POLYPEPTIDES BINDING TO A SPECIFIC EPITOPE OF THE TRANSFERRIN RECEPTOR 1

RELATED APPLICATIONS

This application claims priority to U.S. Application No. 63/571,018, filed March 28, 2024, and EP Application No. 24175277.3, filed May 10, 2024, the disclosure of each is hereby incorporated by reference in their entirety.

1 Technical field of the invention

The present invention relates to polypeptides that are capable of binding to a specific pH-dependent epitope on the (human) transferrin receptor 1 (TfR1).

In particular, the present invention relates to novel and improved immunoglobulin single variable domains (ISVDs), such as heavy-chain single variable domains, which are capable of binding to a specific epitope on TfR1. The invention further relates to polypeptides, nucleic acids, vectors, host cells, conjugates, or pharmaceutical compositions that comprise at least one of these ISVDs binding to a specific epitope on TfR1.

The present invention further relates to methods for producing such ISVDs as well as to uses of such polypeptides for diverse therapeutic, diagnostic and/or medical imaging applications, including but not limited to the delivery of therapeutic, diagnostic and/or medical imaging agents across the blood-brain barrier (BBB), and/or into a cell of a subject.

2 Background

The blood–brain barrier (BBB) is a highly selective semipermeable border of endothelial cells that prevents solutes in the circulating blood from non-selectively crossing into the extracellular fluid of the central nervous system where neurons reside. The blood-brain barrier is formed by endothelial cells of the capillary wall, astrocyte end-feet ensheathing the capillary, and pericytes embedded in the capillary basement membrane. This system allows the passage of some small molecules by passive diffusion, as well as the selective and active transport of various nutrients, ions, organic anions, and macromolecules such as glucose and amino acids that are crucial to neural function.

In its neuroprotective role, the BBB functions to hinder the delivery of many potentially important diagnostic and therapeutic agents to the brain. Therapeutic molecules and antibodies that might

otherwise be effective in diagnosis and therapy do not cross the BBB in adequate amounts to be clinically effective.

Transferrin receptor 1 (TfR1) endocytic pathway for iron homeostasis has been one of the most extensively characterized systems for drug delivery across the BBB. TfR1 mediates influx of iron-loaded transferrin from blood to brain in addition to the transcytosis of iron-depleted transferrin in the reverse direction. Transferrin itself has been used as a vehicle for brain delivery, but transferrin conjugates must compete for the receptor with the high plasma concentration of the endogenous ligand and thus are not sufficiently effective for treatment or diagnosis.

Accordingly, the BBB still represents an obstacle to several potentially successful drugs by preventing these from reaching the brain in a pharmaceutically effective amount. Thus, there remains a need for therapeutic compounds that are able to effectively cross the BBB, such as improved selective TfR1-specific binding compounds, especially ones having one or more advantageous biological properties with therapeutic and/or diagnostic benefit over the currently known anti-TfR1 antibodies and other regulators of iron transport systems.

3 Summary of the Invention

The present invention provides improved ISVDs (immunoglobulin single variable domains) as defined further herein that are able to effectively cross the blood-brain barrier (BBB) in a selective manner (see Examples 18-20). More particularly, the ISVDs of the invention, and the polypeptides and compounds comprising said ISVDs specifically bind to TfR1 and are characterized by several improved features compared to existing TfR1-binders. One of these advantageous characteristics of the TfR1-binding polypeptides according to the present invention is that these bind to a unique epitope on TfR1 (see Example 21), which epitope is different from the epitopes bound by the known natural TfR1 ligands (i.e., transferrin, see Example 11) and which is different from the epitopes bound by the existing TfR1-binding agents. This particular epitope is present only at neutral pH (neutral conformation) while it is not present at acidic pH (acidic conformation), see Example 22. This leads to a pH-dependent binding without the need to engineer TfR1 binders for pH dependence (see Example 7).

The ISVDs of the present invention can therefore be applied for several prophylactic, diagnostic, and therapeutic applications, particularly for those, for which a transport across the blood-brain-barrier is beneficial or required. Since the ISVDs of the present invention bind TfR1, and TfR1 is

expressed on virtually any cell of a subject such as a human subject (albeit at varying degrees), the ISVDs of the present invention can also be used to transport or deliver an agent into a cell of any type, tissue, organ, or body part of the subject.

Accordingly, the present invention relates to an immunoglobulin single variable domain (ISVD),
wherein the ISVD specifically binds to an epitope of a human transferrin receptor 1 (TfR1) homodimer (CD71),
wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,
wherein the epitope comprises amino acid residues on both the first TfR1 monomer and the second TfR1 monomer; and
wherein the amino acid residues of the epitope form a conformation that differs between pH 7.4 (neutral conformation) and pH 6.0 (acidic conformation), wherein the ISVD has a reduced affinity to the TfR1 homodimer at pH 6.0 compared to pH 7.4, wherein optionally binding of the ISVD to the TfR1 homodimer has a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation.

The present invention further relates to an ISVD,
wherein the ISVD specifically binds to an epitope of a human transferrin receptor 1 (TfR1) homodimer,
wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,
wherein the ISVD is characterized in that the epitope comprises:
(i) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, K382, and E383 of the first TfR1 monomer, and
(ii) at least one of amino acid residues K633, E634, M635, G363, L637, R719, N722, N723, G724, A725, F726, N727, E728, T729, and R732 of the second TfR1 monomer,
wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the conformation (or spatial arrangement) of the amino acid residues of the epitope changes when shifting the pH from pH 7.4 to pH 6.0 (or vice versa) resulting in a binding

of the ISVD to the TfR1 with a dissociation rate constant (k_{off}) that is at least 10 times lower at pH 7.4 compared to the k_{off} at pH 6.0.

In one embodiment, the amino acid residues of the epitope form a conformation that differs between pH 7.4 (neutral conformation) and pH 6.0 (acidic conformation), wherein the ISVD has a reduced affinity to the TfR1 homodimer at pH 6.0 compared to pH 7.4, optionally wherein binding of the ISVD to the TfR1 homodimer has a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation.

In one embodiment, the epitope comprises:

(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the epitope comprises:

(i) at least one of amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the epitope comprises:

(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the epitope comprises:

(i) at least one of amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the epitope comprises:

(i) at least one of amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the epitope comprises:

(i) at least one of amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the ISVD specifically binds to TfR1 with a k_{off} rate of less than 10×10^{-3} s^{-1} at a neutral pH, such as pH 7.4. In one embodiment, the ISVD binds to TfR1 with a k_{off} rate of more than 5×10^{-2} s^{-1} at an acidic pH, such as pH 6.0.

In one embodiment, the first TfR1 monomer, the second TfR1 monomer, or the first and the second TfR1 monomer comprises or consists of SEQ ID NO: 1 or (polymorphic) variants or isoforms thereof. In one embodiment, the first TfR1 monomer, the second TfR1 monomer, or the first and the second TfR1 monomer comprises or consists of amino acid positions 89 to 760 of SEQ ID NO: 1.

In one embodiment, the ISVD according to the invention specifically binds to amino acid residues on TfR1 that are not involved in binding of TfR1 to transferrin.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 7 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 7; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 8 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 8; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 9, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 9.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 10 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 10; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 11 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 11; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 12, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 12.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 13 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 13; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 14 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 14; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 15, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 15.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 16 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 16; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 17 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 17; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 18, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 18.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 19 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 19; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 20 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 20; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 21, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 21.

In one embodiment, the ISVD according to the invention comprises or consists of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26.

In one embodiment, the net charge of the CDRs or the paratope of the ISVD according to the invention is not altered by changing the pH from pH 6.0 to 7.4 (and/or from pH 7.4 to 6.0).

In one embodiment, the ISVD according to the invention is cross-reactive to cynomolgus TfR1, mouse TfR1, or both.

In one embodiment, two ISVDs can bind to the same TfR1 homodimer.

In one embodiment, the ISVD is a humanized V_{HH} of an ISVD described herein.

The present invention further relates to a polypeptide comprising the ISVD of the invention.

In one embodiment, the present invention provides polypeptides comprising at least one ISVD according to the invention and at least one other ISVD binding to the same TfR1 molecule as the ISVD according to the invention.

The present invention further relates to a nucleic acid encoding the ISVD of the invention or the polypeptide of the invention.

The present invention further relates to a vector comprising the nucleic acid of the invention.

The present invention further relates to a host cell comprising the nucleic acid of the invention or the vector of the invention.

The present invention further relates to a conjugate comprising the ISVD of the invention or the polypeptide of the invention, and an agent. The agent optionally is covalently attached to the ISVD or the polypeptide.

In one embodiment, the agent is a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, a radioactive isotope, etc.

The present invention further relates to a pharmaceutical composition comprising the ISVD of the invention, the polypeptide of the invention, or the conjugate of the invention. The pharmaceutical composition optionally comprises a pharmaceutically acceptable carrier.

The present invention further relates to the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention for use in a method of delivering an agent across the blood brain barrier (BBB) in a subject. The agent optionally is a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, a radioactive isotope, etc.

The present invention further relates to the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention for use in a method of delivering an agent into a cell in a subject.

In some embodiments, (a therapeutically effective amount of) the ISVD, the polypeptide, the conjugate, or the pharmaceutical composition is administered to the subject. In some embodiments, a therapeutically effective amount of the ISVD, the polypeptide, the conjugate, or the pharmaceutical composition is administered to the subject.

The present invention further relates to a use of the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention for delivery of an agent into a cell in a subject.

The present invention further relates to a use of the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention for delivery of an agent across the blood-brain-barrier in a subject.

In one embodiment, the subject is a human. In some embodiments, the subject has a disease of the nervous system (such as central nervous system, peripheral nervous system, or sciatic nerve) or of muscle tissue (such as skeletal muscle, heart muscle, smooth muscle).

The present invention further relates to a method for increasing the exposure of the CNS to an agent comprising:

(i) administering the conjugate of the invention to a subject.

The present invention further relates to a method of manufacturing a conjugate as defined herein comprising:

(i) Providing an ISVD as defined herein; and

(ii) Conjugating the agent to the ISVD.

4 Brief description of the figures

Figure 1A and 1B show MSD data for binding of ISVD of the invention to human TfR1 at pH 7.4 and 6.0.

Figure 2 shows exemplary Octet® response curves for T0281007D02 binding against human TfR1 (hTfR1), cynomolgus TfR1 (cTfR1), and mouse TfR1 (mTfR1). The y-axis indicates the shift or response in nm while the x-axis shows the time (s). The legend below the x-axis indicates the various concentrations of T0281007D02 that generated the respective response curves (decreasing from top to bottom of the respective figure). The dashed or solid vertical line indicates the switch from association to dissociation phase.

Figure 3A shows internalization of anti-TfR1 ISVD clones in HEK293T cells using flow cytometry-based read-out. IRR00028 is a non-TfR1 binding ISVD clone.

Figure 3B shows internalization of anti-TfR1 ISVD clones in HEK293T cells using Incucyte based read-out. IRR00028 is a non-TfR1 binding ISVD clone. Data is shown for read-out after 11h40.

Figure 4 shows the Schild analysis of anti-TfR1 ISVD clones. Human Tf is titrated out on HEK293T cells in absence and presence of EC30, 10xEC50, and 100xEC50 concentrations of ISVD protein. Results are shown for T0281007D02, T0281047E03 ISVD clones and for a non-TfR1 binding (IRR00028) ISVD clone and anti-TfR1 competing clones T028106B04 as controls.

Figure 5 shows example of Octet® response curves for T0281007D02 binning data competing against other anti-TfR1 ISVD molecules for binding to human TfR1 (hTfR1). The y-axis indicates the shift or response in nm while the x-axis shows the time (s). After saturation of the immobilized hTfR1 by T0281007D02, no ISVD shows an increase in shift during the second anti-TfR1 ISVD

molecule capture step at ~600 s, with the exception of the transferrin competitor T0281006B04. This suggests that all tested ISVD, except T0281006B04, compete with T0281007D02.

Figure 6 shows the binding of oligonucleotide conjugated and corresponding non-conjugated anti-TfR1 ISVD formats to HEK293T cells to assess whether an impact for binding to human TfR1 was observed upon oligonucleotide conjugation.

Figure 7A shows monovalent anti-TfR1 ISVD exposure in the brain.

Figure 7B shows a brain PK study using Iodine 125 radiolabeled molecules in hTfR-KI mice.

Figure 8 shows Malat1 RNA knockdown in Gastrocnemius, heart, brain, and sciatic nerve, after 4 doses over 2 weeks using ISVD-HLE-MALAT1 ASO conjugate in hTfR-KI mice.

Figure 9 shows the conformational change of TfR1 from pH 6.0 (dark grey) to pH 7.4 (light grey) as determined by CryoEM.

Figure 10A shows in dark grey ISVD 30F12 in ribbon diagram and in light grey a Fab molecule aligned in the same orientation as the ISVD in the structure when binding to TfR1 (black).

Figure 10B shows in light grey a Fab molecule aligned in the different orientation compared to ISVD 30F12 in the structure when binding to TfR1 (black).

5 Detailed description

5.1 Definitions

Amino acid residues will be indicated interchangeably herein according to the standard three-letter or one-letter amino acid code, as mentioned in Table B-1 below.

Table B-1: Common amino acids

1-Letter Code	3-Letter Code	Amino Acid Name
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid

E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xaa	Unspecified
Y	Tyr	Tyrosine

When an amino acid residue is indicated as "X" or "Xaa", it means that the amino acid residue is unspecified, unless the context requires a more limited interpretation. For example, if the description provides an amino acid sequence of a CDR wherein one (or more) of the amino acid residue(s) is (are) indicated with “X”, the description may further specify which amino acid residue(s) is (can be) present at that specific position of the CDR.

Amino acids are those L-amino acids commonly found in naturally occurring proteins and are listed in Table B-1. Those amino acid sequences containing D-amino acids are not intended to be embraced by this definition. Any amino acid sequence that contains post-translationally modified amino acids may be described as the amino acid sequence that is initially translated using the symbols shown in the Table B-1 with the modified positions; e.g., hydroxylations or glycosylations, but these modifications shall not be shown explicitly in the amino acid sequence. Any peptide or protein that can be expressed as a sequence modified linkages, cross links and end caps, non-peptidyl bonds, etc., is embraced by this definition. The terms “protein”, “peptide”,

"protein/peptide", and "polypeptide" are used interchangeably throughout the disclosure, and each has the same meaning for purposes of this disclosure. Each term refers to an organic compound made of a linear chain of two or more amino acids. The compound may have ten or more amino acids; twenty-five or more amino acids; fifty or more amino acids; one hundred or more amino acids, two hundred or more amino acids, and even three hundred or more amino acids. The skilled artisan will appreciate that polypeptides generally comprise fewer amino acids than proteins, although there is no art-recognized cut-off point of the number of amino acids that distinguish a polypeptide from a protein; that polypeptides may be made by chemical synthesis or recombinant methods; and that proteins are generally made in vitro or in vivo by recombinant methods as known in the art.

When a nucleotide sequence or amino acid sequence is said to "comprise" another nucleotide sequence or amino acid sequence, respectively, or to "essentially consist of" another nucleotide sequence or amino acid sequence, this may mean that the latter nucleotide sequence or amino acid sequence has been incorporated into the first-mentioned nucleotide sequence or amino acid sequence, respectively, but more usually this generally means that the first-mentioned nucleotide sequence or amino acid sequence comprises within its sequence a stretch of nucleotides or amino acid residues, respectively, that has the same nucleotide sequence or amino acid sequence, respectively, as the latter sequence, irrespective of how the first-mentioned sequence has actually been generated or obtained (which may for example be by any suitable method described herein). By means of a non-limiting example, when an ISVD is said to comprise a CDR sequence, this may mean that said CDR sequence has been incorporated into the ISVD, but more usually this generally means that the ISVD contains within its sequence a stretch of amino acid residues with the same amino acid sequence as said CDR sequence, irrespective of how said ISVD has been generated or obtained. It should also be noted that when the latter amino acid sequence has a specific biological or structural function, it has essentially the same, a similar or an equivalent biological or structural function in the first-mentioned amino acid sequence (in other words, the first-mentioned amino acid sequence is such that the latter sequence is capable of performing essentially the same, a similar or an equivalent biological or structural function). For example, when an ISVD is said to comprise a CDR sequence or framework sequence, respectively, the CDR sequence and framework are capable, in said ISVD, of functioning as a CDR sequence or framework sequence, respectively. Also, when a nucleotide sequence is said to comprise another nucleotide sequence, the first-mentioned nucleotide sequence is such that, when it is expressed into an expression product (e.g. a polypeptide), the amino acid sequence encoded by the latter nucleotide sequence

forms part of said expression product (in other words, that the latter nucleotide sequence is in the same reading frame as the first-mentioned, larger nucleotide sequence).

In the context of the present technology, "binding to" a certain target molecule has the usual meaning in the art as understood in the context of antibodies and their respective antigens.

The term "immunoglobulin single variable domain" (ISVD), interchangeably used with "single variable domain", defines immunoglobulin molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from "conventional" immunoglobulins (e.g., monoclonal antibodies) or their fragments (such as Fab, Fab', $F(ab')_2$, scFv, di-scFv), wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (V_H) and a light chain variable domain (V_L) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both V_H and V_L will contribute to the antigen binding site, i.e., a total of 6 CDRs will be involved in antigen binding site formation.

In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a $F(ab')_2$ fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an immunoglobulin single variable domain, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain variable domains, i.e., by a V_H-V_L pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

In contrast, immunoglobulin single variable domains are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single V_H, a single V_{HH} or single V_L domain.

As such, the single variable domain may be a light chain variable domain sequence (e.g., a V_L-sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g., a V_H-sequence or V_{HH} sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that essentially consists of the

single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit).

An immunoglobulin single variable domain (ISVD) can for example be a heavy chain ISVD, such as a V_H, V_{HH}, including a camelized V_H or humanized V_{HH}. In some embodiments, the ISVD is a V_{HH}, including a camelized V_H or humanized V_{HH}. Heavy chain ISVDs can be derived from a conventional four-chain antibody or from a heavy chain antibody.

For example, the immunoglobulin single variable domain may be a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a NANOBODY® molecule (as defined herein, and including but not limited to a V_{HH}); other single variable domains, or any suitable fragment of any one thereof. In particular, the immunoglobulin single variable domain may be a NANOBODY® immunoglobulin single variable domain (such as a V_{HH}, including a humanized V_{HH} or camelized V_H) or a suitable fragment thereof. [Note: Nanobody® is a registered trademark of Ablynx N.V.]

"V_{HH} domains", also known as V_{HH}s, V_{HH} antibody fragments, and V_{HH} antibodies, have originally been described as the antigen binding immunoglobulin variable domain of "heavy chain antibodies" (i.e., of "antibodies devoid of light chains"; Hamers-Casterman et al. Nature 363: 446-448, 1993). The term "V_{HH} domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_H domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as "V_L domains"). For a further description of V_{HH}s, reference is made to the review article by Muyldermans (Reviews in Molecular Biotechnology 74: 277-302, 2001).
Typically, the generation of immunoglobulins involves the immunization of experimental animals, fusion of immunoglobulin producing cells to create hybridomas and screening for the desired specificities. Alternatively, immunoglobulins can be generated by screening of naïve or synthetic libraries, e.g., by phage display.

The generation of immunoglobulin sequences, such as Nanobody® V_{HH}s, has been described extensively in various publications, among which WO 94/04678, Hamers-Casterman *et al.* 1993 and Muyldermans et al. 2001 (Reviews in Molecular Biotechnology 74: 277-302, 2001) can be

exemplified. In these methods, camelids are immunized with the target antigen in order to induce an immune response against said target antigen. The repertoire of V_{HH}s obtained from said immunization is further screened for V_{HH}s that bind the target antigen. In these instances, the generation of antibodies requires purified antigen for immunization and/or screening. Antigens can be purified from natural sources, or in the course of recombinant production. Immunization and/or screening for immunoglobulin sequences can be performed using peptide fragments of such antigens.

The present technology may use immunoglobulin sequences of different origin, comprising mouse, rat, rabbit, donkey, human and camelid immunoglobulin sequences. The technology also includes fully human, humanized, or chimeric sequences. For example, the invention comprises camelid immunoglobulin sequences and humanized camelid immunoglobulin sequences, or camelized domain antibodies, e.g., camelized dAb as described by Ward et al (see for example WO 94/04678 and Riechmann, Febs Lett., 339:285-290, 1994 and Prot. Eng., 9:531-537, 1996). Moreover, the invention also uses fused immunoglobulin sequences, e.g. forming a multivalent and/or multispecific construct (for multivalent and multispecific polypeptides containing one or more V_{HH} domains and their preparation, reference is also made to Conrath et al., J. Biol. Chem., Vol. 276, 10. 7346-7350, 2001, as well as to for example WO 96/34103 and WO 99/23221), and immunoglobulin sequences comprising tags or other functional moieties, e.g. toxins, labels, radiochemicals, etc., which are derivable from the immunoglobulin sequences of the present technology.

A "humanized V_{HH}" comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_{HH} domain, but that has been "humanized", i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring V_{HH} sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a V_H domain from a conventional 4-chain antibody from a human being (e.g. indicated above). This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art (e.g., WO 2008/020079). Again, it should be noted that such humanized V_{HH}s can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_{HH} domain as a starting material.

A "camelized V_H" comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_H domain, but that has been "camelized", i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring V_H domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_{HH} domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art (e.g., WO 2008/020079). Such "camelizing" substitutions can be inserted at amino acid positions that form and/or are present at the V_H-V_L interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996), supra). In some embodiments, the V_H sequence that is used as a starting material or starting point for generating or designing the camelized V_H is a V_H sequence from a mammal, for example the V_H sequence of a human being, such as a V_H3 sequence. However, it should be noted that such camelized V_H can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_H domain as a starting material.

A preferred structure of an immunoglobulin single variable domain sequence can be considered to be comprised of four framework regions ("FRs"), which are referred to in the art and herein as "Framework region 1" ("FR1"); as "Framework region 2" ("FR2"); as "Framework region 3" ("FR3"); and as "Framework region 4" ("FR4"), respectively; which framework regions are interrupted by three complementary determining regions ("CDRs"), which are referred to in the art and herein as "Complementarity Determining Region 1" ("CDR1"); as "Complementarity Determining Region 2" ("CDR2"); and as "Complementarity Determining Region 3" ("CDR3"), respectively.

As further described in paragraph q) on pages 58 and 59 of WO 08/020079 (incorporated herein by reference), the amino acid residues of an immunoglobulin single variable domain can be numbered according to the general numbering for V_H domains given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to V_{HH} domains from Camelids in the article of Riechmann and Muyldermans, 2000 (J. Immunol. Methods 240 (1-2): 185-195; see for example Figure 2 of this publication). It should be noted that - as is well known in the art for V_H domains and for V_{HH} domains - the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is,

one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering). This means that, generally, the numbering according to Kabat may or may not correspond to the actual numbering of the amino acid residues in the actual sequence. The total number of amino acid residues in a V_H domain and a V_{HH} domain will usually be in the range of from 110 to 120, often between 112 and 115. It should however be noted that smaller and longer sequences may also be suitable for the purposes described herein.

CDR sequences can be determined according to the AbM numbering as described in Kontermann and Dübel (Eds. 2010, Antibody Engineering, vol 2, Springer Verlag Heidelberg Berlin, Martin, Chapter 3, pp. 33-51). According to this method, FR1 comprises the amino acid residues at positions 1-25, CDR1 comprises the amino acid residues at positions 26-35, FR2 comprises the amino acids at positions 36-49, CDR2 comprises the amino acid residues at positions 50-58, FR3 comprises the amino acid residues at positions 59-94, CDR3 comprises the amino acid residues at positions 95-102, and FR4 comprises the amino acid residues at positions 103-113.

Determination of CDR regions may also be done according to different methods. In the CDR determination according to Kabat, which is used throughout this application unless indicated otherwise, FR1 of an immunoglobulin single variable domain comprises the amino acid residues at positions 1-30, CDR1 of an immunoglobulin single variable domain comprises the amino acid residues at positions 31-35, FR2 of an immunoglobulin single variable domain comprises the amino acids at positions 36-49, CDR2 of an immunoglobulin single variable domain comprises the amino acid residues at positions 50-65, FR3 of an immunoglobulin single variable domain comprises the amino acid residues at positions 66-94, CDR3 of an immunoglobulin single variable domain comprises the amino acid residues at positions 95-102, and FR4 of an immunoglobulin single variable domain comprises the amino acid residues at positions 103-113.

In such an immunoglobulin sequence, the framework sequences may be any suitable framework sequences, and examples of suitable framework sequences will be clear to the skilled person, for example on the basis the standard handbooks and the further disclosure and prior art mentioned herein.

The framework sequences can be (a suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from immunoglobulin framework

sequences (for example, by humanization or camelization). For example, the framework sequences may be framework sequences derived from a light chain variable domain (e.g., a V_L-sequence) and/or from a heavy chain variable domain (e.g., a V_H-sequence or V_{HH} sequence). In one particularly preferred aspect, the framework sequences are either framework sequences that have been derived from a V_{HH}-sequence (in which said framework sequences may optionally have been partially or fully humanized) or are conventional V_H sequences that have been camelized (as defined herein).

In particular, the framework sequences present in the ISVD sequence used in the invention may contain one or more of hallmark residues (as defined herein), such that the ISVD sequence is a Nanobody® molecule, such as a V_{HH}, including a humanized V_{HH} or camelized V_H. Some preferred, but non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein.

Again, as generally described herein for the immunoglobulin sequences, it is also possible to use suitable fragments (or combinations of fragments) of any of the foregoing, such as fragments that contain one or more CDR sequences, suitably flanked by and/or linked via one or more framework sequences (for example, in the same order as these CDR's and framework sequences may occur in the full-sized immunoglobulin sequence from which the fragment has been derived).

