant host cell (such as a bacterial, yeast or mammalian cell) under suitable conditions for the production of the antibody fragment and recovering the fragment from the cell culture.

Brain shuttle construct

[00115] An optimized RMT brain delivery platform is developed using the transferrin receptor (Tfr) by enhancing the intrinsic transcytosis efficiency, extending peripheral pharmacokinetics, and engineering for an acceptable safety profile while maintaining efficacy of the therapeutic mAb. The interplay between transcytosis receptor affinity and brain concentration in human Tfr knock-in mice is studied. A thorough study of binding kinetics demonstrate that for optimal brain PK and PD of mAbs, a neutral off-rate that is neither too fast nor too slow is required. The enhanced brain delivery observed in mice was confirmed in cynomolgus monkey.

[00116] It is also discovered that engineered antibody constant region with increased binding to the neonatal Fc receptor (FcRn) resulted in decreased peripheral clearance and enhancement in brain concentration.

[00117] Additional Fc mutations are introduced to abolish binding to Fc gamma receptors (FcγR) and avoid effector function mediated toxicity. When coupled with a high affinity anti-Tau binding mAb, these mutations prevent effector function mediated toxicity in the periphery while maintaining antibody dependent phagocytosis (ADP) through a novel, non-FcγR mechanism for microglial uptake and target degradation. This mechanism is dependent upon internalization through the Tfr receptor and is more efficient in promoting target degradation than traditional FcγR mediated ADP without the stimulating the secretion of pro-inflammatory cytokines. To the knowledge of the inventors, this is the first report of non-FcγR mediated ADP, representing a

novel, efficient, non-inflammatory mechanism for phagocytosis that can be exploited for a variety of therapeutic applications.

[00118] Accordingly, in one general aspect, the application relates to an antibody-targeted brain delivery system comprising an anti-TfR antibody or antigen binding fragment thereof of the application. The anti-TfR antibody or antigen binding fragment thereof can be used to deliver a therapeutic or diagnostic agent into a cell (e.g., a cancer cell) or a BBB system. Agents that can be delivered include any neurological disorder drug or agent that can be used to detect or analyze a neurological disorder drug. For example, such agent can be neurotrophic factors, including, but not limited to, nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-line neurotrophic factor (GDNF) and insulin-like growth factor (IGF); neuropeptides, including, but not limited to, Substance P, neuropeptide Y, vasoactive intestinal peptide (VIP), gamma-amino-butyric acid (GABA), dopamine, cholecystokinin (CCK), endorphins, enkephalins and thyrotropin releasing hormone (TRH); cytokines; anxiolytic agents; anticonvulsants; polynucleotides and transgenes, including, for example, small interfering RNAs and/or antisense oligos; or antibodies or antigen binding fragments thereof that bind to a brain target. An anti-hTfR antibody or antigen binding fragment thereof of the application can be an effective means to enhance the delivery of an agent of interest from the blood into the brain and function there.

[00119] In particular, an agent of interest can be delivered in a combined form or linked to an anti-TfR antibody or antigen binding fragment thereof of the application, parenterally, e.g., intravenously. For example, the agent can be non-covalently attached to the anti-TfR antibody or antigen binding fragment thereof. The agent can also be covalently attached to the anti-TfR antibody or antigen binding fragment thereof to form a conjugate. In certain embodiments, the conjugation is by construction of a protein fusion (i.e., by genetic fusion of the two genes encoding an anti-TfR antibody or antigen binding fragment thereof and a neurological disorder drug and expression as a single protein). Known methods can be used to link an agent to an antibody or antigen binding fragment thereof in view of the present disclosure. See, for example, Wu et al., *Nat Biotechnol.*, 23(9):1137-46, 2005; Trail et al., *Cancer Immunol Immunother.*, 52(5):328-37, 2003; Saito et al., *Adv Drug Deliv Rev.*, 55(2):199-215, 2003; Jones et al., *Pharmaceutical Research*, 24(9):1759-1771, 2007.

[00120] In some embodiments, a therapeutic or diagnostic agent to be delivered to the brain and an anti-TfR antibody or antigen binding fragment thereof can be covalently linked together (or conjugated) via a non-peptide linker or a peptide linker. Examples of non-peptide linkers include, but are not limited to, polyethylene glycol, polypropylene glycol, copolymer of ethylene glycol and propylene glycol, polyoxyethylated polyol, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ether, biodegradable polymer, polymerized lipid, chitins, and hyaluronic acid, or derivatives thereof, or combinations thereof. A peptide linker can be a peptide chain consisting of 1 to 50 amino acids linked by peptide bonds or a derivative thereof, whose N-terminus and C-terminus can be covalently linked to an anti-TfR antibody or an antigen binding fragment thereof.

[00121] In certain embodiments, a conjugate of the application is a multi-specific antibody comprising a first antigen binding region which binds a TfR and a second antigen binding region which binds a brain antigen, such as beta-secretase 1 (BACE1), tau, and the other brain antigens disclosed herein. Techniques for making multi-specific antibodies include, but are not limited to, recombinant co-expression of two immunoglobulin heavy chain-light chain pairs having different specificities (see Milstein and Cuello, *Nature* 305: 537, 1983), WO 93/08829, and Traunecker et al, *EMBO J.* 10: 3655, 1991), and “knob-in-hole” engineering (see, e.g., U.S. Patent No. 5,731,168). Multi-specific antibodies can also be made by engineering electrostatic steering effects (WO 2009/089004A1); cross-linking two or more antibodies or fragments (see, e.g., US Patent No. 4,676,980, and Brennan et al, *Science*, 229: 81, 1985); using leucine zippers (see, e.g., Kostelny et al, *J. Immunol.*, 148(5): 1547-1553, 1992)); using “diabody” technology (see, e.g., Hollinger et al, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448, 1993)); using single-chain Fv (scFv) dimers (see, e.g. Gruber et al, *J. Immunol.*, 152:5368 (1994)); and preparing trispecific antibodies as described, e.g., in Tutt et al. *J. Immunol.* 147: 60, 1991. A multi-specific antibody of the application also encompasses antibodies having three or more functional antigen binding sites, including “Octopus antibodies” or “dual-variable domain immunoglobulins” (DVDs) (see, e.g. US 2006/0025576A1, and Wu et al. *Nature Biotechnology*, 25(11):1290-7, 2007). A multi-specific antibody of the application also encompasses a “Dual Acting Fab” or “DAF” comprising an antigen binding region that binds to TfR as well as the brain antigen (e.g. BACE1 or Tau) (see, US 2008/0069820, for example). In one embodiment, the antibody is an antibody fragment, various such fragments being disclosed herein.

[00122] In one embodiment, a multi-specific antibody of the application is a fusion construct comprising an anti-TfR antibody or antigen-binding fragment thereof of the application covalently linked (or fused) to a second antibody or antigen binding fragment thereof. Preferably, the second antibody or antigen binding fragment thereof binds to a brain target, such as BACE, tau or other brain antigens, such as those described herein. The anti-TfR antibody or antigen-binding fragment thereof can be fused to the carboxy- and/or amino- terminus of a light and/or heavy chain of the second antibody or antigen binding fragment thereof, directly or via a linker.

[00123] In one embodiment, the anti-TfR antibody or antigen-binding fragment thereof is fused to the carboxy-terminus of a light chain of the second antibody or antigen binding fragment thereof, directly or via a linker.

[00124] In another embodiment, the anti-TfR antibody or antigen-binding fragment thereof is fused to the amino-terminus of a light chain of the second antibody or antigen binding fragment thereof, directly or via a linker.

[00125] In another embodiment, the anti-TfR antibody or antigen-binding fragment thereof is fused to the carboxy-terminus of a heavy chain of the second antibody or antigen binding fragment thereof, directly or via a linker.

[00126] In another embodiment, the anti-TfR antibody or antigen-binding fragment thereof is fused to the amino-terminus of a heavy chain of the second antibody or antigen binding fragment thereof, directly or via a linker.

[00127] In a preferred embodiment, a fusion construct of the application comprises an anti-TfR antibody or antigen-binding fragment thereof, preferably an anti-huTfR1 VHH or scFv fragment, of the application covalently linked, via a linker, to the carboxy terminus of only one of the two heavy chains of a second antibody or antigen binding fragment thereof that binds to a brain target. Preferably, the linker has the amino acid sequence of SEQ ID NO: 312 or SEQ ID NO: 313.

[00128] To facilitate the formation of a heterodimer between the two heavy chains, e.g., one with a fusion of the anti-TfR antibody or antigen-binding fragment thereof and one without, or one containing the Fc for the anti-TfR arm and one for the anti-brain target arm, heterodimeric mutations introduced into the Fc of the two heavy chains. Examples of such Fc mutations include, but are not limited to, the Zymework mutations (see, e.g., US 10,457,742) and the “knob

in hole” mutations (see, e.g., Ridgway et al., *Protein Eng.*, 9(7): 617-621, 1996). Other heterodimer mutations can also be used in the invention. In some embodiment, a modified CH3 as described herein is used to facilitate the formation of a heterodimer between the two heavy chains.

[00129] In addition to the heterodimeric mutations, other mutations can also be introduced. In some embodiment, the Fc region of the fusion construct or bispecific antibody further comprises one or more mutations that alter (increase or diminish), preferably eliminate ADCC/CDC (such as the AAS mutations described herein), and/or one or more mutations that alter (increase or diminish), preferably increase, the binding of the fusion construct or bispecific antibody to FcRn (such as the YTE mutations described herein). In some embodiment, one or more cysteine residues in the fusion construct or bispecific antibody are substituted with other amino acids, such as serine.

[00130] In certain embodiments, a fusion construct of the application comprises:

- (1) a first heavy chain having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 301, 304, 307, 285, 288, 298, 10, 13, 16, 19, 22, 25, 35, 38, 41, 44, 47, 50, 53, 63, 66, 69, 72, 75, 78, 81, 91, 94, 97, 100, 103, 106, 116, 119, 122, 125, 128, 131, 141, 144, 147, 150, 153, 156, 159, 169, 172, 175, 178, 181, 184, 187, 197, 200, 203, 206, 209, 212, 215, 225, 228, 231, 234, 237, 240, 250, 252, 256, 259, 269, 272, 275, 320, 327, 334, 341, 351, 361, 371, 381, 391, 401, 411, 421, 431, 441, 451, 461 and 471;
- (2) two light chains each independently having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of 302, 305, 308, 286, 289, 299, 11, 14, 17, 20, 23, 26, 36, 39, 42, 45, 48, 51, 54, 64, 67, 70, 73, 76, 79, 82, 92, 95, 98, 101, 104, 107, 117, 120, 123, 126, 129, 132, 142, 145, 148, 151, 154, 157, 160, 170, 173, 176, 179, 182, 185, 188, 198, 201, 204, 207, 210, 213, 216, 226, 229, 232, 235, 238, 241, 251, 253, 257, 260, 270, 273, 276, 321, 328, 335, 342, 352, 362, 372, 382, 392, 402, 412, 422, 432, 442, 452, 462 and 472, respectively; and
- (3) a second heavy chain having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group

consisting of 303, 306, 309, 287, 290, 300, 12, 15, 18, 21, 24, 27, 37, 40, 43, 46, 49, 52, 55, 65, 68, 71, 74, 77, 80, 83, 93, 96, 99, 102, 105, 108, 118, 121, 124, 127, 130, 133, 143, 146, 149, 152, 155, 158, 161, 171, 174, 177, 180, 183, 186, 189, 199, 202, 205, 208, 211, 214, 217, 227, 230, 233, 236, 239, 242, 252, 254, 258, 261, 271, 274, 277, 322, 329, 336, 343, 353, 363, 373, 383, 393, 403, 413, 423, 433, 443, 453, 463 and 473, respectively.

[00131] A conjugate, such as a multi-specific antibody or fusion construct, of the application can be produced by any of a number of techniques known in the art in view of the present disclosure. For example, it can be expressed from a recombinant host cells, wherein expression vector(s) encoding the heavy and light chains of the fusion construct or multi-specific antibody is (are) transfected into a host cell by standard techniques. The host cells can be prokaryotic or eukaryotic host cells.

[00132] In an exemplary system, one or more recombinant expression vectors encoding the heterodimeric two heavy chains and the light chains of a fusion construct of the application is/are introduced into host cells by transfection or electroporation. The selected transformant host cells are cultured to allow for expression of the heavy and light chains under conditions sufficient to produce the fusion construct, and the fusion construct is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the protein construct from the culture medium.

[00133] The application provides an isolated nucleic acid encoding the amino acid sequence of an anti-TfR antibody or antigen binding fragment thereof alone or as part of a fusion construct or multispecific antibody in any of the embodiments described herein or any of the claims. The isolated nucleic acid can be part of a vector, preferably an expression vector.

[00134] In another aspect, the application relates to a host cell transformed with the vector disclosed herein. In an embodiment, the host cell is a prokaryotic cell, for example, *E. coli*. In another embodiment, the host cell is a eukaryotic cell, for example, a protist cell, an animal cell, a plant cell, or a fungal cell. In an embodiment, the host cell is a mammalian cell including, but not limited to, CHO, COS, NS0, SP2, PER.C6, or a fungal cell, such as *Saccharomyces cerevisiae*, or an insect cell, such as Sf9.

Pharmaceutical composition and related methods

[00135] The invention also relates to pharmaceutical compositions, methods of preparation and methods for use thereof.

[00136] In another general aspect, the invention relates to a pharmaceutical composition, comprising an anti-TfR antibody or antigen binding fragment thereof or a conjugate thereof of the invention and a pharmaceutically acceptable carrier. The anti-TfR antibody or antigen binding fragment thereof or conjugate (such as a multi-specific antibody or fusion construct) of the invention is also useful in the manufacture of a medicament for therapeutic applications mentioned herein. The pharmaceutically acceptable carrier can be any suitable excipient, diluent, filler, salt, buffer, stabilizer, solubilizer, oil, lipid, lipid containing vesicle, microsphere, liposomal encapsulation, or other material well known in the art for use in pharmaceutical formulations. It will be understood that the characteristics of the carrier, excipient or diluent will depend on the route of administration for a particular application.

[00137] Accordingly, in one embodiment, the application relates to a method of transporting a therapeutic or diagnostic agent across the blood-brain barrier (BBB) comprising exposing an anti-TfR antibody or antigen binding fragment thereof coupled to the therapeutic or diagnostic agent to the blood- brain barrier such that the antibody or antigen binding fragment thereof transports the agent coupled thereto across the blood- brain barrier. In one embodiment, the agent is a neurological disorder drug. In another embodiment, the agent is an imaging agent or an agent for detecting a neurological disorder. Preferably, the anti-TfR antibody or antigen binding fragment thereof or conjugate thereof does not impair the binding of the TfR to its native ligand transferrin. The antibody specifically binds to TfR in such a manner that it does not inhibit binding of the TfR to transferrin. In some embodiment, the BBB is in a mammal, preferably a primate, such as a human, more preferably a human having a neurological disorder. In one embodiment, the neurological disorder is selected from the group consisting of Alzheimer's disease (AD), stroke, dementia, muscular dystrophy (MD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), cystic fibrosis, Angelman's syndrome, Liddle syndrome, Parkinson's disease, Pick's disease, Paget's disease, cancer, and traumatic brain injury.

[00138] In one embodiment, an anti-TfR antibody or antigen binding fragment thereof, or a conjugate thereof of the application, is used to detect a neurological disorder before the onset of symptoms and/or to assess the severity or duration of the disease or disorder. The antibody, antigen binding fragment or conjugate thereof permits detection and/or imaging of the

neurological disorder, including imaging by radiography, tomography, or magnetic resonance imaging (MRI).

[00139] In another embodiment, an anti-TfR antibody or antigen binding fragment thereof, or a conjugate thereof, is used in treating a neurological disorder (e.g., Alzheimer's disease), comprising administering to a subject in need of the treatment an effective amount of anti-TfR antibody or antigen binding fragment thereof, or a conjugate thereof. In some embodiments, the method further comprises administering to the subject an effective amount of at least one additional therapeutic agent.

[00140] In another embodiment, the application relates to the use of an anti-TfR antibody or antigen binding fragment or conjugate thereof of the application in the manufacture or preparation of a medicament. In one embodiment, the medicament is for treatment of neurological disease or disorder. In a further embodiment, the medicament is for use in a method of treating neurological disease or disorder comprising administering to an individual having neurological disease or disorder an effective amount of the medicament.

[00141] Another general aspect of the application relates to a method of inducing antibody dependent phagocytosis (ADP) without stimulating secretion of a pro-inflammatory cytokine in a subject in need thereof, comprising administering to the subject a complex comprising a therapeutic antibody or antigen binding fragment thereof coupled to, preferably covalently conjugated to, the antigen-binding fragment thereof of an anti-TfR antibody binding fragment according to an embodiment of the application, wherein the therapeutic antibody or antigen binding fragment thereof does not have effector function. For example, the therapeutic antibody or antigen binding fragment thereof can comprise one or more amino acid modifications that reduces or eliminates the effector function, such as the ADCC or CDC, such as mutations that reduce or abolish the binding to Fc gamma receptor. Such mutations can be at positions L234, L235, D270, N297, E318, K320, K322, P331, and P329, such as one, two or three mutations of L234A, L235A and P331S, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat. In one embodiment, the therapeutic antibody or antigen binding fragment thereof binds specifically to tau aggregates.

[00142] In some embodiments, the method further comprises administering to the subject an effective amount of at least one additional therapeutic agent. In certain embodiments, an additional therapeutic agent is a therapeutic agent effective to treat the same or a different

neurological disorder as the anti-TfR antibody or antigen binding fragment or conjugate thereof is being employed to treat. Exemplary additional therapeutic agents include, but are not limited to: the various neurological drugs described above, cholinesterase inhibitors (such as donepezil, galantamine, rivastigmine, and tacrine), NMDA receptor antagonists (such as memantine), amyloid beta peptide aggregation inhibitors, antioxidants, γ -secretase modulators, nerve growth factor (NGF) mimics or NGF gene therapy, PPAR γ agonists, HMG-CoA reductase inhibitors (statins), ampakines, calcium channel blockers, GABA receptor antagonists, glycogen synthase kinase inhibitors, intravenous immunoglobulin, muscarinic receptor agonists, nicotinic receptor modulators, active or passive amyloid beta peptide immunization, phosphodiesterase inhibitors, serotonin receptor antagonists and anti-amyloid beta peptide antibodies. In certain embodiments, the at least one additional therapeutic agent is selected for its ability to mitigate one or more side effects of the neurological drug. The additional therapeutic agent can be administered in the same or separate formulations and administered together or separately with the anti-TfR antibody or antigen binding fragment or conjugate thereof. The anti-TfR antibody or antigen binding fragment or conjugate of the application can be administered prior to, simultaneously with, and/or following, the administration of the additional therapeutic agent and/or adjuvant. The anti-TfR antibody or antigen binding fragment or conjugate thereof of the application can also be used in combination with other interventional therapies such as, but not limited to, radiation therapy, behavioral therapy, or other therapies known in the art and appropriate for the neurological disorder to be treated or prevented.

[00143] The anti-TfR antibody or antigen binding fragment or conjugate thereof of the application (and any additional therapeutic agent) can be administered by any suitable means, including parenteral, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration, depending in part on whether the administration is brief or chronic. Various dosing schedules including but not limited to single or multiple administrations over various time- points, bolus administration, and pulse infusion are contemplated herein.