However, it should be noted that the invention is not limited as to the origin of the ISVD sequence (or of the nucleotide sequence used to express it), nor as to the way that the ISVD sequence or nucleotide sequence is (or has been) generated or obtained. Thus, the ISVD sequences may be naturally occurring sequences (from any suitable species) or synthetic or semi-synthetic sequences. In a specific but non-limiting aspect, the ISVD sequence is a naturally occurring sequence (from any suitable species) or a synthetic or semi-synthetic sequence, including but not limited to "humanized" (as defined herein) immunoglobulin sequences (such as partially or fully humanized mouse or rabbit immunoglobulin sequences, and in particular partially or fully humanized V_{HH} sequences), "camelized" (as defined herein) immunoglobulin sequences, as well as immunoglobulin sequences that have been obtained by techniques such as affinity maturation (for example, starting from synthetic, random or naturally occurring immunoglobulin sequences), CDR grafting, veneering, combining fragments derived from different immunoglobulin sequences, PCR assembly using overlapping primers, and similar techniques for engineering immunoglobulin sequences well known to the skilled person; or any suitable combination of any of the foregoing.

Similarly, nucleotide sequences may be naturally occurring nucleotide sequences or synthetic or semi-synthetic sequences, and may for example be sequences that are isolated by PCR from a suitable naturally occurring template (e.g. DNA or RNA isolated from a cell), nucleotide sequences that have been isolated from a library (and in particular, an expression library), nucleotide sequences that have been prepared by introducing mutations into a naturally occurring nucleotide sequence (using any suitable technique known per se, such as mismatch PCR), nucleotide sequence that have been prepared by PCR using overlapping primers, or nucleotide sequences that have been prepared using techniques for DNA synthesis known per se.

As described herein, an ISVD may be a Nanobody® V_{HH} or a suitable fragment thereof. For a general description of ISVDs, reference is made to the further description below, as well as to the prior art cited herein. In this respect, it should however be noted that this description and the prior art mainly described ISVDs of the so-called "V_H3 class" (i.e., ISVDs with a high degree of sequence homology to human germline sequences of the V_H3 class such as DP-47, DP-51, or DP-29). It should however be noted that the invention in its broadest sense can generally use any type of ISVD, and for example also uses the ISVDs belonging to the so-called "V_H4 class" (i.e., ISVDs with a high degree of sequence homology to human germline sequences of the V_H4 class such as DP-78), as for example described in WO 2007/118670.

Generally, ISVDs (in particular V_{HH} sequences, including (partially) humanized V_{HH} sequences and camelized V_H sequences) can be characterized by the presence of one or more "Hallmark residues" (as described herein) in one or more of the framework sequences (again as further described herein). Thus, generally, a ISVD can be defined as an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which one or more of the Hallmark residues are as further defined herein.

In particular, an ISVD can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which the framework sequences are as further defined herein.

More in particular, an ISVD can be an immunoglobulin sequence with the (general) structure

FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4

in which FR1 to FR4 refer to framework regions 1 to 4, respectively, and in which CDR1 to CDR3 refer to the complementarity determining regions 1 to 3, respectively, and in which:

5 one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-0 below.

Table A-0: Hallmark Residues in Nanobody® ISVDs

Position	Human V_H3	Hallmark Residues
11	L, V; predominantly L	L, S, V, M, W, F, T, Q, E, A, R, G, K, Y, N, P, I; preferably L
37	V, I, F; usually V	$F^{(1)}$, Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably $F^{(1)}$ or Y
$44^{(8)}$	G	$E^{(3)}$, $Q^{(3)}$, $G^{(2)}$, D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably $G^{(2)}$, $E^{(3)}$ or $Q^{(3)}$;most preferably $G^{(2)}$ or $Q^{(3)}$.
$45^{(8)}$	L	$L^{(2)}$, $R^{(3)}$, P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably $L^{(2)}$ or $R^{(3)}$
$47^{(8)}$	W, Y	$F^{(1)}$, $L^{(1)}$ or $W^{(2)}$ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably $W^{(2)}$, $L^{(1)}$ or $F^{(1)}$
83	R or K; usually R	R, $K^{(5)}$, T, $E^{(5)}$, Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K
84	A, T, D; predominantly A	$P^{(5)}$, S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P
103	W	$W^{(4)}$, $R^{(6)}$, G, S, K, A, M, Y, L, F, T, N, V, Q, $P^{(6)}$, E, C; preferably W
104	G	G, A, S, T, D, P, N, E, C, L; preferably G
108	L, M or T; predominantly L	Q, $L^{(7)}$, R, P, E, K, S, T, M, A, H; preferably Q or $L^{(7)}$
Notes: In particular, but not exclusively, in combination with KERE (SEQ ID NO: 82) or KQRE (SEQ ID NO: 83) at positions 43-46.		

Usually as GLEW (SEQ ID NO: 84) at positions 44-47.

Usually as KERE (SEQ ID NO: 82) or KQRE (SEQ ID NO: 83) at positions 43-46, e.g., as KEREL (SEQ ID NO: 85), KEREF (SEQ ID NO: 86), KQREL (SEQ ID NO: 87), KQREF (SEQ ID NO: 88), KEREG (SEQ ID NO: 89), KQREW (SEQ ID NO: 90) or KQREG (SEQ ID NO: 91) at positions 43-47. Alternatively, also sequences such as TERE (SEQ ID NO: 92) (for example TEREL (SEQ ID NO: 93)), TQRE (SEQ ID NO: 94) (for example TQREL (SEQ ID NO: 95)), KECE (SEQ ID NO: 96) (for example KECEL (SEQ ID NO: 97) or KECER (SEQ ID NO: 98)), KQCE (SEQ ID NO: 99) (for example KQCEL (SEQ ID NO: 100)), RERE (SEQ ID NO: 101) (for example REREG (SEQ ID NO: 102)), RQRE (SEQ ID NO: 103) (for example RQREL (SEQ ID NO: 104), RQREF (SEQ ID NO: 105) or RQREW (SEQ ID NO: 106)), QERE (SEQ ID NO: 107) (for example QEREG (SEQ ID NO: 108)), QQRE (SEQ ID NO: 109), (for example QQREW (SEQ ID NO: 110), QQREL (SEQ ID NO: 111) or QQREF (SEQ ID NO: 112)), KGRE (SEQ ID NO: 113) (for example KGREG (SEQ ID NO: 114)), KDRE (SEQ ID NO: 115) (for example KDREV (SEQ ID NO: 116)) are possible. Some other possible, but less preferred sequences include for example DECKL (SEQ ID NO: 117) and NVCEL (SEQ ID NO: 118).

With both GLEW (SEQ ID NO: 84) at positions 44-47 and KERE (SEQ ID NO: 82) or KQRE (SEQ ID NO: 83) at positions 43-46.

Often as KP or EP at positions 83-84 of naturally occurring V_{HH} domains.

In particular, but not exclusively, in combination with GLEW (SEQ ID NO: 84) at positions 44-47.

With the proviso that when positions 44-47 are GLEW (SEQ ID NO: 84), position 108 is always Q in (non-humanized) V_{HH} sequences that also contain a W at 103.

The GLEW (SEQ ID NO: 84) group also contains GLEW(SEQ ID NO: 84)-like sequences at positions 44-47, such as for example GVEW (SEQ ID NO: 119), EPEW (SEQ ID NO: 120), GLER (SEQ ID NO: 121), DQEW (SEQ ID NO: 122), DLEW (SEQ ID NO: 123), GIEW (SEQ ID NO: 124), ELEW (SEQ ID NO: 125), GPEW (SEQ ID NO: 126), EWLP (SEQ ID NO: 127), GPER (SEQ ID NO: 128), GLER (SEQ ID NO: 129) and ELEW (SEQ ID NO: 130).

5.2 ISVDs of the invention

The present inventors have identified a unique epitope on TfR1, which is different to the epitope of the natural ligands of TfR1, such as transferrin, and which is different from the TfR1 epitopes

of existing antibodies. This newly discovered epitope has many advantages over the presently known epitopes.

As outlined herein, this particular epitope of TfR1 homodimer is present only at neutral pH (neutral conformation) while it is not present at acidic pH (acidic conformation). This leads to a pH-dependent binding without the need to engineer the TfR1 binders for pH dependence (see e.g., Example 7). "pH-dependent binding" relates to a binding property of a ligand such as an ISVD, which specifically binds to a target such as TfR1 homodimer at pH7.4 but binds with a reduced affinity (e.g., higher k_{off} rate, or higher K_D) to the same target at pH 6.0. The epitope thus can be seen as switching from a binding conformation at neutral pH such as pH 7.4 to a non-binding conformation at acidic pH such as pH 6.0. This process is not necessarily without transition, but - without wishing to be bound by theory - the epitope can be described as gradually losing its specific conformation and/or structure by lowering the pH from pH 7.4 to acidic values such as pH 6.0 (see e.g., Example 22). In one embodiment, the binding of the ISVD to the TfR1 homodimer has a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation.

In addition, the novel ISVDs of the invention are capable of binding specifically to TfR1 without interfering with the natural physiological roles of TfR1 (see e.g., Example 11). The "immunoglobulin single variable domain" (ISVD) disclosed herein bind to human TfR1, or (polymorphic) variants or isoforms thereof. Isoforms are alternative protein sequences that can be generated from the same gene by a single biological event or by the combination of biological events such as alternative promoter usage, alternative splicing, alternative initiation, and ribosomal frameshifting, all as known in the art.

The present inventors surprisingly identified a novel and unique conformational epitope of TfR1, being present and available for binding by the ISVD's of the present invention exclusively and/or optimally at a neutral pH. Accordingly, the identification of this specific conformational epitope of TfR1 resulted in the development of improved ISVD sequences that are able to specifically bind and modulate TfR1 under neutral pH conditions but not under acidic pH conditions. Thus, in contrast to the known pH dependent TfR1 binders, the development of the conditionally binding ISVD's of the present invention did not require additional CDR sequence modification steps, which are typically applied in the art to create pH dependent binders.

The novel epitope furthermore can be characterized in that it is predicted to be exclusively accessible for binding by small ISVDs but not by larger binders such as antibodies or large fragments (e.g., Fab fragments) thereof. As shown in Fig. 10, the epitope is located in a cavity on the TfR1, into which larger molecules could not be modeled (see also e.g., Example 23. Thus, larger molecules (i.e., being larger than an ISVD such as a V_{HH}). "Larger molecules" as used herein may relate to molecules having a molecular weight of at least 20 kDa, at least 25 kDa, at least 30 kDa, at least 40 kDa, at least 100 kDa, at least 150 kDa. In one embodiment, a larger molecule has at least 25 kDa. In a further embodiment, a larger molecule has at least 150 kDa.

Finally, the ISVDs of the invention can show cross-reactivity to cynomolgus or mouse TfR1, or both, in addition to their capacity to bind human TfR1 (see, e.g., Example 8). This is a particular feature of this pH-dependent epitope on TfR1.

The present invention generally relates to an immunoglobulin single variable domain (ISVD),

wherein the ISVD specifically binds to an epitope of a transferrin receptor 1 (TfR1) homodimer,

wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,

wherein the epitope comprises amino acid residues of both the first TfR1 monomer and the second TfR1 monomer.

Accordingly, the present invention relates to an immunoglobulin single variable domain (ISVD),

wherein the ISVD specifically binds to an epitope of a transferrin receptor 1 (TfR1) homodimer,

wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,

wherein the epitope comprises amino acid residues of both the first TfR1 monomer and the second TfR1 monomer;

and

wherein the amino acid residues of the epitope form a conformation that allows specific binding of the ISVD at pH 7.4 (neutral conformation) and a different conformation that does not allow specific binding of the ISVD at pH 6.0 (acidic conformation).

The binding of the ISVD to the TfR1 homodimer may have a dissociation rate constant (k_{off}) that is at least 5 times lower for the neutral conformation compared to the acidic conformation. The binding of the ISVD to the TfR1 homodimer may have a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation. The binding of the ISVD to the TfR1 homodimer may have a dissociation rate constant (k_{off}) that is at least 15 times lower for the neutral conformation compared to the acidic conformation. The binding of the ISVD to the TfR1 homodimer may have a dissociation rate constant (k_{off}) that is at least 20 times lower for the neutral conformation compared to the acidic conformation. The binding of the ISVD to the TfR1 homodimer may have a dissociation rate constant (k_{off}) that is at least 50 times lower for the neutral conformation compared to the acidic conformation.

While the amino acid residues being part of the TfR1 epitope, bound by the ISVDs of the invention, are present on both the first and the second TfR1 monomers, they are not necessarily the same amino acid residues of both the first TfR1 monomer and the second TfR1 monomer. Thus, the epitope identified in the present invention may comprise amino acid residues of the first TfR1 monomer and amino acid residues of the second TfR1 monomer, wherein the bound amino acid residues of the first TfR1 monomer are different or do not correspond to the bound amino acid residues of the second TfR1 monomer. In one embodiment, the amino acid residues of the first TfR1 monomer that are comprised in the epitope of the invention are different or do not correspond to the amino acid residues of the second TfR1 monomer that are comprised in the epitope of the invention.

The term “antigenic determinant” refers to the epitope on the antigen recognized by the antigen binding molecule (such as an ISVD of the invention or a polypeptide comprising the ISVD of the invention) and more in particular by the antigen binding site of said molecule. The terms “antigenic determinant” and “epitope’ may also be used interchangeably herein. The antigen binding molecule (such as an antibody, an ISVD, a polypeptide of the invention, or generally an antigen-binding protein or polypeptide or a fragment thereof) that can (specifically) bind to, that has affinity for and/or that has specificity for a specific antigenic determinant, epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be "against" or "directed against" said antigenic determinant, epitope, antigen or protein.

As shown in the examples, the ISVDs of the present invention bind to an epitope of TfR1, which comprises amino acid residues of TfR1 on both TfR1 monomers of the TfR1 homodimer complex.

This specific epitope lies within the groove formed by the apical domains of TfR1 and thus is not likely to be accessible by larger binders such as Fab fragments or antibodies (see, e.g., Examples 21-32). In particular, the epitope thus may comprise

(i) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, K382, and E383 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G363, L637, R719, N722, N723, G724, A725, F726, N727, E728, T729, and R732 of the second TfR1 monomer.

The amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In one embodiment, the conformation (or spatial arrangement) of the amino acid residues of the epitope changes when shifting the pH from pH 7.4 to pH 6.0 (or vice versa), resulting in a binding of the ISVD to the TfR1 with a dissociation rate constant (k_{off}) that is at least 10 times lower at pH 7.4 compared to the k_{off} at pH 6.0. This is shown, e.g., in Example 22.

In one embodiment, the ratio of K_D for binding of the ISVD of the invention to the TfR1 homodimer at pH7.4 compared to pH 6.0 is at least 2, at least 3, at least 5, at least 10, at least 50, at least 89, at least 90, at least 100, or at least 200.

The ISVDs of the present invention can be characterized in that they specifically bind to a region of TfR1, wherein the region comprises amino acid residues 316 to 326 and amino acid residues 379 to 384 of the first TfR1 monomer and amino acid residues 633 to 638 and amino acid residues 718 to 733 of the second TfR1 monomer.

In more specific embodiments of the present invention,

the ISVD specifically binds to an epitope of a transferrin receptor 1 (TfR1) homodimer, wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,

wherein the ISVD is characterized in that the epitope comprises:

(i) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, K382, and E383 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G363, L637, R719, N722, N723, G724, A725, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The amino acid residues of the epitope may be accessible to the ISVD at pH 7.4 but not at pH 6.0 due to a conformational change of the TfR1 homodimer.

The epitope may comprise

(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer,

(ii) amino acid residues E634, M635, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

(iii) at least one of amino acid residues F187, D194, K224, A225, N317, Q320, V380, K382, and E383 of the first TfR1 monomer, and

(iv) at least one of amino acid residues K633, G363, L637, R719, N722, N723, G724, and A725 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) at least one of amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer,

(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

(iii) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, S324, V380, K382, and E383 of the first TfR1 monomer, and

(iv) at least one of amino acid residues K633, G363, L637, R719, N722, N723, G724, A725, F726, and N727 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) at least one of amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) at least one of amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) at least one of amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) at least one of amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may comprise

(i) amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and

(ii) amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer,

(ii) amino acid residues E634, M635, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

(iii) at least one of amino acid residues F187, D194, K224, A225, N317, Q320, V380, K382, and E383 of the first TfR1 monomer, and

(iv) at least one of amino acid residues K633, G363, L637, R719, N722, N723, G724, and A725 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer,

(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer,

(iii) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, S324, V380, K382, and E383 of the first TfR1 monomer, and

(iv) at least one of amino acid residues K633, G363, L637, R719, N722, N723, G724, A725, F726, and N727 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) at least one of amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

The epitope may consist of

(i) amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and

(ii) amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer,

wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

In some embodiments, the present ISVD of the invention bind to at least one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) or all of F321, R325, and E383 of hTfR1 (SEQ ID NO: 1) (for example ISVD T02810030F12).

In some embodiments, the present ISVD of the invention bind to at least one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) or all of K189, S324, E728, and T729 of hTfR1 (SEQ ID NO: 1) (for example ISVD T02810023B05).

In some embodiments, the present ISVD of the invention bind to at least one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) or all of K189, K224, F321, R325, K633, E634, M365, G636, N723, E728, T729, and E759 of hTfR1 (SEQ ID NO: 1) (for example ISVD T0281007D02). In some embodiments, E759 of SEQ ID NO: 1 is N-acetylglutamate (NAG).

In some embodiments, the present ISVD of the invention bind to at least one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) or all of K189, Y309, S324, E634, E728, T729, and R732 of hTfR1 (SEQ ID NO: 1) (for example ISVD T0281001D02).

In some embodiments, the present ISVD of the invention bind to at least one (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or 17) or all of D194, F321, R325, E383, E634, M635, G636, E728, T729, R732, and E759 of hTfR1 (SEQ ID NO: 1) (for example ISVD T0281002F11). In some embodiments, E759 of SEQ ID NO: 1 is N-acetylglutamate (NAG).

In certain embodiments, UCSF Chimera software (available at the University of California, San Francisco server) is used for the visualization of the paratope/epitope structure and the determination of the distance in Å and interactions between paratope residues and epitope

residues. The term "paratope" refers to residues of an antibody involved in recognition of and binding to the epitope of an antigen. In some embodiments, paratope/epitope pairs may be characterized by, e.g., distance between antibody and antigen atoms in the bound antibody/antigen complex (such as within 3.5Å). In some embodiments, paratope/epitope pairs may be characterized by participation in a hydrogen bond interaction, and/or a salt bridge interaction, between antibody and antigen residues.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue Y32 that is within 2.4 Å of epitope residue F321 of SEQ ID NO: 1;
paratope residue D30 that is within 3.7 Å of epitope residue R325 of SEQ ID NO: 1; and
paratope residue R56 that is within 2.9 Å of epitope residue E383 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue T33 that is within 3.5 Å of epitope residue T729 of SEQ ID NO: 1;
paratope residue Y35 that is within 2.7 Å of epitope residue T729 of SEQ ID NO: 1;
paratope residue Y37 that is within 2.7 Å of epitope residue E728 of SEQ ID NO: 1;
paratope residue N56 that is within 3.8 Å of epitope residue S324 of SEQ ID NO: 1;
paratope residue N56 that is within 2.5 Å of epitope residue K189 of SEQ ID NO: 1;
paratope residue T57 that is within 3.8 Å of epitope residue K189 of SEQ ID NO: 1;
paratope residue D98 that is within 3.4 Å of epitope residue E728 of SEQ ID NO: 1; and
paratope residue K96 that is within 2.4 Å of epitope residue E728 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue R47 that is within 3.8 Å of epitope residue M635 of SEQ ID NO: 1;
paratope residue A56 that is within 2.7 Å of epitope residue K189 of SEQ ID NO: 1;
paratope residue Y59 that is within 2.2 Å of epitope residue F321 of SEQ ID NO: 1;
paratope residue T61 that is within 3.0 Å of epitope residue E634 of SEQ ID NO: 1;
paratope residue N62 that is within 3.8 Å of epitope residue K633 of SEQ ID NO: 1;
paratope residue K65 that is within 2.8 Å of epitope residue G636 of SEQ ID NO: 1;
paratope residue D101 that is within 3.7 Å of epitope residue T729 of SEQ ID NO: 1;
paratope residue D101 that is within 3.2 Å of epitope residue NAG759 of SEQ ID NO: 1;

paratope residue R103 that is within 3.5 Å of epitope residue N723 of SEQ ID NO: 1;
paratope residue P105 that is within 3.6 Å of epitope residue N723 of SEQ ID NO: 1;
paratope residue R47 that is within 3.9 Å of epitope residue E728 of SEQ ID NO: 1;
paratope residue D54 that is within 2.6 Å of epitope residue E728 of SEQ ID NO: 1; and
paratope residue D54 that is within 3.9 Å of epitope residue R325 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue Y35 that is within 2.6 Å of epitope residue T729 of SEQ ID NO: 1;
paratope residue Y37 that is within 2.7 Å of epitope residue E728 of SEQ ID NO: 1;
paratope residue Y37 that is within 3.9 Å of epitope residue R732 of SEQ ID NO: 1;
paratope residue Q44 that is within 3.6 Å of epitope residue E634 of SEQ ID NO: 1;
paratope residue D55 that is within 2.6 Å of epitope residue Y309 of SEQ ID NO: 1;
paratope residue N56 that is within 3.5 Å of epitope residue S324 of SEQ ID NO: 1;
paratope residue N56 that is within 2.3 Å of epitope residue K189 of SEQ ID NO: 1;
paratope residue T57 that is within 2.9 Å of epitope residue K189 of SEQ ID NO: 1;
paratope residue D98 that is within 3.1 Å of epitope residue E728 of SEQ ID NO: 1;
paratope residue D98 that is within 2.4 Å of epitope residue T729 of SEQ ID NO: 1;
paratope residue K96 that is within 3.7 Å of epitope residue E728 of SEQ ID NO: 1; and
paratope residue D55 that is within 3.8 Å of epitope residue R325 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue Y32 that is within 2.4 Å of epitope residue F321 of SEQ ID NO: 1;
paratope residue Y32 that is within 3.8 Å of epitope residue R732 of SEQ ID NO: 1;
paratope residue R35 that is within 3.1 Å of epitope residue M635 of SEQ ID NO: 1;
paratope residue Y37 that is within 2.6 Å of epitope residue E634 of SEQ ID NO: 1;
paratope residue L47 that is within 3.5 Å of epitope residue E634 of SEQ ID NO: 1;
paratope residue G54 that is within 3.4 Å of epitope residue E383 of SEQ ID NO: 1;
paratope residue R56 that is within 3.7 Å of epitope residue F321 of SEQ ID NO: 1;
paratope residue N58 that is within 3.6 Å of epitope residue G636 of SEQ ID NO: 1;
paratope residue T74 that is within 3.7 Å of epitope residue D194 of SEQ ID NO: 1;
paratope residue E99 that is within 3.6 Å of epitope residue T729 of SEQ ID NO: 1;
paratope residue Y100 that is within 3.3 Å of epitope residue T729 of SEQ ID NO: 1;

paratope residue S101 that is within 3.2 Å of epitope residue NAG759 of SEQ ID NO: 1;
paratope residue Q104 that is within 2.6 Å of epitope residue E728 of SEQ ID NO: 1;
paratope residue D30 that is within 3.6 Å of epitope residue R325 of SEQ ID NO: 1;
paratope residue R35 that is within 2.6 Å of epitope residue E728 of SEQ ID NO: 1; and
paratope residue R56 that is within 3.4 Å of epitope residue E383 of SEQ ID NO: 1.

In certain embodiments, UCSF Chimera, PISA, or UCSF ChimeraX software, or any combination thereof, is used for the visualization of the paratope/epitope structure and the determination of the distance in Å and interactions between paratope residues and epitope residues.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue Y32 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;
paratope residue D30 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1; and
paratope residue R56 that can form a salt bridge with epitope residue E383 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue T33 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;
paratope residue Y35 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;
paratope residue Y37 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;
paratope residue N56 that can form a hydrogen bond with epitope residue S324 of SEQ ID NO: 1;
paratope residue N56 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;
paratope residue T57 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

paratope residue D98 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1; and

paratope residue K96 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue R47 that can form a hydrogen bond with epitope residue M635 of SEQ ID NO: 1;

paratope residue A56 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

paratope residue Y59 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

paratope residue T61 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

paratope residue N62 that can form a hydrogen bond with epitope residue K633 of SEQ ID NO: 1;

paratope residue K65 that can form a hydrogen bond with epitope residue G636 of SEQ ID NO: 1;

paratope residue D101 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

paratope residue D101 that can form a hydrogen bond with epitope residue NAG759 of SEQ ID NO: 1;

paratope residue R103 that can form a hydrogen bond with epitope residue N723 of SEQ ID NO: 1;

paratope residue P105 that can form a hydrogen bond with epitope residue N723 of SEQ ID NO: 1;

paratope residue R47 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1;

paratope residue D54 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1; and

paratope residue D54 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue Y35 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

paratope residue Y37 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

paratope residue Y37 that can form a hydrogen bond with epitope residue R732 of SEQ ID NO: 1;

paratope residue Q44 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

paratope residue D55 that can form a hydrogen bond with epitope residue Y309 of SEQ ID NO: 1;

paratope residue N56 that can form a hydrogen bond with epitope residue S324 of SEQ ID NO: 1;

paratope residue N56 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

paratope residue T57 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

paratope residue D98 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

paratope residue D98 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

paratope residue K96 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1; and

paratope residue D55 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

paratope residue Y32 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

paratope residue Y32 that can form a hydrogen bond with epitope residue R732 of SEQ ID NO: 1;

paratope residue R35 that can form a hydrogen bond with epitope residue M635 of SEQ ID NO: 1;

paratope residue Y37 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

paratope residue L47 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

paratope residue G54 that can form a hydrogen bond with epitope residue E383 of SEQ ID NO: 1;

paratope residue R56 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

paratope residue N58 that can form a hydrogen bond with epitope residue G636 of SEQ ID NO: 1;

paratope residue T74 that can form a hydrogen bond with epitope residue D194 of SEQ ID NO: 1;

paratope residue E99 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

paratope residue Y100 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

paratope residue S101 that can form a hydrogen bond with epitope residue NAG759 of SEQ ID NO: 1;

paratope residue Q104 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

paratope residue D30 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1;

paratope residue R35 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1; and

paratope residue R56 that can form a salt bridge with epitope residue E383 of SEQ ID NO: 1.

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 19, 20, and 21, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue Y32 that is within 2.4 Å of epitope residue F321 of SEQ ID NO: 1;

b) paratope residue D30 that is within 3.7 Å of epitope residue R325 of SEQ ID NO: 1; and

c) paratope residue R56 that is within 2.9 Å of epitope residue E383 of SEQ ID NO: 1,

or any combination (e.g., any 1, 2, or 3 of a)-c).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 7, 8, and 9, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue T33 that is within 3.5 Å of epitope residue T729 of SEQ ID NO: 1;

b) paratope residue Y35 that is within 2.7 Å of epitope residue T729 of SEQ ID NO: 1;

c) paratope residue Y37 that is within 2.7 Å of epitope residue E728 of SEQ ID NO: 1;

d) paratope residue N56 that is within 3.8 Å of epitope residue S324 of SEQ ID NO: 1;

e) paratope residue N56 that is within 2.5 Å of epitope residue K189 of SEQ ID NO: 1;

f) paratope residue T57 that is within 3.8 Å of epitope residue K189 of SEQ ID NO: 1;

g) paratope residue D98 that is within 3.4 Å of epitope residue E728 of SEQ ID NO: 1; and

h) paratope residue K96 that is within 2.4 Å of epitope residue E728 of SEQ ID NO: 1,

or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, or 8 of a)-h).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 16, 17, and 18, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue R47 that is within 3.8 Å of epitope residue M635 of SEQ ID NO: 1;

b) paratope residue A56 that is within 2.7 Å of epitope residue K189 of SEQ ID NO: 1;

c) paratope residue Y59 that is within 2.2 Å of epitope residue F321 of SEQ ID NO: 1;

d) paratope residue T61 that is within 3.0 Å of epitope residue E634 of SEQ ID NO: 1;

e) paratope residue N62 that is within 3.8 Å of epitope residue K633 of SEQ ID NO: 1;

f) paratope residue K65 that is within 2.8 Å of epitope residue G636 of SEQ ID NO: 1;

g) paratope residue D101 that is within 3.7 Å of epitope residue T729 of SEQ ID NO: 1;

h) paratope residue D101 that is within 3.2 Å of epitope residue NAG759 of SEQ ID NO: 1;

i) paratope residue R103 that is within 3.5 Å of epitope residue N723 of SEQ ID NO: 1;

j) paratope residue P105 that is within 3.6 Å of epitope residue N723 of SEQ ID NO: 1;

k) paratope residue R47 that is within 3.9 Å of epitope residue E728 of SEQ ID NO: 1;

l) paratope residue D54 that is within 2.6 Å of epitope residue E728 of SEQ ID NO: 1; and

m) paratope residue D54 that is within 3.9 Å of epitope residue R325 of SEQ ID NO: 1,

or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13 of a)-m).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 10, 11, and 12, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue Y35 that is within 2.6 Å of epitope residue T729 of SEQ ID NO: 1;

b) paratope residue Y37 that is within 2.7 Å of epitope residue E728 of SEQ ID NO: 1;

c) paratope residue Y37 that is within 3.9 Å of epitope residue R732 of SEQ ID NO: 1;

d) paratope residue Q44 that is within 3.6 Å of epitope residue E634 of SEQ ID NO: 1;

e) paratope residue D55 that is within 2.6 Å of epitope residue Y309 of SEQ ID NO: 1;

f) paratope residue N56 that is within 3.5 Å of epitope residue S324 of SEQ ID NO: 1;

g) paratope residue N56 that is within 2.3 Å of epitope residue K189 of SEQ ID NO: 1;

h) paratope residue T57 that is within 2.9 Å of epitope residue K189 of SEQ ID NO: 1;

i) paratope residue D98 that is within 3.1 Å of epitope residue E728 of SEQ ID NO: 1;

j) paratope residue D98 that is within 2.4 Å of epitope residue T729 of SEQ ID NO: 1;

k) paratope residue K96 that is within 3.7 Å of epitope residue E728 of SEQ ID NO: 1; and

l) paratope residue D55 that is within 3.8 Å of epitope residue R325 of SEQ ID NO: 1,

or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 of a)-l).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 13, 14, and 15, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue Y32 that is within 2.4 Å of epitope residue F321 of SEQ ID NO: 1;

b) paratope residue Y32 that is within 3.8 Å of epitope residue R732 of SEQ ID NO: 1;

c) paratope residue R35 that is within 3.1 Å of epitope residue M635 of SEQ ID NO: 1;

d) paratope residue Y37 that is within 2.6 Å of epitope residue E634 of SEQ ID NO: 1;

e) paratope residue L47 that is within 3.5 Å of epitope residue E634 of SEQ ID NO: 1;

f) paratope residue G54 that is within 3.4 Å of epitope residue E383 of SEQ ID NO: 1;

g) paratope residue R56 that is within 3.7 Å of epitope residue F321 of SEQ ID NO: 1;

h) paratope residue N58 that is within 3.6 Å of epitope residue G636 of SEQ ID NO: 1;

i) paratope residue T74 that is within 3.7 Å of epitope residue D194 of SEQ ID NO: 1;

j) paratope residue E99 that is within 3.6 Å of epitope residue T729 of SEQ ID NO: 1;

k) paratope residue Y100 that is within 3.3 Å of epitope residue T729 of SEQ ID NO: 1;

l) paratope residue S101 that is within 3.2 Å of epitope residue NAG759 of SEQ ID NO: 1;

m) paratope residue Q104 that is within 2.6 Å of epitope residue E728 of SEQ ID NO: 1;

n) paratope residue D30 that is within 3.6 Å of epitope residue R325 of SEQ ID NO: 1;

o) paratope residue R35 that is within 2.6 Å of epitope residue E728 of SEQ ID NO: 1; and

p) paratope residue R56 that is within 3.4 Å of epitope residue E383 of SEQ ID NO: 1,

or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 of a)-p).

In certain embodiments, UCSF Chimera, PISA, or UCSF ChimeraX software, or any combination thereof is used for the visualization of the paratope/epitope structure and the determination of the distance in Å and interactions between paratope residues and epitope residues.

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 19, 20, and 21, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue Y32 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

b) paratope residue D30 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1; and

c) paratope residue R56 that can form a salt bridge with epitope residue E383 of SEQ ID NO: 1, or any combination (e.g., any 1, 2, or 3) of a)-c).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 7, 8, and 9, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue T33 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

b) paratope residue Y35 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

c) paratope residue Y37 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

d) paratope residue N56 that can form a hydrogen bond with epitope residue S324 of SEQ ID NO: 1;

e) paratope residue N56 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

f) paratope residue T57 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

g) paratope residue D98 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1; and

h) paratope residue K96 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1, or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, or 8) of a)-h).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 16, 17, and 18, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue R47 that can form a hydrogen bond with epitope residue M635 of SEQ ID NO: 1;

b) paratope residue A56 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

c) paratope residue Y59 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

d) paratope residue T61 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

e) paratope residue N62 that can form a hydrogen bond with epitope residue K633 of SEQ ID NO: 1;

f) paratope residue K65 that can form a hydrogen bond with epitope residue G636 of SEQ ID NO: 1;

g) paratope residue D101 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

h) paratope residue D101 that can form a hydrogen bond with epitope residue NAG759 of SEQ ID NO: 1;

i) paratope residue R103 that can form a hydrogen bond with epitope residue N723 of SEQ ID NO: 1;

j) paratope residue P105 that can form a hydrogen bond with epitope residue N723 of SEQ ID NO: 1;

k) paratope residue R47 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1;

l) paratope residue D54 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1; and

m) paratope residue D54 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1, or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13) of a)-m).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 10, 11, and 12, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue Y35 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

b) paratope residue Y37 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

c) paratope residue Y37 that can form a hydrogen bond with epitope residue R732 of SEQ ID NO: 1;

d) paratope residue Q44 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

e) paratope residue D55 that can form a hydrogen bond with epitope residue Y309 of SEQ ID NO: 1;

f) paratope residue N56 that can form a hydrogen bond with epitope residue S324 of SEQ ID NO: 1;

g) paratope residue N56 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

h) paratope residue T57 that can form a hydrogen bond with epitope residue K189 of SEQ ID NO: 1;

i) paratope residue D98 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

j) paratope residue D98 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

k) paratope residue K96 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1; and

l) paratope residue D55 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1, or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12) of a)-l).

In some embodiments, the ISVD of the invention comprises CDR1-3 of SEQ ID NOs: 13, 14, and 15, respectively, wherein the ISVD of the invention, when bound to hTfR1, comprises, as defined by IMGT numbering:

a) paratope residue Y32 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

b) paratope residue Y32 that can form a hydrogen bond with epitope residue R732 of SEQ ID NO: 1;

c) paratope residue R35 that can form a hydrogen bond with epitope residue M635 of SEQ ID NO: 1;

d) paratope residue Y37 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

e) paratope residue L47 that can form a hydrogen bond with epitope residue E634 of SEQ ID NO: 1;

f) paratope residue G54 that can form a hydrogen bond with epitope residue E383 of SEQ ID NO: 1;

g) paratope residue R56 that can form a hydrogen bond with epitope residue F321 of SEQ ID NO: 1;

h) paratope residue N58 that can form a hydrogen bond with epitope residue G636 of SEQ ID NO: 1;

i) paratope residue T74 that can form a hydrogen bond with epitope residue D194 of SEQ ID NO: 1;

j) paratope residue E99 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

k) paratope residue Y100 that can form a hydrogen bond with epitope residue T729 of SEQ ID NO: 1;

l) paratope residue S101 that can form a hydrogen bond with epitope residue NAG759 of SEQ ID NO: 1;

m) paratope residue Q104 that can form a hydrogen bond with epitope residue E728 of SEQ ID NO: 1;

n) paratope residue D30 that can form a salt bridge with epitope residue R325 of SEQ ID NO: 1;

o) paratope residue R35 that can form a salt bridge with epitope residue E728 of SEQ ID NO: 1; and

p) paratope residue R56 that can form a salt bridge with epitope residue E383 of SEQ ID NO: 1, or any combination (e.g., any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, or 16) of a)-p).

The ISVD of the invention may comprise 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively). The ISVD of the invention may consist of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively).

In one embodiment,

a) CDR1 (according to Kabat) has the amino acid sequence of $IX_2X_3X_4X_5$, wherein X_2 is N or Y, X_3 is T or L, X_4 is M or T, and X_5 is Y or R;

b) CDR2 (according to Kabat) has the amino acid sequence of $X_1X_2X_3X_4GX_6X_7TX_9YAX_{12}X_{13}VKG$ (SEQ ID NO: 131), wherein X_1 is W or G, X_2 is I, S, or V, X_3 is T or A, X_4 is G, R, or H, X_6 is D or G, X_7 is N, S, or R, X_9 is N or S, X_{12} is S, P, H, or D, and X_{13} is S or F; and

c) CDR3 (according to Kabat) has the amino acid sequence of $DX_2X_3X_4$ (SEQ ID NO:) or LEX_5SGSQY (SEQ ID NO: 132), wherein X_2 is an optional G, X_3 is T, V, or I, X_4 is Y or D, and X_5 is Y or F.

In one embodiment,

a) CDR1 (according to Kabat) has the amino acid sequence of INTMY (SEQ ID NO: 133);

b) CDR2 (according to Kabat) has the amino acid sequence of $WX_2TX_4GDX_7TX_9YAX_{12}SVKG$ (SEQ ID NO: 134), wherein X_2 is I, S, or V, X_4 is G or R, X_7 is N or S, X_9 is N or S, and X_{12} is S, P, or H; and

c) CDR3 (according to Kabat) has the amino acid sequence of $DX_2X_3X_4$ (SEQ ID NO:), wherein X_2 is an optional G, X_3 is T, V, or I, and X_4 is Y or D.

In one embodiment,

a) CDR1 (according to Kabat) has the amino acid sequence of IYLTR (SEQ ID NO: 135);

b) CDR2 (according to Kabat) has the amino acid sequence of GVAHGGRTNYADFVKG (SEQ ID NO: 136); and

c) CDR3 (according to Kabat) has the amino acid sequence of LEX_3SGSQY (SEQ ID NO: 132), wherein X_3 is Y or F.

In one embodiment,

a) CDR1 (according to IMGT) has the amino acid sequence of $GX_2X_3X_4X_5X_6X_7TMY$ (SEQ ID NO: 137), wherein X_2 is S, I, or D (optionally X_2 is S), X_3 is G, S, or T (optionally X_3 is T), X_4 is G, I, or S (optionally X_4 is S), X_5 is S or V (optionally X_5 is S), X_6 is I or G (optionally X_6 is I), X_7 is N or E (optionally X_7 is N);

b) CDR2 (according to IMGT) has the amino acid sequence of $WX_2TX_4GDX_7TX_9$ (SEQ ID NO: 138), wherein X_2 is I, S, or V, X_4 is G or R, X_7 is N or S (optionally X_7 is N), and X_9 is N, R or S (optionally X_9 is N); and

c) CDR3 (according to IMGT) has the amino acid sequence of $X_1X_2X_3X_4X_5X_6X_7$ (SEQ ID NO:), wherein X_1 is N or K (optionally X_1 is K), X_2 is A, D, T, or G (optionally X_2 is T), X_3 is D or not present (optionally X_3 is not present), X_5 is G, S, or D (optionally X_5 is D), X_6 is G, S, or D (optionally, X_6 is D), and X_7 is I, R, T, or V (optionally X_7 is V).

In one embodiment,

a) CDR1 (according to IMGT) has the amino acid sequence of GTGLDIYLTR (SEQ ID NO: 139);

b) CDR2 (according to IMGT) has the amino acid sequence of GVAHGGRTN (SEQ ID NO: 140); and

c) CDR3 (according to IMGT) has the amino acid sequence of NVLEX$_3$SGSQY (SEQ ID NO: 141), wherein X_3 is Y or F.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 7 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 7; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 8 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 8; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 9, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 9.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 10 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 10; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 11 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 11; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 12, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 12.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 13 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 13; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 14 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 14; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 15, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 15.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 16 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 16; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 17 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 17; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 18, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 18.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 19 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 19; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 20 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 20; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 21, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 21.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 28 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 28; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 30 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 30; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 32, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 32.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 39 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 39; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 41 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 41; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 43, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 43.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 50 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 50; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 52 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 52; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 54, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 54.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 61 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 61; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 63 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 63; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 65, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 65.

In one embodiment, the ISVD of the invention consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 68 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 68; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 70 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 70; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 72, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 72.

When comparing two immunoglobulin single variable domains, the term "amino acid difference" refers to an insertion, deletion, or substitution of a single amino acid residue on a position of the first sequence, compared to the second sequence; it being understood that two immunoglobulin single variable domains can contain one, two, three, or four such amino acid differences. In one embodiment, amino acid difference refers to a substitution.

For the purposes of comparing two or more immunoglobulin single variable domains or other amino acid sequences such e.g. the polypeptides of the invention etc., the percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence (also referred to herein as "amino acid identity") may be calculated or determined as described in paragraph f) on pages 49 and 50 of WO 08/020079 (incorporated herein by reference), such as by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (position), i.e., as an "amino acid difference" as defined herein; alternatively, the degree of sequence identity between two amino acid sequences may be calculated using a known computer algorithm for sequence alignment, such as NCBI Blast v2.0, using standard settings. Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0 967 284, EP 1 085 089, WO 00/55318, WO 00/78972, WO 98/49185, and GB 2 357 768-A.

Usually, for the purpose of determining the percentage of “sequence identity” between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the “first” amino acid sequence, and the other amino acid sequence will be taken as the “second” amino acid sequence. Also, in determining the degree of sequence identity between two immunoglobulin single variable domains, the skilled person may take into account so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure, and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example

from WO 04/037999, GB-A-3 357 768, WO 98/49185, WO 00/46383 and WO 01/09300; and (preferred) types and/or combinations of such substitutions may be selected on the basis of the pertinent teachings from WO 04/037999 as well as WO 98/49185 and from the further references cited therein. Examples of conservative substitutions are described herein further below.

Any amino acid substitutions applied to the polypeptides described herein may also be based on the analysis of the frequencies of amino acid variations between homologous proteins of different species developed by Schulz et al. 1978 (Principles of Protein Structure, Springer-Verlag), on the analyses of structure forming potentials developed by Chou and Fasman 1975 (Biochemistry 13: 211) and 1978 (Adv. Enzymol. 47: 45-149), and on the analysis of hydrophobicity patterns in proteins developed by Eisenberg et al. 1984 (Proc. Natl. Acad. Sci. USA 81: 140-144), Kyte & Doolittle 1981 (J Molec. Biol. 157: 105-132), and Goldman et al. 1986 (Ann. Rev. Biophys. Chem. 15: 321-353), all incorporated herein in their entirety by reference. Information on the primary, secondary and tertiary structure of ISVDs is given in the description herein and in the general background art cited above. Also, for this purpose, the crystal structure of a V_{HH} domain from a llama is for example given by Desmyter et al. 1996 (Nature Structural Biology, 3: 803), Spinelli et al. 1996 (Natural Structural Biology 3: 752-757), and Decanniere et al. 1999 (Structure, 7: 361). Further information about some of the amino acid residues that in conventional V_H domains form the V_H/V_L interface and potential camelizing substitutions on these positions can be found in the prior art cited above.

Immunoglobulin single variable domains and nucleic acid sequences are said to be "exactly the same" if they have 100% sequence identity (as defined herein) over their entire length.

The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26, and optionally has an amino acid sequence having 4, 3, 2 or 1 “amino acid difference(s)” (as defined herein) with the sequence of SEQ ID NOs: 2-6 and 22-26, respectively. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26, and optionally has an amino acid sequence having more than 4 such as 5, 6, 7, 8, 9, or 10 “amino acid difference(s)” (as defined herein) with the sequence of SEQ ID NOs: 2-6 and 22-26, respectively. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 2. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 3. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 4. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 5. The ISVD of the invention may

comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 6. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 2. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 3. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 4. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 5. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 6. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 2. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 3. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 4. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 5. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 6.

The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 22. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 23. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 24. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 25. The ISVD of the invention may comprise or consist of an amino acid sequence as depicted in any one of SEQ ID NO: 26. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 22. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 23. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 24. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 25. The ISVD of the invention may comprise an amino acid sequence as depicted in any one of SEQ ID NO: 26. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 22. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 23. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 24. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 25. The ISVD of the invention may consist of an amino acid sequence as depicted in any one of SEQ ID NO: 26.

Table 1: ISVDs disclosed herein.

ISVD	Amino acid sequence
T0281007D02	EVQLVESGGGLVQPGGSLRLSCAASGFIISGYWMYWVRQAPGKGLE RVAVIDTDGAFTYYTNSVKGRFTISRDNAKNAVYLQMNSLQPEDTA VYYCHANGDLRWPRDRSSGYWGQGTLVTVSS (SEQ ID NO: 5)
T0281012D06	EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIAWFRQAPGKERE GVSCISINSSPTRYLGSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIY YCAARNAYDYCIDYERSEYRYWGQGTLVTVSS (SEQ ID NO: 22)
T0281005A01	EVQLVESGGGLVQPGGSLRLSCAASGISISGETMYWYRQAPGSQREF VAWITGGDNTRYGDSVKGRFTISSDNAKNTVFLQMNSLKPEDTAVY YCKDSRFWGQGTLVTVSS (SEQ ID NO: 23)
T0281001C08	EVQLVESGGGLVQPGGSLRLSCVASGSTSSINTMYWYRQAPGKQRE WVAWSTRGDNTNYAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAI YYCKGDVYWGQGTLVTVSS (SEQ ID NO: 24)
T0281002A09	EVQLVESGGGLVQPGGSLRLSCVASGDTSSINTVYWYRQAPGKQRE WVAWSTRGDNTNYAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAI YYCKTDVHWGQGTLVTVSS (SEQ ID NO: 25)
T0281008E07	EVQLVESGGGLVQPGESLRLSCAVSGSGGSINTMYWYRQAPGKQRE WVAWVTGGDSTSYAHSVKGRFTISTDNDKNNMYLQMNSLKPEDTA VYYCNADGIYWGQGTLVTVSS (SEQ ID NO: 26)
T0281001D02	EVQLVESGGGLVQPGGSLRLSCVASGSTSVINTMYWYRQAPGKQRE WVAWITGGDNTNYASSVKGRFTISGDNAKNSVYLQMNDLKPEDTAI YYCKTDTYWGQGTLVTVSS (SEQ ID NO: 3)
T0281002F11	EVQLVESGGGLVQPGGSLRLSCAASGTGLDIYLTRWYRQAAEKSRE LVAGVAHGGRTNYADFVKGRFTISRDNTKNTVYLQMDSLKPEDTA VYYCNVLEYSGSQYWGQGTLVTVSS (SEQ ID NO: 4)
T02810023B0 5	EVQLVESGGGLVQPGGSLRLSCVASGSTSSINTMYWYRQAPGKQRE WVAWSTRGDNTNYAPSVKGRFTMSRDDAKNSVYLQMNDLKPEDT AIYYCKTDVDWGQGTLVTVSS (SEQ ID NO: 2)
T02810030F1 2	EVQLVESGGGLVQPGGSLRLSCAASGTGLDIYLTRWYRQAAEKSRE LVAGVAHGGRTNYADFVKGRFTISRDNTKNTVYLQMDSLKPEDTA VYYCNVLEFSGSQYWGQGTLVTVSS (SEQ ID NO: 6)

Table 2A: Framework regions (FR) and Complementarity-determining regions (CDR) according to IMGT of ISVDs of the invention.

ISVD	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
T0281001C08	EVQLVESGGGLVQPGGSLRLSCVAS (SEQ ID NO: 27)	GSTSSINTMY (SEQ ID NO: 28)	WYRQAPGKQREWVA (SEQ ID NO: 29)	WSTRGDNTN (SEQ ID NO: 30)	YAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAIYYC (SEQ ID NO: 31)	KGDVY (SEQ ID NO: 32)	WGQGTLVTVSS (SEQ ID NO: 33)
T0281001D02	EVQLVESGGGLVQPGGSLRLSCVAS (SEQ ID NO: 34)	GSTSVINTMY (SEQ ID NO: 10)	WYRQAPGKQREWVA (SEQ ID NO: 35)	WITGGDNTN (SEQ ID NO: 11)	YASSVKGRFTISGDNAKNSVYLQMNDLKPEDTAIYYC (SEQ ID NO: 36)	KTDTY (SEQ ID NO: 12)	WGQGTLVTVSS (SEQ ID NO: 37)
T0281002A09	EVQLVESGGGLVQPGGSLRLSCVAS (SEQ ID NO: 38)	GDTSSINTVY (SEQ ID NO: 39)	WYRQAPGKQREWVA (SEQ ID NO: 40)	WSTRGDNTN (SEQ ID NO: 41)	YAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAIYYC (SEQ ID NO: 42)	KTDVH (SEQ ID NO: 43)	WGQGTLVTVSS (SEQ ID NO: 44)
T0281002F11	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 45)	GTGLDIYLTR (SEQ ID NO: 13)	WYRQAAEKSRELVA (SEQ ID NO: 46)	GVAHGGRTN (SEQ ID NO: 14)	YADFVKGRFTISRDNTKNTVYLQMDSLKPEDTAVYYC (SEQ ID NO: 47)	NVLEYSGSQY (SEQ ID NO: 15)	WGQGTLVTVSS (SEQ ID NO: 48)
T0281005A01	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 49)	GISISGETMY (SEQ ID NO: 50)	WYRQAPGSQREFVA (SEQ ID NO: 51)	WITGGDNTR (SEQ ID NO: 52)	YGDSVKGRFTISSDNAKNTVFLQMNSLKPEDTAVYYC (SEQ ID NO: 53)	KDSRF (SEQ ID NO: 54)	WGQGTLVTVSS (SEQ ID NO: 55)
T0281007D02	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 56)	GFIISGYWMY (SEQ	WVRQAPGKGLERVA (SEQ ID NO: 57)	VIDTDGAFTY (SEQ	YTNSVKGRFTISRDNAKNAVYLQMNSLQPEDTAVYYC (SEQ ID NO: 58)	HANGDLRWPRDRSSGY (SEQ ID NO: 18)	WGQGTLVTVSS (SEQ

		ID NO: 16)		ID NO: 17)			ID NO: 59)
T028 1008 E07	EVQLVESG GGLVQPGE SLRLSCAVS (SEQ ID NO: 60)	GSG GSI NTM Y (SEQ ID NO: 61)	WYRQ APGKQ REWV A (SEQ ID NO: 62)	WV TGG DST S (SEQ ID NO: 63)	YAHSVKGRFTIST DNDKNNMYLQM NSLKPEDTAVYYC (SEQ ID NO: 64)	NADGIY (SEQ ID NO: 65)	WGQ GTL VTV SS (SEQ ID NO: 66)
T028 1012 D06	EVQLVESG GGLVQPGG SLRLSCAAS (SEQ ID NO: 67)	GFT LDY YAI A (SEQ ID NO: 68)	WFRQ APGKE REGVS (SEQ ID NO: 69)	CISI NSS PTR (SEQ ID NO: 70)	YLGSVKGRFTISR DNAKNTVYLQMN SLKPEDTAIYYC (SEQ ID NO: 71)	AARNAY DYCIDY ERSEYR Y (SEQ ID NO: 72)	WGQ GTL VTV SS (SEQ ID NO: 73)
T028 1030 F12	EVQLVESG GGLVQPGG SLRLSCAAS (SEQ ID NO: 74)	GTG LDI YLT R (SEQ ID NO: 19)	WYRQ AAEKS RELVA (SEQ ID NO: 75)	GVA HGG RTN (SEQ ID NO: 20)	YADFVKGRFTISR DNTKNTVYLQMD SLKPEDTAVYYC (SEQ ID NO: 76)	NVLEFS GSQY (SEQ ID NO: 21)	WGQ GTL VTV SS (SEQ ID NO: 77)
T028 1002 3B05	EVQLVESG GGLVQPGG SLRLSCVAS (SEQ ID NO: 78)	GST SSIN TMY (SEQ ID NO: 7)	WYRQ APGKQ REWV A (SEQ ID NO: 79)	WST RGD NTN (SEQ ID NO: 8)	YAPSVKGRFTMSR DDAKNSVYLQMN DLKPEDTAIYYC (SEQ ID NO: 80)	KTDVD (SEQ ID NO: 9)	WGQ GTL VTV SS (SEQ ID NO: 81)

Table 2B: Framework regions (FR) and Complementarity-determining regions (CDR) according to AbM of ISVDs of the invention.