[00144] For the prevention or treatment of a disease, the appropriate dosage of an anti-TfR antibody or antigen binding fragment or conjugate thereof of the application (when used alone or in combination with one or more other additional therapeutic agents) will depend on various

factors, such as the type of disease to be treated, the type of antibody or conjugate, the severity and course of the disease, whether the antibody, antigen binding fragment or conjugate thereof is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, the physiological state of the subject (including, e.g., age, body weight, health), and the discretion of the attending physician. Treatment dosages are optimally titrated to optimize safety and efficacy. The antibody, antigen binding fragment or conjugate thereof is suitably administered to the patient at one time or over a series of treatments.

[00145] According to particular embodiments, a therapeutically effective amount refers to the amount of therapy which is sufficient to achieve one, two, three, four, or more of the following effects: (i) reduce or ameliorate the severity of the disease, disorder or condition to be treated or a symptom associated therewith; (ii) reduce the duration of the disease, disorder or condition to be treated, or a symptom associated therewith; (iii) prevent the progression of the disease, disorder or condition to be treated, or a symptom associated therewith; (iv) cause regression of the disease, disorder or condition to be treated, or a symptom associated therewith; (v) prevent the development or onset of the disease, disorder or condition to be treated, or a symptom associated therewith; (vi) prevent the recurrence of the disease, disorder or condition to be treated, or a symptom associated therewith; (vii) reduce hospitalization of a subject having the disease, disorder or condition to be treated, or a symptom associated therewith; (viii) reduce hospitalization length of a subject having the disease, disorder or condition to be treated, or a symptom associated therewith; (ix) increase the survival of a subject with the disease, disorder or condition to be treated, or a symptom associated therewith; (xi) inhibit or reduce the disease, disorder or condition to be treated, or a symptom associated therewith in a subject; and/or (xii) enhance or improve the prophylactic or therapeutic effect(s) of another therapy.

[00146] In another aspect, the application relates to an article of manufacture (such as a kit) containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers can be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous

solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is an antibody, antigen binding fragment thereof or a conjugate of the application. The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture can include (a) a first container with a composition contained therein, wherein the composition comprises an antibody, antigen binding fragment thereof or a conjugate of the application; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention can further include a package insert indicating that the compositions can be used to treat a particular condition. Optionally, the article of manufacture can further comprise a second (or third) container comprising a pharmaceutically acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It can further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

EMBODIMENTS

[00147] The invention provides also the following non-limiting embodiments.

1. An anti-TfR antibody or antigen-binding fragment thereof for delivering an agent to the brain of a subject in need thereof, wherein the anti-TfR antibody or antigen-binding fragment thereof binds to a transferrin receptor (TfR), preferably human TfR1, with a dissociation constant K_D of at least 1 nM at a neutral pH and an off-rate constant k_d of at least 10^{-4} sec^{-1} at an acidic pH, preferably the pH 5.

1a. The anti-TfR antibody or antigen-binding fragment thereof of embodiment 1, having a dissociation constant K_D of 1 nM to 500 nM, such as 1 nM, 10 nM, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, or any value in between, at the neutral pH.

1b. The anti-TfR antibody or antigen-binding fragment thereof of embodiment 1 or 1a, having an off-rate constant k_d of 10^{-4} sec^{-1} to 10^{-1} sec^{-1} , such as 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} sec^{-1} or any value in between, at the acidic pH.

2. The anti-TfR antibody or antigen-binding fragment thereof of any one of embodiments 1 to 1b, having an off-rate constant k_d of 2×10^{-2} to $2 \times 10^{-4} \text{ sec}^{-1}$, preferably $2.0 \times 10^{-3} \text{ sec}^{-1}$, at the neutral pH.

2a. The anti-TfR antibody or antigen-binding fragment thereof of embodiment 2, wherein the off-rate constant k_d at the neutral pH is 2×10^{-2} to $2 \times 10^{-4} \text{ sec}^{-1}$, such as 2×10^{-2} , 1×10^{-2} , 9×10^{-3} , 8×10^{-3} , 7×10^{-3} , 6×10^{-3} , 5×10^{-3} , 4×10^{-3} , 3×10^{-3} , 2×10^{-3} , 1×10^{-3} , 9×10^{-4} , 8×10^{-4} , 7×10^{-4} , 6×10^{-4} , 5×10^{-4} , 4×10^{-4} , 3×10^{-4} , $2 \times 10^{-4} \text{ sec}^{-1}$, or any value in between.

3. The anti-TfR antibody or antigen-binding fragment thereof of any one of embodiments 1 to 2a, comprising:

(1) a heavy chain variable region comprising heavy chain complementarity determining regions (HCDRs) HCDR1, HCDR2 and HCDR3, and a light chain variable region comprising light chain complementarity determining regions (LCDRs) LCDR1, LCDR2 and LCDR3, wherein the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 have the amino acid sequences of:

- i. SEQ ID NOs: 292, 293, 294, 295, 296, and 297, respectively;
- ii. SEQ ID NOs: 279, 280, 281, 282, 283 and 284, respectively;
- iii. SEQ ID NOs: 29, 30, 31, 32, 33 and 34, respectively;
- iv. SEQ ID NOs: 57, 58, 59, 60, 61 and 62, respectively;
- v. SEQ ID NOs: 85, 86, 87, 88, 89 and 90, respectively;
- vi. SEQ ID NOs: 110, 111, 112, 113, 114 and 115, respectively;
- vii. SEQ ID NOs: 135, 136, 137, 138, 139 and 140, respectively;
- viii. SEQ ID NOs: 191, 192, 193, 194, 195 and 196, respectively;
- ix. SEQ ID NOs: 244, 245, 246, 247, 248 and 249, respectively;
- x. SEQ ID NOs: 263, 264, 265, 266, 267 and 268, respectively;
- xi. SEQ ID NOs: 345, 346, 347, 348, 349 and 350, respectively;
- xii. SEQ ID NOs: 355, 356, 357, 358, 359 and 360, respectively;
- xiii. SEQ ID NOs: 365, 366, 367, 368, 369 and 370, respectively;
- xiv. SEQ ID NOs: 375, 376, 377, 378, 379 and 380, respectively;
- xv. SEQ ID NOs: 385, 386, 387, 388, 389 and 390, respectively;
- xvi. SEQ ID NOs: 395, 396, 377, 398, 399 and 400, respectively;
- xvii. SEQ ID NOs: 405, 406, 407, 408, 409 and 410, respectively;
- xviii. SEQ ID NOs: 415, 416, 417, 418, 419 and 420, respectively;
- xix. SEQ ID NOs: 425, 426, 427, 428, 429 and 430, respectively;
- xx. SEQ ID NOs: 435, 436, 437, 438, 439 and 440, respectively;

- xxi. SEQ ID NOs: 445, 446, 447, 448, 449 and 450, respectively;
- xxii. SEQ ID NOs: 455, 456, 457, 458, 459 and 460, respectively;
- xxiii. SEQ ID NOs: 465, 466, 467, 468, 469 and 470, respectively;
- xxiv. SEQ ID NOs: 475, 476, 477, 478, 479 and 480, respectively;
- xxv. SEQ ID NOs: 485, 486, 487, 488, 489 and 490, respectively;
- xxvi. SEQ ID NOs: 495, 496, 497, 498, 499 and 500, respectively;
- xxvii. SEQ ID NOs: 505, 506, 507, 508, 509 and 510, respectively;
- xxviii. SEQ ID NOs: 515, 516, 517, 518, 519 and 520, respectively;
- xxix. SEQ ID NOs: 525, 526, 527, 528, 529 and 530, respectively;
- xxx. SEQ ID NOs: 535, 536, 537, 538, 539 and 540, respectively; or
- xxxi. SEQ ID NOs: 545, 546, 547, 548, 549 and 550, respectively; or

(2) a single variable domain on a heavy chain (VHH) comprising heavy chain complementarity determining regions (HCDRs) HCDR1, HCDR2 and HCDR3 having the amino acid sequences of:

- i. SEQ ID NOs: 7, 8 and 9, respectively;
- ii. SEQ ID NOs: 317, 318 and 319, respectively;
- iii. SEQ ID NOs: 324, 325 and 326, respectively;
- iv. SEQ ID NOs: 331, 332 and 333, respectively; or
- v. SEQ ID NOs: 338, 339 and 340, respectively

4. The antibody or antigen-binding fragment thereof of embodiment 3, being a VHH fragment comprising an amino acid sequence having at least 80%, such as at least 85%, 90%, 95% or 100%, sequence identity to SEQ ID NO: 6, 316, 323, 330, or 337.

4a. The antibody or antigen-binding fragment thereof of embodiment 2, wherein the VHH fragment comprises the amino acid sequence of SEQ ID NO: 6, 316, 323, 330, or 337.

5. The antibody or antigen-binding fragment thereof of embodiment 3, being a single-chain variable fragment (scFv) comprising the heavy chain variable region (VH) covalently linked to the light chain variable region (VL) via a linker, such as a peptide linker having about 10 to about 25 amino acids in length.

5a. The antibody or antigen-binding fragment thereof of embodiment 5, wherein the VH is linked to the amino-terminus of the VL via the linker in the scFv.

5b. The antibody or antigen-binding fragment thereof of embodiment 5, wherein the VH is linked to the carboxy-terminus of the VL via the linker in the scFv.

5c. The antibody or antigen-binding fragment thereof of embodiment 5a or 5b, wherein the linker comprises one or more of Gly and Ser, with one or more interspersed Glu, Thr and Lys residues, preferably the linker has the amino acid sequence of SEQ ID NO: 314.

5d. The antibody or antigen-binding fragment thereof of embodiment 5c, wherein the scFv comprises the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 having the amino acid sequences of SEQ ID NOs: 279, 280, 281, 282, 283 and 284, respectively, or SEQ ID NOs: 292, 293, 294, 295, 296, and 297, respectively;

5e. The antibody or antigen-binding fragment thereof of embodiment 5, wherein the scFv comprises an amino acid sequence having at least 80%, such as at least 85%, 90%, 95% or 100%, sequence identity to the amino acid sequences of SEQ ID NO: 278, 291, 28, 56, 84, 109, 134, 162, 190, 218, 243, 262, 344, 354, 364, 374, 384, 394, 404, 414, 424, 434, 444, 454, 464, 474, 484, 494, 504, 514, 524, 534 or 544.

5f. The antibody or antigen-binding fragment thereof of embodiment 5e, wherein the scFv comprises the amino acid sequence of SEQ ID NO: 278, 291, 28, 56, 84, 109, 134, 162, 190, 218, 243, 262, 344, 354, 364, 374, 384, 394, 404, 414, 424, 434, 444, 454, 464, 474, 484, 494, 504, 514, 524, 534 or 544.

5g. The antibody or antigen-binding fragment thereof of embodiment 5e, wherein the scFv comprises the amino acid sequence of SEQ ID NO: 278, 291, 162 or 218.

5h. An antibody or antigen-binding fragment thereof that binds to the same epitope of the antibody or antigen-binding fragment thereof of any one of embodiments 3 to 5g.

5i. An antibody or antigen-binding fragment thereof that competes with the antibody or antigen-binding fragment thereof of any one of embodiments 3 to 5g in binding to the Tfr.

5j. The antibody or antigen-binding fragment thereof of any one of embodiments 3 to 5i, binding to human Tfr1 with a dissociation constant K_D of 1 to 500 nM, such as 1 nM, 10 nM, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, or any value in between, at pH7.4.

5k. The antibody or antigen-binding fragment thereof of any one of embodiments 3 to 5j, binding to human Tfr1 with an off-rate constant k_d of 10^{-4} to 10^{-1} sec^{-1} , such as 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} sec^{-1} or any value in between, at pH5.

6. A complex comprising the antibody or antigen-binding fragment thereof of any one of embodiments 1 to 5k coupled to a therapeutic or diagnostic agent.

6a. The complex of embodiment 6, wherein the antibody or antigen-binding fragment thereof is coupled to the therapeutic or diagnostic agent noncovalently.

6b. The complex of embodiment 6, wherein the antibody or antigen-binding fragment thereof is coupled to the therapeutic or diagnostic agent covalently to form a conjugate.

6c. The complex of embodiment 6, wherein the antibody or antigen-binding fragment thereof is covalently linked to the therapeutic or diagnostic agent via a linker.

6d. The complex of embodiment 6c, wherein the linker is a non-peptide linker, such as polyethylene glycol, polypropylene glycol, copolymer of ethylene glycol and propylene glycol, polyoxyethylated polyol, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ether, biodegradable polymer, polymerized lipid, chitins, and hyaluronic acid, or derivatives thereof, or combinations thereof.

6e. The complex of embodiment 6c, wherein the linker is a peptide linker, such as a peptide chain consisting of 1 to 50 amino acids linked by peptide bonds or a derivative thereof.

6f. The complex of any one of embodiments 6 to 6e, wherein the antibody or antigen-binding fragment thereof is coupled to the diagnostic agent for detecting a neurological disorder, preferably, the diagnostic agent is an agent for positron emission tomography (PET), or an agent for IDK.

6g. The complex of any one of embodiments 6 to 6e, wherein the antibody or antigen-binding fragment thereof is coupled to a therapeutic agent, preferably a neurological disorder drug.

6h. The complex of embodiment 6g, wherein the neurological disorder drug is selected from the group consisting of small molecule compounds, antibodies, peptides, proteins, natural ligands of one or more CNS target(s), modified versions of natural ligands of one or more CNS target(s), aptamers, inhibitory nucleic acids (i.e., small inhibitory RNAs (siRNA) and short hairpin RNAs (shRNA)), ribozymes, and active fragments of the foregoing.

6i. The complex of embodiment 6g, wherein the neurological disorder drug is selected from the group consisting of antibodies, aptamers, proteins, peptides, inhibitory nucleic acids and small molecules and active fragments of any of the foregoing that either are themselves or specifically recognize and/or act upon (i.e., inhibit, activate, or detect) a CNS antigen or target

molecule such as, but not limited to, amyloid precursor protein or portions thereof, amyloid beta, beta-secretase, gamma-secretase, tau, alpha-synuclein, parkin, huntingtin, DR6, presenilin, ApoE, glioma or other CNS cancer markers, and neurotrophins. Non-limiting examples of neurological disorder drugs and the corresponding disorders they may be used to treat: Brain-derived neurotrophic factor (BDNF), Chronic brain injury (Neurogenesis), Fibroblast growth factor 2 (FGF-2), Anti-Epidermal Growth Factor Receptor Brain cancer, (EGFR)-antibody, Glial cell-line derived neural factor Parkinson's disease, (GDNF), Brain-derived neurotrophic factor (BDNF) Amyotrophic lateral sclerosis, depression, Lysosomal enzyme Lysosomal storage disorders of the brain, Ciliary neurotrophic factor (CNTF) Amyotrophic lateral sclerosis, Neuregulin-1 Schizophrenia, Anti-HER2 antibody (e.g. trastuzumab) Brain metastasis from HER2-positive cancer.

7. The complex of embodiment 6, being a multi-specific antibody comprising a first antigen binding region which binds a Tfr and a second antigen binding region which binds a brain antigen (or brain target), wherein the first antigen binding region comprises the antigen-binding fragment thereof of any one of embodiments 1 to 5k.

7a. The multi-specific antibody of embodiment 7, wherein the brain target is selected from the group consisting of beta-secretase 1 (BACE1), amyloid beta (Abeta), epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), Tau, apolipoprotein E4 (ApoE4), alpha-synuclein, CD20, huntingtin, prion protein (PrP), leucine rich repeat kinase 2 (LRRK2), parkin, presenilin 1, presenilin 2, gamma secretase, death receptor 6 (DR6), amyloid precursor protein (APP), p75 neurotrophin receptor (p75NTR), and caspase 6.

7b. The multi-specific antibody of embodiment 7a, wherein the second antigen binding region binds a BACE1 or Tau.

7c. The multi-specific antibody of any one of embodiments 7 to 7b, wherein the first antigen binding region is covalently linked to a first Fc, and the second antigen binding region is covalently linked to a second Fc.

7d. The multi-specific antibody of embodiment 7c, wherein the first Fc is different from the second Fc in one or more amino acid residues to facilitate the formation of a heterodimer between the first Fc and the second Fc.

8. The multi-specific antibody of any one of embodiments 7 to 7d, being a fusion construct comprising the antibody or antigen-binding fragment thereof of any one of

embodiments 1 to 5k covalently linked a second antibody or antigen binding fragment thereof that binds the brain antigen (or brain target).

8a. The fusion construct of embodiment 8, wherein the antibody or antigen-binding fragment thereof of any one of embodiments 1 to 5k is covalently linked, preferably via a linker, to the amino-terminus of a heavy chain of the second antibody or antigen binding fragment thereof.

8b. The fusion construct of embodiment 8, wherein the antibody or antigen-binding fragment thereof of any one of embodiments 1 to 5k is covalently linked, preferably via a linker, to the amino-terminus of a light chain of the second antibody or antigen binding fragment thereof.

8c. The fusion construct of embodiment 8, wherein the antibody or antigen-binding fragment thereof of any one of embodiments 1 to 5k is covalently linked, preferably via a linker, to the carboxy-terminus of a light chain of the second antibody or antigen binding fragment thereof.

8d. The fusion construct of embodiment 8, wherein the antibody or antigen-binding fragment thereof of any one of embodiments 1 to 5k is covalently linked, preferably via a linker, to the carboxy-terminus of a heavy chain of the second antibody or antigen binding fragment thereof.

9. The fusion construct of embodiment 8d, wherein the antibody or antigen-binding fragment thereof any one of embodiments 1 to 5k is covalently linked, via a linker, to the carboxy terminus of only one of the two heavy chains of the second antibody or antigen binding fragment.

9a. The fusion construct of any one of embodiments 8a to 9, wherein the linker is a peptide linker comprising one or more of Gly and Ser, preferably the linker has the amino acid sequence of SEQ ID NO: 312 or SEQ ID NO: 313.

9b. The fusion construct of any one of embodiments 8 to 9a, wherein the second antibody or antigen binding fragment thereof comprise a first Fc in its first heavy chain and a second Fc in its second heavy chain, and the first Fc is different from the second Fc in one or more amino acid residues to facilitate the formation of a heterodimer between the first Fc and the second Fc.

9c. The multi-specific antibody of embodiment 7d or the fusion construct of embodiment 9b, wherein the first Fc contains one or more “knob” mutations and the second Fc contains more or more corresponding “hole” mutations, or vice versa (see, e.g., U.S. Patent No. 5,731,168, Ridgway et al., *Protein Eng.*, 9(7): 617-621, 1996, for the “knob-in-hole” mutations, which are incorporated herein by reference in its entirety), preferably a Knob mutation of T366W and a hole mutation of T366S, L368A or Y407V.

9d. The multi-specific antibody of embodiment 7d or the fusion construct of embodiment 9b, wherein each of the first Fc and the second Fc comprises a modified heterodimeric CH3 domain as compared to a wild-type CH3 domain polypeptide, preferably, the modified heterodimeric CH3 domain comprises one or more mutations as described in US10,457,742.

9e. The multi-specific antibody or fusion construct of embodiment 9d, wherein the modified heterodimeric CH3 domain of the first Fc comprises amino acid modifications at positions T350, L351, F405, and Y407, and the modified heterodimeric CH3 domain of the second Fc comprises amino acid modifications at positions T350, T366, K392 and T394.

9f. The multi-specific antibody or fusion construct of embodiment 9e, wherein the amino acid modification at position T350 is T350V, T350I, T350L or T350M; the amino acid modification at position L351 is L351Y; the amino acid modification at position F405 is F405A, F405V, F405T or F405S; the amino acid modification at position Y407 is Y407V, Y407A or Y407I; the amino acid modification at position T366 is T366L, T366I, T366V or T366M, the amino acid modification at position K392 is K392F, K392L or K392M, and the amino acid modification at position T394 is T394W.

9g. The multi-specific antibody or fusion construct of embodiment 9e, wherein the modified heterodimeric CH3 domain of the first Fc comprises mutations T350V, L351Y, F405A and Y407V, and the modified heterodimeric CH3 domain of the second Fc comprises mutations T350V, T366L, K392L and T394W, or vice versa.

10. The multi-specific antibody or fusion construct of any one of embodiments 7-9g, wherein the Fc region of the multi-specific antibody or fusion construct further comprises substitutions that alter (increase or diminish), preferably increase, the binding of the second antibody or antigen binding fragment thereof to neonatal Fc receptor (FcRn).

10a. The multi-specific antibody or fusion construct of embodiment 10, wherein the second antibody or antigen binding fragment thereof comprises one or more mutations in the Fc domain that enhance binding of the fusion to the neonatal Fc receptor (RcRn).

10b. The multi-specific antibody or fusion construct of embodiment 10 or 10a, wherein the one or more mutations enhance the binding at an acidic pH.

10c. The multi-specific antibody or fusion construct of embodiment 10b, wherein the Fc of the second antibody has the M252Y/S254T/T256E (YTE) mutations, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat.

11. The multi-specific antibody or fusion construct of any one of embodiments 7-10c, wherein the Fc region of the multi-specific antibody or fusion construct further comprises substitutions that alter (increase or diminish), preferably reduces or eliminates the effector function.

11a. The multi-specific antibody or fusion construct of embodiment 11, wherein the second antibody or antigen binding fragment thereof comprises one or more mutations in the Fc domain that reduce or eliminate the effector function, such antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC).

11b. The multi-specific antibody or fusion construct of embodiment 11a, wherein the Fc of the second antibody has one or more amino acid modifications at positions L234, L235, D270, N297, E318, K320, K322, P331, and P329, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat.

11c. The multi-specific antibody or fusion construct of embodiment 11b, wherein the Fc of the second antibody has one, two or three mutations of L234A, L235A and P331S (the AAS mutations).

12. The multi-specific antibody or fusion construct of any one of embodiments 7 to 11c, wherein the first antigen binding region or the antibody or antigen-binding fragment thereof does not contain cysteine.

13. The multi-specific antibody or fusion construct of embodiments 7 to 12, wherein the second antigen binding region or the second antibody or antigen binding fragment thereof binds Tau, preferably comprising the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 having the amino acid sequences of SEQ ID Nos: 554 to 559, respectively, preferably, the second antibody is a monoclonal antibody comprising a heavy chain having the amino acid

sequence of SEQ ID NO: 310 and a light chain having the amino acid sequence of SEQ ID NO: 311.

14. The fusion construct of embodiment 9, comprising:

- (1) a first heavy chain having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 301, 304, 307, 285, 288, 298, 10, 13, 16, 19, 22, 25, 35, 38, 41, 44, 47, 50, 53, 63, 66, 69, 72, 75, 78, 81, 91, 94, 97, 100, 103, 106, 116, 119, 122, 125, 128, 131, 141, 144, 147, 150, 153, 156, 159, 169, 172, 175, 178, 181, 184, 187, 197, 200, 203, 206, 209, 212, 215, 225, 228, 231, 234, 237, 240, 250, 252, 256, 259, 269, 272, 275, 320, 327, 334, 341, 351, 361, 371, 381, 391, 401, 411, 421, 431, 441, 451, 461 and 471;
- (2) two light chains each independently having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of 302, 305, 308, 286, 289, 299, 11, 14, 17, 20, 23, 26, 36, 39, 42, 45, 48, 51, 54, 64, 67, 70, 73, 76, 79, 82, 92, 95, 98, 101, 104, 107, 117, 120, 123, 126, 129, 132, 142, 145, 148, 151, 154, 157, 160, 170, 173, 176, 179, 182, 185, 188, 198, 201, 204, 207, 210, 213, 216, 226, 229, 232, 235, 238, 241, 251, 253, 257, 260, 270, 273, 276, 321, 328, 335, 342, 352, 362, 372, 382, 392, 402, 412, 422, 432, 442, 452, 462 and 472, respectively; and
- (3) a second heavy chain having an amino acid sequence that is at least 80%, such as at least 85%, 90%, 95% or 100%, identical to an amino acid sequence selected from the group consisting of 303, 306, 309, 287, 290, 300, 12, 15, 18, 21, 24, 27, 37, 40, 43, 46, 49, 52, 55, 65, 68, 71, 74, 77, 80, 83, 93, 96, 99, 102, 105, 108, 118, 121, 124, 127, 130, 133, 143, 146, 149, 152, 155, 158, 161, 171, 174, 177, 180, 183, 186, 189, 199, 202, 205, 208, 211, 214, 217, 227, 230, 233, 236, 239, 242, 252, 254, 258, 261, 271, 274, 277, 322, 329, 336, 343, 353, 363, 373, 383, 393, 403, 413, 423, 433, 443, 453, 463 and 473, respectively.

14a. The fusion construct of embodiment 14, wherein the two light chains have an identical amino acid sequence.

14b. The fusion construct of embodiment 14, wherein the two light chains have different amino acid sequences.

14c. The fusion construct of embodiment 14, wherein:

- (1) the first heavy chain has the amino acid sequence selected from the group consisting of SEQ ID NOs: 301, 304, 307, 285, 288, 298, 10, 13, 16, 19, 22, 25, 35, 38, 41, 44, 47, 50, 53, 63, 66, 69, 72, 75, 78, 81, 91, 94, 97, 100, 103, 106, 116, 119, 122, 125, 128, 131, 141, 144, 147, 150, 153, 156, 159, 169, 172, 175, 178, 181, 184, 187, 197, 200, 203, 206, 209, 212, 215, 225, 228, 231, 234, 237, 240, 250, 252, 256, 259, 269, 272, 275, 320, 327, 334, 341, 351, 361, 371, 381, 391, 401, 411, 421, 431, 441, 451, 461 and 471;
- (2) the two light chains each have the amino acid sequence selected from the group consisting of 302, 305, 308, 286, 289, 299, 11, 14, 17, 20, 23, 26, 36, 39, 42, 45, 48, 51, 54, 64, 67, 70, 73, 76, 79, 82, 92, 95, 98, 101, 104, 107, 117, 120, 123, 126, 129, 132, 142, 145, 148, 151, 154, 157, 160, 170, 173, 176, 179, 182, 185, 188, 198, 201, 204, 207, 210, 213, 216, 226, 229, 232, 235, 238, 241, 251, 253, 257, 260, 270, 273, 276, 321, 328, 335, 342, 352, 362, 372, 382, 392, 402, 412, 422, 432, 442, 452, 462 and 472, respectively; and
- (3) the second heavy chain has the amino acid sequence selected from the group consisting of 303, 306, 309, 287, 290, 300, 12, 15, 18, 21, 24, 27, 37, 40, 43, 46, 49, 52, 55, 65, 68, 71, 74, 77, 80, 83, 93, 96, 99, 102, 105, 108, 118, 121, 124, 127, 130, 133, 143, 146, 149, 152, 155, 158, 161, 171, 174, 177, 180, 183, 186, 189, 199, 202, 205, 208, 211, 214, 217, 227, 230, 233, 236, 239, 242, 252, 254, 258, 261, 271, 274, 277, 322, 329, 336, 343, 353, 363, 373, 383, 393, 403, 413, 423, 433, 443, 453, 463 and 473, respectively.

14d. The fusion construct of embodiment 14, wherein:

- (1) the first heavy chain has the amino acid sequence of SEQ ID NOs: 285, 288, 298, or 301;
- (2) the two light chains each have the amino acid sequence of 286, 289, 299 or 302, respectively; and
- (3) the second heavy chain has the amino acid sequence of 287, 290, 300 or 303, respectively.

15. An isolated nucleic acid encoding the antibody or antigen-binding fragment of any one of embodiments 1-5k or the fusion construct of any one of embodiments 7-14d.

16. A vector comprising the isolated nucleic acid of claim 15.
17. A host cell comprising the nucleic acid of embodiment 15 or the vector of embodiment 16.
18. A method of producing the antibody or antigen-binding fragment of any one of embodiments 1-5k or the fusion construct of any one of embodiments 7-14d, comprising culturing a cell comprising a nucleic acid encoding the antibody or antigen-binding fragment or the fusion construct under conditions to produce the antibody or antigen-binding fragment the fusion construct, and recovering the antibody or antigen-binding fragment, the conjugate or the fusion construct from the cell or cell culture.
19. A pharmaceutical composition comprising the antibody or antigen-binding fragment of any one of embodiments 1-5k, the complex of any one of embodiments 6-6i, or the multi-specific antibody or fusion construct of any one of embodiments 7-14d, and a pharmaceutically acceptable carrier.
20. A method of treating or detecting a neurological disorder in a subject in need thereof, comprising administering to the subject an effective amount of the antibody or antigen-binding fragment of any one of embodiments 1-5k, the complex of any one of embodiments 6-6i, or the multi-specific antibody or fusion construct of any one of embodiments 7-14d, or the pharmaceutical composition of embodiment 19.
21. A method of increasing delivery of a therapeutic or diagnostic agent to the brain of a subject in need thereof, comprising administering to the subject a conjugate comprising the therapeutic or diagnostic agent coupled to the antibody or antigen-binding fragment thereof of any one of embodiments 1-5k.
22. A method of transporting a therapeutic or diagnostic agent across the blood-brain barrier (BBB) comprising exposing an anti-TfR antibody or antigen binding fragment thereof of any one of embodiments 1-5k coupled to the therapeutic or diagnostic agent to the blood- brain barrier such that the antibody or antigen binding fragment thereof transports the agent coupled thereto across the blood- brain barrier.
23. A method of delivering a therapeutic or diagnostic agent across the blood-brain barrier (BBB) of a subject in need thereof, comprising administering to the subject a complex comprising the therapeutic or diagnostic agent coupled to, preferably covalently conjugated to, the antibody or antigen-binding fragment thereof of any one of embodiments 1 to 5.

24. A method of inducing antibody dependent phagocytosis (ADP) without stimulating secretion of a pro-inflammatory cytokine in a subject in need thereof, comprising administering to the subject a complex comprising a therapeutic antibody or antigen binding fragment thereof coupled to, preferably covalently conjugated to, the antigen-binding fragment thereof of any one of embodiments 1 to 5, wherein the therapeutic antibody or antigen binding fragment thereof comprises one or more mutations in the Fc domain that reduce or eliminate the effector function, such antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC).

24a. The method of embodiment 23, wherein the therapeutic antibody or antigen binding fragment thereof comprises one or more amino acid modifications at positions L234, L235, D270, N297, E318, K320, K322, P331, and P329, wherein the numbering of amino acid residues is according to the EU index as set forth in Kabat.

24b. The method of embodiment 24a, wherein the therapeutic antibody or antigen binding fragment thereof comprises one, two or three mutations of L234A, L235A and P331S.

25. The method of any one of embodiments 20 -24b, wherein the subject is in need of a treatment of a neurological disorder, preferably the neurological disorder is selected from the group consisting of neurodegenerative diseases (such as Lewy body disease, postpoliomyelitis syndrome, Shy-Draeger syndrome, olivopontocerebellar atrophy, Parkinson's disease, multiple system atrophy, striatonigral degeneration, spinocerebellar ataxia, spinal muscular atrophy), tauopathies (such as Alzheimer disease and supranuclear palsy), prion diseases (such as bovine spongiform encephalopathy, scrapie, Creutz-feldt-Jakob syndrome, kuru, Gerstmann-Straussler-Scheinker disease, chronic wasting disease, and fatal familial insomnia), bulbar palsy, motor neuron disease, and nervous system heterodegenerative disorders (such as Canavan disease, Huntington's disease, neuronal ceroid-lipofuscinosis, Alexander's disease, Tourette's syndrome, Menkes kinky hair syndrome, Cockayne syndrome, Halervorden-Spatz syndrome, lafora disease, Rett syndrome, hepatolenticular degeneration, Lesch-Nyhan syndrome, and Unverricht-Lundborg syndrome), dementia (such as Pick's disease, and spinocerebellar ataxia), and cancer of the CNS and/or brain (such as brain metastases resulting from cancer elsewhere in the body).

26. The method of any one of embodiments 20 to 25, wherein the antibody or antigen-binding fragment thereof, the complex, the multispecific antibody, the fusion construct or the pharmaceutical composition is administered intravenously.

27. The method of any one of embodiments 21 to 26, wherein the therapeutic agent or therapeutic antibody or antigen binding fragment thereof is a neurological disorder drug.

28. The method of any one of embodiments 21 to 23, wherein the agent is an imaging agent or an agent for detecting a neurological disorder.

29. The method of any one of embodiments 20-28, wherein the anti-TfR antibody or antigen binding fragment thereof, complex or fusion thereof, does not impair the binding of the TfR to its native ligand transferrin.

30. The method of any one of embodiments 20 to 29, wherein the administration reduces Fc-mediated effector function.

31. The method of any one of embodiments 21 to 30, wherein the administration does not induce rapid reticulocyte depletion.

32. The method of embodiment 31, wherein the therapeutic antibody or antigen binding fragment thereof binds specifically to tau aggregates

33. The method of any one of embodiments 20-32, wherein the subject is a primate, such as a human, more preferably a human having a neurological disorder.

34. The method of embodiment 33, wherein the neurological disorder is selected from the group consisting of Alzheimer's disease (AD), stroke, dementia, muscular dystrophy (MD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), cystic fibrosis, Angelman's syndrome, Liddle syndrome, Parkinson's disease, Pick's disease, Paget's disease, cancer, and traumatic brain injury.

[00148] The following examples of the invention are to further illustrate the nature of the invention. It should be understood that the following examples do not limit the invention and the scope of the invention is to be determined by the appended claims.

EXAMPLES

Example 1

[00149] While the blood-brain barrier (BBB) prevents harmful substances from entering the brain and is essential for brain homeostasis, it presents a formidable obstacle for efficiently delivering drugs to the brain. Towards this end, a monoclonal antibody (mAb) brain shuttle platform was developed that penetrates the BBB and results in substantially higher brain concentrations than mAb alone.

Antibody Generation (OMT rats and Ablexis Mice)

[00150] OMT rats (OmniRat® from Ligand Pharmaceuticals) and Ablexis mice (Ablexis, LLC, San Diego, CA) were immunized with human (SEQ ID NO: 1), cyno (SEQ ID NO: 2) and marmoset (SEQ ID NO: 3) transferrin receptor (TfR) using repetitive immunizations multiple sites (RIMMS) protocols for 46 days (Ablexis), 49 days (OMT) or 50 days (OMT). Briefly, animals were repeatedly immunized at multiple subcutaneous sites proximal to regional draining lymph nodes. Serum titration (ELISA, enzyme-linked immunosorbent assay) was done at day 32 (OMT) or day 35 (Ablexis) and all animals show generally low to modest titers on human, cyno, and marmoset TfR, and no titers on the negative control. Lymph nodes were harvested from sera-positive rats and mice and fused to generate hybridomas.

[00151] Hybridomas were first screened by Meso Scale Discovery (MSD) or ELISA for binding to HEK293T huTfR (human transferrin receptor) expressing cells. All these hits were then tested in the confirmatory screen. In the confirmatory screen on fluorescence-activated cell sorting (FACS) MDCK-huTfR cells (Madin-Darby canine kidney cells) and pBECs (Microvascular Endothelial Cells, endogenous huTfR expression) were used and MDCK (parental) cells were used as negative cell line. After confirmatory screen, 616 TfR specific cell binders were identified (binding either/or/both huTfR expressing cells). From these 616 hits, 340 were binding on pBECs and MDCK-huTfR cells, 16 were binding on pBECs only and 260 were binding on MDCK-huTfR only.

[00152] The hybridomas that bound pBECs and MDCK-huTfR cells were then assessed for binding to rat TfR (SEQ ID NO: 4) and mouse TfR (SEQ ID NO: 5), checked for internalization in pBECs and competition with TfR. RNA lysates were prepared for those mAbs that were human, cyno and marmoset cross-reactive and internalized without competing for TfR. Antibody V-region sequencing data was obtained.

Antibody Generation (Llama)

[00153] For the generation of single domain (VHH) antibodies against human TfR with cross reactivity to cyno, mouse and rat, two llamas were used for immunizations at Abcore (animal 1663L and 1663L) in project 452L. Antibody titers were determined by ELISA using TfR protein (1 µg/ml). Three bleeds from the two animals were tested and both animals showed good early titers.

[00154] Phage display was done at Abcore using their standard protocol. Two libraries were made: Library 1 (452L-1) from the second bleed of both animals, and Library 2 (452L-2) from the second and third bleed. Plasmid DNA from 12 random individual clones were sequenced and >80% contained VHH inserts with the correct reading frame. Both phage display libraries were screened with human TfR using standard Abcore panning procedures. Three rounds of panning with human TfR at 10 µg/ml were done. After panning, 94 individual clones were screened by phage ELISA for specific binding to protease-activated receptor 1 (Par1) N-terminal domain and non-specific binding to BSA (bovine serum albumin). Cross reactivity with cyno, mouse, and rat TfR was measured. 94 clones were selected for sequence analysis.

Phage Antibody Generation

[00155] Phage libraries were panned against biotinylated huTfR, complexed with transferrin. The biotinylated complex was captured on streptavidin magnetic beads (Dynal) and exposed to the de novo pIX Fab libraries which were pre-incubated with transferrin protein at a final concentration of 100 nM (round 1 and 2) or 50 nM (round 3 and 4). Non-specific phages were washed away in PBS-Tween and bound phages were recovered by infection of MC1061F' E. coli cells. Phages were amplified from these cells overnight and panning was repeated for a total of four rounds. Following four rounds of biopanning, monoclonal Fabs were screened for binding to human transferrin receptor in an ELISA. Clones that demonstrated binding to transferrin receptor were sequenced in the heavy and light chain variable regions.

[00156] Examples of TfR antibodies or antigen binding fragments of the invention are summarized in Table 1a below.

[00157] The binding affinities (KD, kon or ka and koff or kdis or kd) of the anti-TfR mAbs, as part of the tripod fusion constructs (BBBB constructs) described in more detail below, to TfR at neutral pH (7.4) and acidic pH (5) were measured using the following biolayer interferometry method. The results are shown in Table 1b below.