ISV D	**FR1**	**CDR 1**	**FR2**	**CDR 2**	**FR3**	**CDR3**	**FR4**
T02 8100 1C0 8	EVQLVESG GGLVQPGG SLRLSCVAS (SEQ ID NO: 27)	GST SSIN TMY (SEQ ID	WYRQ APGKQ REWV A (SEQ ID NO: 29)	WST RGD NTN (SEQ ID	YAPSVKGRFTISRD DAKNSVYLQMNDL KPEDTAIYYCKG (SEQ ID NO: 182)	DVY	WGQ GTL VTVS S (SEQ ID

		NO: 28)		NO: 30)			NO: 33)
T028100 1D02	EVQLVESGGGLVQPGGSLRLSCVAS (SEQ ID NO: 34)	GSTSVINTMY (SEQ ID NO: 10)	WYRQAPGKQREWVA (SEQ ID NO: 35)	WITGGDNTN (SEQ ID NO: 11)	YASSVKGRFTISGDNAKNSVYLQMNDLKPEDTAIYYCKT (SEQ ID NO: 183)	DTY	WGQGTLVTVSS (SEQ ID NO: 37)
T028100 2A09	EVQLVESGGGLVQPGGSLRLSCVAS (SEQ ID NO: 38)	GDTSSINTVY (SEQ ID NO: 39)	WYRQAPGKQREWVA (SEQ ID NO: 40)	WSTRGDNTN (SEQ ID NO: 41)	YAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAIYYCKT (SEQ ID NO: 184)	DVH	WGQGTLVTVSS (SEQ ID NO: 44)
T028100 2F11	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 45)	GTGLDIYLTR (SEQ ID NO: 13)	WYRQAAEKSRELVA (SEQ ID NO: 46)	GVAHGGRTN (SEQ ID NO: 14)	YADFVKGRFTISRDNTKNTVYLQMDSLKPEDTAVYYCNV (SEQ ID NO: 185)	LEYSGSQY (SEQ ID NO: 191)	WGQGTLVTVSS (SEQ ID NO: 48)
T028100 5A01	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 49)	GISSGETMY (SEQ ID NO: 50)	WYRQAPGSQREFVA (SEQ ID NO: 51)	WITGGDNTR (SEQ ID NO: 52)	YGDSVKGRFTISSDNAKNTVFLQMNSLKPEDTAVYYCKD (SEQ ID NO: 186)	SRF	WGQGTLVTVSS (SEQ ID

							NO: 55)
T028100 7D02	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 56)	GFIISGYWMY (SEQ ID NO: 16)	WVRQAPGKGLERVA (SEQ ID NO: 57)	VIDTDGAFTY (SEQ ID NO: 17)	YTNSVKGRFTISRDNAKNAVYLQMNSLQPEDTAVYYCHA (SEQ ID NO: 187)	NGDLRWPRDRSSGY (SEQ ID NO: 192)	WGQGTLVTVSS (SEQ ID NO: 59)
T028100 8E07	EVQLVESGGGLVQPGESLRLSCAVS (SEQ ID NO: 60)	GSGGSINTMY (SEQ ID NO: 61)	WYRQAPGKQREWVA (SEQ ID NO: 62)	WVTGGDSTS (SEQ ID NO: 63)	YAHSVKGRFTISTDNDKNNMYLQMNSLKPEDTAVYYCNA (SEQ ID NO: 188)	DGIY (SEQ ID NO: 193)	WGQGTLVTVSS (SEQ ID NO: 66)
T028101 2D06	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 67)	GFTLDYYAIA (SEQ ID NO: 68)	WFRQAPGKEREGVS (SEQ ID NO: 69)	CISINSSPTR (SEQ ID NO: 70)	YLGSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIYYCAA (SEQ ID NO: 189)	RNAYDYCIDYERSEYRY (SEQ ID NO: 194)	WGQGTLVTVSS (SEQ ID NO: 73)
T028103 0F12	EVQLVESGGGLVQPGGSLRLSCAAS (SEQ ID NO: 74)	GTGLDIYLTR (SEQ ID	WYRQAAEKSRELVA (SEQ ID NO: 75)	GVAHGGRTN (SEQ ID NO: 20)	YADFVKGRFTISRDNTKNTVYLQMDSLKPEDTAVYYCNV (SEQ ID NO: 190)	LEFSGSQY (SEQ ID NO: 195)	WGQGTLVTVSS (SEQ ID

		NO: 19)					NO: 77)

As outlined herein, the novel epitope identified by the present inventors is present at neutral pH but not at acidic pH. Thus, already the specific binding to the epitope of the invention is sufficient for pH dependent binding and there is no need to further alter the CDRs or the paratope of the ISVD of the invention, e.g., by introducing a histidine. In one embodiment, the charge of the CDRs of the ISVD of the invention is not altered by changing the pH from 6 to 7.4 (or from pH 7.4 to 6). In one further embodiment, the charge of the paratope of the ISVD is not altered by changing the pH from pH 6.0 to 7.4 (or from pH 7.4 to 6). In one embodiment, the net charge of the CDRs of the ISVD of the invention is not altered by changing the pH from 6 to 7.4 (or from pH 7.4 to 6). In one further embodiment, the net charge of the paratope of the ISVD is not altered by changing the pH from pH 6.0 to 7.4 (or from pH 7.4 to 6). "Charge" in this context may relate to only the charge of the (functional) group of the variable side chain of a given amino acid. In some embodiments, the CDR or the paratope of the ISVD of the invention does not contain a histidine.

Transferrin receptor protein 1 (TfR1), also known as Cluster of Differentiation 71 (CD71), is a protein that in humans is encoded by the TFRC gene. TfR1 is required for iron import from transferrin into cells by endocytosis. TfR1 is a transmembrane glycoprotein composed of two disulfide-linked monomers joined by two disulfide bonds. Each monomer binds one holo-transferrin molecule creating an iron-Tf-TfR1complex which enters the cell by endocytosis. An exemplary human TfR1 monomer amino acid sequence is depicted UniProt database entry P02786, dated 2006-05-30, version 2 and as also depicted in SEQ ID NO: 1. In one embodiment, both the first and the second TfR1 polypeptide comprise an amino acid sequence as depicted in SEQ ID NO: 1. In one embodiment, both the first and the second TfR1 polypeptide consist of an amino acid sequence as depicted in SEQ ID NO: 1. In one embodiment, both the first and the second TfR1 polypeptide comprise an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1. In one embodiment, both the first and the second TfR1 polypeptide consist of an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1. Amino acid residues 1-61 of TfR1 are considered cytoplasmic. Amino acid residues 62-89 are considered transmembrane. Amino acid residues 90-760 are considered extracellular. The extracellular portion (ectodomain) has three domains: the helical domain (residues 606-760), the protease-like domain (residues 121-183, 384-605), and the apical domain (residues 184-383) domain 35 (Sjöström, D., Linnaeus University Dissertations, No. 406/2021; Lawrence et al., Science (1999) 286(5440):779-82).

Exemplary sequence of human TfR1 (SEQ ID NO: 1):

MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAVDEEENADNNTKANVT
KPKRCSGSICYGTIAVIVFFLIGFMIGYLGYCKGVEPKTECERLAGTESPVREEPGEDFPA
ARRLYWDDLKRKLSEKLDSTDFTGTIKLLNENSYVPREAGSQKDENLALYVENQFREFK
LSKVWRDQHFVKIQVKDSAQNSVIIVDKNGRLVYLVENPGGYVAYSKAATVTGKLVH
ANFGTKKDFEDLYTPVNGSIVIVRAGKITFAEKVANAESLNAIGVLIYMDQTKFPIVNAE
LSFFGHAHLGTGDPYTPGFPSFNHTQFPPSRSSGLPNIPVQTISRAAAEKLFGNMEGDCPS
DWKTDSTCRMVTSESKNVKLTVSNVLKEIKILNIFGVIKGFVEPDHYVVVGAQRDAWG
PGAAKSGVGTALLLKLAQMFSDMVLKDGFQPSRSIIFASWSAGDFGSVGATEWLEGYL
SSLHLKAFTYINLDKAVLGTSNFKVSASPLLYTLIEKTMQNVKHPVTGQFLYQDSNWAS
KVEKLTLDNAAFPFLAYSGIPAVSFCFCEDTDYPYLGTTMDTYKELIERIPELNKVARAA
AEVAGQFVIKLTHDVELNLDYERYNSQLLSFVRDLNQYRADIKEMGLSLQWLYSARGD
FFRATSRLTTDFGNAEKTDRFVMKKLNDRVMRVEYHFLSPYVSPKESPFRHVFWGSGS
HTLPALLENLKLRKQNNGAFNETLFRNQLALATWTIQGAANALSGDVWDIDNEF

A “monomer” as used herein relates to a single polypeptide chain. It can associate with a further polypeptide chain with identical sequence to form a “homodimer”, which in some embodiments comprises or consists of an amino acid sequence as depicted in SEQ ID NO: 1. Two TfR1 monomers form a TfR1 homodimer. In some embodiments, the TfR1 monomer(s) comprise or consist of an amino acid sequence as depicted in SEQ ID NO: 1 or (polymorphic) variants or isoforms thereof. Unless indicated otherwise, TfR1 as herein used relates to a TfR1 homodimer (TfR1 and TfR1 homodimer are used interchangeably). In one embodiment, the TfR1 homodimer is embedded in a membrane. In one embodiment, the epitope is located on the same side of the membrane as the apical domain of the TfR1.

In some embodiments, the TfR1 homodimer is a human TfR1 homodimer. In some embodiments, the first TfR1 monomer and the second TfR1 monomer are human. In some embodiments, the first TfR1 monomer, the second TfR1 monomer, or the first and the second TfR1 monomer comprises or consists of an amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the first TfR1 comprises an amino acid sequence as depicted in SEQ ID NO: 1. In some embodiments, the first TfR1 monomer, the second TfR1 monomer consists of an amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the second TfR1 monomer comprises an

amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the second TfR1 monomer consists of an amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments the first and the second TfR1 monomer comprises an amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the first and the second TfR1 monomer consists of an amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments the first and the second TfR1 monomer comprises an amino acid sequence as depicted in SEQ ID NO: 1. In some embodiments, the first and the second TfR1 monomer consists of an amino acid sequence as depicted in SEQ ID NO: 1.

In some embodiments, the first TfR1 monomer, the second TfR1 monomer, or the first and the second TfR1 monomer comprises or consists of an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the first TfR1 comprises an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1. In some embodiments, the first TfR1 monomer, the second TfR1 monomer consists of an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the second TfR1 monomer comprises an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the second TfR1 monomer consists of an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments the first and the second TfR1 monomer comprises an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments, the first and the second TfR1 monomer consists of an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof. In some embodiments the first and the second TfR1 monomer comprises an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1. In some embodiments, the first and the second TfR1 monomer consists of an amino acid sequence as depicted in positions 89 to 760 of SEQ ID NO: 1.

The ISVDs of the invention bind to amino acid residues of both the first TfR1 monomer and the second TfR1 monomer of a TfR1 homodimer. These amino acid residues on the first TfR1 monomer and the second TfR1 monomer typically are not identical, i.e., the ISVDs of the present invention typically do not bind the same set of amino acid residues on both the first TfR1 monomer and the second TfR1 monomer. Since TfR1 is a homodimer, the epitope of the present invention

can occur twice on the TfR1 homodimer. Accordingly, two identical ISVDs according to the invention may bind the same TfR1 homodimer simultaneously. The "same" TfR1 homodimer within the context of the invention relates to a single TfR1 (homodimer) consisting of two TfR1 monomers, wherein two ISVDs of the invention bind to both TfR1 monomers of said TfR1 homodimer.

The terms "specificity", "binding specifically" or "specific binding" refer to the number of different target molecules, such as antigens, from the same organism to which a particular binding unit, such as an ISVD, can bind with sufficiently high affinity (see below). "Specificity", "binding specifically" or "specific binding" are used interchangeably herein with "selectivity", "binding selectively" or "selective binding". Binding units, such as ISVDs, specifically bind to their designated targets. The specificity/selectivity of a binding unit can be determined based on affinity. The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given as by the K_D, or dissociation constant, which is expressed in units of mol/liter (or M). The affinity can also be expressed as an association constant, K_A, which equals $1/K_D$ and is expressed in units of $(mol/liter)^{-1}$ (or M^{-1}). The affinity is a measure for the binding strength between a moiety and a binding site on the target molecule: the lower the value of the K_D, the stronger the binding strength between a target molecule and a targeting moiety. Typically, binding units used in the present technology (such as ISVDs) will bind to their targets with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (i.e., with an association constant (K_A) of 10^5 to 10^{12} liter/moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles). Any K_D value greater than 10^{-4} mol/liter (or any K_A value lower than 10^4 liters/mol) is generally considered to indicate non-specific binding. The K_D for biological interactions, such as the binding of immunoglobulin sequences to an antigen, which are considered specific are typically in the range of 10^{-5} moles/liter (10000 nM or 10μM) to 10^{-12}moles/liter (0.001 nM or 1 pM) or less. Accordingly, specific/selective binding may mean that – using the same measurement method, e.g., SPR – a binding unit (or polypeptide comprising the same) binds to TfR1 with a K_D value of 10^{-5} to 10^{-12} moles/liter or less and binds to related targets with a K_D value greater than 10^{-4} moles/liter. Specific binding to a certain target from a certain species does not exclude that the binding unit can also specifically bind to the analogous target from a different species. For example, specific binding to human TfR1 does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to TfR1 from cynomolgus monkeys.

In one embodiment, the amino acid residues of the epitope form a conformation that differs between pH 7.4 (neutral conformation) and pH 6.0 (acidic conformation), wherein the ISVD has a reduced affinity to the TfR1 homodimer at pH6 compared to pH 7.4, optionally wherein binding of the ISVD to the TfR1 homodimer has a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation.

Specific binding of a binding unit to its designated target can be determined in any suitable manner known per se, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned herein. The dissociation constant may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned below. In this respect, it will also be clear that it may not be possible to measure dissociation constants of more than 10^{-4} moles/liter or 10^{-3} moles/liter (e.g., of 10^{-2} moles/liter). Optionally, as will also be clear to the skilled person, the (actual or apparent) dissociation constant may be calculated on the basis of the (actual or apparent) association constant (K_A), by means of the relationship [$K_D = 1/K_A$]. The affinity of a molecular interaction between two molecules can be measured via different techniques known per se, such as the well-known surface plasmon resonance (SPR) biosensor technique (see for example Ober et al. 2001, Intern. Immunology 13: 30 1551-1559). The term "surface plasmon resonance", as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding k_{on}, k_{off} measurements and hence K_D (or K_A) values. This can for example be performed using the well-known BIAcore® system (BIAcore International AB, a GE Healthcare company, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Jonsson et al. (1993, Ann. Biol. Clin. 51: 19-26), Jonsson et al. (1991 Biotechniques 11: 620-627), Johnsson et al. (1995, J. Mol. Recognit. 8: 125- 131), and Johnnson et al. (1991, Anal. Biochem. 198: 268-277). Another well-known biosensor technique to determine affinities of biomolecular interactions is bio-layer interferometry (BLI) (see for example Abdiche et al. 2008, Anal. Biochem. 377: 209-217). The term "bio-layer Interferometry" or "BLI", as used herein, refers to a label-free optical technique that analyzes the interference pattern of light reflected from two surfaces: an internal reference layer (reference beam) and a layer of immobilized protein on the biosensor tip (signal beam). A change in the number of

molecules bound to the tip of the biosensor causes a shift in the interference pattern, reported as a wavelength shift (nm), the magnitude of which is a direct measure of the number of molecules bound to the biosensor tip surface. Since the interactions can be measured in real-time, association and dissociation rates and affinities can be determined. BLI can for example be performed using the well-known Octet® Systems (ForteBio, a division of Pall Life Sciences, Menlo Park, USA). Alternatively, affinities can be measured in Kinetic Exclusion Assay (KinExA) (see for example Drake et al. 2004, Anal. Biochem., 328: 35-43), using the KinExA® platform (Sapidyne Instruments Inc, Boise, USA). The term "KinExA", as used herein, refers to a solution-based method to measure true equilibrium binding affinity and kinetics of unmodified molecules. Equilibrated solutions of an antibody/antigen complex are passed over a column with beads precoated with antigen (or antibody), allowing the free antibody (or antigen) to bind to the coated molecule. Detection of the antibody (or antigen) thus captured is accomplished with a fluorescently labeled protein binding the antibody (or antigen). The GYROLAB® immunoassay system provides a platform for automated bioanalysis and rapid sample turnaround (Fraley et al. 2013, Bioanalysis 5: 1765-74).

One advantage of the ISVD of the invention is its cross-reactivity (see e.g., Examples 8 & 9). The TfR1 binding polypeptides of the present invention are, in certain embodiments, such that they are cross-reactive between human TfR1 and TfR1 from at least one, from at least two, from at least three and up to essentially all of the following species of mammal: mouse, dog, rat, rabbit, guinea pig, pig, sheep, cow and cynomolgus monkey. Thus, the ISVD of the invention can specifically bind to an epitope of human TfR1 and optionally specifically bind to an epitope of cynomolgus TfR1 and/or mouse TfR1.

When an ISVD is said to exhibit "(improved) cross-reactivity for binding to human and non-human primate TfR1" compared to another ISVD, it means that for said ISVD the ratio of the binding activity (such as expressed in terms of K_D or k_{off}) for human TfR1 and for non-human primate (such as cynomolgus) TfR1 is lower than that same ratio calculated for the other ISVD in the same assay. Good cross-reactivity for binding to human and non-human primate (such as cynomolgus) TfR1 allows for the assessment of toxicity of an TfR1 binding polypeptide according to the present invention in preclinical studies conducted on non-human primates. In one embodiment, the ISVD of the invention are cross-reactive at pH 7.4.

In particular embodiments, the ISVDs of the present invention are such that they are (at least) cross-reactive between human TfR1 and cynomolgus monkey TfR1, and also between either human TfR1 and/or cynomolgus monkey TfR1 on the one hand, and at least one or both of rat TfR1 and mouse TfR1 on the other hand. Without being limited to any specific mechanism or hypothesis, it is assumed that the polypeptides of the invention are (essentially) capable of binding to (one or more amino acid residues within) the corresponding stretches of amino acid residues that are present within the amino acid sequence of those mammalian TfR1 proteins, with which the polypeptides of the invention are cross-reactive. In one embodiment, the ISVD is cross-reactive to cynomolgus TfR1, mouse TfR1, or both. In one embodiment, the ISVD is cross-reactive to cynomolgus TfR1. In one embodiment, the ISVD is cross-reactive to mouse TfR1. In one embodiment, the ISVD is cross-reactive to cynomolgus TfR1 and mouse TfR1.

As shown in the Examples, the ISVDs of the present invention bind to TfR1 with greater affinity at neutral pH (such as pH 7.4) than at acidic pH (such as pH 6.0). Generally, the dissociation rate constant k_{off} is higher at acidic pH than at neutral pH. In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 10 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 20 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 30 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 40 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 50 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 60 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 70 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 80 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 90 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 100 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding

of the ISVD of the invention to TfR1 is at least 125 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 150 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 200 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4). In one embodiment, the k_{off} rate for the binding of the ISVD of the invention to TfR1 is at least 250 times higher at acidic pH (such as pH 6.0) compared to neutral pH (such as pH 7.4).

A "neutral" pH as used herein generally relates to a pH within the range of 6.5 to 8.0. In one embodiment, a neutral pH relates to a pH value of 7. In one embodiment, a neutral pH value relates to pH 7.4, also known as physiological pH. An "acidic" pH as used herein generally relates to a pH value of less than 6.5. In one embodiment, an acidic pH relates to a pH value of 6.0.

In one embodiment, the ISVD specifically binds to TfR1 with a k_{off} rate of less than 10×10^{-3} s^{-1} at a neutral pH, such as pH 7.4. In one embodiment, the ISVD specifically binds to TfR1 with a k_{off} rate of less than 5×10^{-3} s^{-1} at a neutral pH, such as pH 7.4. In one embodiment, the ISVD binds to TfR1 with a k_{off} rate of more than 5×10^{-2} s^{-1} at an acidic pH, such as pH 6.0. In one embodiment, the ISVD binds to TfR1 with a k_{off} rate of more than 10×10^{-2} s^{-1} at an acidic pH, such as pH 6.0. The kinetic rate constant k_{off} and k_{on} can be measured by methods described herein, e.g., by surface plasmon resonance. The kinetic rate constant k_{off} and k_{on} can be measured by Bio-Layer Interferometry (BLI), e.g., using an Octet® HTX system (Sartorius), e.g., as described in the examples.

As mentioned herein, TfR1 is involved in the uptake of Transferrin-bound iron into cells. It is thus beneficial, if ISVDs binding to TfR1 do not interfere with the physiological function of TfR1. As shown in Example 11, there is no or negligible influence on transferrin binding. This is mainly due to the inventive epitope identified by the present inventors. Without wishing to be bound by theory, the epitope of the ISVDs of the invention differs from that of transferrin. In one embodiment, the ISVDs of the invention specifically binds to amino acid residues that are not involved in binding of TfR1 to transferrin. In one embodiment, the ISVDs of the invention do not compete with transferrin. "Do not compete" in this context describes that the fold difference of the EC_{50} value for binding of transferrin to TfR1 is below 2 between an experiment without an ISVD of the invention and an ISVD of the invention added at a concentration of 100x EC_{50} for binding of said

ISVD to TfR1. In one embodiment, the binding of Transferrin to TfR1 homodimer is not impacted by the binding of the ISVD of the invention to the TfR1 homodimer.

The present invention further relates to ISVDs that compete for binding to TfR1 with an ISVD of the invention. The present invention further relates to ISVDs that bind to the same epitope as defined herein. Competing ISVDs and ISVDs that recognize the same or an overlapping epitope can be identified using routine techniques such as an immunoassay, for example, by showing the ability of one antibody to block the binding of another antibody to a target antigen, i.e., a competitive binding assay. Competitive binding may be determined using an assay such as described in the Examples below, e.g., epitope binning.

5.3 Polypeptides

The ISVD of the present invention can be comprised in a polypeptide. This polypeptide may comprise one or more further ISVDs of the invention, and/or any other peptide or protein, or both.

Generally, polypeptides according to the invention that comprise or essentially consist of a single building block, single immunoglobulin single variable domain or single Nanobody® ISVD will be referred to herein as "monovalent" proteins or polypeptides, as "monovalent constructs", as "monovalent building block", as "monovalent immunoglobulin single variable domain", "monovalent Nanobody® ISVD" or as "monovalent Nanobody® V_{HH}", respectively.

Polypeptides that comprise or essentially consist of two or more immunoglobulin single variable domains (such as at least two immunoglobulin single variable domains of the invention) will be referred to herein as "multivalent" polypeptides, (fusion) proteins, compounds or as "multivalent constructs". Some non-limiting examples of such multivalent constructs will become clear from the further description herein.

Polypeptides of the invention that contain at least two building blocks, ISVDs, Nanobody® ISVDs or Nanobody® V_{HH}s, in which at least one building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} is directed against a first antigen (i.e., against the first target, such as e.g. TfR1 in the ISVDs of the invention) and at least one building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} is directed against a second antigen (i.e., against the second target which is different from the first target, such as e.g. a serum albumin or a therapeutic target other than TfR1), will also be referred to as "multispecific" polypeptides of the invention, and the building blocks,

ISVDs, Nanobody® ISVDs or Nanobody® V_{HH}s present in such polypeptides will also be referred to herein as being in a "multivalent format" or "multispecific format". Thus, for example, a "bispecific" polypeptide of the invention is a polypeptide that comprises at least one building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} directed against a first target (e.g. TfR1) and at least one further building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} directed against a second target (i.e., directed against a second target different from said first target, such as e.g. serum albumin), whereas a "trispecific" polypeptide of the invention is a polypeptide that comprises at least one building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} directed against a first target (e.g. TfR1), a second building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} directed against a second target different from said first target (e.g. serum albumin) and at least one further building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} directed against a third antigen (i.e., different from both the first and the second target, such as a therapeutic target other than TfR1 and serum albumin); etc. As will be clear from the description, the invention is not limited to bispecific polypeptides, in the sense that a multispecific polypeptide of the invention may comprise at least a first building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} against a first target, a second building block, ISVD, Nanobody® ISVD or Nanobody® V_{HH} against a second target and any number of building blocks, ISVDs, Nanobody® ISVDs or Nanobody® V_{HH}s directed against one or more targets, which may be the same or different from the first and/or second target, respectively.