Table 1a

Name	Sequence	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
TTR1 (VHH)	SEQ ID NO: 6	SEQ ID NO: 7 GLTFSNYA	SEQ ID NO: 8 IGGSGGTW	SEQ ID NO: 9 AADQRAGSYSS GWYTRSSDSL			
TTR2	SEQ ID NO: 28	SEQ ID NO: 29 GFTFRNAW	SEQ ID NO: 30 IKRKIDGGT	SEQ ID NO: 31 TTDPSRIPVAGAFDY	SEQ ID NO: 32 QSVSSTY	SEQ ID NO: 33 GASSRAT	SEQ ID NO: 34 QQYGSSPYT
TTR3	SEQ ID NO: 56	SEQ ID NO: 57 GFTFSSYNMNV	SEQ ID NO: 58 SSISSSSYI	SEQ ID NO: 59 AREGISAYDALNV	SEQ ID NO: 60 SSSNIGNNY	SEQ ID NO: 61 DNNKRPS	SEQ ID NO: 62 GTWDSLSAVV
TTR4	SEQ ID NO: 84	SEQ ID NO: 85 GFTFSSYAMNW	SEQ ID NO: 86 SGISGSGVSTN	SEQ ID NO: 87 AKEDYDSSGYYPFDY	SEQ ID NO: 88 HKLGDKF	SEQ ID NO: 89 QDRKRPS	SEQ ID NO: 90 QTWYSSTVI
TTR5	SEQ ID NO: 109	SEQ ID NO: 110 GFTFSSDAMHW	SEQ ID NO: 111 AVIWDGSKNY	SEQ ID NO: 112 ARDRQWLAFDY	SEQ ID NO: 113 SSDVGGYNF	SEQ ID NO: 114 EVSKRPS	SEQ ID NO: 115 SYRDSNNFDVL
TTR6	SEQ ID NO: 134	SEQ ID NO: 135 GFTFNNYVMNV	SEQ ID NO: 136 ISGSGGTT	SEQ ID NO: 137 AKEDDDSTGYYPFDY	SEQ ID NO: 138 KLGDKFV	SEQ ID NO: 139 QDSKRPS	SEQ ID NO: 140 QTDWDRSTVV
TTR7	SEQ ID NO: 162	SEQ ID NO: 163 GFTFSSYAMNW	SEQ ID NO: 164 ISGSGGHT	SEQ ID NO: 165 AREGYDSSGYNPFY	SEQ ID NO: 166 KLGDKYA	SEQ ID NO: 167 QDSKRPS	SEQ ID NO: 168 QAWDSSTVV
TTR8	SEQ ID NO: 190	SEQ ID NO: 191 GGSISSSTSY	SEQ ID NO: 192 IYYSGNT	SEQ ID NO: 193 ARHDWYGSGYGWFDY	SEQ ID NO: 194 SLRSY	SEQ ID NO: 195 AKNNRPS	SEQ ID NO: 196 NSRDSSGNHNV
TTR9	SEQ ID NO: 218	SEQ ID NO: 219 GGSFSGYY	SEQ ID NO: 220 FIHSGST	SEQ ID NO: 221 ARGMDSSGFYSFDF	SEQ ID NO: 222 KLGDKF	SEQ ID NO: 223 QDRKKRP	SEQ ID NO: 224 QTWYSSTVI
TTR10	SEQ ID NO: 243	SEQ ID NO: 244 GYSFTSYW	SEQ ID NO: 245 IDPSDSYT	SEQ ID NO: 246 ARMYKGRGHLEFDY	SEQ ID NO: 247 QSVSKA	SEQ ID NO: 248 AASNRA	SEQ ID NO: 249 QQYYRAPYT
TTR11	SEQ ID NO: 262	SEQ ID NO: 263 GYSFTSYW	SEQ ID NO: 264 IDPSDSYT	SEQ ID NO: 265 ARMYKGRGHLEFDY	SEQ ID NO: 266 QSVSKA	SEQ ID NO: 267 AASNRA	SEQ ID NO: 268 QQYYRAPYT
TTR12	SEQ ID NO: 278	SEQ ID NO: 279 GGSFSGYY	SEQ ID NO: 280 FIHSGST	SEQ ID NO: 281 ARGMDSSGFYSFDF	SEQ ID NO: 282 KLGDKF	SEQ ID NO: 283 QDRKKRP	SEQ ID NO: 284 QTWYSSTVI
TTR13	SEQ ID NO: 291	SEQ ID NO: 292 GFTFSSYAMNW	SEQ ID NO: 293 ISGSGGHT	SEQ ID NO: 294 AREGYDSSGYNPFY	SEQ ID NO: 295 KLGDKYA	SEQ ID NO: 296 QDSKRPS	SEQ ID NO: 297 QAWDSSTVV
TTR14 (VHH)	SEQ ID NO: 316	SEQ ID NO: 317 GLTFSNYA	SEQ ID NO: 318 IGGSGGTW	SEQ ID NO: 319 AADQRAGSYSSGWYTR SDSLY			

TTR15 (VHH)	SEQ ID NO: 323	SEQ ID NO: 324 GLTFSSYV	SEQ ID NO: 325 INGDGKF	SEQ ID NO: 326 ASDQRAAGSLSSGWYSRR SYDTLY			
TTR16 (VHH)	SEQ ID NO: 330	SEQ ID NO: 331 GFTFSSYA	SEQ ID NO: 332 ISWSGRW	SEQ ID NO: 333 VSDQRPGLSSGWYSRS SDTLY			
TTR17 (VHH)	SEQ ID NO: 337	SEQ ID NO: 338 VRISSANV	SEQ ID NO: 339 SISGGDP	SEQ ID NO: 340 NYWNEGIRY			
TTR18	SEQ ID NO: 344	SEQ ID NO: 345 GFTFSSYW	SEQ ID NO: 346 INNIGNSR	SEQ ID NO: 347 ARAGNWDRDTEDI	SEQ ID NO: 348 QSVLYSSNNKIY	SEQ ID NO: 349 WAS	SEQ ID NO: 350 QQYYSTPYT
TTR19	SEQ ID NO: 354	SEQ ID NO: 355 GFTFSRY'S	SEQ ID NO: 356 ISSSSTNI	SEQ ID NO: 357 ARDYMWKVFDY	SEQ ID NO: 358 QSLDSDDDGNIF	SEQ ID NO: 359 TLS	SEQ ID NO: 360 MQRIEFPIT
TTR20	SEQ ID NO: 364	SEQ ID NO: 365 GFTFSRY'S	SEQ ID NO: 366 ISSSSTNI	SEQ ID NO: 367 AREYMWKVFDY	SEQ ID NO: 368 QSLDSDDDGNIF	SEQ ID NO: 369 TVS	SEQ ID NO: 370 MQRIEFPIT
TTR21	SEQ ID NO: 374	SEQ ID NO: 375 GFTFNSYD	SEQ ID NO: 376 IDTAGDT	SEQ ID NO: 377 ARDRLGYGGLDV	SEQ ID NO: 378 QSVSSSY	SEQ ID NO: 379 GAS	SEQ ID NO: 380 QQYDRSPIT
TTR22	SEQ ID NO: 384	SEQ ID NO: 385 GLTFNNHN	SEQ ID NO: 386 ISSSSSYK	SEQ ID NO: 387 ARDGLAAFDADF	SEQ ID NO: 388 QSLVYSDDGITY	SEQ ID NO: 389 KVS	SEQ ID NO: 390 MQGTHWPPT
TTR23	SEQ ID NO: 394	SEQ ID NO: 395 GFLSTSGMS	SEQ ID NO: 396 IDWRDDK	SEQ ID NO: 397 AGIPGY	SEQ ID NO: 398 QNVATN	SEQ ID NO: 399 SAS	SEQ ID NO: 400 QQYNNYPPT
TTR24	SEQ ID NO: 404	SEQ ID NO: 405 GFTFSTYD	SEQ ID NO: 406 IWYDGTNR	SEQ ID NO: 407 ARDHGYTKFSDAFDF	SEQ ID NO: 408 QGINSY	SEQ ID NO: 409 AAS	SEQ ID NO: 410 LQHNSYPLT
TTR25	SEQ ID NO: 414	SEQ ID NO: 415 GGSISAYY	SEQ ID NO: 416 IYASGIT	SEQ ID NO: 417 ARQETDITGYDYFDY	SEQ ID NO: 418 KLGDKF	SEQ ID NO: 419 QDN	SEQ ID NO: 420 QTWDRSDAV
TTR26	SEQ ID NO: 424	SEQ ID NO: 425 GFTFSY'W	SEQ ID NO: 426 ISSDGSST	SEQ ID NO: 427 AREQRWLKSY'YGMVDV	SEQ ID NO: 428 QGINSY	SEQ ID NO: 429 AAS	SEQ ID NO: 430 QQLNSYPLT
TTR27	SEQ ID NO: 434	SEQ ID NO: 435 AFRFSNFN	SEQ ID NO: 436 ITSTGT'YI	SEQ ID NO: 437 ARQGIPAWDAFDL	SEQ ID NO: 438 QGINSY	SEQ ID NO: 439 ASS	SEQ ID NO: 440 LQHNSYPT
TTR28	SEQ ID NO: 444	SEQ ID NO: 445 GFTFSSYN	SEQ ID NO: 446 ISSSSSYI	SEQ ID NO: 447 AREGISAYDALNV	SEQ ID NO: 448 QGINSF	SEQ ID NO: 449 AAS	SEQ ID NO: 450 LQHDSYPLT
TTR29	SEQ ID NO: 454	SEQ ID NO: 455 GFTFSSYN	SEQ ID NO: 456 ISSSSSYI	SEQ ID NO: 457 AREGISAYDALNV	SEQ ID NO: 458 SSNIGNNY	SEQ ID NO: 459 DNN	SEQ ID NO: 460 GTWDSLSAVV
TTR30	SEQ ID NO: 464	SEQ ID NO: 465 GFTFSDYY	SEQ ID NO: 466 ISSSSVTI	SEQ ID NO: 467 AREDSDTSGYDPPFDH	SEQ ID NO: 468 QSVSSN	SEQ ID NO: 469 GAS	SEQ ID NO: 470 QQYNNWPYT
TTR31	SEQ ID NO: 474	SEQ ID NO: 475 GFTLSDDYD	SEQ ID NO: 476 INSGGNII	SEQ ID NO: 477 ARLYYDTSYSSFDY	SEQ ID NO: 478 KLGDKF	SEQ ID NO: 479 QDR	SEQ ID NO: 480 QTWYSSTVI

TTR32	SEQ ID NO: 484	SEQ ID NO: 485	SEQ ID NO: 486	SEQ ID NO: 487	SEQ ID NO: 488	SEQ ID NO: 489	SEQ ID NO: 490
	NO: 484	GFTFSSYA	ISAGGGST	AKREDDTTGYHYFDY	KLGDKF	QDT	QAWDRTTVV
TTR33	SEQ ID NO: 494	SEQ ID NO: 495	SEQ ID NO: 496	SEQ ID NO: 497	SEQ ID NO: 498	SEQ ID NO: 499	SEQ ID NO: 500
	NO: 494	GYSISSNWW	IYFSGST	ARKKGAYDYADAFDI	KIGSKS	DDS	QVWDSSSDHVV
TTR34	SEQ ID NO: 504	SEQ ID NO: 505	SEQ ID NO: 506	SEQ ID NO: 507	SEQ ID NO: 508	SEQ ID NO: 509	SEQ ID NO: 510
	NO: 504	GDSITNSNFY	IFHGGNT	ARYVAAPDYFDY	NLGKGF	QDR	QAWDSSTVV
TTR35	SEQ ID NO: 514	SEQ ID NO: 515	SEQ ID NO: 516	SEQ ID NO: 517	SEQ ID NO: 518	SEQ ID NO: 519	SEQ ID NO: 520
	NO: 514	GFTFSSYV	ISGSGGNT	AKLDYDTSGYDPDF	KLGDKF	QDN	QAWVSSTAI
TTR36	SEQ ID NO: 524	SEQ ID NO: 525	SEQ ID NO: 526	SEQ ID NO: 527	SEQ ID NO: 528	SEQ ID NO: 529	SEQ ID NO: 530
	NO: 524	GFTFSSDG	IWYDGSNK	ARDROWLAFDY	SSDVGGYNF	EVS	SSYTDSNNFDVL
TTR37	SEQ ID NO: 534	SEQ ID NO: 535	SEQ ID NO: 536	SEQ ID NO: 537	SEQ ID NO: 538	SEQ ID NO: 539	SEQ ID NO: 540
	NO: 534	GYTFTSYD	MNPNSGDT	MKMYDDTTGYHSFDS	RLGDRF	QDT	QWVATTVV
TTR38	SEQ ID NO: 544	SEQ ID NO: 545	SEQ ID NO: 546	SEQ ID NO: 547	SEQ ID NO: 548	SEQ ID NO: 549	SEQ ID NO: 550
	NO: 544	GGISISNSNY	IFHGGNT	ARYVAAPDYFDY	KLGDKF	QDR	QAWDSSTVV

Table 1b

scFv	mAb	Neutral pH			Acidic pH		
		KD (M)	kon(1/Ms)	kdis(1/s)	KD (M)	kon(1/Ms)	kdis(1/s)
TIR14 SEQ ID: 316	BBBB432 SEQ ID: 320, 321, 322	1.13E-08	1.67E+05	1.89E-03	1.44E-08	1.81E+05	2.59E-03
TIR15 SEQ ID: 323	BBBB435 SEQ ID: 327, 328, 329	1.52E-08	1.63E+05	2.46E-03	7.76E-09	1.83E+05	1.42E-03
TIR16 SEQ ID: 330	BBBB436 SEQ ID: 334, 335, 336	9.88E-09	1.50E+05	1.48E-03	6.87E-09	1.72E+05	1.18E-03
TIR17 SEQ ID: 337	BBBB439 SEQ ID: 341, 342, 343	3.65E-08	2.06E+05	7.51E-03	4.02E-08	2.01E+05	8.07E-03
TIR18 SEQ ID: 344	BBBB459 SEQ ID: 351, 352, 353	1.08E-09	1.24E+05	1.34E-04	9.44E-09	1.44E+05	1.35E-03
TIR19 SEQ ID: 354	BBBB464 SEQ ID: 361, 362, 363	5.29E-09	5.09E+04	2.69E-04	6.44E-08	5.29E+04	3.40E-03
TIR20 SEQ ID: 364	BBBB467 SEQ ID: 371, 372, 373	1.03E-08	6.95E+04	7.14E-04	9.25E-08	6.66E+04	6.16E-03
TIR21 SEQ ID: 374	BBBB476 SEQ ID: 381, 382, 383	6.98E-09	1.67E+05	1.17E-03	1.13E-08	1.79E+05	2.02E-03
TIR22 SEQ ID: 384	BBBB478 SEQ ID: 391, 392, 393	1.17E-09	1.03E+05	1.21E-04	3.58E-08	1.08E+05	3.85E-03
TIR23 SEQ ID: 394	BBBB479 SEQ ID: 401, 402, 403	2.68E-08	3.01E+04	8.05E-04	6.2E-08	3.94E+04	2.44E-03
TIR24 SEQ ID: 404	BBBB482 SEQ ID: 411, 412, 413	4.63E-09	2.68E+04	1.24E-04	2.78E-08	3.75E+04	1.04E-03
TIR25 SEQ ID: 414	BBBB486 SEQ ID: 421, 422, 423	<1.0E-12	9.79E+04	<1.0E-07	1.36E-08	1.14E+05	1.56E-03

Tripod construct design

TIR26 SEQ ID: 424	BBBB500 SEQ ID: 431, 432, 433	8.72E-09	6.96E+04	6.07E-04	2.62E-08	8.03E+04	2.11E-03
TIR27 SEQ ID: 343	BBBB504 SEQ ID: 441, 442, 443	6.96E-09	8.82E+04	6.14E-04	5.58E-08	8.51E+04	4.75E-03
TIR28 SEQ ID: 444	BBBB508 SEQ ID: 451, 452, 453	5.64E-09	1.09E+05	6.14E-04	5.57E-08	1.12E+05	6.24E-03
TIR29 SEQ ID: 454	BBBB509 SEQ ID: 63, 64, 65	6.45E-08	7.10E+03	4.58E-04	1.53E-05	3.41E+02	5.22E-03
TIR30 SEQ ID: 464	BBBB522 SEQ ID: 471, 472, 473	5.29E-08	5.27E+03	2.79E-04	1.66E-05	3.80E+02	6.30E-03
TIR31 SEQ ID: 474	BBBB529 SEQ ID: 481, 482, 483	2.11E-09	1.10E+05	2.31E-04	4.6E-08	1.09E+05	5.01E-03
TIR32 SEQ ID: 484	BBBB530 SEQ ID: 491, 492, 493	3.92E-09	1.21E+05	4.76E-04	2.92E-07	6.79E+04	1.98E-02
TIR33 SEQ ID: 494	BBBB532 SEQ ID: 501, 502, 503	5.1E-09	2.00E+05	1.02E-03	2.24E-08	2.14E+05	4.78E-03
TIR34 SEQ ID: 504	BBBB538 SEQ ID: 511, 512, 513	1.62E-08	2.07E+05	3.35E-03	2.96E-07	1.07E+05	3.17E-02
TIR35 SEQ ID: 514	BBBB539 SEQ ID: 521, 522, 523	2.76E-10	9.35E+04	2.59E-05	5.03E-08	8.92E+04	4.49E-03
TIR36 SEQ ID: 524	BBBB540 SEQ ID: 531, 532, 533	1.19E-08	7.15E+04	8.48E-04	1.2E-07	6.32E+04	7.56E-03
TIR37 SEQ ID: 534	BBBB546 SEQ ID: 541, 542, 543	1.68E-09	8.51E+04	1.43E-04	3.62E-08	9.40E+04	3.40E-03
TIR38 SEQ ID: 544	BBBB547 SEQ ID: 551, 552, 553	3.18E-09	1.90E+05	6.05E-04	5.98E-08	1.77E+05	1.06E-02

[00158] Antibodies were generated against the TfR by immunizing rodents and llamas. The resultant mAbs were screened for binding competition with transferrin and non-competitive mAbs formatted as scFv or nanobodies in a tripod mAb (also named TTP mAb) architecture and characterized. The tripod is used to deliver a substance of interest (e.g., a monoclonal antibody) to the brain. More specifically, a tripod construct (FIG. 1) containing a fusion of an antigen binding fragment of an antibody against TfR and a monoclonal antibody of interest (mAb) was developed to help the mAb penetrate the BBB and result in substantially higher brain concentrations of the mAb than the mAb alone.

[00159] For example, a tripod mAb consists of the therapeutic mAb with a TfR binding scFv or nanobody appended to the C-terminus of one antibody heavy chain using a short, flexible linker. Tripod mAbs were analysed for characteristics that have been previously described to enhance transcytosis (reviewed in Goulatis and Shusta 2017): valency, binding affinity, pH dependent binding, and rapid internalization in brain endothelial cells (Figures 2-4).