The terms bispecific polypeptide, bispecific format, bispecific construct, bispecific Nanobody® construct, bispecific and bispecific ISVD construct are used interchangeably herein.

As will be clear from the further description above and herein, the immunoglobulin single variable domains of the invention can be used as "building blocks" to form polypeptides of the invention, e.g., by suitably combining them with other groups, residues, moieties or binding units, in order to form compounds or constructs as described herein (such as, without limitations, the bi-/tri-/tetra-/ multivalent and bi-/tri-/tetra-/multispecific polypeptides of the invention described herein) which combine within one molecule one or more desired properties or biological functions.

In further specific embodiments, the present invention provides polypeptides wherein said at least one ISVD and said at least one further binding moiety are directly linked to each other or are linked via linkers.

In one embodiment, the polypeptide of the invention comprises two ISVDs of the invention. In one embodiment, the present invention provides polypeptides comprising at least one ISVD

according to the invention and at least one other ISVD binding to the same TfR1 molecule as the ISVD according to the invention.

The polypeptides of the invention can generally be prepared by a method which comprises at least one step of suitably linking the one or more immunoglobulin single variable domains of the invention to the one or more further groups, residues, moieties, or binding units, optionally via one or more suitable linkers, so as to provide the polypeptide of the invention. Polypeptides of the invention can also be prepared by a method which generally comprises at least the steps of providing a nucleic acid that encodes a polypeptide of the invention, expressing said nucleic acid in a suitable manner, and recovering the expressed polypeptide of the invention. Such methods can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the methods and techniques further described herein. The process of designing/selecting and/or preparing a polypeptide of the invention, starting from a polypeptide comprising at least one ISVD of the invention, is also referred to herein as "formatting" said polypeptide of the invention; and a polypeptide of the invention that is made part of a compound, construct or polypeptide of the invention is said to be "formatted" or to be "in the format of" said compound, construct or polypeptide of the invention. Examples of ways in which a polypeptide of the invention can be formatted, and examples of such formats will be clear to the skilled person based on the disclosure herein; and such formatted immunoglobulin single variable domains or polypeptides form a further aspect of the invention.

For example, such further groups, residues, moieties, or binding units may be one or more additional immunoglobulins, such that the compound or construct is a fusion protein or fusion polypeptide. In a preferred but non-limiting aspect, said one or more other groups, residues, moieties, or binding units are immunoglobulin single variable domains. In some embodiments, said one or more other groups, residues, moieties or binding units are chosen from the group consisting of domain antibodies, immunoglobulin single variable domains that are suitable for use as a domain antibody, single domain antibodies, immunoglobulin single variable domains (ISVDs) that are suitable for use as a single domain antibody, "dAbs", immunoglobulin single variable domains that are suitable for use as a dAb, V_{HH}s, humanized V_{HH}s, camelized V_Hs, or Nanobody® V_{HH}S. Alternatively, such groups, residues, moieties, or binding units may for example be chemical groups, residues, moieties, which may or may not by themselves be biologically and/or pharmacologically active. For example, and without limitation, such groups may be linked to the

one or more immunoglobulin single variable domains or polypeptides of the invention so as to provide a "derivative" of an ISVD or polypeptide of the invention, as further described herein.

The polypeptide of the invention may be a fusion protein. The term “fusion protein” refers in some embodiments to polypeptides comprising at least one ISVD of the invention and at least one other peptide, protein such as an ISVD, enzyme or the like characterized in that the fusion protein is expressed from the same open reading frame.

In some embodiments, the polypeptides comprise at least two or more immunoglobulin single variable domains disclosed herein. In some embodiments, the polypeptides essentially consist of two or more immunoglobulin single variable domains disclosed herein. A polypeptide that "essentially consists of" two or more immunoglobulin single variable domains, is a polypeptide that in addition to the two or more immunoglobulin single variable domains disclosed herein does not have additional immunoglobulin single variable domains. For instance, a polypeptide that essentially consists of two immunoglobulin single variable domains does not include any additional immunoglobulin single variable domains. However, it should be appreciated that a polypeptide that essentially consists of two or more immunoglobulin single variable domains may include additional functionalities, such as a label, a toxin, one or more linkers, a binding sequence, etc. These additional functionalities include both amino acid based and non-amino acid-based groups. In some embodiments, the polypeptides consist of one or more immunoglobulin single variable domains disclosed herein. It should be appreciated that the terms "polypeptide construct" and "polypeptide" can be used interchangeably herein (unless the context clearly dictates otherwise).

5.4 Nucleic acids

The present invention further relates to a nucleic acid or nucleotide sequence that encodes an ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention (also referred to as "nucleic acid of the invention" or "nucleotide sequence of the invention"). A nucleic acid of the invention can be in the form of single or double stranded DNA or RNA, and may be in the form of double stranded DNA. For example, the nucleotide sequences of the invention may be genomic DNA, cDNA, or synthetic DNA (such as DNA with a codon usage that has been specifically adapted for expression in the intended host cell or host organism).

According to one embodiment of the invention, the nucleic acid of the invention is in essentially isolated form, as defined herein. The nucleic acid of the invention may also be in the form of, be present in and/or be part of a vector, such as for example a plasmid, cosmid or YAC, which again may be in essentially isolated form. A nucleic acid sequence is considered to be "(in) essentially isolated (form)" – for example, compared to its native biological source and/or the reaction medium or cultivation medium from which it has been obtained – when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another nucleic acid, another protein/polypeptide, another biological component or macromolecule or at least one contaminant, impurity or minor component. In particular, a nucleic acid sequence or amino acid sequence is considered "essentially isolated" when it has been purified at least 2-fold, in particular at least 10-fold, more in particular at least 100-fold, and up to 1000-fold or more. A nucleic acid sequence that is "in essentially isolated form" is essentially homogeneous, as determined using a suitable technique, such as a suitable chromatographical technique, such as polyacrylamide-gel electrophoresis.

For the purposes of comparing two or more nucleotide sequences, the percentage of "sequence identity" between a first nucleotide sequence and a second nucleotide sequence may be calculated by dividing [the number of nucleotides in the first nucleotide sequence that are identical to the nucleotides at the corresponding positions in the second nucleotide sequence] by [the total number of nucleotides in the first nucleotide sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of a nucleotide in the second nucleotide sequence – compared to the first nucleotide sequence - is considered as a difference at a single nucleotide (position). Alternatively, the degree of sequence identity between two or more nucleotide sequences may be calculated using a known computer algorithm for sequence alignment such as NCBI Blast v2.0, using standard settings. Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0 967 284, EP 1 085 089, WO 00/55318, WO 00/78972, WO 98/49185 and GB 2 357 768-A. Usually, for the purpose of determining the percentage of "sequence identity" between two nucleotide sequences in accordance with the calculation method outlined hereinabove, the nucleotide sequence with the greatest number of nucleotides will be taken as the "first" nucleotide sequence, and the other nucleotide sequence will be taken as the "second" nucleotide sequence.

The nucleic acids of the invention can be prepared or obtained in a manner known per se, based on the information on the polypeptides or protein constructs of the invention given herein, and/or

can be isolated from a suitable natural source. Also, as will be clear to the skilled person, to prepare a nucleic acid of the invention, also several nucleotide sequences, such as at least one nucleotide sequence encoding an immunoglobulin single variable domain of the invention and for example nucleic acids encoding one or more linkers can be linked together in a suitable manner.

Techniques for generating the nucleic acids of the invention will be clear to the skilled person and may for instance include, but are not limited to, automated DNA synthesis; site-directed mutagenesis; combining two or more naturally occurring and/or synthetic sequences (or two or more parts thereof), introduction of mutations that lead to the expression of a truncated expression product; introduction of one or more restriction sites (e.g. to create cassettes and/or regions that may easily be digested and/or ligated using suitable restriction enzymes), and/or the introduction of mutations by means of a PCR reaction using one or more "mismatched" primers. These and other techniques will be clear to the skilled person, and reference is again made to the standard handbooks.

The nucleic acid of the invention may also be in the form of, be present in and/or be part of a genetic construct, as will be clear to the person skilled in the art. Such genetic constructs generally comprise at least one nucleic acid of the invention that is optionally linked to one or more elements of genetic constructs known per se, such as for example one or more suitable regulatory elements (such as a suitable promoter(s), enhancer(s), terminator(s), etc.) and the further elements of genetic constructs referred to herein. Such genetic constructs comprising at least one nucleic acid of the invention will also be referred to herein as “genetic constructs of the invention”.

The genetic constructs of the invention may be DNA or RNA, and may be double-stranded DNA. The genetic constructs of the invention may also be in a form suitable for transformation of the intended host cell or host organism, in a form suitable for integration into the genomic DNA of the intended host cell or in a form suitable for independent replication, maintenance and/or inheritance in the intended host organism. For instance, the genetic constructs of the invention may be in the form of a vector, such as for example a plasmid, cosmid, YAC, a viral vector or transposon. In particular, the vector may be an expression vector, i.e., a vector that can provide for expression in vitro and/or in vivo (e.g., in a suitable host cell, host organism and/or expression system).

In a preferred but non-limiting embodiment, a genetic construct of the invention comprises a) at least one nucleic acid of the invention; operably connected to b) one or more regulatory elements, such as a promoter and optionally a suitable terminator; and optionally also c) one or more further elements of genetic constructs known per se; in which the terms "regulatory element", "promoter", "terminator" and "operably connected" have their usual meaning in the art (as further described herein); and in which said "further elements" present in the genetic constructs may for example be 3'- or 5'-UTR sequences, leader sequences, selection markers, expression markers/reporter genes, and/or elements that may facilitate or increase (the efficiency of) transformation or integration. These and other suitable elements for such genetic constructs will be clear to the skilled person, and may for instance depend upon the type of construct used; the intended host cell or host organism; the way the nucleotide sequences of the invention of interest are to be expressed (e.g., via constitutive, transient, or inducible expression); and/or the transformation technique to be used. For example, regulatory sequences, promoters, and terminators known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

In some embodiments, in the genetic constructs of the invention, said at least one nucleic acid of the invention and said regulatory elements, and optionally said one or more further elements, are "operably linked" to each other, by which is generally meant that they are in a functional relationship with each other. For instance, a promoter is considered "operably linked" to a coding sequence if said promoter can initiate or otherwise control/regulate the transcription and/or the expression of a coding sequence (in which said coding sequence should be understood as being "under the control of" said promoter). Generally, when two nucleotide sequences are operably linked, they will be in the same orientation and usually also in the same reading frame. They will usually also be essentially contiguous, although this may also not be required.

Accordingly, the present invention relates to a nucleic acid encoding the ISVD of the invention. The present invention further relates to a nucleic acid encoding the polypeptide of the invention. The present invention further relates to a vector comprising the nucleic acid of the invention.

5.5 Host cells

The nucleic acids of the invention, the genetic constructs of the invention, and/or the vectors of the invention may be used to transform a host cell or host organism, e.g., for expression and/or production of the polypeptide or protein construct of the invention. The host can be a non-human

host. Suitable hosts or host cells will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism, for example: a bacterial strain, including but not limited to gram-negative strains such as strains of *Escherichia coli*; of *Proteus*, for example of *Proteus mirabilis*; of *Pseudomonas*, for example of *Pseudomonas fluorescens*; and gram-positive strains such as strains of *Bacillus*, for example of *Bacillus subtilis* or of *Bacillus brevis*; of *Streptomyces*, for example of *Streptomyces lividans*; of *Staphylococcus*, for example of *Staphylococcus carnosus*; and of *Lactococcus*, for example of *Lactococcus lactis*; a fungal cell, including but not limited to cells from species of *Trichoderma*, for example from *Trichoderma reesei*; of *Neurospora*, for example from *Neurospora crassa*; of *Sordaria*, for example from *Sordaria macrospora*; of *Aspergillus*, for example from *Aspergillus niger* or from *Aspergillus sojae*; or from other filamentous fungi; a yeast cell, including but not limited to cells from species of *Saccharomyces*, for example of *Saccharomyces cerevisiae*; of *Schizosaccharomyces*, for example of *Schizosaccharomyces pombe*; of *Pichia*, for example of *Pichia pastoris* (also known as *Komagataella phaffii*) or of *Pichia methanolica*; of *Hansenula*, for example of *Hansenula polymorpha*; of *Kluyveromyces*, for example of *Kluyveromyces lactis*; of *Arxula*, for example of *Arxula adeninivorans*; of *Yarrowia*, for example of *Yarrowia lipolytica*; an amphibian cell or cell line, such as *Xenopus* oocytes; an insect-derived cell or cell line, such as cells/cell lines derived from *lepidoptera*, including but not limited to *Spodoptera* SF9 and Sf21 cells or cells/cell lines derived from *Drosophila*, such as Schneider and Kc cells; a plant or plant cell, for example in tobacco plants; and/or a mammalian cell or cell line, for example a cell or cell line derived from a human, a cell or a cell line from mammals including but not limited to CHO-cells, BHK-cells (for example BHK-21 cells) and human cells or cell lines such as HeLa, COS (for example COS-7) and PER.C6 cells; as well as all other hosts or host cells known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments), which will be clear to the skilled person. Reference is also made to the general background art cited hereinabove, as well as to for example WO 94/29457; WO 96/34103; WO 99/42077; Frenken et al. 1998 (Res. Immunol. 149: 589-99); Riechmann and Muyldermans 1999 (J. Immunol. Met. 231: 25-38); van der Linden 2000 (J. Biotechnol. 80: 261-70); Joosten et al. 2003 (Microb. Cell Fact. 2: 1); Joosten et al. 2005 (Appl. Microbiol. Biotechnol. 66: 384-92); and the further references cited herein.

For expression of the polypeptides, ISVDs, compounds, or constructs in a cell, they may also be expressed as so-called "intrabodies", as for example described in WO 94/02610, WO 95/22618,

and US 7004940; WO 03/014960; in Cattaneo and Biocca 1997 (Intracellular Antibodies: Development and Applications. Landes and Springer-Verlag); and in Kontermann 2004 (Methods 34: 163-170).

According to one preferred, but non-limiting embodiment of the invention, the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention is produced in a bacterial cell, in particular a bacterial cell suitable for large scale pharmaceutical production, such as cells of the strains mentioned above.

According to another preferred, but non-limiting embodiment of the invention, the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention is produced in a yeast cell, in particular a yeast cell suitable for large scale pharmaceutical production, such as cells of the species mentioned above. In one embodiment, the host cell is a *Pichia pastoris* host cell.

According to yet another preferred, but non-limiting embodiment of the invention, the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention is produced in a mammalian cell, in particular in a human cell or in a cell of a human cell line, and more in particular in a human cell or in a cell of a human cell line that is suitable for large scale pharmaceutical production, such as the cell lines mentioned hereinabove.

Suitable techniques for transforming a host or host cell of the invention will be clear to the skilled person and may depend on the intended host cell/host organism and the genetic construct to be used. Reference is again made to the handbooks and patent applications mentioned above.

The transformed host cell (which may be in the form or a stable cell line) or host organisms (which may be in the form of a stable mutant line or strain) form further aspects of the present invention.

In some embodiments, these host cells or host organisms are such that they express, or are (at least) capable of expressing (e.g., under suitable conditions), the the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention (and in case of a host organism: in at least one cell, part, tissue, or organ thereof). The invention also

includes further generations, progeny and/or offspring of the host cell or host organism of the invention, for instance obtained by cell division or by sexual or asexual reproduction.

Accordingly, in another aspect, the invention relates to a host or host cell that expresses (or that under suitable circumstances can express) an the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention; and/or that contains a nucleic acid (or vector) encoding the same. Some preferred but non-limiting examples of such hosts or host cells can be as generally described in WO 04/041867, WO 04/041865, or WO 09/068627. For example, the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention may with advantage be expressed, produced, or manufactured in a yeast strain, such as a strain of *Pichia pastoris*. Reference is also made to WO 04/25591, WO 10/125187, WO 11/003622, and WO 12/056000 which also describes the expression/production in *Pichia* and other hosts/host cells of immunoglobulin single variable domains and polypeptides comprising the same.

To produce/obtain expression of the polypeptides, the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention, the transformed host cell or transformed host organism may generally be kept, maintained and/or cultured under conditions such that the (desired) the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention is expressed/produced. Suitable conditions will be clear to the skilled person and will usually depend upon the host cell/host organism used, as well as on the regulatory elements that control the expression of the (relevant) nucleotide sequence of the invention. Again, reference is made to the handbooks and patent applications mentioned above in the paragraphs on the genetic constructs of the invention.

Generally, suitable conditions may include the use of a suitable medium, the presence of a suitable source of food and/or suitable nutrients, the use of a suitable temperature, and optionally the presence of a suitable inducing factor or compound (e.g., when the nucleotide sequences of the invention are under the control of an inducible promoter); all of which may be selected by the skilled person. Again, under such conditions, the ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention may be expressed in a constitutive manner, in a transient manner, or only when suitably induced.

The ISVD, polypeptide, compound, protein such as a fusion protein, or construct such as a multispecific construct of the invention may then be isolated from the host cell/host organism and/or from the medium in which said host cell or host organism was cultivated, using protein isolation and/or purification techniques known per se, such as (preparative) chromatography and/or electrophoresis techniques, differential precipitation techniques, affinity techniques (e.g. using a specific, cleavable amino acid sequence fused with the polypeptide or construct of the invention) and/or preparative immunological techniques (i.e. using antibodies against the amino acid sequence to be isolated).

An polypeptide or protein is considered to be "*(in) essentially isolated (form)*" – for example, compared to its native biological source and/or the reaction medium or cultivation medium from which it has been obtained – when it has been separated from at least one other component with which it is usually associated in said source or medium, such as another protein/polypeptide, another biological component or macromolecule or at least one contaminant, impurity or minor component. In particular, a polypeptide or protein is considered "essentially isolated" when it has been purified at least 2-fold, in particular at least 10-fold, more in particular at least 100-fold, and up to 1000-fold or more. A polypeptide or protein that is "in essentially isolated form" is essentially homogeneous, as determined using a suitable technique, such as a suitable chromatographical technique, such as polyacrylamide-gel electrophoresis.

5.6 Conjugates

The ISVD (or polypeptides) of the present invention may be covalently or non-covalently attached to an agent to form a conjugate. The agent typically has therapeutic or diagnostic function. Such conjugates may thus be used for treating or diagnosing a disease in a subject. Such diseases particularly relate to diseases of the nervous system or of muscle tissues. Accordingly, the present invention relates to a conjugate comprising the ISVD of the invention or the polypeptide of the invention, and an agent, wherein the agent optionally is covalently attached to the ISVD or the polypeptide. The covalent attachment might be direct or via a linker.

The "agent" as used herein relates to any compound, e.g., a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, a radioactive isotope, etc. In some embodiments, the agent is useful for treating a disease or for diagnosis of a disease.

The agent may be a small molecule.

The agent may be a nucleotide such as an oligonucleotide (e.g., single, double or more stranded RNA and/or DNA molecules, and analogs and derivatives thereof); small regulatory RNA such as shRNA, miRNA, siRNA and the like; and a plasmid or fragment thereof. The agent may be an antisense oligonucleotide. The agent may be an siRNA.

The agent may be a peptide or a protein, such as an antibody or antibody fragment; a therapeutic peptide such as a hormone, cytokine, growth factor, enzyme, antigen or antigenic peptide, transcription factor, or any functional domain thereof.

The agent, as used herein, may be any chemical substance which may be used to provide a signal or contrast in imaging or in other words, a "imaging agent". A signal enhancing domain may be an organic molecule, metal ion, salt or chelate, a particle (e.g., iron particle), or a labeled peptide, protein, glycoprotein, polymer, or liposome. For example, an imaging agent may include one or more of a radionuclide, a paramagnetic metal, a fluorochrome, a dye, and an enzyme substrate.

For x-ray imaging, the imaging agent may comprise iodinated organic molecules or chelates of heavy metal ions of atomic numbers 57 to 83. In certain embodiments, the imaging agent is I^{125} labeled IgG (see, e.g., M. Sovak, ed., "Radiocontrast Agents," Springer Verlag, pp. 23-125 (1984).

For ultrasound imaging, an imaging agent may comprise gas-filled bubbles or particles or metal chelates where the metal ions have atomic numbers 21-29, 42, 44 or 57-83. See e.g., Tyler et al., Ultrasonic Imaging, 3, pp. 323-29 (1981) and D. P. Swanson, "Enhancement Agents for Ultrasound: Fundamentals," Pharmaceuticals in Medical Imaging, pp. 682-87. (1990) for other suitable compounds.

For nuclear radiopharmaceutical imaging or radiotherapy, an imaging agent may comprise a radioactive molecule. In certain embodiments, chelates of Tc, Re, Co, Cu, Au, Ag, Pb, Bi, In and Ga may be used. In certain embodiments, chelates of Tc-99m may be used. See e.g., Rayudu GVS, Radiotracers for Medical Applications, I, pp. 201 and D. P. Swanson et al., ed., Pharmaceuticals in Medical Imaging, pp. 279-644 (1990) for other suitable compounds.

For ultraviolet/visible/infrared light imaging, an imaging agent may comprise any organic or inorganic dye or any metal chelate.

For MRI, an imaging agent may comprise a metal-ligand complex of a paramagnetic form of a metal ion with atomic numbers 21-29, 42, 44, or 57-83. In certain embodiments, the paramagnetic metal is selected from: Cr(III), Cu(II), Dy(III), Er(III) and Eu(III), Fe(III), Gd(III), Ho(III), Mn(II and III), Tb(III). A variety of chelating ligands useful as MRI agents are well known in the art.

The agent may be linked to the ISVD of the invention through a peptide linker. In some embodiments, the peptide linker may predominantly include the following amino acid residues: Gly, Ser, Ala, or Thr. The peptide linker may have a length that is adequate to link two molecules in such a way that they assume the correct conformation relative to one another so that they retain their respective desired activity. In some embodiments, the linker is 1 to 50 (e.g., 1 to 30, 1 to 20, 1 to 10 or 1 to 5) amino acids in length. Useful linkers include glycine-serine pharmaceutical compositions polymers, including for example, (GS)n, (GSGGS)n (SEQ ID NO: 142), (GGGGS)n (SEQ ID NO: 143), and (GGGS)n (SEQ ID NO: 144), where n is an integer of at least one; glycine alanine polymers; alanine-serine polymers; XTEN linkers; and other flexible linkers. In some embodiments, the linker is GGGG (SEQ ID NO: 145) or SGSGGGG (SEQ ID NO: 146). Additional exemplary linkers for linking antibody fragments or single-5 chain variable fragments can include AAEPKSS (SEQ ID NO: 147), AAEPKSSDKTHTCPPCP (SEQ ID NO: 148), GGGG (SEQ ID NO: 145), or GGGGDKTHTCPPCP (SEQ ID NO: 149).

The conjugates of the invention can be manufactures using techniques known by a person skilled in the art. Exemplary methods are described in the Examples. Accordingly, the present invention also relates to a method of manufacturing a conjugate of the invention comprising: (i) Providing an ISVD the invention; and (ii) Conjugating the agent to the ISVD.

5.7 Pharmaceutical compositions

The invention also relates to a pharmaceutical composition comprising the ISVD of the invention, the polypeptide of the invention, or the conjugate of the invention.

The pharmaceutical composition of the invention may comprise a pharmaceutically acceptable carrier.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without

excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition, or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. “Pharmaceutically acceptable carrier” as used herein include, but are not limited to, appropriate solvents, dispersion media, antibacterial and antifungal agents, isotonic agents, and the like. In some embodiments, the pharmaceutical composition is a sterile aqueous solution, and may comprise a buffer; a surfactant; a polyol; an antioxidant; and/or a chelating agent.

Methods of preparing these formulations or compositions include the step of bringing into association the ISVDs of the invention, the polypeptides (including the fusion proteins of the invention) of the invention, or the conjugates of the invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the ISVDs of the invention, the polypeptides (including the fusion proteins of the invention) of the invention, or the conjugates of the invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

5.8 Uses

The ISVD of the present invention can be used for treating or diagnosing several diseases in a subject such as a human subject. The unique properties of the ISVD of the invention not only allow the transport of an agent (in)to any cell expressing TfR1 on its surface but also allows the transport across the blood-brain-barrier (BBB). The unique and inventive epitope recognized by the ISVD of the invention allows binding at neutral pH (in the plasma) and release at acidic pH (e.g., in the lysosome after internalization, or in endosomes for transcytosis across the BBB). As shown in the examples, the conjugation of an agent to an ISVD of the invention (forming a conjugate) does not influence binding of the ISVD to TfR1. The inventors could further show that conjugates comprising the ISVD of the invention are able to transport an agent across the BBB.

Accordingly, the present invention relates to the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention for use in a method of delivering an agent across the blood brain barrier (BBB) in a subject.

The present further relates to a method of delivering an agent across the blood brain barrier (BBB) in a subject, optionally comprising a step of administering a (therapeutically) effective amount of the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention to the subject.

"Across the blood brain barrier" as used herein describes the process of the translocation (e.g., by transcytosis) of an agent or the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention from the circulatory system of a subject into the central nervous system. A successful translocation across the BBB can be assessed by a person skilled in the art using standard methods such as direct methods (e.g., fluorescent or radioactive labeling) or indirect methods (e.g., reduction of gene expression in the central nervous system in response to an antisense oligonucleotide), e.g., as described in the Examples.

The present invention further relates to the ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention for use in a method of delivering an agent across the blood nerve barrier in a subject.

The present further relates to a method of delivering an agent across the blood nerve barrier in a subject, optionally comprising a step of administering a therapeutically effective amount of the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention to the subject.

"Across the blood nerve barrier" as used herein describes the process of the translocation (e.g., by transcytosis) of an agent or the ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention from the circulatory system of a subject into the peripheral nervous system. A successful translocation across the blood nerve barrier can be assessed by a person skilled in the art using standard methods such as direct methods (e.g., fluorescent or radioactive labeling) or indirect methods (e.g., reduction of gene expression in the peripheral nervous system in response to an antisense oligonucleotide), e.g., as described in the Examples.

The present invention further relates to the ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention for use in a method of delivering an agent into a cell in a subject. The present invention further relates to a method of delivering an agent into a cell in a subject, optionally comprising a comprising a step of administering a therapeutically effective amount of the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention to the subject.

In some embodiments of the methods and uses described herein, an effective amount of the ISVD, the polypeptide, the conjugate, or the pharmaceutical composition is administered to the subject. In some embodiments of the methods described herein, a therapeutically effective amount of the ISVD, the polypeptide, the conjugate, or the pharmaceutical composition is administered to the subject.

The ISVDs of the invention, the polypeptides of the invention, or the conjugates of the invention, or pharmaceutical compositions comprising the same can be administered in any suitable manner, depending on the specific pharmaceutical formulation or composition to be used. Thus, the ISVDs of the invention, the polypeptides of the invention, or the conjugates of the invention can for example be administered orally, intraperitoneally, intravenously, subcutaneously, intramuscularly, or via any other route of administration that circumvents the gastrointestinal tract, intranasally, transdermally, topically, by means of a suppository, by inhalation, again depending on the specific pharmaceutical formulation or composition to be used. The clinician will be able to select a suitable route of administration and a suitable pharmaceutical formulation or composition to be used in such administration, depending on the disease or disorder to be prevented or treated and other factors well known to the clinician.

As used herein, a "therapeutically effective amount" in the present context refers to the amount of a therapy alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment and/or management of a disease and/or disorder. In one aspect, a therapeutically effective amount refers to the amount of a therapy sufficient to ameliorate, modify, stabilize, or control a disease and/or disorder, or one or more symptoms thereof. In another aspect, a therapeutically effective amount refers to the amount of a therapy sufficient to reduce the symptoms of a disease and/or disorder. In another aspect, a therapeutically effective amount refers to the amount of a therapy sufficient to delay or minimize the spread of a disease and/or disorder.

Used in connection with an amount of a multispecific polypeptide of the invention, the term can encompass an amount that improves overall therapy, reduces, or avoids unwanted effects, or enhances the therapeutic efficacy of or synergies with another therapy. In one embodiment, a therapeutically effective amount of a therapy reduces or avoids unwanted effects, or enhances the therapeutic efficacy of or synergies with another therapy by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100% relative to a control (e.g., a negative control such as phosphate buffered saline) in an assay known in the art or described herein.

As outlined herein, the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention can be used to deliver into a cell in a subject. Accordingly, the present invention relates to the use of the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention for delivering an agent into a cell in a subject. Since the transport into a cell comprises first binding to the cell surface, "into a cell" can also include "to a cell" or "to the surface of a cell". It is also envisioned that the cell is not in a subject but in some embodiments outside of a subject, e.g., an isolated cell or a cell in a cell culture medium. In this context, the "cell" generally is any cell, which expresses TfR1 on its surface. TfR1 is expressed in varying degrees depending on the cell type. TfR1 is highly expressed, e.g., on muscle tissue and cells of the BBB.

Accordingly, the cell may be a cell of the nervous system (such as central nervous system, peripheral nervous system, or sciatic nerve) or of muscle tissue (such as skeletal muscle, heart muscle, smooth muscle). In one embodiment, the cell is a cell of the nervous system. In one embodiment, the cell is a cell of the central nervous system. In one embodiment, the cell is a cell of the peripheral nervous system. In one embodiment, the cell is a cell of the sciatic nerve. In one embodiment, the cell is a cell of the muscle tissue. In one embodiment, the cell is a cell of skeletal muscle. In one embodiment, the cell is a cell of heart muscle. In one embodiment, the cell is a cell of smooth muscle.

As outlined herein, the ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention can be used to deliver (or translocate) an agent across the BBB. Accordingly, the present invention relates to the use of the

ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention for delivering an agent across the BBB in a subject.

The ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention can be used to increase the exposure of nervous system to agent. This use may be therapeutic or diagnostic. Accordingly, the present invention relates to a method for increasing the exposure of the central nervous system (CNS) to an agent comprising: (i) administering the ISVD of the invention, the polypeptide of the invention, the conjugate of the invention, or the pharmaceutical composition of the invention.

As used herein, the terms "subject" and "patient" are used interchangeably. As used herein, a subject can be a mammal, such as a non-primate (e.g., cows, pigs, horses, cats, dogs, rats, etc.) or a primate (e.g., monkey and human). In certain embodiments, the term "subject," as used herein, refers to a vertebrate, such as a mammal. Mammals include, without limitation, humans, non-human primates, wild animals, feral animals, farm animals, sport animals, and pets. The subject may be human.

As outlined herein, the ISVDs of the invention, the polypeptides of the invention, the conjugates of the invention, or the pharmaceutical compositions of the invention can be used for treating diseases. Accordingly, the subject may have a disease of the nervous system (such as central nervous system, peripheral nervous system, or sciatic nerve) or of muscle tissue (such as skeletal muscle, heart muscle, smooth muscle).

5.9 Production

The invention also relates to methods for preparing the polypeptides, ISVDs, compounds and constructs described herein. The polypeptides, ISVDs, compounds and constructs of the invention can be prepared in a manner known per se, as will be clear to the skilled person from the further description herein. For example, polypeptides, ISVDs, compounds and constructs of the invention can be prepared in any manner known per se for the preparation of antibodies and in particular for the preparation of antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments) such as ISVDs. Some preferred, but non-limiting methods for preparing the polypeptides and constructs include the methods and techniques described herein.

The methods for producing a polypeptide, ISVD, compound and construct of the invention may

comprise the following steps: the expression, in a suitable host cell or host organism (also referred to herein as a "host of the invention") or in another suitable expression system of a nucleic acid that encodes said ISVD, polypeptide or protein construct of the invention, optionally followed by: isolating and/or purifying the polypeptide, ISVD, compound and construct of the invention thus obtained.

In particular, such a method may comprise the steps of: cultivating and/or maintaining a host cell or host organism of the invention under conditions that are such that said host cell or host organism of the invention expresses and/or produces at least one polypeptide, ISVD, compound and/or construct of the invention; optionally followed by: isolating and/or purifying the polypeptide, ISVD, compound and/or construct of the invention thus obtained.

An exemplary method for generating further ISVDs binding to the epitope of the invention described herein, is as follows: First, ISVDs against TfR1 are generated as, e.g., described herein. In a next step, a competition setup could be employed: For example, one could add EC_{30} concentration of an ISVD with known epitope to TfR1 expressing cells in absence or presence of ISVDs (generated, e.g., with the first step) with unknown epitope (e.g., purified or *E. coli* periplasmic extracts). Competing Nanobodies would be assigned to the same epitope bin, indicating overlapping epitopes. In a further step, confirmation on identical epitopes can be done via X-ray analysis or cryo-EM structural analysis, e.g., as described herein.

***

Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks mentioned in paragraph a) on page 46 of WO 2008/020079.

It must be noted that as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a reagent" includes one or more of such different reagents and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain,

using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

The term “about” used in the context of the parameters or parameter ranges of the provided herein shall have the following meanings. Unless indicated otherwise, where the term “about” is applied to a particular value or to a range, the value or range is interpreted as being as accurate as the method used to measure it. If no error margins are specified in the application, the last decimal place of a numerical value indicates its degree of accuracy. Where no other error margins are given, the maximum margin is ascertained by applying the rounding-off convention to the last decimal place, e.g., for a pH value of about pH 2.7, the error margin is 2.65-2.74. However, for the following parameters, the specific margins shall apply: a temperature specified in °C with no decimal place shall have an error margin of ± 1°C (e.g., a temperature value of about 50°C means 50°C ± 1°C); a time indicated in hours shall have an error margin of 0.1 hours irrespective of the decimal places (e.g., a time value of about 1.0 hours means 1.0 hours ± 0.1 hours; a time value of about 0.5 hours means 0.5 hours ± 0.1 hours).
In the present application, any parameter indicated with the term “about” is also contemplated as being disclosed without the term “about”. In other words, embodiments referring to a parameter value using the term “about” shall also describe an embodiment directed to the numerical value of said parameter as such. For example, an embodiment specifying a pH of “about pH 2.7” shall also disclose an embodiment specifying a pH of “pH 2.7” as such; an embodiment specifying a pH range of “between about pH 2.7 and about pH 2.1” shall also describe an embodiment specifying a pH range of “between pH 2.7 and pH 2.1”, etc.

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".

5.10 Embodiments

The present invention further relates to the following items:

A1. An immunoglobulin single variable domain (ISVD),
wherein the ISVD specifically binds to an epitope of a transferrin receptor 1 (TfR1) homodimer, optionally a human TfR1 homodimer,
wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,
optionally wherein the epitope comprises amino acid residues of both the first TfR1 monomer and the second TfR1 monomer.

1. An immunoglobulin single variable domain (ISVD),
wherein the ISVD specifically binds to an epitope of a transferrin receptor 1 (TfR1) homodimer, optionally a human TfR1 homodimer,
wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,
wherein the ISVD is characterized in that the epitope comprises:
(i) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, K382, and E383 of the first TfR1 monomer, and
(ii) at least one of amino acid residues K633, E634, M635, G363, L637, R719, N722, N723, G724, A725, F726, N727, E728, T729, and R732 of the second TfR1 monomer,
wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

2. The ISVD of item 1 or A1, wherein the amino acid residues of the epitope form a conformation that differs between pH 7.4 (neutral conformation) and pH 6.0 (acidic conformation), wherein the ISVD has a reduced affinity to the TfR1 homodimer at pH 6.0 compared to pH 7.4, optionally wherein binding of the ISVD to the TfR1 homodimer has a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation.

3. The ISVD of any one of the preceding items, wherein the epitope comprises:
(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and
(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer.

4. The ISVD of any one of the preceding items,

(a) wherein the epitope comprises:

(i) at least one of amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer;

(b) wherein the epitope comprises:

(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer;

(c) wherein the epitope comprises:

(i) at least one of amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) at least one of amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer;

(d) wherein the epitope comprises:

(i) at least one of amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer; or

(e) wherein the epitope comprises:

(i) at least one of amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and

(ii) at least one of amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer.

5. The ISVD of any one of the preceding items, wherein the ISVD specifically binds to TfR1 with a k_{off} rate of less than 10×10^{-3} s^{-1} at a neutral pH, such as pH 7.4.

6. The ISVD of any one of the preceding items, wherein the ISVD binds to TfR1 with a k_{off} rate of more than 5 x 10^{-2} s^{-1} at an acidic pH, such as pH 6.0.

7. The ISVD of any one of the preceding items, wherein the first TfR1 monomer, the second TfR1 monomer, or the first and the second TfR1 monomer comprises or consists of an amino acid sequence as depicted in SEQ ID NO: 1, or (polymorphic) variants or isoforms thereof.

9. The ISVD of any one of the preceding items, wherein the ISVD consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

(i) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 7 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 7; and
b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 8 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 8; and
c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 9, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 9;

(ii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 10 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 10; and
b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 11 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 11; and
c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 12, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 12;

(iii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 13 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 13; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 14 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 14; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 15, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 15;

(iv) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 16 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 16; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 17 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 17; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 18, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 18;

(v) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 19 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 19; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 20 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 20; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 21, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 21;

(vi) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 28 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 28; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 30 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 30; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 32, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 32;

(vii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 39 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 39; and
b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 41 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 41; and
c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 43, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 43;

(viii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 50 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 50; and
b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 52 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 52; and
c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 54, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 54;

(ix) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 61 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 61; and
b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 63 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 63; and
c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 65, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 65;

or

(x) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 68 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 68; and
b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 70 and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 70; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 72, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NO: 72.

10. The ISVD of any one of the preceding items, wherein the ISVD comprises or consists of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26, and optionally has an amino acid sequence having 4, 3, 2 or 1 "amino acid difference(s)" (as defined herein) with the sequence of SEQ ID NOs: 2-6 and 22-26, respectively.

10A. The ISVD of any one of the preceding items, wherein the ISVD comprises or consists of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26.

10B. The ISVD of any one of the preceding items, wherein the ISVD comprises of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26.

10C. The ISVD of any one of the preceding items, wherein the ISVD consists of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26.

11. The ISVD of any one of the preceding items, wherein the net charge of the CDRs or the paratope of the ISVD is not altered by changing the pH from pH 6.0 to 7.4 (or from pH 7.4 to 6).

12. The ISVD of any one of the preceding items, wherein the ISVD is cross-reactive to cynomolgus TfR1, mouse TfR1, or both.

13. The ISVD of any one of the preceding items, wherein two ISVDs can bind to the same TfR1 homodimer.

14. A polypeptide comprising the ISVD of any one of the preceding items.

15. A nucleic acid encoding the ISVD of any one of items 1-13 or the polypeptide of item 14.

16. A vector comprising the nucleic acid of item 15.

17. A host cell comprising the nucleic acid of item 16 or the vector of item 17.

18. A conjugate comprising the ISVD of any one of items 1-13 or the polypeptide of item 14, and an agent, wherein the agent optionally is covalently attached to the ISVD or the polypeptide.

19. The conjugate of item 18, wherein the agent is a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, a radioactive isotope, etc.

20. A pharmaceutical composition comprising the ISVD of any one of items 1-13, the polypeptide of item 14, or the conjugate of item 18 or 19, optionally comprising a pharmaceutically acceptable carrier.

21. The ISVD of any one of items 1-13, the polypeptide of item 14, the conjugate of item 18 or 19, or the pharmaceutical composition of item 20 for use in a method of delivering an agent across the blood brain barrier (BBB) in a subject, wherein optionally the agent is a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, a radioactive isotope , etc.

22. The ISVD of any one of items 1-13, the polypeptide of item 14, the conjugate of item 18 or 19, or the pharmaceutical composition of item 20 for use in a method of delivering an agent into a cell in a subject.

23. The ISVD, the polypeptide, the conjugate, or the pharmaceutical composition for use of any one of items 21 or 22, wherein (a therapeutically effective amount of) the ISVD, the polypeptide, the conjugate, or the pharmaceutical composition is administered to the subject.

24. Use of the ISVD of any one of items 1-13, the polypeptide of item 14, the conjugate of item 18 or 19, or the pharmaceutical composition of item 20 for delivery of an agent into a cell in a subject.

25. Use of the ISVD of any one of items 1-13, the polypeptide of item 14, the conjugate of item 18 or 19, or the pharmaceutical composition of item 20 for delivery of an agent across the blood-brain-barrier in a subject.

26. The ISVD for use of any one of items 21 to 23 or the use of item 24 or 25, wherein the subject is a human.

27. The ISVD for use of any one of items 21 to 23 or 26 or the use of any one of items 24 to 26, wherein the subject has a disease of the nervous system (such as central nervous system, peripheral nervous system, or sciatic nerve) or of muscle tissue (such as skeletal muscle, heart muscle, smooth muscle).

28. A method for increasing the exposure of the CNS to an agent comprising:
    (i) administering the conjugate of item 18 or 19 to a subject.

29. A method of manufacturing a conjugate as defined in item 18 or 19 comprising:
    (i) Providing an ISVD as defined in any one of items 1-13; and
    (ii) Conjugating the agent to the ISVD.

6 Examples

6.1 Example 1: Immunizations

Two llamas and one alpaca were immunized with recombinant human transferrin receptor 1 (TfR1) (in house produced; amino acid residues 89-760; UniProt ID P02786, version 2 of 30 May 2006) ectodomain protein according to standard protocols, with the aim to induce a heavy-chain antibody dependent humoral immune response.

Immune blood (PBL) samples were taken at regular intervals, and total RNA was prepared from the isolated B-cells. The humoral immune response was monitored during the immunization process by comparing the antigen specific serum titers of a sample collected prior to initiation of immunization and a serum sample typically collected after multiple antigen administrations. Briefly, different regions of MagPlex®-C MICROSPHERES beads (Luminex®) were functionalized with recombinant His8 tagged human, cynomolgus (in house produced; amino acid residues 87-760; XP_045243212.1, version of 18 August 2023), or mouse (in house produced;

amino acid residues 89-763; UniProt ID Q62351, version 1 of 1 November 1996) ectodomain TfR1 protein and human ectodomain TfR2 peptide (Abcam, ab87613). The different bead regions were added to each well of a 384 F-bottom plate (Thermo Scientific, 262160) and serially diluted camelid serum was applied. The presence of anti-TfR1 immunoglobulins was detected via both anti-alpaca IgG (Jackson ImmunoResearch, 128-065-160) and anti-alpaca IgG, subclasses 2+3 (Jackson ImmunoResearch, 128-065-229) specific detection tools, followed by secondary Streptavidin-Phycoerythrin (BD – Pharmingen, 554061) detection and readout with the FlexMAP® 3D (Luminex Corporation).

XP_045243212.1

MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLGVDEEENTDNNTKANGT
KPKRCGGNICYGTIAVIIFFLIGFMIGYLGYCKGVEPKTECERLAGTESPAREEPEEDFPA
APRLYWDDLKRKLSEKLDTTDFTSTIKLLNENLYVPREAGSQKDENLALYIENQFREFK
LSKVWRDQHFVKIQVKDSAQNSVIIVDKNGGLVYLVENPGGYVAYSKAATVTGKLVH
ANFGTKKDFEDLDSPVNGSIVIVRAGKITFAEKVANAESLNAIGVLIYMDQTKFPIVKAD
LSFFGHAHLGTGDPYTPGFPSFNHTQFPPSQSSGLPNIPVQTISRAAAEKLFGNMEGDCPS
DWKTDSTCKMVTSENKSVKLTVSNVLKETKILNIFGVIKGFVEPDHYVVVGAQRDAWG
PGAAKSSVGTALLLKLAQMFSDMVLKDGFQPSRSIIFASWSAGDFGSVGATEWLEGYLS
SLHLKAFTYINLDKAVLGTSNFKVSASPLLYTLIEKTMQDVKHPVTGRSLYQDSNWASK
VEKLTLDNAAFPFLAYSGIPAVSFCFCEDTDYPYLGTTMDTYKELVERIPELNKVARAA
AEVAGQFVIKLTHDTELNLDYERYNSQLLLFLRDLNQYRADVKEMGLSLQWLYSARGD
FFRATSRLTTDFRNAEKRDKFVMKKLNDRVMRVEYYFLSPYVSPKESPFRHVFWGSGS
HTLSALLESLKLRRQNNSAFNETLFRNQLALATWTIQGAANALSGDVWDIDNEF (SEQ ID NO: 150)

UniProt ID Q62351

MMDQARSAFSNLFGGEPLSYTRFSLARQVDGDNSHVEMKLAADEEENADNNMKASVR
KPKRFNGRLCFAAIALVIFFLIGFMSGYLGYCKRVEQKEECVKLAETEETDKSETMETED
VPTSSRLYWADLKTLLSEKLNSIEFADTIKQLSQNTYTPREAGSQKDESLAYYIENQFHE
FKFSKVWRDEHYVKIQVKSSIGQNMVTIVQSNGNLDPVESPEGYVAFSKPTEVSGKLVH
ANFGTKKDFEELSYSVNGSLVIVRAGEITFAEKVANAQSFNAIGVLIYMDKNKFPVVEA
DLALFGHAHLGTGDPYTPGFPSFNHTQFPPSQSSGLPNIPVQTISRAAAEKLFGKMEGSCP
ARWNIDSSCKLELSQNQNVKLIVKNVLKERRILNIFGVIKGYEEPDRYVVVGAQRDALG
AGVAAKSSVGTGLLLKLAQVFSDMISKDGFRPSRSIIFASWTAGDFGAVGATEWLEGYL

SSLHLKAFTYINLDKVVLGTSNFKVSASPLLYTLMGKIMQDVKHPVDGKSLYRDSNWIS
KVEKLSFDNAAYPFLAYSGIPAVSFCFCEDADYPYLGTRLDTYEALTQKVPQLNQMVRT
AAEVAGQLIIKLTHDVELNLDYEMYNSKLLSFMKDLNQFKTDIRDMGLSLQWLYSARG
DYFRATSRLTTDFHNAEKTNRFVMREINDRIMKVEYHFLSPYVSPRESPFRHIFWGSGSH
TLSALVENLKLRQKNITAFNETLFRNQLALATWTIQGVANALSGDIWNIDNEF (SEQ ID NO: 151)

6.2 Example 2: Library construction and phage display selections

Peripheral blood mononuclear cells were prepared from the blood samples using Ficoll-Hypaque according to the instructions of the manufacturer. Total RNA extracted from these cells and from lymph nodes was used as starting material for RT-PCR to amplify ISVD encoding gene fragments. These fragments were cloned into phagemid vector pAX212. Phage was prepared according to standard protocols (Phage Display of Peptides and Proteins: A Laboratory Manual, Academic Press; 1st edition (October 28, 1996) Brian K. Kay, Jill Winter, John McCafferty) and stored after filtering with a 0.22 µm filter at 4°C until further use. Three phage libraries were constructed, with library sizes between 0.95 $x10^8$ and 1.68 $x10^8$, and a percentage of insert ranging from 87 to 100%.

To identify ISVD molecules recognizing human, cynomolgus and mouse TfR1, the phage libraries were incubated with 0.5 or 50 nM recombinant biotinylated His8-AviTag human, cynomolgus or mouse TfR1. Complexes of TfR1 and phage were captured from solution on streptavidin coated magnetic beads. After extensive washing with PBS/ 0.05% Polysorbate-20, bound phages were eluted by addition of trypsin (1 mg/ml). Outputs of these round 1 selections were incubated with 0.01, 0.05, 0.5 or 5 nM recombinant biotinylated human, cynomolgus or mouse TfR1. Bound phages were eluted by addition of trypsin (1 mg/ml). Individual clones from round 1 and round 2 selections were picked.

All individual clones were grown in 96 deep well plates (1 ml volume). Expression of monovalent ISVD clones was induced by adding IPTG to a final concentration of 1 mM. Periplasmic extracts were prepared by freezing the cell pellets and dissolving them in 100 µl PBS. Cell debris was removed by centrifugation.

6.3 Example 3: Screening for human, cynomolgus, mouse TfR1 binding ISVD molecules in periplasmic extracts

In order to determine the binding capacity of the ISVD clones, crude periplasmic extract was screened using the xMAP technology (Luminex™). Different regions of MagPlex®-C MICROSPHERES beads (Luminex®) were functionalized with recombinant human, cynomolgus, or mouse ectodomain TfR1 protein and human ectodomain TfR2 protein. A bead mix containing 2000 beads of each region was added to each well of a 384-well flat bottom Nunc plate (ThermoFisher Scientific, 262160) and periplasmic extracts added to each well. ISVD proteins were detected using anti-FLAG-PE (BioLegend, 637310) and read-out for each bead region was done using the FLEXMAP 3D (Luminex™).

Alternatively, crude periplasmic extracts were screened in an ELISA setup. In short, 384-well flat clear bottom microplates (Corning, 3765) were coated with human or mouse ectodomain TfR1 protein. After a blocking step, the periplasmic extracts were added and bound ISVD protein was detected using mouse anti-FLAG-HRP (Sigma, A8592). After washing, QuantaBlu™ substrate (ThermoScientific, 15169) was added and fluorescence at 420 nm with excitation at 325 nm was measured using the CLARIOstar microplate reader (BMG Labtech).

6.4 Example 4: Expression and purification of anti-TfR1 monovalent ISVD clones

FLAG-6xHis tagged anti-TfR1 ISVD clones were selected for expression and purification. The ISVD sequences are shown in Table 3. A 3x FLAG-tag (1x FLAG tag: DYKDDDDK, SEQ ID NO: 152) followed by a 6x His-tag (HHHHHH, SEQ ID NO: 153) were added C-terminally via an AAA linker.

Table 3: ID and amino acid sequence of ISVD tested.