[00160] Heavy and light chain variable sequences of an antibody against TfR were fused in a single genetic construct as the single-chain variable fragment (scFv) using the following format, Hc_GTEGKSSGSGSESKST (SEQ ID NO: 314)_Lc. The scFv or a VHH against TfR was then fused to the C-terminus of the heavy chain (Hc) of an antibody of interest using either GGSGGS (SEQ ID NO: 312) or GGAGGA (SEQ ID NO: 313) linker. The Zymeworks heterodimerization mutations in CH3 were utilized in the antibody Hc (Hc A: T350V_L351Y_F405A_Y407V; Hc B: T350V_T366L_K392L_T394W) to generate the tripod construct (Figure 1), which is also referred to as a tripod mAb. A tripod mAb contains two light chains with the identical amino acid sequence and two heavy chains with different amino acid sequences. Only one of the two heavy chains is fused to a scFv or VHH of a TfR antibody of the invention and the two heavy chains also differ in their constant regions to facilitate heterodimerization between the two heavy chains. Thus, each tripod mAb according to an embodiment of the application is associated with three amino acid sequences: the amino acid sequence of the first heavy chain fused to the antigen binding fragment of a TfR antibody, the amino acid sequence of the light chain, and the amino acid sequence of the second heavy chain not fused to the antigen binding fragment of a TfR antibody.

Tripod expression and purification

[00161] Tripod mAbs were expressed in CHO-Expi cells and purified using Protein A affinity chromatography followed by Size Exclusion chromatography or Ion-exchange chromatography.

[00162] Examples of the Tripod mAbs made are provided in Table 2a:

Table 2a

Tripod name	mAb component name (target)	scFv component name	VHH component name	VL SEQ ID NO	VH SEQ ID NO	VHH/scFv SEQ ID NO
BBBB434	B21M (IgG1 AAS)		TfR1	11	12/10	6
BBBB978	BACE (IgG1AAS)		TfR1	14	13/15	6
BBBB1009	Tau (IgG1)		TfR1	17	16/18	6
BBBB1011	Tau (IgG1 AASYTE)		TfR1	20	19/21	6
BBBB1073	B-amyloid		TfR1	23	22/24	6
BBBB1215	B21M (IgG4 PAA)		TfR1	26	25/27	6
BBBB501	B21M (IgG1 AAS)	TfR2		36	35/37	28
BBBB951	B21M (IgG1)	TfR2		39	38/40	28
BBBB979	BACE (IgG1AAS)	TfR2		42	41/43	28
BBBB1520	Tau (IgG1 AASYTE)	TfR2		45	44/46	28
BBBB1018	Tau (IgG1)	TfR2		48	47/49	28
BBBB1076	B-amyloid	TfR2		51	50/52	28
BBBB1216	B21M (IgG4 PAA)	TfR2		54	53/55	28
BBBB509	B21M (IgG1 AAS)	TfR3		64	65/65	56
BBBB946	B21M (IgG1)	TfR3		67	66/68	56
BBBB947	BACE (IgG1AAS)	TfR3		70	69/71	56
BBBB1023	Tau (IgG1 AASYTE)	TfR3		73	72/74	56
BBBB1021	Tau (IgG1)	TfR3		76	75/77	56
BBBB1079	B-amyloid	TfR3		79	78/80	56
BBBB1217	B21M (IgG4 PAA)	TfR3		82	81/83	56
BBBB520	B21M (IgG1 AAS)	TfR4		92	91/93	84

Tripod name	mAb component name (target)	scFv component name	VHH component name	VL SEQ ID NO	VH SEQ ID NO	VHH/scFv SEQ ID NO
BBBB975	BACE (IgG1AAS)	TfR4		95	94/96	84
BBBB1026	Tau (IgG1 AASYTE)	TfR4		98	97/99	84
BBBB1024	Tau (IgG1)	TfR4		101	100/102	84
BBBB1082	B-amyloid	TfR4		104	103/105	84
BBBB1218	B21M (IgG4 PAA)	TfR4		107	106/108	84
BBBB534	B21M (IgG1 AAS)	TfR5		117	116/118	109
BBBB945	B21M (IgG1)	TfR5		120	119/121	109
BBBB973	BACE (IgG1AAS)	TfR5		123	122/124	109
BBBB1035	Tau (IgG1 AASYTE)	TfR5		126	125/127	109
BBBB1033	Tau (IgG1)	TfR5		129	128/130	109
BBBB1219	B21M (IgG4 PAA)	TfR5		132	131/133	109
BBBB537	B21M (IgG1 AAS)	TfR6		142	141/143	134
BBBB989	B21M (IgG1)	TfR6		145	144/156	134
BBBB977	BACE (IgG1AAS)	TfR6		148	147/149	134
BBBB1038	Tau (IgG1 AASYTE)	TfR6		151	150/152	134
BBBB1036	Tau (IgG1)	TfR6		154	153/155	134
BBBB1085	B-amyloid	TfR6		157	156/158	134
BBBB1220	B21M (IgG4 PAA)	TfR6		160	159/161	134
BBBB543	B21M (IgG1 AAS)	TfR7		170	169/171	162
BBBB1112	B21M (IgG1)	TfR7		173	172/174	162
BBBB969	BACE (IgG1AAS)	TfR7		176	175/177	162
BBBB1048	Tau (IgG1 AASYTE)	TfR7		179	178/180	162
BBBB1046	Tau (IgG1)	TfR7		182	181/183	162
BBBB1088	B-amyloid	TfR7		185	184/186	162
BBBB1221	B21M (IgG4 PAA)	TfR7		188	187/189	162
BBBB556	B21M (IgG1 AAS)	TfR8		198	197/199	190

Tripod name	mAb component name (target)	scFv component name	VHH component name	VL SEQ ID NO	VH SEQ ID NO	VHH/scFv SEQ ID NO
BBBB993	B21M (IgG1)	TfR8		201	200/202	190
BBBB983	BACE (IgG1AAS)	TfR8		204	203/205	190
BBBB1051	Tau (IgG1 AASYTE)	TfR8		207	206/208	190
BBBB1049	Tau (IgG1)	TfR8		210	209/211	190
BBBB1091	B-amyloid	TfR8		213	212/214	190
BBBB1222	B21M (IgG4 PAA)	TfR8		216	215/217	190
BBBB557	B21M (IgG1 AAS)	TfR9		226	225/227	218
BBBB970	BACE (IgG1AAS)	TfR9		229	228/230	218
BBBB1055	Tau (IgG1 AASYTE)	TfR9		232	231/233	218
BBBB1053	Tau (IgG1)	TfR9		235	234/236	218
BBBB1094	B-amyloid	TfR9		238	237/239	218
BBBB1223	B21M (IgG4 PAA)	TfR9		241	240/242	218
BBBB354	B21M (IgG1 AAS)	TfR10		251	250/252	243
BBBB932	B21M (IgG1)	TfR10		254	253/255	243
BBBB383	BACE (IgG1AAS)	TfR10		257	256/258	243
BBBB1224	B21M (IgG4 PAA)	TfR10		260	259/261	243
BBBB368	B21M (IgG1 AAS)	TfR11		270	269/271	262
BBBB934	B21M (IgG1)	TfR11		273	273/274	262
BBBB426	BACE (IgG1AAS)	TfR11		276	275/277	262
BBBB1136	Tau (IgG1 AASYTE)	TfR12		286	285/287	278
BBBB1134	Tau (IgG1)	TfR12		289	288/290	278
BBBB1133	Tau (IgG1 AASYTE)	TfR13		299	298/300	291
BBBB1131	Tau (IgG1)	TfR13		302	301/303	291
BBBB1166	B-amyloid	TfR13		305	304/306	291
BBBB432	B21M (IgG1 AAS)	TfR14		321	320/322	316
BBBB435	B21M (IgG1 AAS)	TfR15		328	327/329	323

Tripod name	mAb component name (target)	scFv component name	VHH component name	VL SEQ ID NO	VH SEQ ID NO	VHH/scFv SEQ ID NO
BBBB436	B21M (IgG1 AAS	TfR16		335	334/336	330
BBBB439	B21M (IgG1 AAS	TfR17		342	341/343	337
BBBB459	B21M (IgG1 AAS	TfR18		352	351/353	344
BBBB464	B21M (IgG1 AAS	TfR19		362	361/363	354
BBBB467	B21M (IgG1 AAS	TfR20		372	371/373	364
BBBB476	B21M (IgG1 AAS	TfR21		382	381/383	374
BBBB478	B21M (IgG1 AAS	TfR22		392	391/393	384
BBBB479	B21M (IgG1 AAS	TfR23		402	401/403	394
BBBB482	B21M (IgG1 AAS	TfR24		412	411/413	404
BBBB486	B21M (IgG1 AAS	TfR25		422	421/423	414
BBBB500	B21M (IgG1 AAS	TfR26		432	431/433	424
BBBB504	B21M (IgG1 AAS	TfR27		442	441/443	434
BBBB508	B21M (IgG1 AAS	TfR28		452	451/453	444
BBBB509	B21M (IgG1 AAS	TfR29		462	461/463	454
BBBB522	B21M (IgG1 AAS	TfR30		472	471/473	464
BBBB529	B21M (IgG1 AAS	TfR31		482	481/483	474
BBBB530	B21M (IgG1 AAS	TfR32		492	491/493	484
BBBB532	B21M (IgG1 AAS	TfR33		502	501/503	494
BBBB538	B21M (IgG1 AAS	TfR34		512	511/513	504
BBBB539	B21M (IgG1 AAS	TfR35		522	521/523	514
BBBB540	B21M (IgG1 AAS	TfR36		532	531/533	524

Tripod name	mAb component name (target)	scFv component name	VHH component name	VL SEQ ID NO	VH SEQ ID NO	VHH/scFv SEQ ID NO
BBBB546	B21M (IgG1 AAS)	TfR37		542	541/543	534
BBBB547	B21M (IgG1 AAS)	TfR38		552	551/553	544

Cell binding and TfR specificity

[00163] Tripod mAbs were analysed for characteristics that have been previously described to enhance transcytosis (reviewed in Goulatis and Shusta 2017): valency, binding affinity, pH dependent binding, and rapid internalization in brain endothelial cells (Figures 2-4).

[00164] Human brain endothelial cells (hCMECD3, 50,00 cells) were incubated with 10 ug/mL purified tripod mAb and allowed to incubate overnight at 4°C in either the presence or absence of 10x molar concentration of huTfR1 ECD (SEQ ID NO: 1). Cells were fixed and washed the following morning, incubated with secondary antibody (Jackson Immunosciences Cat# 109-546-170), washed again and then analyzed by FACS. Positive binders were defined as having binding signal greater than 2-fold over isotype control and a ratio of binding signal/binding signal with TfR ECD ≥ 2 (Table 2b).

Table 2b: hCMECD3 cell binding and specificity for tripod mAbs.

Protein Batch ID	SEQ ID NOs:	Antibody Binding without TfR			Antibody Binding with TfR			Ratio
		Rep 1	Rep 2	Ave Signal	Rep 1	Rep 2	Ave Signal	
BBBB434	10, 11, 12	1749	1405	1577	808	834	821	1.9
BBBB501	35, 36, 37	2281	1848	2065	507	790	649	3.18
BBBB509	63, 64, 65	1921	1814	1868	632	626	629	2.97
BBBB520	91, 92, 93	3413	3588	3501	766	874	820	4.27
BBBB534	116, 117, 118	3378	3246	3312	782	849	816	4.06
BBBB537	141, 142, 143	3667	3531	3599	841	803	822	4.38
BBBB543	169, 170, 171	1490	1291	1391	776	738	757	1.84
BBBB556	197, 198, 199	3262	3539	3401	711	749	730	4.66
BBBB557	225, 226, 227	2394	2204	2299	327	361	344	6.68

[00165] Additional hCMECD3 cell binding assay was conducted to measure specificity for additional tripod mAbs, and the results of which are shown in Table 2c below:

Table 2c.

IgG4 versions:						
Name	Binding Target	Description	ELN	ka (1/Ms)	kd (1/s)	KD (M)
BBBB1215 (n=6)	TfR	B21M IgG4 ZW BBBB434	BBB-00735	(4.56±0.26)E+05	(2.83±0.25)E-03	(6.24±0.75)E-09
BBBB1217 (n=3)	TfR	B21M IgG4 ZW BBBB509	BBB-00735	No Binding		
BBBB1224 (n=6)	TfR	B21M IgG4 ZW BBBB354	BBB-00735	(5.90±0.95)E+05	(1.10±0.21)E-02	(1.11±0.23)E-08
BBBB1227 (n=3)	CD98hc	B21M IgG4 ZW BBBB445	BBB-00718	(4.40±0.17)E+05	(2.42±0.64)E-03	(5.49±1.23)E-09
BBBB1228 (n=3)	CD98hc	B21M IgG4 ZW BBBB448	BBB-00718	(4.26±1.13)E+05	(1.77±0.71)E-03	(4.21±2.34)E-09
BBBB1229 (n=3)	CD98hc	B21M IgG4 ZW BBBB449	BBB-00718	(4.82±1.34)E+05	(3.36±2.38)E-03	(6.88±3.14)E-09
Results are Average ± 95%CI						

Transferrin competition

[00166] MDCK cells expressing recombinant human transferrin receptor were plated at 10,000 cells per well in a MA6000 384 HB plate and cultured for 18 hours in DMEM media supplemented with 10% FBS and 500 µg/mL Geneticin. Prior to the assay the cells were incubated in serum-free DMEM media supplemented with 5µM Monensin for 1h in a 37°C CO₂ incubator and then for 30 minutes at room temperature with StartingBlock (PBS) supplemented with 5µM Monensin. The cells in alternate rows of the plate were incubated for 30 minutes at room temperature with 2.7mg/mL human holo transferrin prepared in serum-free DMEM media supplemented with 5µM Monensin. Test antibodies were diluted to 5µg/mL in serum-free DMEM media supplemented with 5µM Monensin and added to duplicate wells containing the holo transferrin or to duplicate wells that received no transferrin and then incubated for 1h at room temperature. The supernatants were removed and 2 µg/mL Sulfo-TAG labeled anti-human antibody was added to each well and incubated for 30 minutes at room temperature. All wells were washed with PBS and Surfactant-Free MSD Read Buffer T was added. The plates were read on an MSD SECTOR® S600 imager.

[00167] Statistical analysis including mean, standard deviation and RSD were performed in Excel. Any samples with an RSD >25% were excluded. The mean values of the test antibodies incubated in the presence of transferrin were compared against the mean values in the absence of transferrin. Antibodies with values in the presence of transferrin that were ≤70% of the values in the absence of transferrin were considered ligand competitive (Table 3).

Table 3: Selected tripod mAbs are not competitive with transferrin.

Sample ID	SEQ ID NO:	w/o Tf avg value	with Tf avg value	Relative % Ab binding
BBBB434	10, 11, 12	1758	1762	100.3
BBBB501	35, 36, 37	1348	1618	120.0
BBBB509	63, 64, 65	282	447	158.6
BBBB520	91, 92, 93	1369	1798	131.3
BBBB534	116, 117, 118	1350	1141	84.5
BBBB537	141, 142, 143	1504	1851	123.1
BBBB543	169, 170, 171	225	233	103.8
BBBB556	197, 198, 199	1246	1531	122.9
BBBB557	225, 226, 227	598	530	88.7

Internalization

[00168] Human brain endothelial cells (hCMEC/D3) were plated at 10,000 cells/well in Collagen-coated 384-well Cell Carrier Ultra plates (Perkin Elmer) and allowed to attach for 16 hours at 37°C in a humidified incubator. The cells (50,00 cells) were then incubated with 200 ug/mL purified tripod mAb and allowed to incubate at 37°C for one hour. The cells were fixed, washed and incubated with a fluorescently labeled secondary antibody for one hour. The cells were then washed again and incubated with fluorescently labeled actin stain, Phalloidin, and nuclear stain, Hoechst 33342. Cells were washed again and imaged using the ImageXpress Micro (Molecular Devices) with a 40x objective. Internalizing mAbs were identified on the basis of colocalization with Phalloidin using MetaXpress 6.0. All mAbs from Tables 2 and 3 were positive for internalization.

Affinity Analysis & pH-dependent binding, species cross-reactivity

[00169] Affinity and pH dependency were initially measured using a Forte Bio Octet Platform. Biotinylated huTfR was immobilized on streptavidin sensors and mAbs associated for 180 seconds in 0.1M Phosphate pH 7.4. Dissociation was completed for 300 seconds in either 0.1M Phosphate pH 7.4 or 0.1M Phosphate pH 5 (Table 4). Preferably, a tripod mAb of interest has a high binding affinity at pH7.4 and low binding affinity at pH5, e.g., $K_D \geq 1\text{nM}$ and $k_d \geq 10^{-4}\text{ sec}^{-1}$, preferably about 10^{-3} at pH 5, such that the tripod mAb binds to TfR at a neutral pH (e.g.,

pH7.4) and dissociates from TfR at an acidic pH (e.g., pH5). Preferably, the KD at the acidic pH and the neutral pH and are similar, such as at a ratio of KD acidic/KD neutral of about 1.5.

Table 4: Kinetic rate constants measured using the Octet platform to huTfR.

Sample ID	pH 7.4 Dissociation			pH 5 Dissociation			Ratio
	KD (M)	k _{on} (1/Ms)	k _{dis} (1/s)	KD (M)	k _{on} (1/Ms)	k _{dis} (1/s)	
BBBB434	1.73E-08	1.53E+05	2.64E-03	2.45E-08	1.66E+05	4.06E-03	1.54
BBBB501	1.60E-08	1.47E+05	2.35E-03	1.68E-08	1.53E+05	2.57E-03	1.09
BBBB509	6.45E-08	7.10E+03	4.58E-04	1.53E-05	3.41E+02	5.22E-03	11.39
BBBB520	3.28E-09	1.53E+05	5.00E-04	7.32E-08	1.39E+05	1.02E-02	20.41
BBBB534	1.15E-08	7.52E+04	8.63E-04	7.14E-08	7.67E+04	5.48E-03	6.35
BBBB537	3.77E-10	1.38E+05	5.22E-05	4.42E-08	1.37E+05	6.05E-03	115.81
BBBB543	8.29E-08	2.63E+05	2.18E-02	1.62E-05	9.86E+03	1.60E-01	7.33
BBBB556	<1.0E-12	8.57E+04	<1.0E-07	1.99E-08	1.07E+05	2.13E-03	Faster
BBBB557	2.94E-08	2.28E+05	6.69E-03	2.13E-06	2.97E+04	6.33E-02	9.46

[00170] To gain additional accuracy for affinity measurements, tripod mAb affinity for huTfR was determined using surface plasmon resonance (SPR) on a BioRad Proteon instrument, ProteOn XPR36 system. An Fc capture surface was generated by coupling anti-IgG Fc mAb (Jackson ImmunoResearch) to a GLC chip (BioRad) using the amine-coupling chemistry (BioRad). Tripod mAbs were captured using a concentration of 0.3 ug/mL, flowed for 30 seconds at 60 uL/min for a target density of 120 RU huTfR was then flowed over the immobilized tripod mAbs at concentrations from 3.125 – 800 nM (in a 4-fold serial dilution) for 3 min (at 50 uL/min) association followed by dissociation for 10 minutes at 50 uL/min. The chip surface was regenerated with two 18 second pulses of 100 mM H₃PO₄ (Sigma) at 100 uL/min. The collected data were processed using ProteOn Manager software V3.1.0.6 (BioRad). First, the data was corrected for background using inter-spots. Then, double reference subtraction of the data was performed by using the buffer injection for analyte injections. The kinetic analysis of the data was performed using a Langmuir 1:1 binding model. The result for each mAb was reported in the format of K_a (On-rate), K_d (Off-rate) and K_D (equilibrium dissociation constant) (Table 5).