ISVD	Amino acid sequence
T0281007D02	EVQLVESGGGLVQPGGSLRLSCAASGFIISGYWMYWVRQAPGKGLE RVAVIDTDGAFTYYTNSVKGRFTISRDNAKNAVYLQMNSLQPEDTA VYYCHANGDLRWPRDRSSGYWGQGTLVTVSS (SEQ ID NO: 5)
T0281012D06	EVQLVESGGGLVQPGGSLRLSCAASGFTLDYYAIAWFRQAPGKERE GVSCISINSSPTRYLGSVKGRFTISRDNAKNTVYLQMNSLKPEDTAIY YCAARNAYDYCIDYERSEYRYWGQGTLVTVSS (SEQ ID NO: 22)

T0281005A01	EVQLVESGGGLVQPGGSLRLSCAASGISISGETMYWYRQAPGSQREF VAWITGGDNTRYGDSVKGRFTISSDNAKNTVFLQMNSLKPEDTAVY YCKDSRFWGQGTLVTVSS (SEQ ID NO: 23)
T0281001C08	EVQLVESGGGLVQPGGSLRLSCVASGSTSSINTMYWYRQAPGKQRE WVAWSTRGDNTNYAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAI YYCKGDVYWGQGTLVTVSS (SEQ ID NO: 24)
T0281002A09	EVQLVESGGGLVQPGGSLRLSCVASGDTSSINTVYWYRQAPGKQRE WVAWSTRGDNTNYAPSVKGRFTISRDDAKNSVYLQMNDLKPEDTAI YYCKTDVHWGQGTLVTVSS (SEQ ID NO: 25)
T0281008E07	EVQLVESGGGLVQPGESLRLSCAVSGSGGSINTMYWYRQAPGKQRE WVAWVTGGDSTSYAHSVKGRFTISTDNDKNNMYLQMNSLKPEDTA VYYCNADGIYWGQGTLVTVSS (SEQ ID NO: 26)
T0281001D02	EVQLVESGGGLVQPGGSLRLSCVASGSTSVINTMYWYRQAPGKQRE WVAWITGGDNTNYASSVKGRFTISGDNAKNSVYLQMNDLKPEDTAI YYCKTDTYWGQGTLVTVSS (SEQ ID NO: 3)
T0281002F11	EVQLVESGGGLVQPGGSLRLSCAASGTGLDIYLTRWYRQAAEKSRE LVAGVAHGGRTNYADFVKGRFTISRDNTKNTVYLQMDSLKPEDTA VYYCNVLEYSGSQYWGQGTLVTVSS (SEQ ID NO: 4)
T02810023B05	EVQLVESGGGLVQPGGSLRLSCVASGSTSSINTMYWYRQAPGKQRE WVAWSTRGDNTNYAPSVKGRFTMSRDDAKNSVYLQMNDLKPEDT AIYYCKTDVDWGQGTLVTVSS (SEQ ID NO: 2)
T02810030F12	EVQLVESGGGLVQPGGSLRLSCAASGTGLDIYLTRWYRQAAEKSRE LVAGVAHGGRTNYADFVKGRFTISRDNTKNTVYLQMDSLKPEDTA VYYCNVLEFSGSQYWGQGTLVTVSS (SEQ ID NO: 6)

Monovalent ISVD molecules were cloned in an expression vector and expressed in *E. coli* TG1 cells as 3xFLAG, His6-tagged proteins. *E. coli* cells were grown in "ZYM-5052" auto-induction medium (2 hours at 37°C followed by 29 hours at 30°C). After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets and resuspension in dPBS. These extracts were used as starting material for immobilized metal affinity chromatography (IMAC) using High Affinity Ni-Charged resins (Genscript, L00223) with 0.2M Na acetate pH 4 as elution buffer followed by a desalting step with PD columns with Sephadex G25 resin (GE Healthcare, 28918008).

Monovalent GGCGGS (SEQ ID NO: 154) fused anti-TfR1 ISVD clones with C-tag (EPEA, SEQ ID NO: 155) were expressed in Expi293F cells (Invitrogen/Life Technologies, A14527) after being cloned into pcDNA3.4 expression vectors. Materials required for transfection included OptiMEM (Invitrogen/Life Technologies, 31985062) and Expi293F transfection kit reagents (ExpiFectamine and Enhancers; Invitrogen/Life Technologies, A14524).

Expi293F cells were diluted to a density of 1.8 million cells/mL the day before transfection using fresh Expi293F expression media (Invitrogen/Life Technologies, A14351). The cells were then allowed to grow overnight (37°C, 8% CO_2, 110-120 rpm). On the day of transfection, the cells were diluted to 2.5 million cells/mL. ExpiFectamine™ was diluted in Opti-MEM™ and incubated at room temperature for 5 min. For each clone, the DNA was diluted in Opti-MEM™ and added to the ExpiFectamine™/ Opti-MEM™ solution. The final solution of DNA/ ExpiFectamine™ in Opti-MEM™ was incubated at room temperature for 10-20 min before being added to the cells. The transfected cultures were incubated at 37°C and 8% CO_2 while shaking at 110-120 rpm. One day post transfection, Enhancers 1 and 2 were added. The cells were harvested 4-5 days post transfection by spinning down at 3000 rpm for 20 min at room temperature. The resulting supernatant was filtered and used as the starting material for purification. The ISVD clones were first purified by C-Tag-affinity chromatography followed by size exclusion chromatography.

6.5 Example 5: Thermal shift assay (TSA)

The thermal stability of monovalent ISVD proteins was assessed by the thermal shift assay (TSA). In short, the TSA was performed in a 96-well plate and per well 5 µL of ISVD protein sample (0.8 mg/mL in D-PBS) was added to 5 µL of Sypro™ Orange (40× in Milli-Q® water; Invitrogen, Cat. No. S6551) and 10 µL of buffer (100 mM phosphate, 100 mM borate, 100 mM citrate and 115 mM NaCl with a pH ranging from 4 to 9). A temperature gradient (37 to 99°C at a rate of 0.03°C/s) was applied, which induced unfolding of the ISVD proteins, and hence exposure of hydrophobic patches. Binding of Sypro™ Orange to those hydrophobic patches, caused increase in fluorescence intensity, which was measured on a qPCR machine (LightCycler 480II, Roche) (Ex/Em = 465/580 nm). The inflection point of the first derivative of the fluorescence intensity curve at pH 7 served as a measure of the melting temperature (Tm) that are summarized in **Table 4**.

6.6 Example 6: Oligomerization assay

Oligomerization propensity of monovalent ISVD proteins under stressed conditions (1 week at 45 °C) was investigated by analytical size exclusion chromatography (SE-HPLC). For this, ISVD

proteins with a 3xFLAG-His6 tag, produced in *E. coli*, purified via IMAC followed by preparative SEC, filtered (0.22 µm), at a concentration of 1 mg/mL (D PBS) were used. The SE-HPLC profiles of two 100 µL aliquots were compared: one sample was incubated for 1 week at -20 °C and the other sample for 1 week at 45 °C. Samples were cleared by centrifugation for 5 minutes at 20000 RCF and subsequently analysed on a Waters Xbridge column. The difference in relative pre-peak areas of stressed (+45 °C) and non-stressed samples (-20 °C) was calculated and reported as Δ% oligo (= % oligo 1W 45°C - % oligo T0) in **Table 4**.

Table 4: Summary of thermal shift assay and oligomerization assay data

ISVD	Delta % Oligo (1w at 45°C)	Tm pH 7.0 (°C)
T0281001D02	0	64
T0281002F11	0	66
T0281007D02	0	84
T0281030F12	0	ND
T0281023B05	0	67

6.7 Example 7: Binding affinity of purified anti-TfR1 ISVD molecules to human TfR1 at pH 7.4 and pH 6.0

The binding affinity (K_D value) for the human TfR1 binding of the FLAG-6xHis tagged anti-TfR1 ISVDs of the invention was determined at neutral and low pH values (7.4 and 6.0) using Meso Scale Discovery (MSD) technology. A dilution series of recombinant human TfR1 ectodomain protein was incubated with a fixed concentration of purified anti-TfR1 ISVD molecules in 1x PBS + 1% BSA (either pH 7.4 or pH 6.0) assay buffer for 48 hours at 25°C. Afterwards the ISVD-TfR1 mixtures were applied over MSD GOLD 96-well Small Spot Streptavidin SECTOR Plate (Meso Scale Discovery, L45SA-1) that were blocked using MSD Blocker A Kit (Meso Scale Discovery, R93AA-1) and coated with 1µg/ml biotinylated human ectodomain TfR1 protein. After 10 minutes of incubation the plates were washed and unbound ISVD molecules were detected using in house Sulfo tagged (Sulfo kit: GOLD MSD SULFO-TAG NHS Ester (Meso Scale Discovery, cat#R91AO-1)) ANTI-FLAG® M2 (Sigma-Aldrich, F3165) and MSD GOLD Read Buffer (Meso Scale Discovery, R92TG-2) with a read-out on a MESO QuickPlex SQ 120 device. MSD data for binding to human TfR1 at pH 7.4 and 6.0 is depicted in figure 1 and K_D values summarized in **Table 5**.

Table 5: Overview MSD determined KD values for binding of ISVD clones to hTfR1 at pH 7.4 and 6.0. Some clones show a show a reduced K_D at pH 6.0 but no sigmoidal fit could be obtained.

ISVD	K_D hTfR1 pH7.4 (M)	K_D hTfR1 pH6.0 (M)	Ratio K_D pH6.0/pH7.4
T0281008E07	5.6E-11	1.2E-08	214
T0281030F12	5.9E-09	No fit possible	-
T0281023B05	1.5E-09	3.0E-07	200
T0281001C08	3.3E-08	No fit possible	-
T0281001D02	4.1E-10	3.7E-08	90
T0281002A09	6.3E-10	No fit possible	-
T0281002F11	2.3E-10	No fit possible	-
T0281005A01	4.5E-10	No fit possible	-
T0281007D02	5.5E-10	No fit possible	-
T0281012D06	5.5E-09	1.6E-08	3

The dissociation rate constant (k_{off}) from human TfR1 was determined for each anti-TfR1 ISVD molecule (GGCGGS (SEQ ID NO: 154) fused with C-tag) by Bio-Layer Interferometry (BLI) using an Octet® HTX system (Sartorius). Biotinylated human TfR1 was immobilized on streptavidin (SA) biosensors (Sartorius, 18-5019) in HBS-P+ buffer (10mM HEPES, 150mM NaCl, 0.05 % P20, pH 7.4; Cytiva, BR100671). After loading (120 s) and baseline establishment (60 s), the biosensors were dipped into different concentrations (ranging from 7.81 nM – 500 nM) of the anti-TfR1 ISVD molecules diluted into HBS-P+ buffer for the association phase (80-90 s). The association phase was followed by a dissociation phase (180 s) that was either executed at pH 7.4 or pH 6.0. The koff for each molecule at pH 7.4 was determined by using a 1:1 binding model to globally fit the response curves for both the association and dissociation phases. The koff for each molecule at pH 6.0 was determined by a 1:1 binding model to locally fit the dissociation phase of each response curve. The koff at pH 6.0 was then calculated by taking the average of the koff values at each anti-TfR1 ISVD concentration for each analyte/ligand pair. The values for koff from both pH 6.0 and pH 7.4 dissociation measurements are reported in **Table 6**. Increases in koff for dissociation at pH 6.0 compared to pH 7.4 indicate the pH dependent nature of anti-TfR1 ISVD molecules when binding to human TfR1.

Table 6: Dissociation rates (koff) determined for anti-TfR1 ISVD molecules (GGCGGS (SEQ ID NO: 154) fused with C-tag) at pH 7.4 and pH 6.0.

ISVD ID	koff hTfR1 at pH 7.4 (1/s)	koff hTfR1 pH 6.0 (1/s)	Ratio koff pH6.0/pH7.4
T0281007D02	3.43E-04	8.79E-02	256
T0281012D06	4.83E-03	7.74E-02	16
T0281005A01	1.62E-03	1.12E-01	69

T0281001C08	1.59E-03	1.42E-01	89
T0281002A09	7.70E-04	1.44E-01	187
T0281008E07	6.18E-04	1.09E-01	176
T0281001D02	1.07E-03	1.07E-01	100
T0281002F11	1.27E-03	1.13E-01	89

6.8 Example 8: Binding affinity of purified anti-TfR1 ISVD molecules to human, cynomolgus, and mouse TfR1

The kinetic values (ka, koff, and K_D) for each anti-TfR1 ISVD molecule (GGCGGS (SEQ ID NO: 154) fused with C-tag) were determined for binding against human, cynomolgus, and mouse TfR1 by Bio-Layer Interferometry (BLI) using an Octet® HTX system (Sartorius). Biotinylated TfR1 from each species was immobilized on streptavidin (SA) biosensors (Sartorius, 18-5019) in HBS-P+ buffer (10mM HEPES, 150mM NaCl, 0.05 % P20, pH 7.4; Cytiva, BR100671). After loading (120 s) and baseline establishment (60 s), the biosensors were dipped into different concentrations (ranging from 3.91 nM – 1000 nM) of the anti-TfR1 ISVD molecules diluted into HBS-P+ buffer for the association phase (90 s). The association phase was followed by a dissociation phase (180 s) in HBS-P+ buffer. The kinetic values for each anti-TfR1 ISVD molecule against each species of TfR1 were determined by using a 1:1 binding model to globally fit the response curves for both the association and dissociation phases. Fig. 2 shows an exemplary response curve for T0281007D02. All kinetic values are reported below in **Table 7**.

Table 7: Kinetic values for anti-TfR1 ISVD molecules binding against human, cynomolgus, and mouse TfR1 at pH 7.4.

	Human TfR1			**Cynomolgus TfR1**			**Mouse TfR1**		
ISVD ID	**ka (1/Ms)**	**koff (1/s)**	**K_D (M)**	**ka (1/Ms)**	**koff (1/s)**	**K_D (M)**	**ka (1/Ms)**	**koff (1/s)**	**K_D (M)**
T0281007D02	4.4E+05	8.7E-04	**2.0E-09**	3.4E+05	7.3E-03	**2.1E-08**	5.0E+05	3.7E-03	**7.4E-09**
T0281012D06	2.4E+05	1.8E-03	**7.3E-09**	2.4E+05	1.3E-03	**5.4E-09**	2.8E+05	1.3E-02	**4.9E-08**
T0281005A01	2.7E+05	4.7E-04	**1.7E-09**	1.5E+05	2.7E-03	**1.8E-08**	1.1E+05	5.8E-02	**5.2E-07**
T0281001C08	5.5E+04	1.8E-04	**3.3E-09**	8.7E+04	9.6E-04	**1.1E-08**	6.7E+04	6.2E-03	**9.2E-08**
T0281002A09	9.2E+04	5.7E-04	**6.2E-09**	1.0E+05	1.3E-03	**1.3E-08**	1.0E+05	6.2E-03	**6.2E-08**
T0281008E07	1.1E+05	2.6E-04	**2.3E-09**	7.0E+03	1.7E-03	**2.4E-07**	4.8E+04	2.2E-03	**4.5E-08**
T0281001D02	1.6E+05	9.3E-05	**6.0E-10**	6.8E+04	1.6E-03	**2.4E-08**	7.3E+04	6.5E-03	**8.9E-08**
T0281002F11	3.6E+05	7.7E-04	**2.2E-09**	2.9E+05	4.5E-03	**1.6E-08**	4.4E+05	1.6E-02	**3.6E-08**

T02810023B05	9.4E+04	3.4E-05	**3.7E-10**	9.3E+04	4.3E-04	**4.6E-09**	1.0E+05	2.1E-03	**2.1E-08**

Cross-species target reactivity was assessed by measuring the binding to human, cynomolgus and mouse TfR1. Single-cycle kinetics data for the anti-TfR1 ISVD molecules were also obtained on the Biacore 8K instrument (Cytiva, 29722782) using the Biotin CAPture Kit (Cytiva, 28920234). In short, Biotin CAPture reagent was loaded on the CAP sensor chip surface to capture about 600-900 RU of biotinylated human, cynomolgus or mouse TfR1 recombinant ectodomain protein. Single-cycle kinetics data was obtained by injecting increasing concentrations of anti-TfR1 ISVD molecule (from 2.46 to 1500 nM) over the chip surface with 120 seconds contact time and a final 600 second dissociation time. In between different ISVD clones the chip surface was regenerated using 8 M GuHCl/1 M NaOH (3:1).

Evaluation of the single-cycle binding curves was done using the Biacore Insight Evaluation Software (Cytiva, version 5.0.18.22102. Kinetic analysis was performed by fitting a 1:1 interaction model (Langmuir binding) (Rmax = global; RI = constant = 0, drift = constant = 0). Kinetic data for binding towards human, cynomolgus, and mouse TfR1 are shown in **Table 8**.

Table 8: Single-cycle kinetic parameters for binding of anti-TfR1 ISVD clones to human, cynomolgus, or mouse TfR1 at pH 7.4.

	Human TfR1			**Cynomolgus TfR1**			**Mouse TfR1**		
ISVD ID	**ka (1/Ms)**	**kd (1/s)**	**K_D (M)**	**ka (1/Ms)**	**kd (1/s)**	**K_D (M)**	**ka (1/Ms)**	**kd (1/s)**	**K_D (M)**
T0281002F11	6.5E+05	7.3E-04	**1.1E-09**	4.4E+05	5.6E-03	**1.3E-08**	4.1E+05	1.4E-02	**3.5E-08**
T0281007D02	8.0E+05	5.8E-04	**7.3E-10**	5.0E+05	9.0E-03	**1.8E-08**	6.6E+05	4.3E-03	**6.5E-09**
T0281023B05	5.3E+04	7.1E-05	**1.3E-09**	6.4E+04	2.8E-04	**4.4E-09**	6.3E+04	2.0E-03	**3.2E-08**
T0281030F12	6.0E+05	9.8E-03	**1.6E-08**	4.2E+05	3.9E-02	**9.2E-08**	2.7E+05	4.2E-02	**1.6E-07**

6.9 Example 9: Binding of purified anti-TfR1 ISVD molecules to mouse pre-B cells, parental or overexpressing human or cynomolgus TFR1, and HEK293 cells

Binding of purified ISVD proteins to mouse pre-B cells, either parental or overexpressing human or cynomolgus TfR1, and HEK293T cells was determined using flow cytometry. HEK293T cells show endogenous expression of human TfR1, and parental mouse pre-B cells show endogenous expression of mouse TfR1. In short, $4x10^4$ cells per well were seeded in 384-well Bio-One V-

bottom plates (Greiner, 781280). Serial dilutions of 3xFLAG-His6 tagged ISVD proteins were added and incubated for 30 minutes at 4 °C. Detection was performed using anti-FLAG-BV421 (BioLegend, 637322) and PI (Sigma-Aldrich, P4170) was used as dead stain to gate out living cells. Cell suspensions were analyzed with iQue® 3 (Intellicyt) and EC50 values estimated by dose response modelling using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.). The results are shown in **Table 9**.

Table 9: Overview of EC50 values monovalent ISVD proteins binding to mouse pre-B cells (parental, expressing human TfR1 or cynomolgus TfR1) and HEK293T cells.

ISVD ID	EC50 binding to mouse pre-B cells expressing human TfR1 (M)	EC50 binding to mouse pre-B cells expressing cynomolgus TfR1 (M)	EC50 binding to mouse pre-B cells (M)	EC50 binding to HEK293T cells (M)
T0281006B04	7.1E-09	9.0E-09	2.7E-08	3.6E-09
T0281008E07	5.1E-09	1.9E-08	Incomplete DRC	3.2E-09
T0281005A01	3.1E-09	9.4E-09	Incomplete DRC	1.8E-09
T0281002F11	3.2E-09	7.9E-09	3.8E-09	1.5E-09
T0281007D02	3.3E-09	3.0E-08	7.6E-09	1.1E-09
T0281001D02	5.3E-09	1.7E-08	Incomplete DRC	3.0E-09
T0281012D06	7.5E-09	2.9E-09	Incomplete DRC	4.5E-09
T0281030F12	Incomplete DRC	Incomplete DRC	Incomplete DRC	Incomplete DRC
T0281023B05	7.5E-09	8.6E-09	weak binding	3.3E-08

DRC: dose response curve

6.10 Example 10: Internalization of anti-TfR1 ISVD clones in HEK293T cells

To select anti-TfR1 ISVD clones displaying good internalization behavior in TfR1 expressing target cells a HEK293T based characterization assay in flow cytometry was setup (HEK293T cells show endogenous expression of human TfR1). In short, purified 3FLAG-HIS6 anti-TfR1 ISVD clones were titrated out over HEK293T cells (seeded at 1.5E04 cells/well) together with 500 nM mouse anti-FLAG mAb (clone M2) (Sigma, F1804) labeled in house with pHAb Amine Reactive Dye (Promega, G9841 0000512694). pHAb is a pH sensitive dye that has very low fluorescence at neutral pH and show an increase in fluorescence as the pH of the solution becomes acidic. After an incubation of 4 hours the cells were washed and DAPI (BD Biosciences, 564907) was added as dead stain to gate out living cells, subsequently a flow cytometry readout on the iQue® 3 was

performed. Resulting internalization graphs are shown in figure 3A with the top values for the fitted curves summarized in **Table 10**.

Table 10: Summary of top values for the fitted curves of the internalization data shown in Figure 3A.

ISVD ID	Top value
T0281001D02	231555
T0281002F11	506339
T0281005A01	292843
T0281007D02	255289
T0281008E07	180911
T0281012D06	491854
T0281023B05	216858
T0281030F12	729687

To confirm the internalization behavior, an Incucyte SX5® (Sartorius) based read-out was performed in real time for selected clones. Similar, HEK293T cells (seeded at $2x10^4$ cells/well) were plated and allowed adherence overnight. After that, purified 3FLAG-HIS6 anti-TfR1 ISVD clones were titrated out over together with 500 nM mouse anti-FLAG mAb (clone M2) (Sigma, F1804) pHAb Amine Reactive Dye labeled in house (Promega, G9841 0000512694). Imaging acquisition was performed with the Incucyte SX5® (Sartorius) every hour (37°C, 5% CO_2) at 10x magnification. The analysis was performed at selected time-points by using the AI-driven Confluence Analysis (in which the orange-fluorescence from pHAb is masked and normalized to the total of confluency seen in Phase Contrast images). The Orange (positive cells, showing pHAb internalization)/Phase area (monolayer, total of cells per field of view) is plotted for each compound in figure 3B.

In correspondence with data above (see Fig. 3A), the Orange area / Phase area for T0281030F12 is the highest of all tested ISVD compounds.

6.11 Example 11: Competition of monovalent anti-TfR1 ISVD molecules against human transferrin

Purified anti-TfR1 ISVD clones were tested for competition against the human TfR1 ligand transferrin (Tf). EC30 concentration of recombinant biotinylated human Tf (Sigma-Aldrich, T3915) was co-incubated with a titration series of each ISVD molecule before adding them to HEK293 cells. After 90 minutes incubation the cells were washed and binding of the Tf ligand

was detected with streptavidin-PE (BD – Pharmingen, 554061). Cell suspensions were analyzed with iQue Screener PLUS 3 (Intellicyt) and dose response modelling was performed using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.). **Table 11** shows an overview if competition was observed.

Schild analysis of Tf binding in presence of high concentrations of ISVD protein demonstrated no significant impact on Tf binding. A non-TfR1 binding (IRR00028) ISVD and a TfR1 inhibiting T028106B04 ISVD served as negative and positive ctrl, respectively. In short, human transferrin was titrated out on HEK293T cells in absence and presence of EC30, 10xEC50, and 100xEC50 concentrations of ISVD protein (pre-mixing Tf and Nb). After 90 minutes incubation the cells were washed, binding of the Tf ligand was detected with streptavidin-PE (BD – Pharmingen, 554061) and DAPI (BD Biosciences, 564907) was used as dead stain to gate out living cells. Cell suspensions were analyzed with iQue Screener PLUS 3 (Intellicyt) and dose response modelling was performed using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.).

Significant shifts in EC50 value for the Tf binding to hTfR1 are observed upon increasing the concentration of the Tf competing T0281006B04 (respectively about 6-fold and 50-fold shifts to weaker Tf binding in presence of 10x or 100x EC50 of T0281006B04) while no shifts observed for the non-TfR1 binding ISVD. For both T0281047E03 and T0281007D02 no significant shifts in EC50 values are observed (respectively 0.9-fold and 1.3-fold difference when working in presence of 100xEC50 T0281047E03 or T0281007D02).

Table 11: Summary of competition profile of anti-TfR1 ISVD clones with human Tf.

	cFACS	Schild Analysis
T0281030F12	Limited	ND
T0281023B05	No	ND
T0281001D02	Limited	No
T0281002F11	Limited	ND
T0281005A01	No	ND
T0281007D02	Limited	No
T0281008E07	Limited	ND
T0281012D06	Limited	ND
T0281047E03	No	No
IRR00028 (negative control)	No	No
T0281006B04 (positive control)	Yes	Yes

6.12 Example 12: Epitope binning of monovalent anti-TfR1 ISVD molecules on HEK293T cells

Epitope binning was performed for C-tagged anti-TfR1 ISVD molecules (see Example 4) with bio-layer interferometry (BLI) using an Octet® HTX system (Sartorius). Biotinylated human TfR1 at 10 nM was captured on streptavidin (SA) biosensors (Sartorius, 18-5019) in HBS-P+ buffer (10mM HEPES, 150mM NaCl, 0.05 % P20, pH 7.4; Cytiva, BR100671). A baseline signal (60 s) was established after loading and capture of the first anti-TfR1 ISVD molecule was performed to saturate the available TfR1 binding positions (300 s with 400 nM molecule). Another baseline step (60 s) was completed followed by a second anti-TfR1 ISVD molecule capture (300 s with 400 nM molecule). A final dissociation step (600 s) was then performed. Competition was determined by the absence of a significant difference in RU level during the second anti-TfR1 ISVD molecule capture step (see figure 5 for exemplary curves). On the other hand, a significant increase in RU level indicated that the anti-TfR1 ISVD molecules targeted different epitopes. Conclusions from the binning data are reported below in **Table 12**.

Table 12: Binning of anti-TfR1 ISVD clones on immobilized human TfR1 using BLI*.

C-tag formats:	T0281007D02	T0281012D06	T0281005A01	T0281001C08	T0281002A09	T0281008E07	T0281006B04	T0281001D02	T0281002F11
T0281007D02	S	C	C	C	C	C	NC	C	C
T0281012D06	C	S	C	C	C	C	A	C	C
T0281005A01	C	C	S	C	C	C	NC	C	C
T0281001C08	C	C	C	S	C	C	NC	C	C
T0281002A09	C	C	C	C	S	C	NC	C	A
T0281008E07	C	C	C	C	C	S	NC	C	C
T0281006B04	NC	NC	NC	NC	NC	NC	S	NC	NC
T0281001D02	C	C	C	C	C	C	NC	S	C
T0281002F11	C	C	C	C	C	C	NC	C	S

*C = competing; NC = non-competing; A = ambiguous; S = self

Purified FLAG-His tagged anti-TfR1 ISVD clones were binned against GGC tagged ISVD formats to assess their direct competition profile. In short, GGC tagged ISVD clones were titrated out over HEK293T cells, followed by addition of 3xFLAG-His6 tagged ISVD formats at EC30 concentration. Detection was performed using anti-FLAG-BV421 (BioLegend, 637322). Cell suspensions were analyzed with iQue® 3 (Intellicyt) and EC50 values estimated by dose response modelling using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.). **Table 13** shows an overview if competition was observed.