Table 5: Binding affinity of anti-TfR brain shuttles for TfR when fused to B21M mAb or BACE mAb.

mAb (B21M/BA CE)	B21M mAb			BACE mAb		
	ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)
BBBB434/ BBBB978	5.39E+05	2.34E-03	4.34E-09	(4.23±0.14)E+05	(2.67±0.06)E-03	(6.31±0.10)E-09
BBBB501/ BBBB979	2.45E+05	2.45E-03	1.00E-08	(2.60±0.15)E+05	(2.67±0.05)E-03	(1.03±0.06)E-08
BBBB520/ BBBB975	6.31E+05	6.28E-04	9.95E-10	(5.91±0.44)E+05	(6.59±0.11)E-04	(1.12±0.09)E-09
BBBB534/ BBBB973	1.03E+05	5.50E-04	5.35E-09	(1.08±0.06)E+05	(4.34±0.35)E-04	(4.03±0.46)E-09
BBBB537/ BBBB977	7.34E+05	2.55E-04	3.48E-10	(6.75±0.50)E+05	(1.52±0.08)E-04	(2.27±0.28)E-10
BBBB543/ BBBB969	3.27E+05	1.90E-02	5.80E-08	(2.73±0.03)E+05	(2.23±0.21)E-02	(8.16±0.92)E-08
BBBB556/ BBBB983	1.94E+05	1.70E-04	8.78E-10	(1.99±0.10)E+05	<8.67E-05	<4.36E-10
BBBB557/ BBBB970	4.22E+05	6.43E-03	1.52E-08	(3.45±0.09)E+05	(7.21±0.30)E-03	(2.09±0.05)E-08
BBBB354/ BBBB383	(14.4±0.5 9)E+05	(2.21±0.1 9)E-02	(1.54±0.2)E-08	(11.1±0.29)E+05	(2.00±0.18)E-02	(1.79±0.2)E-08
BBBB368/ BBBB426	(5.98±0.2 7)E+05	(6.06±1.4 9)E-02	(1.1±0.21)E-07	(8.55±0.15)E+05	(11.1±0.65)E-02	(1.3±0.68)E-07

[00171] pH dependent binding was assessed using the SPR (proteon) method described above, except during the dissociation the buffer pH was stepped down from 7.4 to 6.5 to 6.0. The individual sensorgrams were evaluated and scored for pH dependent binding if the off-rate was faster as the pH decreased (example in Figure 3).

[00172] Species cross-reactivity was assessed using the same method as for determining binding affinity, except the TfRs used were cyno (SEQ ID NO: 2), marmoset (SEQ ID NO: 3), rat (SEQ ID NO: 4) and mouse (SEQ ID NO: 5). No rat or mouse cross-reactive mAbs were identified. Cyno and marmoset cross-reactive tripod mAbs were identified (Table 6).

Table 6: Species cross-reactivity for selected tripod mAbs

mAB	Cynomolgus			Marmoset		
	ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)
BBBB520	5.29E+05	2.52E-04	4.77E-10	3.73E+05	3.66E-04	9.81E-10
BBBB534	2.44E+05	7.78E-03	3.19E-08	2.10E+05	4.99E-04	2.38E-09
BBBB537	6.18E+05	8.83E-05	1.43E-10	2.66E+05	6.56E-05	2.46E-10
BBBB543	2.50E+05	2.91E-02	1.17E-07	2.04E+05	7.73E-03	3.80E-08
BBBB556	8.68E+04	2.05E-03	2.36E-08	4.73E+04	1.04E-03	2.20E-08
BBBB557	2.95E+05	1.86E-02	6.31E-08	1.91E+05	1.53E-02	8.03E-08

[00173] An anti-TfR antibody or antigen-binding fragment of the invention can be used to deliver any type of immunoglobulin. Similar results have been observed with IgG1 and IgG4 therapeutic mAbs delivered by the tripod structure (data not shown).

Mouse pharmacokinetics and pharmacodynamics and anti-BACE mAb brain shuttles

[00174] To analyze the impact of binding properties on transcytosis an in vivo PK/PD study was completed in mice. C57BL/6-Tfrctm2618(TFRC)Arte mice (Taconic Artemis) were administered with test articles by IV bolus injection (13 mg/kg, 10mL/kg). At the scheduled timepoints mice were anesthetized by inhalation isoflurane. Blood collected via cardiac puncture, and plasma processed. Mouse brain was collected following whole-body perfusion with 5 mLs of 0.9% saline solution. The collected brain sample (minus cerebellum) was split into right/left

hemispheres, snap frozen in liquid nitrogen, and stored at -70°C until tissue homogenization and capillary depletion processing.

[00175] None of the TfR binding molecules were cross-reactive with murine TfR so human TfR KI mice were used to assess transcytosis. The tripod mAb was formatted with an anti-beta secretase 1 (BACE1) antagonist mAb to allow for pharmacodynamic assessment of the mAb following transcytosis into the brain. BACE1 cleaves beta-amyloid to release A β ₁₋₄₀. The inhibition of BACE1 is measured by quantitating the concentration of the product A β ₁₋₄₀ in the brain. Mice were dosed intravenously with two tripod mAbs, BBBB383 and BBBB426, along with the control mAb, BBBB456. BBBB456 is the anti-BACE1 antagonist mAb alone. BBBB383 and BBBB426 differ only in their affinity for TfR, K_D = 18 nM and 130 nM respectively. Brain exposure was determined following perfusion and capillary depletion to reduce interference from mAb in blood or retained within the vascular endothelium (Johnsen, Burkhart et al. 2017). The brain concentration of both BBBB383 and BBBB426 was enhanced over BBBB456 at all timepoints, with BBBB383 having greater mAb brain concentration than BBBB426. A strong PK/PD relationship was observed with mAb brain concentration correlative with a reduction in A β ₁₋₄₀ levels. The lower plasma exposure of both TfR containing mAbs is attributed to TMDD through binding to TfR in the periphery.

[00176] Selected anti-TfR brain shuttles were then fused to a prototypical anti-BACE (β -secretase) mAb and binding affinity was reassessed using same method as described above. As shown in Table 5, the affinity of the anti-TfR brain shuttles was similar when fused to B21M mAb (anti-human respiratory syncytial virus) and anti-BACE antagonist mAb. Internalization was assessed for selected molecules and found unchanged from internalization observed when the anti-TfR brain shuttle was fused to B21M mAb.

[00177] Since none of the anti-TfR brain shuttles bound to mouse or rat TfR, in vivo rodent studies were conducted in huTfR knock-in mice (C57BL/6-Tfrc^{tm2618}(TFRC)Arte mice (Taconic Artemis)) using the prototypical anti-BACE antagonist mAb (BBBB456, SEQ ID NOs: 307, 308 and 309). The anti-BACE antagonist mAb was selected as a model PD system for measuring inhibition of BACE1 (through the concentration of its product peptide, A β ₁₋₄₀), a reflection of the amount of mAb that was trafficked to the brain.

[00178] The first in vivo study assessed the PK/PD relationship in the brains of huTfR mice. The knock-in (KI) mice were dosed at 13 mg/kg *i.v.* with BBBB383 (SEQ ID NOs: 256, 257 and

258), BBBB426 (SEQ ID NOs: 275, 276 and 277) and BBBB456 (SEQ ID NO: 307, 308 and 309). Brains and plasma were harvested at 4, 24 and 72 hours. At the scheduled timepoints, mice were anesthetized by inhalation of isoflurane. Mouse brains from KI mice were collected following whole-body perfusion with 5 mL of 0.9% saline solution. The collected brain sample (minus cerebellum) was split into right/left hemispheres, snap frozen in liquid nitrogen, and stored at -70°C until tissue homogenization and capillary depletion processing.

[00179] For sample preparation of the capillary-depleted brain tissue lysates, individual weights were obtained for the brain hemispheres to measure drug concentration. The brain tissue samples were added to a calculated volume of modified dPBS buffer (2.5-3 µL buffer per 1 mg tissue) containing protease inhibitor (Pierce; A32955) and transferred to Lysing Matrix D (MP Biomedicals™; 6913-100) tubes. Tissue samples were homogenized at 2.9 m/s for 15 seconds using a Bead Ruptor 24 Elite (Omni International). The total cell suspension was transferred into a new tube and mixed with an equal volume of a 26% dextran buffer (13% final dextran concentration). Mixed tissue homogenate was centrifuged at 2,000 g for 10 minutes at 4°C. Carefully, the upper layer (capillary-depleted fraction) was separated from the remaining sample and transferred to a new tube containing 10x RIPA lysis buffer (Millipore™; 20-188). Capillary-depleted samples plus lysis buffer were vortexed well, centrifuged at 14,000 rpm for 30 minutes at 4°C, and supernatant transferred to a new tube. Brain tissue sample lysates were either stored frozen at -70°C or measured for protein concentrations using BCA protein assay kit (Pierce™; 23227). Final brain tissue sample lysates were normalized to 7 mg/mL total protein concentration prior to immunoassay determination of BBB-enabled mAbs.

[00180] The concentration of BBB-enabled mAbs in mouse brain tissue for PK assessment was determined using MesoScale Discovery (MSD®; Gaithersburg, MD) ECLIA technology developed in a typical sandwich immunoassay format. The assay was performed on MSD Gold™ Small Spot Streptavidin 96-well plates (Cat: L45SA). Briefly, streptavidin-coated plates were blocked with 1% bovine serum albumin (BSA) in 1x phosphate buffered saline (PBS) for 30 minutes at room temperature. The standard curve was prepared fresh in 50% naïve C57BL/6 mouse brain tissue lysates by serial dilution. Frozen quality controls (QCs) prepared in naïve C57BL/6 mouse brain tissue lysates at 2x of the working assay concentration were diluted and tested with each assay. Master mix containing the capture (biotinylated anti-human Fc mAb, 1 µg/mL) and detection (ruthenium-labeled anti-human Fc mAb, 0.5 µg/mL) reagents was

combined with diluted standards, QCs, and samples at a 1:1 volume ratio in the assay plate. The mixture was incubated for 1 hour with shaking at room temperature. Assay plates were washed, and MSD T read buffer (1x) was added to all wells. Raw data values were read on an MSD SECTOR® S600 imager. The standard curve range for the assay was tested at 1-512 ng/mL with a minimum required sample dilution (MRD) of 1:2, yielding a limit of sensitivity of 2 ng/mL in brain tissue lysates. The MSD output files with the raw ECL counts were imported into Watson LIMS (Thermo Scientific) and then regressed with a 5-parameter logistic fit with 1/F2 weighting.

[00181] The concentration of BBB-enabled mAbs in mouse plasma for PK assessment was determined using a similar protocol as described above. The standard curve was prepared fresh in 10% pooled mouse plasma by serial dilution. Frozen QCs prepared in pooled mouse plasma at 10x of the working assay concentration were diluted and tested with each assay. Master mix containing the capture (biotinylated anti-human Fc mAb, 1 µg/mL) and detection (ruthenium-labeled anti-human Fc mAb, 0.5 µg/mL) reagents was combined with diluted standards, QCs, and samples at a 1:1 volume ratio in the assay plate. The mixture was incubated with shaking for 1 hour at room temperature. Assay plates were washed, and MSD T read buffer (1x) was added to all wells. Raw data values were read on an MSD SECTOR® S600 imager. The standard curve range for the assay was tested at 2-512 ng/mL with an MRD of 1:10, yielding a limit of sensitivity of 20 ng/mL in plasma matrix. The MSD output files with the raw ECL counts were imported into Watson LIMS (Thermo Scientific) and the regressed with a 5-parameter logistic fit with 1/F2 weighting.

[00182] BACE activity measurements were made by homogenizing mouse brains in 2 ml lysing matrix D tube (8 µl of 0.4% DEA/50mM NaCl per mg of brain weight, Fast Prep-24 at 6/shakes/sec for 20 sec). Tubes were then centrifuged at 4°C for 5 min in an Eppendorf Centrifuge set to a maximum speed. Homogenate (supernatant) was then transferred to precooled tubes which were then centrifuged for 70 minutes at 13,000 rpm at 4°C. Supernatant was then transferred to a tube containing 10% of 0.5 M Tris/HCL and frozen at -80°C until assayed. Aβ 1-40 peptide standards and thawed processed brain homogenate are pre-complexed at 1:1 with ruthenium (Meso Scale Discovery (MSD), R91AN-1) labeled anti-Aβ antibody. 50 ul of complex was added to blocked plate containing capture antibody to Aβ 1-40. After overnight incubation at 2-8°C with no shaking, plates were washed and 2x read buffer (MSD, R92TC-1) added. Plate was read using Meso Sector S 600 (MSD, IC0AA).

[00183] Brain shuttle containing mAbs BBBB383 and BBBB426 were observed to have faster plasma clearance than the anti-BACE mAb BBBB456 alone (Figure 5A). However, the converse was true in the brain with BBBB383 and BBBB426 observed to have increased brain concentration at all timepoints over the control BBBB456. When the PD effect of BACE inhibition was measured, both brain shuttle mAbs were observed to inhibit the activity of BACE to a greater extent than the control anti-BACE mAb alone. (Figure 5B).

[00184] Additional brain shuttle containing mAbs were similarly assessed at 4 and 24 hrs following 13 mg/kg *i.v.* dosing (Figure 7A-B). Similar to the first study, all brain shuttle mAbs were observed to have faster plasma clearance than the control anti-BACE mAb. A range of brain concentration was observed for the brain shuttle mAbs with enhancement in brain concentration for all except BBBB974. It was hypothesized that BBBB974 did not traffic efficiently to the brain due to its binding kinetics. Specifically, BBBB974 has a slow neutral on-rate that may prevent efficient association with the TfR *in vivo*. A tripod mAb concentration dependent decrease in A β 1-40 levels were also observed for all tripod mAbs except BBBB983, which had an increase in brain concentration over the control BBBB456 but no impact on A β 1-40 concentration (Figure 8). This observation may be due to the binding kinetics, as BBBB983 has a very slow neutral off-rate which may prevent the efficient diffusion in the brain that is necessary for BACE inhibition. These data underscore the importance of TfR binding kinetics for both the delivery and function of a therapeutic mAb.

[00185] The relationship between affinity and transcytosis efficiency has been described previously as improved transcytosis with decreased affinity for TfR (Yu, Zhang et al. 2011), a conclusion that is discrepant with the above data. To probe the transcytosis affinity relationship in more detail, nine tripod mAb were assessed for brain PK/PD in the mouse model described above. These tripod mAbs differed in affinity for the TfR by approximately 100-fold (K_D ranged from 0.2nM-81nM). Brain concentration was measured at 24 hours ($C_{max \text{ brain}}$) following IV dosing (Figure 17). As expected, a range of transcytosis efficiency was observed from no enhancement to ten-fold improvement over the control mAb. The data indicate a more nuanced relationship between affinity and transcytosis efficiency than what has been previously described, with influence from both on- and off-rates impacting brain concentration. For example, no enhancement in brain exposure for BBBB946 over the control mAb was observed, although it had a K_D = 65nM and a fast off-rate at pH 6. This mAb was unique though, having a

slower on-rate than other mAbs under study ($k_a \approx 10^3 \text{ M}^{-1} \text{ s}^{-1}$ compared with $k_a \approx 10^5 \text{ M}^{-1} \text{ s}^{-1}$). In fact, when compared with another tripod antibody, BBBB969, with similar K_D ($K_D = 81 \text{ nM}$) but 100x faster on-rate the contrast is apparent. BBBB969 enhanced brain concentration by 5.5-fold demonstrating the importance of a sufficiently fast on-rate for efficient brain delivery. The efficiency of transcytosis for the other eight mAbs studied can be best described by their off-rates, with optimal brain delivery occurring at an off-rate that is neither too fast nor too slow (optimal neutral k_d of $2 \times 10^{-3} \text{ s}^{-1}$). A strong PK/PD relationship was observed for all tripod mAbs except for BBBB983, which had 5.5x enhancement in brain concentration but no impact on $A\beta_{1-40}$ levels. This mAb has a slow neutral off-rate ($< 8 \times 10^{-5} \text{ s}^{-1}$) which we hypothesize impacts its ability to diffuse in the brain to the target. Taken together the data demonstrate the importance of optimizing both the neutral on- and off-rates for optimal brain PK and PD. We observed no influence of binding epitope on TfR in the study (data not shown).

Selection of mAbs for cyno studies and assessment of brain shuttles fused to anti-Tau mAb

[00186] Critical to confirming the ability of the TfR targeting tripod mAbs to enhance therapeutic antibody brain exposure in humans is demonstrating enhanced brain delivery in non-human primates. The best performing tripod mAbs in the mouse study (BBBB979 and BBBB978) did not bind to the cyno TfR and were therefore excluded from further study. The next best, BBBB970 and BBBB969, both contained a free cysteine residue in the light chain of the anti-TfR brain shuttle (SEQ ID NO: 162 and SEQ ID NO: 218). Since free cysteine residues can contribute to nonideal biophysical properties during manufacturing, the free cysteines were mutated to serine residues (SEQ ID NO: 278 and SEQ ID NO: 291).

[00187] The new scFvs were fused to the anti-Tau mAb, PT1B844 (SEQ ID NOs: 310 and 311) to generate BBBB1136 (SEQ ID NOs: 285, 286 and 287)/BBBB1134 (SEQ ID NOs: 288, 289 and 290), and BBBB1133 (SEQ ID NOs: 298, 299 and 300)/BBBB1131 (SEQ ID NOs: 301, 302 and 303) (IgG1 AAS YTE/IgG1). The affinity for huTfR was measured (Table 7).

Table 7: Binding affinity of anti-TfR brain shuttles for huTfR with Cys-Ser mutations fused to the anti-Tau mAb

	k_a (1/Ms)	k_a (1/Ms)	K_D (M)
BBBB1131	(2.53±0.08)E+05	(3.17±0.05)E-02	(1.25±0.05)E-07

BBBB1133	(2.38±0.04)E+05	(3.23±0.29)E-02	(1.36±0.15)E-07
BBBB1134	(2.12±0.10)E+05	(2.49±0.11)E-03	(1.18±0.10)E-08
BBBB1136	(2.08±0.15)E+05	(2.26±0.06)E-03	(1.09±0.11)E-08

[00188] BBBB1134/BBBB1136 maintained very similar binding to huTfR as BBBB557/BBBB970, indicating that neither the Cys-Ser mutation or fusion to the anti-Tau mAb perturbed the binding affinity for huTfR. However, BBBB1131/BBBB1133 was approximately 2-fold weaker in binding affinity compared with BBBB543/BBBB969. To determine if the shift in affinity was due to the cys-ser mutation or to fusion to the anti-Tau mAb, the brain shuttle without the mutation but fused to anti-Tau was generated and binding assessed (BBBB1048 (SEQ ID NOs: 178, 179 and 180)/BBBB1046 (SEQ ID NOs: 181, 182 and 183)). The affinity of the non-mutated BBBB1048/BBBB1046 was very similar to BBBB543/BBBB969, indicating that the loss of affinity was due to the cys-ser mutation and not due to fusion to the Tau mAb (Table 8).

Table 8: Binding affinity of anti-TfR brain shuttles for huTfR fused to the anti-Tau mAb

	ka (1/Ms)	ka (1/Ms)	KD (M)
BBBB1046	(2.63±0.03)E+05	(1.82±0.15)E-02	(6.91±0.50)E-08
BBBB1048	(2.57±0.14)E+05	(2.06±0.25)E-02	(7.99±0.71)E-08

[00189] Similar to previous studies, internalization was also assessed and fusion of the brain shuttle to anti-Tau mAb did not impact its ability to internalize in human brain endothelial cells (example in Figure 9, mAbs tested are in Table 9).

Table 9: Anti-TfR brain shuttles assessed for internalization in human brain endothelial cells.

mAb	Internalized
BBBB1046	Yes
BBBB1047	Yes
BBBB1048	Yes
BBBB1052	Yes
BBBB1053	Yes
BBBB1054	Yes

BBBB1055	Yes
BBBB1131	Yes
BBBB1132	Yes
BBBB1133	Yes
BBBB1134	Yes
BBBB1135	Yes
BBBB1136	Yes

Cyno pharmacokinetics of anti-Tau brain shuttle mAbs

[00190] Cynomolgus monkeys were administered with test articles by IV injection (slow bolus) with the indicated dose. At the scheduled timepoints, cynomolgus monkey brain was collected and rinsed with cold saline solution following upper body perfusion with saline for minimum of 5 minutes. Predefined brain locations were isolated, snap frozen in liquid nitrogen, and stored at -80°C until tissue homogenization and capillary depletion processing.

[00191] BBBB1133, BBBB1136 and BBBB1134 were dosed *i.v.* at 10 mg/kg along with the non-brain shuttle-enabled mAbs PT1B844 (Figure 18) and PT1B916 in cynomolgus monkeys. Plasma was sampled at 4, 24 and 72 hours. Cynomolgus monkey brain was collected and rinsed with cold saline solution following upper body perfusion with saline for minimum of 5 minutes. Predefined brain locations were isolated, snap frozen in liquid nitrogen, and stored at -80°C until tissue homogenization and capillary depletion processing.

[00192] For sample preparation of the capillary-depleted brain tissue lysates, individual tissue weights were obtained for the brain locations collected. The brain tissue samples were added to a calculated volume of modified dPBS buffer (2.5 µL buffer/1 mg tissue) containing protease inhibitor (Pierce; A32955) and transferred to Lysing Matrix D (MP Biomedicals™; 6913-100) tubes. Tissue samples were homogenized at 2.9 m/s for 15 seconds using a Bead Ruptor 24 Elite (Omni International). The total cell suspension was transferred into a new tube and mixed with an equal volume of a 26% dextran buffer (13% final dextran concentration). Mixed tissue homogenate was centrifuged at 2,000g for 10 minutes at 4°C. Carefully, the upper layer (capillary-depleted fraction) was separated from the remaining sample and transferred to a new tube containing 10x RIPA lysis buffer (Millipore™; 20-188). Capillary-depleted samples plus lysis buffer were vortexed well, centrifuged at 14,000 rpm for 30 minutes at 4°C, and supernatant

transferred to a new tube. Brain tissue sample lysates were either stored frozen at -70°C or measured for protein concentrations using BCA protein assay kit (Pierce™; 23227). Final brain tissue sample lysates were normalized to 7 mg/mL total protein concentration prior to immunoassay determination of BBB-enabled mAbs.

[00193] The concentration of BBB-enabled mAbs in cynomolgus monkey brain tissue for PK assessment was determined using MSD® ECLIA technology developed in a typical sandwich immunoassay format. The assay was performed on MSD Gold™ Small Spot Streptavidin 96-well plates. The streptavidin-coated plates were blocked with 1% bovine serum albumin (BSA) in 1x phosphate buffered saline (PBS) for 30 minutes at room temperature. The standard curve was prepared fresh in 50% naïve cyno brain tissue lysates by serial dilution. Frozen QCs prepared in naïve cyno brain tissue lysates at 2x of the working assay concentration were diluted and tested with each assay. Master mix containing the capture (biotinylated anti-human Fc mAb, 1 µg/mL) and detection (ruthenium-labeled anti-human Fc mAb, 0.5 µg/mL) reagents was combined with diluted standards, QCs, and samples at a 1:1 volume ratio in the assay plate. The mixture was incubated for 1 hour with shaking at room temperature. Assay plates were washed, and MSD T read buffer (1x) was added to all wells. Raw data values were read on an MSD SECTOR® S600 imager. The standard curve range for the assay was tested at 1 – 512 ng/mL with a minimum required sample dilution (MRD) of 1:2, yielding a limit of sensitivity of 2 ng/mL in brain tissue lysates. The MSD output files with the raw ECL counts were imported into Watson LIMS (Thermo Scientific) and then regressed with a 5-parameter logistic fit with 1/F2 weighting.

[00194] The concentration of BBB-enabled mAbs in cynomolgus monkey plasma for PK assessment was determined using MSD® ECLIA technology developed in a typical sandwich immunoassay format. The assay was performed on MSD Gold™ Streptavidin 96-well plates, the streptavidin-coated plates blocked with 1% bovine serum albumin (BSA) + 0.5% Tween-20 in 1x phosphate buffered saline (PBS) for 30 minutes at room temperature. The standard curve was prepared fresh in 10% pooled cyno plasma by serial dilution. Frozen QCs prepared in pooled cyno plasma at 10x of the working assay concentration were diluted and tested with each assay. Master mix containing the capture (biotinylated anti-human Fc mAb, 1 µg/mL) and detection (ruthenium-labeled anti-human Fc mAb, 1 µg/mL) reagents was combined with diluted standards, QCs, and samples at a 1:1 volume ratio in the assay plate. The mixture was incubated

with shaking for 1 hour at room temperature. Assay plates were washed, and MSD T read buffer (1x) was added to all wells. Raw data values were read on an MSD SECTOR® S600 imager. The standard curve range for the assay was tested at 2–512 ng/mL with a minimum required sample dilution (MRD) of 1:10, yielding a limit of sensitivity of 20 ng/mL in plasma matrix. The MSD output files with the raw ECL counts were imported into Watson LIMS (Thermo Scientific) and then regressed with a 5-parameter logistic fit with 1/y² weighting.

[00195] Brain concentration was determined for the mAbs across a variety of areas (Figure 10). Brain concentration data were averaged across animals, and each symbol represents a region of the brain. A 7×, 11× and 11× greater brain concentration was observed for BBBB1134, BBBB1136 and BBBB1133 respectively, compared with the control mAb. All brain shuttle containing mAbs had increased brain exposure over the non-brain shuttle containing mAbs in every region of the brain (Figure 11).

[00196] The concentration of mAb in plasma was also determined (Figure 12). Evidence for TMDD was observed in the periphery, with the tripod mAbs having accelerated clearance over the control mAb (Figure 18). The impact of binding to the neonatal Fc receptor (FcRn) was evaluated in this study with BBBB1134 and BBBB1136 being identical except in the Fc domain with BBBB1136 having the “YTE” mutation (Dall’Acqua, K, et al. 2006). The “YTE” mutation enhances binding to FcRn at acidic pH and has been demonstrated to increase the half-life of mAbs in multiple species, including humans (Robbie, C, et al. 2013). As would be anticipated, the addition of the “YTE” mutation resulted in an increased plasma concentration for BBBB1136 compared with BBBB1134. While FcRn is a critical receptor in maintaining IgG homeostasis and extending the serum half-life of IgG in humans (Roopenian and Akilesh 2007) it has also been implicated as a reverse transcytosis, or efflux, receptor from the brain (Cooper, C, et al. 2013). We were interested in understanding the interplay between these two functions for FcRn, as improving half-life by increasing the binding affinity for FcRn may have come at the expense of brain exposure with increased brain efflux. Interestingly, the 2-fold increase in plasma concentration was mirrored by a 2-fold increase in brain concentration suggesting that any potential increase efflux is negligible in this system.

[00197] BBBB1133 had a peripheral half-life most like the mAbs without the brain shuttle, PT1B844 and PT1B916.

Reticulocyte depletion in cynomolgus monkey

[00198] A known liability for TfR targeting to enhance brain exposure is reticulocyte depletion due to antibody-dependent cell-mediated cytotoxicity (ADCC) of reticulocytes in an Fc-dependent fashion (*Science Translational Medicine* 2013: Vol. 5, 183). mAbs were tested with WT IgG1 (BBBB1134) and the mutations “AAS” (BBBB1136 and BBBB1133) to reduce FcγR binding for reticulocyte depletion in the cyno PK study. As expected, rapid reticulocyte depletion was observed for the WT IgG1 tripod mAb, BBBB1134, but not observed for BBBB1136, BBBB1133 or the non-brain shuttle mAbs PT1B844 and PT1B916 (Figure 13), confirming the impact of Fc function on TfR binding mAbs and reticulocyte depletion.

[00199] A third tripod mAb, BBBB1133, was selected for dose-response and repeat dosing cynomolgus monkey PK. Cynomolgus monkeys were dosed intravenously at 2, 10 and 30 mg/kg and brain PK determine 48 hours, 7- and 14-days later. Plasma PK was assessed over two weeks (Figure 18A and B). Linear brain PK was observed between 2 and 10 mg/kg and nonlinear brain PK between 10 and 30 mg/kg. The proposed mechanism of delivery is receptor-mediated, which will be saturable, and the data indicated that 30mg/kg is a saturating dose in cynomolgus. Linear PK was observed in plasma and CSF with a half-life of approximately 6 days. Repeat dosing was also completed using the same dose ranges dosed weekly for three weeks (Figure 18C and D). Evidence for accumulation with repeated dosing of 30mg/kg was observed and is aligned with the previous observation that 30mg/kg is a saturating dose. Linear PK was observed once again the periphery with no evidence for PK tolerance with repeat dosing.

[00200] The reticulocyte data indicated that effector silent Fc mAbs are required for the safe dosing of this brain delivery platform. While avoiding reticulocyte depletion is an important characteristic for the safety of the therapeutic mAb, this requirement though would prevent using anti-TfR mediated brain delivery for any therapeutic mAb that requires effector function, like ADP, for the therapeutic mechanism of action. For example, one potential therapeutic mechanism of action relies on Fc-dependent microglia phagocytosis of Tau aggregates. By inhibiting the ability of the brain shuttle mAb to bind to FcγR to prevent reticulocyte depletion, the mAb would not be able to bind FcγR on microglia cells to promote phagocytosis of Tau aggregates.

[00201] To explore alternative pathways for ADP, we assessed the ability of the effector silent tripod mAbs, BBBB1133 and BBBB1136, to induce phagocytosis of Tau oligomers in human IPSC derived microglia cells. Both tripod mAbs induced greater phagocytosis of Tau oligomers

than the control anti-Tau mAb, PT1B844, an IgG1 mAb (Figure 19A). The ADP of Tau oligomers by BBBB1133 has been demonstrated to occur through TfR mediated internalization and can be blocked with the addition of an excess of soluble TfR extracellular domain. Addition of excess of soluble Fc does not impact ADP, confirming that non-classical ADP utilizes the TfR and not FcγRs (Figure 19B). Similar intracellular trafficking of Tau was observed for BBBB1133 as the control mAb (PT1B844) through early endosomes (EEA1) to intermediate endosomes (Rab17) and finally the lysosome (LAMP1) (Figure 19C).

[00202] To further validate non-classical ADP as a physiological relevant mechanism for Tau degradation by microglia, we assessed the ability of the tripod mAbs to induce phagocytosis of human postmortem Alzheimer's disease brain-derived tau fibrils (PHF-Tau). ADP of PHF-Tau was measured in both human monocyte derived macrophages and human iPSC derived microglia cells (Figure 20). Both PT1B844 and BBBB1133 induced the phagocytosis of PHF-Tau at early timepoints. However, at the later timepoints BBBB1133 continues to induce ADP of PHF-Tau while PT1B844 mediated ADP stalls. This could be evidence for macrophage and microglial exhaustion as has been described for classical ADCP (Church, VanDerMeid et al. 2016) and a potential advantage of non-classical ADP mechanism utilized by BBBB1133. Similar to the previously described experiment using Tau oligomers, uptake of Tau is blocked with addition of an excess amount of soluble TfR demonstrating that this is a TfR-dependent mechanism. To probe another potential advantage of non-classical ADP over classical ADP, pro-inflammatory cytokines were measured in the PHF Tau phagocytosis experiment. As expected, classical ADP mediated by PT1B844 resulted in the secretion of proinflammatory cytokines while non-classical ADP mediated by BBBB1133 did not.

[00203] To assess the potential of the AAS IgG1 tripod mAbs to promote uptake of Tau aggregates in microglia cell, human microglia derived from Induced Pluripotent Stem Cells (iPSC) were plated onto 384 well Perkin Elmer Cell Carrier Ultra plates at a dilution of 7000 cells per well and maintained in advanced DMEM/F12 media with Glutamax+, Penicillin/Streptomycin, IL34 (100ng/ml), and GMCSF (10ng/ml). On the day of the assay, biotinylated phospho-tau oligomers [sequence: SCBiot-(dPEG4)GTPGSRSR(pT)PSLP(pT)PPTREPLL (SEQ ID NO: 315)-amide] were allowed to complex with streptavidin Alexa Fluor 488 (AF488) at 15-fold molar excess. Labelled phospho-tau oligomers were then allowed to bind test mAbs at approximately 2X molar excess at room

temperature for 30 minutes. The mAb:tau oligomer complex was then delivered to microglia at 20 ul/well. At 2, 4, and 8 hours post incubation, cells were washed twice with phosphate buffered saline (PBS) and fixed in the presence of 4% paraformaldehyde for 15 minutes at room temperature. Following fixation, cells were once again washed twice in PBS and incubated overnight with LAMP1 primary antibody, a marker for lysosomes, at a concentration of 4 ug/ml in permeabilization buffer (0.1% saponin+1% Fish skin gelatin) at 4°C. Post incubation, cells were washed twice with PBS and stained with 1 ug/ml secondary antibody conjugated to Alexa Fluor 647 in permeabilization buffer for 1 hour at 4°C. Post incubation, cells were washed twice with PBS, counter-stained with Hoechst DNA stain at 1 ug/ml for 10 minutes at room temperature in PBS. The cells were then washed one final time in PBS, resuspended in 20 ul of PBS per well and imaged on the Opera Phenix confocal high content microscope. Acquired images were analyzed using Harmony and ImageJ analysis software. Approximately 500 cells per condition were scored for the presence of Tau oligomers within phagolysosomal structures, and labelled with LAMP1 antibody.

[00204] All brain shuttle mAbs promoted more efficient uptake into phagosomes than the non-brain shuttle mAb, PT1B844 (Figure 15). Within the brain shuttle mAbs those with full effector function (BBBB1131, 1134 and 1046) were more efficient than those without effector function. These data demonstrate that eliminating binding to FcγR to reduce the risk of reticulocyte depletion should not impact therapeutic efficacy of the anti-Tau mAbs. In fact, TfR-mediated internalization and trafficking to the phagolysosome appears more efficient in microglia than did traditional FcγR mediated phagocytosis.

[00205] To explore if the observation could be repeated using other targets and cells, the uptake of RSV F-protein was assessed in human macrophages. Primary human macrophages were plated onto 384 well Perkin Elmer Cell Carrier Ultra plates at a dilution of approximately 6000 cells per well and cultured in X-VIVO 10 serum-free hematopoietic cell medium supplemented with 10% FBS, 50 mg/ml macrophage colony-stimulating factor (mCSF) CSF and 25 ng/ml interferon gamma (IFNγ). On the day of the assay, approximately 7-fold molar excess RSV-F protein (His-tagged F protein complexed with anti-His biotinylated antibody and streptavidin Alexa Fluor 488) was allowed to bind anti-RSV mAbs (1 ug/ml) at room temperature for 30 minutes. The mAb:F protein complex was then delivered to macrophages at 20 ul/well. Alexa Fluor 488 labelled E. Coli served as a positive control for phagocytosis. 3

hours post incubation, cells were washed twice with phosphate buffered saline (PBS) and fixed in the presence of 4% paraformaldehyde for 15 minutes at RT. Following fixation, cells were once again washed twice in PBS and incubated overnight with LAMP1 primary antibody, a marker for lysosomes, at a concentration of 4 ug/ml in permeabilization buffer (0.1% saponin+1% Fish skin gelatin) at 4°C. Post incubation, cells were washed twice with PBS and stained with 1 ug/ml secondary antibody conjugated to Alexa Fluor 647 in permeabilization buffer for 1 hour at 4°C. Post incubation, cells were washed twice with PBS, counter-stained with Hoechst DNA stain at 1 ug/ml for 10 minutes at room temperature in PBS. The cells were then washed one final time in PBS, resuspended in 20 ul of PBS per well and imaged on the Opera Phenix confocal high content microscope. Acquired images were analyzed using Harmony and ImageJ analysis software. Approximately 300 cells per condition were scored for the presence of F protein foci within phagolysosomal structures, labelled with LAMP1 antibody.

[00206] As was observed for Tau and microglia cells, all brain shuttle mAbs promoted more efficient uptake into phagosomes than the non-brain shuttle mAb, B21M-IgG1 (Figure 16). However, a difference in uptake between IgG1 (BBBB932 and BBBB934) and IgG1 AAS (BBBB354 and BBBB368) brain shuttle mAbs was not observed. It remains to be determined if a difference between the B21M experiment and the Tau experiment (Figure 15 and Figure 16) is due to the target or the cells. Regardless, the data confirm the robustness of the mechanism where Tfr-mediated internalization and trafficking to the phagolysosome appears at least as efficient as traditional FcγR mediated phagocytosis.

[00207] To the knowledge of the inventors, there has not been any publication describing exploiting this non-classical ADP mechanism for a therapeutic mAb. While not wishing to be bound by theories, it is believed that phagocytosis and endocytosis can both lead to degradation through the convergence of the phagolysosomal paths, such that regardless of the internalization trigger (FcγR mediated phagocytosis or Tfr-mediated endocytosis), the internalized cargo is trafficked to and degraded by the phagolysosome.

Assessment of the PK/PD relationship in the retina of huTfr mice

[00208] Selected anti-Tfr brain shuttles were then fused to a prototypical anti-BACE (β-secretase) mAb and binding affinity was reassessed using same method as described above. As shown in Table 5, the affinity of the anti-Tfr brain shuttles was similar when fused to B21M

mAb (anti-human respiratory syncytial virus) and anti-BACE antagonist mAb. Internalization was assessed for selected molecules (Figure 4) and found unchanged from internalization observed when the anti-TfR brain shuttle was fused to B21M mAb.

[00209] Since none of the anti-TfR brain shuttles bound to mouse or rat TfR, in vivo rodent studies will be conducted in huTfR knock-in mice (C57BL/6-Tfrc^{tm2618}(TFRC)Arte mice (Taconic Artemis)) using the prototypical anti-BACE antagonist mAb (BBBB970, BBBB978, BBBB983). The anti-BACE antagonist mAb was selected as a model PD system for measuring inhibition of BACE1 (through the concentration of its product peptide, A β 1-40), a reflection of the amount of mAb that was trafficked to the brain.

[00210] The first in vivo study will assess the PK/PD relationship in the retina of huTfR mice. The knock-in (KI) mice will be dosed at 10 mg/kg *i.v.* with the BBBB970, BBBB978, BBBB983 and the control BBBB456. Eyes and plasma will be harvested at 4- and 24-hours following dosing. At the scheduled timepoints, mice will be anesthetized by inhalation of isoflurane. Mouse eyes from KI mice will be collected following whole-body perfusion with 5 mL of 0.9% saline solution. The collected eye sample (minus the optic nerve) will be snap frozen in liquid nitrogen, and stored at -70°C until tissue homogenization or prepared for immunohistochemistry.

[00211] BACE activity measurements will be made by homogenizing mouse eyes in lysing matrix D tube (8 μ l of 0.4% DEA/50mM NaCl per mg of brain weight, Fast Prep-24 at 6/shakes/sec for 20 sec). Tubes will then be centrifuged at 4°C for 5 min in an Eppendorf Centrifuge set to a maximum speed. Homogenate (supernatant) will then transferred to precooled tubes which were then centrifuged for 70 minutes at 13,000 rpm at 4°C. Supernatant will then be transferred to a tube containing 10% of 0.5 M Tris/HCL and frozen at -80°C until assayed. A β 1-40 peptide standards and thawed processed eye homogenate are then pre-complexed at 1:1 with ruthenium (Meso Scale Discovery (MSD), R91AN-1) labeled anti-A β antibody. 50 μ l of complex will be added to blocked plate containing capture antibody to A β 1-40. After overnight incubation at 2-8°C with no shaking, plates will be washed and 2x read buffer (MSD, R92TC-1) added. Plate will be read using Meso Sector S 600 (MSD, IC0AA).

Cytokine secretion analysis

[00212] After different treatments on human iPSC-derived microglia, the relative concentrations of secreted proteins in cell supernatants were measured using antibody-based 29-

plex immunoassays (Luminex, R&D systems, Cat.# LXSAM-29). The 29 secreted proteins were: BDNF, CCL3/MIP1 α , CCL20/MIP3 α , Gro β /MIP2, CXCL10/IP10/CRG2, GCSF, IFN α , IL1 α , IL2, IL6, IL10, IL17/IL17 α , MCSF, RAGE/AGER, TNF α , CCL2/JE/MCP1, CCL4/MIP1 β , CXCL9/MIG, FGFb/FGF2, GMCSF, IFN γ , IL1 β , IL4, IL8/CXCL8, IL12p70, IL23, MMP9, Resistin.

PHF Tau

[00213] Postmortem tissue from the cortex obtained from 5 histologically confirmed AD patient (Braak stage V-VI) was used to generate a pool of partially purified PHF by a modified method of (Mercken et al., *Acta Neuropathologica* (1992) 84: 265–272; Greenberg, et al. *J. biol. Chem.* (1992) 267: 564-569). Typically, 5 g of parietal or frontal cortex was homogenized in 10 volumes of cold buffer H (10 mM Tris, 800 mM NaCl, 1 mM EGTA and 10% sucrose/ pH 7.4) using a glass/Teflon Potter tissue homogenizer (IKA Works, Inc; Staufen, Germany) at 1000 rpm. The homogenized material was centrifuged at 27000 $\times$ g for 20 min at 4°C. The pellet was discarded, and the supernatant was adjusted to a final concentration of 1% (w/v) N-lauroylsarcosine and incubated for 2 h at 37°C. Subsequently, the supernatant was centrifuged at 184000 $\times$ g for 90 min at 20°C. The pellet was carefully washed in PBS and resuspended in 750 μ L PBS, aliquoted and frozen at –80°C. The quality of the PHF-tau preparations was evaluated by the use of AT8/AT8 phospho-aggregate selective MSD ELISA. Tau content was determined by western blotting using hTau10 (Janssen R&D) with recombinant 2N4R tau as calibrant.

Study re the ability of the TfR TTP mAb to potentiate ADP in vivo

[00214] The ability of the TfR TTP mAb to potentiate ADP *in vivo* was studied in a mouse model of Tau seeding. The mouse model employed transgenic Tau-P301L mice, expressing the longest human tau isoform with the P301L mutation (tau-4R/2N-P301L) (Terwel, et al. (2005) *J Biol Chem*; 280(5): 3963-73). Due to the lack of mouse TfR cross-reactivity of the TTPs, a mouse surrogate TTP was developed to have similar binding properties to the lead human TfR TTP and used in this study. The model of Tau seeding involves stereotactic hippocampal injections of PHF-Tau, which induces a dose-dependent increase in tau aggregation (Vandermeeren, et al., *J Alzheimers Dis.* (2018); 65(1): 265-281). Following co-injection of mAbs, neutralization of Tau seeding by different anti-tau mAbs has demonstrated that the model

is partially dependent upon Fc-mediated ADP of Tau (Figure 21A). While both anti-Tau mAbs neutralized Tau seeding compared with the isotype control, a statistically significant difference is observed between the mAb with effector function (mouse IgG2a) and the mAb without effector function (mouse IgG2a σ (Vafa, et al., Methods. 2014 Jan 1; 65(1): 114-26)), demonstrating the partial dependence of the model on mAb effector function.

[00215] A similar study was completed comparing the anti-Tau mAb, PT1B844 with a mouse IgG2a Fc, to the PT1B844 TTP mAb with a human IgG1 AAS Fc. Co-injection of mAbs was utilized to normalize for any differences in PK properties between the mAb and the TTP mAb. Both anti-Tau mAbs neutralized Tau seeding compared with the isotype control. TTP mAb displayed at least the same compared to the mAb with full Fc effector function, suggesting that non-classical ADP mechanism is functional *in vivo* (Figure 21B).

Stereotactic injection of PHF in P301L mice

[00216] PHF tau seeding studies, including the currently described study, are performed in compliance with protocols approved by the local ethical committee (628-Tau Spread, Janssen Pharmaceutica) and national institutions adhering to AAALAC guidelines. Mice expressing the longest human tau isoform with the P301L mutation (tau-4R/2N-P301L) (Terwel et al., 2005; Peeraer et al., 2015) were single housed in an enriched environment, individually ventilated cages and under 12/12 h light/dark cycles (light on at 6:00 AM). At the age of 90 +/- 7 days, mice were randomized over treatment groups and gender and received a unilateral injection in the right hippocampus (CA1) of AD-derived PHFs (in the presence of anti-IgG2a (n= 19); anti phospho Tau mouse IgG2a (n= 20) or anti-phospho Tau-TTE (n = 20).

[00217] Tau.P301L mice were deeply anaesthetized with isoflurane (5% in 36% oxygen) and fixed in a stereotactic frame (Stoelting-Neurostar combination). During the further procedure a 2% isoflurane level was maintained. A 30G syringe (Hamilton) was used for injecting 3 μ L in the right hemisphere at a speed of 0.25 μ L/min at the selected coordinates: anteroposterior -2.0, mediolateral +1.6 from bregma, dorsoventral 1.4 mm from dura. Body weight was monitored before and weekly after injection, and no differences were observed between treatment and control groups for all injection experiments (not shown).

[00218] Two months after injection, mice were sacrificed by decapitation and brain tissue from the *ipsilateral* hemisphere was snap frozen. Before extraction, tissue was weighed and

homogenized in 600 μ L of buffer H per 100 mg tissue (10 mM Tris, 800 mM NaCl, 1 mM EGTA and 10% sucrose/ pH 7.4). The homogenate was centrifuged at 27 000 x g for 20 min and supernatant was frozen at -80 °C.

Biochemical analysis MesoScale Discovery (MSD)

[00219] Coating antibody (AT8) was diluted in PBS (1 μ g/mL) and aliquoted into MSD plates (30 μ L per well) (L15XA, MSD, Rockville, MD, USA), which were incubated overnight at 4°C. After washing with 5 x 200 μ L of PBS/0.5% Tween-20, the plates were blocked with 0.1% casein in PBS and washed again with 5 x 200 μ L of PBS/0.5% Tween-20. After adding samples and standards (both diluted in 0.1% casein in PBS), the plates were incubated overnight at 4°C. Subsequently, plates were washed with 5 x 200 μ L of PBS/0.5% Tween-20, and SULFO-TAG™ conjugated detection antibodies (AT8) in 0.1% casein in PBS were added and incubated for 2 h at room temperature while shaking at 600 rpm. After a final wash (5 x 200 μ L of PBS/0.5% Tween-20), 150 μ L of 2 x buffer T (MSD) was added, and plates were read with an MSD imager. Raw signals were normalized against a standard curve consisting of 16 dilutions of a sarcosyl-insoluble prep from postmortem AD brain (PHF) and were expressed as arbitrary units (AU) PHF. Statistical analysis (ANOVA with Bonferroni correction for multiple testing) was performed with the GraphPad prism software. P-values < 0.05 were considered as significantly different.

Discussion

[00220] To achieve an optimized brain delivery platform based on receptor mediated transcytosis, mAbs were generated that bind specifically to the human transferrin receptor (huTfR) with a range of affinities in a pH-dependent manner. The relationship between TfR binding affinity and transcytosis efficiency has been covered extensively in numerous publications with a focus on the equilibrium dissociation constant, K_D . While K_D is an important measure, it has been surprisingly demonstrated in the invention the criticality of the binding kinetics, k_a and k_d , for transcytosis. Inventors discovered that both on- and off-rates need to be optimized for efficient transcytosis and pharmacodynamic activity of the therapeutic mAb delivered. Based on the results, optimal transcytosis occurs when, for example, the $k_a \geq 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and neutral $k_d = 2 \times 10^{-3} \text{ sec}^{-1}$. While not wishing to be bound by theories, it is hypothesized

the interplay between on-rate and off-rate is critical to ensuring efficient transcellular transport through the diverse intracellular vesicles responsible for protein trafficking in polarized cells

[00221] It has been demonstrated that dosing tripod mAbs in cynomolgus monkeys results in 6-12x enhancement in brain concentration over a control mAb. Increasing acidic FcRn binding resulted in reduced peripheral clearance and enhanced brain concentration. Under normal physiological conditions, FcRn-mediated efflux of antibodies from the brain is likely crucial in maintaining brain homeostasis by avoiding unwanted inflammation and immune responses in the brain (Schlachetzki, Zhu et al. 2002, Roopenian and Akilesh 2007). While the preponderance of evidence suggests a strong role for FcRn-mediated efflux of antibodies, there does remain some debate about this clearance mechanism (Garg and Balthasar 2009, Abuqayyas and Balthasar 2013). Inventors discovered that increasing the binding affinity for FcRn has a positive impact on both peripheral and brain concentration, suggesting that any enhanced efflux is insignificant in this system. Dose response experiments in cynomolgus monkeys using a tripod mAb demonstrated the saturability of the mechanism of transport, which occurs at 30mg/kg in this species. Extensive repeat dosing, dose response characterization was also completed in cynomolgus and will aid greatly in predicting human doses and the utility of this platform for specific therapeutic applications.

[00222] Reticulocyte depletion is a known safety liability for TfR binding antibodies. It was observed by the inventors that indeed acute and nearly complete reticulocyte depletion can be observed with an effector function competent mAb. Numerous approaches have been described to avoid this depletion, including reducing effector function {Couch, 2013 #589} and through molecular architecture {Weber, 2018 #590}. While inventors utilized a very similar architecture to one that has been described as sterically capable of attenuating peripheral effector function, they observed robust reticulocyte depletion with the effector function competent mAb.

[00223] The clear disadvantage to Fc mutagenesis is the elimination of effector function from the therapeutic mAb. For many therapeutic targets in the brain, like beta-amyloid and Tau, ADP is believed critical to efficacy. Previous work has demonstrated that recycling receptors, including TfR, can be excluded from sorting tubules and diverted to lysosomes by multivalent cargo binding (Marsh, 1995, *J Cell Biol* (1995) 129 (6): 1509–1522; Wefflen, 2013 *Mol Biol Cell*. 2013 Aug 1; 24(15): 2398–2405). Inventors demonstrated that this endogenous diversion of multivalent cargo can be used as an alternative, non-classical, non-FcγR mechanism of ADP.

Tau internalized through non-classical and classical ADP are trafficked similarly in microglia, with Tau aggregates trafficking through the endolysosomal system to the lysosome for degradation. The described non-classical ADP can be exploited for a variety of therapeutic applications where ADP is necessary for efficacy but classical ADP harmful for safety.

[00224] The data indicate that non-classical ADP is more efficient than classical ADP, potentially due to inherent differences in the binding and internalization between FcγRs and TfR. FcγR mediated internalization requires receptor clustering by the mAb, while TfR is rapidly internalizing and recycling independent of mAb binding. A second potential explanation is macrophage and microglial exhaustion (Zent, 2017 FEBS J. 2017 Apr; 284(7):1021-1039). Macrophage exhaustion appears to be dependent upon the length of time the macrophage is exposed to the target (Church, VanDerMeid et al. 2016, Clin Exp Immunol. 2016 Jan;183(1):90-101) (Mukundan, 2009, Nat Med. 2009 Nov;15(11):1266-72), which is aligned with our observation of that classical ADP stalls with time. Observations for macrophage exhaustion have been made in vitro and in patients, indicating that this exhaustion phenotype may impact therapeutic efficacy of mAbs with effector function. The non-classical ADP offers an efficacy advantage, avoiding this exhaustion phenotype by mediating ADP without activating microglia by binding FcγRs.

[00225] Another advantage of non-classical ADP is that by avoiding microglial activation ADP occurs without stimulating the production pro-inflammatory cytokines. There remains debate on the safety of using effector-function competent mAbs in treating diseases in the brain, particularly around increasing neuroinflammation in patients who already suffer from chronic neuroinflammation (reviewed in {Heneka, 2015 #591}). In addition, there is increasing attention to the role that inflammation plays in the pathogenesis of neurodegenerative disease with the implications of increasing inflammation, as well as, the ability to engage or further activate potentially already exhausted microglia under debate. For example, the toxic impact of classic ADP on neurons was been demonstrated and hypothesized that effector function competent mAbs may pose safety risks {Lee, 2016 #592}. The non-classical ADP mechanism described here avoids the potential neuroinflammation liabilities by potentiating efficient clearance of Tau without needing to activate microglia or stimulate release of proinflammatory cytokines. In conclusion, a robust brain delivery platform has been characterized for pharmacokinetics,

pharmacodynamics and safety establishing the robust preclinical characterization needed to advance to clinical trials.

[00226] When formatted as scFv brain shuttles and fused to a prototypical anti-BACE (beta-secretase) antagonist mAb, a 4-10x improvement in brain concentration was observed over the anti-BACE mAb alone following *i.v.* dosing of transgenic mice expressing huTfR. A strong PK:PD relationship was also noted, with a dose dependent decrease in beta-amyloid detected. The best performing brain shuttles enhanced brain delivery more than competitor molecules, achieving best-in-class delivery through optimized binding interactions between the brain shuttle and huTfR.

[00227] The optimized brain shuttles were then fused to PT1B844, a Tau binding mAb. Brain shuttles fused PT1B844 demonstrated 6 to 16-fold improvement in brain concentration when dosed *i.v.* in cynomolgus monkey. Similar to the mouse data, the enhancement in brain concentration exceeded the best brain shuttles reported in literature. In addition to superior brain PK, the brain shuttles are engineered to reduce Fc-mediated effector function and do not induce rapid reticulocyte depletion in cynomolgus as has been reported by competitors. Importantly, the loss of Fc-function doesn't impact the effectiveness of the therapeutic Tau mAb, as the brain shuttle is more efficient at mediating microglial uptake of Tau than PT1B844 alone.

[00228] It will be appreciated by those skilled in the art that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications within the spirit and scope of the present invention as defined by the appended claims.

Citations:

- Abuqayyas, L. and J. P. Balthasar (2013). "Investigation of the role of FcγR and FcRn in mAb distribution to the brain." *Mol Pharm* **10**(5): 1505-1513.
- Church, A. K., K. R. VanDerMeid, N. A. Baig, A. M. Baran, T. E. Witzig, G. S. Nowakowski and C. S. Zent (2016). "Anti-CD20 monoclonal antibody-dependent phagocytosis of chronic lymphocytic leukaemia cells by autologous macrophages." *Clin Exp Immunol* **183**(1): 90-101.
- Cooper, P. R., G. J. Ciambrone, C. M. Kliwinski, E. Maze, L. Johnson, Q. Li, Y. Feng and P. J. Hornby (2013). "Efflux of monoclonal antibodies from rat brain by neonatal Fc receptor, FcRn." *Brain Res* **1534**: 13-21.
- Dall'Acqua, W. F., P. A. Kiener and H. Wu (2006). "Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn)." *J Biol Chem* **281**(33): 23514-23524.
- Garg, A. and J. P. Balthasar (2009). "Investigation of the influence of FcRn on the distribution of IgG to the brain." *AAPS J* **11**(3): 553-557.
- Goulatis, L. I. and E. V. Shusta (2017). "Protein engineering approaches for regulating blood-brain barrier transcytosis." *Curr Opin Struct Biol* **45**: 109-115.
- Johnsen, K. B., A. Burkhardt, F. Melander, P. J. Kempen, J. B. Vejlebo, P. Siupka, M. S. Nielsen, T. L. Andresen and T. Moos (2017). "Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma." *Sci Rep* **7**(1): 10396.
- Robbie, G. J., R. Criste, W. F. Dall'acqua, K. Jensen, N. K. Patel, G. A. Losonsky and M. P. Griffin (2013). "A novel investigational Fc-modified humanized monoclonal antibody, motavizumab-YTE, has an extended half-life in healthy adults." *Antimicrob Agents Chemother* **57**(12): 6147-6153.
- Roopenian, D. C. and S. Akilesh (2007). "FcRn: the neonatal Fc receptor comes of age." *Nat Rev Immunol* **7**(9): 715-725.
- Schlachetzki, F., C. Zhu and W. M. Pardridge (2002). "Expression of the neonatal Fc receptor (FcRn) at the blood-brain barrier." *J Neurochem* **81**(1): 203-206.
- Vandermeeren, M., M. Borgers, K. Van Kolen, C. Theunis, B. Vasconcelos, A. Bottelbergs, C. Wintmolders, G. Dancels, R. Willems, K. Dockx, L. Delbroek, A. Marreiro, L. Ver Donck, C. Sousa, R. Nanjunda, E. Lacy, T. Van De Castele, D. Van Dam, P. P. De Deyn, J. A. Kemp, T. J. Malia and M. H. Mercken (2018). "Anti-Tau Monoclonal Antibodies Derived from Soluble and Filamentous Tau Show Diverse Functional Properties in vitro and in vivo." *J Alzheimers Dis* **65**(1): 265-281.
- Yu, Y. J., J. K. Atwal, Y. Zhang, R. K. Tong, K. R. Wildsmith, C. Tan, N. Bien-Ly, M. Hersom, J. A. Maloney, W. J. Meilandt, D. Bumbaca, K. Gadkar, K. Hoyte, W. Luk, Y. Lu, J. A. Ernst, K. Scarce-Levie, J. A. Couch, M. S. Dennis and R. J. Watts (2014). "Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates." *Sci Transl Med* **6**(261): 261ra154.
- Yu, Y. J., Y. Zhang, M. Kenrick, K. Hoyte, W. Luk, Y. Lu, J. Atwal, J. M. Elliott, S. Prabhu, R. J. Watts and M. S. Dennis (2011). "Boosting brain uptake of a therapeutic antibody by reducing its affinity for a transcytosis target." *Sci Transl Med* **3**(84): 84ra44.
- Zent, C. S. and M. R. Elliott (2017). "Maxed out macs: physiologic cell clearance as a function of macrophage phagocytic capacity." *FEBS J* **284**(7): 1021-1039.