Table 13: Binning of anti-TfR1 ISVD clones on HEK293T cells.

FLAG-His formats:	Competition against GGC tagged T0281030F12?	Competition against GGC tagged T0281007D02?	Competition against GGC tagged T0281023B05?
T0281002F11	Yes	Yes	Yes
T0281007D02	Yes	Yes	Yes
T0281023B05	Yes	Yes	Yes

6.13 Example 13: Generation of GGC tagged anti-TfR1 formats with 3xFLAG-HIS6 tag

Selected anti-TfR1 ISVD clones were formatted as glycine-glycine-cysteine (GGC) tagged ISVD formats to allow covalent conjugation of oligonucleotides. GGC tagged ISVD formats were expressed as 3xFLAG3-HIS6-tagged protein in *E. coli* (amino acid sequences are shown in **Table 14**. *E. coli* cells were grown in "5052" auto-induction medium (2 hours at 37°C followed by 29 hours at 30°C). After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets and resuspension in dPBS. These extracts were used as starting material for immobilized metal affinity chromatography (IMAC) using High Affinity Ni-Charged resins (Genscript, L00223) with 0.2M Na acetate pH 4 followed by a desalting step via Size exclusion chromatography.

Table 14: Description, and amino acid sequence of GGC tagged anti-TfR1 ISVD formats.

ISVD ID	ISVD Description	Amino acid sequence
A045300007	T0281007D02-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCAASGFII SGYWMYWVRQAPGKGLERVAVIDTDG AFTYYTNSVKGRFTISRDNAKNAVYLQ MNSLQPEDTAVYYCHANGDLRWPRDR SSGYWGQGTLVTVSSAAAGGCDYKDH DGDYKDHDIDYKDDDDKGAAHHHHHH (SEQ ID NO: 156)
A045300008	T0281012D06-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCAASGFT LDYYAIAWFRQAPGKEREGVSCISINSSP TRYLGSVKGRFTISRDNAKNTVYLQMN SLKPEDTAIYYCAARNAYDYCIDYERSE YRYWGQGTLVTVSSAAAGGCDYKDHD GDYKDHDIDYKDDDDKGAAHHHHHH (SEQ ID NO: 157)
A045300009	T0281005A01-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCAASGISI SGETMYWYRQAPGSQREFVAWITGGD NTRYGDSVKGRFTISSDNAKNTVFLQM NSLKPEDTAVYYCKDSRFWGQGTLVTV SSAAAGGCDYKDHDGDYKDHDIDYKD DDDKGAAHHHHHH (SEQ ID NO: 158)
	T0281008E07-GGC-FLAG3-His6	EVQLVESGGGLVQPGESLRLSCAVSGSG GSINTMYWYRQAPGKQREWVAWVTGG DSTSYAHSVKGRFTISTDNDKNNMYLQ

		MNSLKPEDTAVYYCNADGIYWGQGTL VTVSSAAAGGCDYKDHDGDYKDHDID YKDDDDKGAAHHHHHH (SEQ ID NO: 159)
A045300013	T0281001D02-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCVASGST SVINTMYWYRQAPGKQREWVAWITGG DNTNYASSVKGRFTISGDNAKNSVYLQ MNDLKPEDTAIYYCKTDTYWGQGTLVT VSSAAAGGCDYKDHDGDYKDHDIDYK DDDDKGAAHHHHHH (SEQ ID NO: 160)
A045300014	T0281002F11-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCAASGT GLDIYLTRWYRQAAEKSRELVAGVAHG GRTNYADFVKGRFTISRDNTKNTVYLQ MDSLKPEDTAVYYCNVLEYSGSQYWG QGTLVTVSSAAAGGCDYKDHDGDYKD HDIDYKDDDDKGAAHHHHHH (SEQ ID NO: 161)
A045300028	T0281023B05-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCVASGST SSINTMYWYRQAPGKQREWVAWSTRG DNTNYAPSVKGRFTMSRDDAKNSVYLQ MNDLKPEDTAIYYCKTDVDWGQGTLV TVSSAAAGGCDYKDHDGDYKDHDIDY KDDDDKGAAHHHHHH (SEQ ID NO: 162)
A045300038	T0281030F12-GGC-FLAG3-His6	EVQLVESGGGLVQPGGSLRLSCAASGT GLDIYLTRWYRQAAEKSRELVAGVAHG GRTNYADFVKGRFTISRDNTKNTVYLQ MDSLKPEDTAVYYCNVLEFSGSQYWGQ GTLVTVSSAAAGGCDYKDHDGDYKDH DIDYKDDDDKGAAHHHHHH (SEQ ID NO: 163)

6.14 Example 14: Conjugation of GGC tagged ISVD formats with oligonucleotide

GGC tagged ISVD formats were conjugated to Succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)-activated oligonucleotides and the resulting conjugates purified by ion exchange chromatography. In short, reduction of GGC-ISVD dimers was performed by adding a molar excess of TCEP for 3.5h at room temperature. The excess TCEP was removed by desalting using 2mL Zeba desalting columns (Thermo Scientific, 89889). Subsequently, ~~a~~ molar excess of the oligonucleotide was added. To remove excess oligonucleotide

and unreacted ISVD formats, an AEX is performed. Selected fractions were desalted with 2mL Zebaspin columns (Thermo Scientific, 89889). Final concentration of the ISVD-oligonucleotide formats was determined by performing a BCA assay and quality assessed by mass spectrometry and SDS-PAGE.

6.15 Example 15: No impact of oligonucleotide conjugation on hTfR1 binding

The impact of covalent oligonucleotide conjugation to the GGC tagged ISVD formats on TfR binding was verified by assessing the binding to HEK293T cells. In short, $4x10^4$ cells per well were seeded in 384-well Bio-One V-bottom plates (Greiner, 781280). Serial dilutions of 3xFLAG-His6 tagged ISVD proteins or GGC fused, 3xFLAG-His6 tagged ISVD proteins were added and incubated for 30 minutes at 4 °C. Detection was performed using anti-FLAG-PE (BioLegend, 637310) and DAPI (BD Biosciences, 564907) used as dead stain to gate out living cells. Cell suspensions were analyzed with iQue® 3 (Intellicyt) and EC50 values estimated by dose response modelling using 4 parameter logistic regression in GraphPad (GraphPad Software Inc.).

Figure 6 shows similar TfR binding profiles for both unconjugated and conjugated formats.

6.16 Example 16: Generation of untagged anti-TfR1 formats

Selected anti-TfR1 ISVD clones were formatted as either untagged formats or untagged glycine-glycine-cysteine (GGC) fused ISVD formats, either monovalent or combined with an anti-serum albumin (SA) VHH building block ALB23002 for half-life extension (amino acid sequences are shown in Table 15).

ISVD formats A045300062, A045300063 and A045300072 were produced in *Komagataella phaffii* at 2 L or 5 L scale using a general fed-batch methanol-free fermentation process as previously described in De Groeve et al. (2023): Optimizing expression of Nanobody® molecules in Pichia pastoris through co-expression of auxiliary proteins under methanol and methanol-free conditions. Microb Cell Fact 22, 135, hereby incorporated by reference in its entirety. The temperature in the bioreactor was controlled at 30°C, dissolved oxygen at 30% and pH at 6.0 during the ISVD production phase. Expression of the ISVD molecules was derepressed by addition of an 80% (w/w) glycerol feed for 80-96 hours at a limiting and decreasing feeding rate (at start of derepression phase: 15 g/h/L initial volume, 4.5 h after start: 8 g/h/L, 9 h after start: 4 g/h/L, 62 h after start until end of fermentation: 2 g/h/L).

The harvest is pH adjusted to pH 7.0 + 0.2. The harvest is clarified via microfiltration to remove cells and cell debris using tangential flow filtration with a nominal molecular weight cut off of 0.2µm, Hydrosart (Sartorius 3081860702W—SW). The ISVD is then purified with Protein A chromatography. The eluate is then pH adjusted depending on the pI of the molecule to allow for subsequent binding on ion exchange chromatography. The ISVD formats are further purified to remove any truncated and self-associated forms by ion exchange chromatography with elution of the target by salt gradient. Fractions of interest are then pooled and up concentrated via vivaspin spin column of appropriate nominal molecular weight cutoff to allow for further purification via size exclusion chromatography. The size exclusion chromatography is to further purify the ISVD format from residual levels of truncated and self-associated formats and to exchange into D-PBS. To reduce the chance of endotoxin an optional filtration with a Mustang E filter can be performed (Cytiva MSTG25E3) and finally 0.22 µm filtered prior to storage at ≤-20°C.

Table 15: ISVD ID, description, and amino acid sequence of untagged GGC fused anti-TfR1 ISVD formats.

ISVD ID	**ISVD Description**	**Amino acid sequence**
A045300063	T0281007D02(E1D)	DVQLVESGGGLVQPGGSLRLSCAASGFIIS GYWMYWVRQAPGKGLERVAVIDTDGAF TYYTNSVKGRFTISRDNAKNAVYLQMNS LQPEDTAVYYCHANGDLRWPRDRSSGY WGQGTLVTVSS (SEQ ID NO: 164)
A045300062	T0281007D02(E1D)-35GS-ALB23002	DVQLVESGGGLVQPGGSLRLSCAASGFIIS GYWMYWVRQAPGKGLERVAVIDTDGAF TYYTNSVKGRFTISRDNAKNAVYLQMNS LQPEDTAVYYCHANGDLRWPRDRSSGY WGQGTLVTVSSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSGGGGSEVQLVESGG GVVQPGGSLRLSCAASGFTFRSFGMSWV RQAPGKGPEWVSSISGSGSDTLYADSVKG RFTISRDNSKNTLYLQMNSLRPEDTALYY CTIGGSLSRSSQGTLVTVSS (SEQ ID NO: 165)
A045300071	T0281007D02(E1D)-GGC	DVQLVESGGGLVQPGGSLRLSCAASGFIIS GYWMYWVRQAPGKGLERVAVIDTDGAF TYYTNSVKGRFTISRDNAKNAVYLQMNS LQPEDTAVYYCHANGDLRWPRDRSSGY WGQGTLVTVSSGGC (SEQ ID NO: 166)
A045300077	T0281023B05(E1D)-GGC	DVQLVESGGGLVQPGGSLRLSCVASGSTS SINTMYWYRQAPGKQREWVAWSTRGDN TNYAPSVKGRFTMSRDDAKNSVYLQMN

		DLKPEDTAIYYCKTDVDWGQGTLVTVSS GGC (SEQ ID NO: 167)
A045300073	T0281030F12(E1D)-GGC	DVQLVESGGGLVQPGGSLRLSCAASGTG LDIYLTRWYRQAAEKSRELVAGVAHGGR TNYADFVKGRFTISRDNTKNTVYLQMDS LKPEDTAVYYCNVLEFSGSQYWGQGTLV TVSSGGC (SEQ ID NO: 168)
A045300093	T0281002F11(E1D)-GGC	DVQLVESGGGLVQPGGSLRLSCAASGTG LDIYLTRWYRQAAEKSRELVAGVAHGGR TNYADFVKGRFTISRDNTKNTVYLQMDS LKPEDTAVYYCNVLEYSGSQYWGQGTLV TVSSGGC (SEQ ID NO: 169)
A045300072	T0281007D02(E1D)-35GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCAASGFIIS GYWMYWVRQAPGKGLERVAVIDTDGAF TYYTNSVKGRFTISRDNAKNAVYLQMNS LQPEDTAVYYCHANGDLRWPRDRSSGY WGQGTLVTVSSGGGGSGGGGSGGGGSG GGGSGGGGSGGGGSGGGGSEVQLVESGG GVVQPGGSLRLSCAASGFTFRSFGMSWV RQAPGKGPEWVSSISGSGSDTLYADSVKG RFTISRDNSKNTLYLQMNSLRPEDTALYY CTIGGSLSRSSQGTLVTVSSGGC (SEQ ID NO : 170)
A045300587	T0281023B05(E1D)-9GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCVASGSTS SINTMYWYRQAPGKQREWVAWSTRGDN TNYAPSVKGRFTMSRDDAKNSVYLQMN DLKPEDTAIYYCKTDVDWGQGTLVTVSS GGGGSGGGSEVQLVESGGGVVQPGGSLR LSCAASGFTFRSFGMSWVRQAPGKGPEW VSSISGSGSDTLYADSVKGRFTISRDNSKN TLYLQMNSLRPEDTALYYCTIGGSLSRSS QGTLVTVSSGGC (SEQ ID NO: 171)
A045300582	T0281023B05(E1D)-35GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCVASGSTS SINTMYWYRQAPGKQREWVAWSTRGDN TNYAPSVKGRFTMSRDDAKNSVYLQMN DLKPEDTAIYYCKTDVDWGQGTLVTVSS GGGGSGGGGSGGGGSGGGGSGGGGSGG GGSGGGGSEVQLVESGGGVVQPGGSLRL SCAASGFTFRSFGMSWVRQAPGKGPEWV SSISGSGSDTLYADSVKGRFTISRDNSKNT LYLQMNSLRPEDTALYYCTIGGSLSRSSQ GTLVTVSSGGC (SEQ ID NO: 172)
A045300586	T0281030F12(E1D)-9GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCAASGTG LDIYLTRWYRQAAEKSRELVAGVAHGGR TNYADFVKGRFTISRDNTKNTVYLQMDS LKPEDTAVYYCNVLEFSGSQYWGQGTLV TVSSGGGGSGGGSEVQLVESGGGVVQPG GSLRLSCAASGFTFRSFGMSWVRQAPGK

		GPEWVSSISGSGSDTLYADSVKGRFTISRD NSKNTLYLQMNSLRPEDTALYYCTIGGSL SRSSQGTLVTVSSGGC (SEQ ID NO: 173)
A045300593	T0281030F12(E1D)-35GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCAASGTG LDIYLTRWYRQAAEKSRELVAGVAHGGR TNYADFVKGRFTISRDNTKNTVYLQMDS LKPEDTAVYYCNVLEFSGSQYWGQGTLV TVSSGGGGSGGGGSGGGGSGGGGSGGGG SGGGGSGGGGSEVQLVESGGGVVQPGGS LRLSCAASGFTFRSFGMSWVRQAPGKGPE WVSSISGSGSDTLYADSVKGRFTISRDNSK NTLYLQMNSLRPEDTALYYCTIGGSLSRS SQGTLVTVSSGGC (SEQ ID NO: 174)
A045300591	T0281002F11(E1D)-9GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCAASGTG LDIYLTRWYRQAAEKSRELVAGVAHGGR TNYADFVKGRFTISRDNTKNTVYLQMDS LKPEDTAVYYCNVLEYSGSQYWGQGTLV TVSSGGGGSGGGSEVQLVESGGGVVQPG GSLRLSCAASGFTFRSFGMSWVRQAPGK GPEWVSSISGSGSDTLYADSVKGRFTISRD NSKNTLYLQMNSLRPEDTALYYCTIGGSL SRSSQGTLVTVSSGGC (SEQ ID NO: 175)
A045300094	T0281002F11(E1D)-35GS-ALB23002-GGC	DVQLVESGGGLVQPGGSLRLSCAASGTG LDIYLTRWYRQAAEKSRELVAGVAHGGR TNYADFVKGRFTISRDNTKNTVYLQMDS LKPEDTAVYYCNVLEYSGSQYWGQGTLV TVSSGGGGSGGGGSGGGGSGGGGSGGGG SGGGGSGGGGSEVQLVESGGGVVQPGGS LRLSCAASGFTFRSFGMSWVRQAPGKGPE WVSSISGSGSDTLYADSVKGRFTISRDNSK NTLYLQMNSLRPEDTALYYCTIGGSLSRS SQGTLVTVSSGGC (SEQ ID NO: 176)

6.17 Example 17: Generation of asymmetric monovalent anti-TfR1 ISVD-IgG1 NNAS format

A selected anti-TfR1 ISVD clone was formatted as asymmetric monovalent anti-TfR1 ISVD-IgG1-Fc format with NNAS mutations using knob-in-hole technology. The ISVD-IgG1 NNAS formats was expressed in CHOEBNALT-85-1E9 cells (amino acid sequences for both chains are shown in **Table 16**). In brief, CHOEBNALT-85-1E9 cells were grown in CHO TF (Xell AG) medium and the ISVD-IgG1 NNAS format purified by protein A affinity chromatography, followed by a further purification-by size exclusion chromatography.

Table 16: Format ID, description and amino acid sequence of ISVD-IgG1 NNAS format.

ISVD ID	ISVD description	Amino acid sequence
T-0281-00_TP023	Asymmetric ISVD-IgG1-Fc NNAS format (knob-in-hole)	Chain 1: EVQLVESGGGLVQPGGSLRLSCAASGFIISGYWMYW VRQAPGKGLERVAVIDTDGAFTYYTNSVKGRFTISRD NAKNAVYLQMNSLQPEDTAVYYCHANGDLRWPRDR SSGYWGQGTLVTVSSDKTHTCPPCPAPELLGGPSVFL FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV DGVEVHNAKTKPREEQYNNASRVVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS CSVMHEALHNHYTQKSLSLSPG (SEQ ID NO: 177) Chain 2: DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYNNASRVVSVLTVLHQDWLNGKEYKCKVSNKAL PAPIEKTISKAKGQPREPQVCTLPPSRDELTKNQVSLS CAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS FFLVSKLTVDKSRWQQGNVFSCSVMHEALHNRFTQK SLSLSPG (SEQ ID NO: 178)

6.18 Example 18: ASO-mediated knockdown of Malat1 *in vivo*

To evaluate if certain moieties could be efficiently targeted to the brain, skeletal muscle, heart, and sciatic nerve, we assessed antisense oligonucleotides (ASO)-mediated knockdown of RNA transcripts of *Malat1*, a long non-coding RNA that localizes in the nucleus. For this study, the following reagents were prepared.

A045300072 was conjugated to SMCC linked ASO (sequence below). To generate A045300072-ASO conjugate, the ISVD was reduced with a molar excess TCEP for 3 hrs at room temperature, followed by TCEP removal over a HiPrep 26/10 desalting column. The reduced ISVD was then

mixed with molar excess mouse Malat1 ASO (GCATTCTAATAGCAGC; SEQ ID NO: 179) with an SMCC linker. Malat1 ASO sequence is shown below with modifications:

5'-
(SMCC)(NHC6)GbsCbsAbsdTsdTs(5MdC)sdTsdAsdAsdTsdAsdGs(5MdC)sAbsGbsCb-3'
(SEQ ID NO: 179)

Nb: LNA residues (including LNA-5MeC and LNA T/LNA-5MeU)
dN: DNA residues
(5MdC): 5-Methyl DNA C
s: phosphorothioate backbone modification
(NHC6): Aminohexyl linker
(SMCC): SMCC NHS ester

The conjugation progress was monitored with SDS-PAGE gel until completion, then the conjugate complex was purified from excess unreacted ASO using AEX chromatography followed by buffer exchange via SEC into PBS.

To evaluate tissue-specific knockdown of *Malat1* mRNA, hTfR-KI mice were dosed with four IV doses of 400 nmol/kg SMCC-Malat1 ASO alone (N=5), or 400 nmol/kg molar equivalent of ASO in the form of the ISVD-ASO conjugate (DAR1) (N=3) over two weeks. Doses were given at day 0, 3, 7 and 10. 3 days after the last dose, mice were euthanized with CO2 and transcardially perfused with cold PBS. Tissues (Gastrocnemius, heart, sciatic nerve, and brain) were harvested and weighted for quantitative PCR for Malat1, with β-Actin acting as house-keeping gene.

RNA was isolated from frozen tissue samples. Tissue was homogenized using a bead mill homogenizer in TRIzol/chloroform in 2 mL tubes containing 2.8 mm ceramic beads. Subsequently, RNA was isolated following Qiagen's RNAEasy Mini kit instructions. cDNA was generated using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor. Finally, qPCR was run on a QuantStudio 7 Flex, using PrimeTime® Gene Expression Master Mix (IDT) and the following TaqMan qPCR primer sets: β-Actin: forward 5'-GTACGACCAGAGGCATACAG-3' (SEQ ID NO: 180); reverse 5'-ACCGTGAAAAGATGACCCAG-3' (SEQ ID NO: 181); Malat1: Mm01227912_s1 FAM-MGB (ThermoFisher).

Quantification of *Malat1* expression in gastrocnemius, heart, sciatic nerve, and brain of Malat1 ASO-treated mice, resulted in lack of KD when compared to saline group. Mice dosed with C-

tagged T0281007D02 (see Example 4)-HLE-Malat1 ASO conjugates presented a statistically significant *Malat1* KD in gastrocnemius, heart, sciatic nerve, and brain (see Figure 8).

These findings demonstrate that the anti-TfR1 ISVDs of the invention can be used to target oligonucleotides for gene knockdown in skeletal muscle, heart, sciatic nerve, and brain *in vivo*.

6.19 Example 19: Pharmacokinetics and biodistribution of ISVDs of the invention

To evaluate the pharmacokinetics (PK) and biodistribution (BioD) of anti-TfR1 moieties in various tissues including brain we radiolabelled proteins with iodine-125 (^{125}I) and conducted an in vivo PK/BioD study. For this study, the following reagents were prepared:

4 proteins were compared. These are T0281007D02 (7D02 for short), 7D02 fused to anti-albumin nanobody (A045300062), 7D02 fused to Fc (T-0281-00_TP023) and an anti-TfR1 reference antibody in monovalent Fab-Fc- format (Reference Ab 1).

The individual proteins were labelled with ^{125}I by indirect iodination through lysine residues using N-succinimidyl 3-^{125}I-iodobenzoate (^{125}ISIB) reagent. The ^{125}I labelled proteins were tested to confirm the retention of binding to TfR1 receptor. ^{125}I-proteins and the unlabelled proteins were combined to obtain desired specific activity for in vivo experiment.

For conducting in vivo studies, mice were anesthetized with isoflurane (5% isoflurane, 2 L/min air) and subsequently intravenously injected via the retro-orbital venous with the appropriate treatment using a 0.3 mL syringe fitted with a 29-gauge needle. For sample collection at each timepoint, mice were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg) and were rapidly sacrificed by exsanguination *via* intracardiac puncture also to collect blood samples. Organs of interest including brain were harvested, rinsed with saline and weighed using a precision balance.

To evaluate the concentration of radiolabelled protein in tissues the counting of tissue radioactivity was performed in an automatic gamma counter (Hidex AMG) calibrated for ^{125}I (LLOQ: 100 cpm). The ^{125}I activity was counted in a channel with windows set for 15-80 keV. The radioactivity in plasma and tissue samples was expressed as percentage of the injected dose per gram (%ID/mL) and was subsequently used to calculate the absolute molar concentration to plot against the time.

ISVD ‘7D02 with Fc or with Albumin binding Nb fusion have similar pharmacokinetic and biodistribution profile in brain resulting in similar overall brain exposure as an anti-TfR1 antibody (see Figure 9).

These findings demonstrate that the anti-TfR1 ISVDs of the Invention can efficiently biodistributed to brain tissues and can be used for tissue targeting *in vivo*.

6.20 Example 20: pH dependent binding and brain biodistribution

To evaluate the impact of pH dependent binding to TfR1 in brain biodistribution of monovalent anti-TfR1 binders, in addition to their affinity to TfR1. For this purpose, brain exposure of a reference Ab1 monovalent antibody with affinity to TfR1 Kd=14.07 nM was compared with reference Ab2 monovalent antibody with sub-nanomolar affinity to TfR1 (Kd= 0.26nM); while none of these IgG binders have pH-dependent binding to TfR1, this experimental set up allows for the better understanding of the brain exposure of non-pH dependent high or medium affinity binders to TfR1. Data obtained from this experiment will enable comparing the brain exposure of high affinity TfR1 binders with or without pH-dependent binding to TfR1, thus ISVD or reference monovalent IgG.

Reference anti-TfR1 antibodies in monovalent Fab-Fc format were produced by transient transfection of expi293 cells using expi293 transfection kit and following manufacturer’s protocol. Supernatants were harvested 5 days post transfection. Antibodies were purified by Protein A capture step followed by SEC polishing step.

To evaluate brain exposure of anti-TfR1 binders, hTfR1-KI mice were dosed with a single IV dose of 70 nmol/kg per IgG molecule (N=9 per group). IgG control corresponds to Southern Biotech’s human IgG1 Kappa-LE/AF (Cat. Number 0151K-14). 1 hour, 3 hours and 24 hours post-dosing, 3 mice from each group were anesthetized with ketamine/xylazine and transcardially perfused with ice-cold heparinated DBPS with Ca/Mg. Brain cortex was harvested and weighted for IgG quantification through MSD Human/NHP IgG kit (Cat. Number K150JLD-4).

Brain tissues were homogenized in 5 v/w of 1% NP-40 in PBS without Ca/Mg, in the presence of protease inhibitors, by mechanical disruption with 2.8mm ceramic beads in Qiagen’s Tissue Lyser LT. Homogenized tissues were centrifuged at maximum speed for 20 minutes, and supernatants were collected for IgG quantification.

Brain homogenates were diluted 1/10 in homogenesis buffer. MSD plate was blocked with 150uL of 5% Blocker A (2.5g of Blocker BSA into 50mL PBST) per well shaking at RT for 30min. Standard curve was prepared in 1/10 tissue homogenate from a non-dosed mouse to account for matrix effect. 25uL of each standard or experimental sample were loaded per well, and incubated shaking at RT for 2 hours. Plate was washed with 200 µl/well of PBST 3 times. 50x detection antibody (SULFO-TAG Anti-Hu/NHP IgG) was diluted with Diluent 100 to 1x. 25uL of detection antibody were added into each well and incubated shaking at RT for 2 hours. Plate was washed with 200uL/well of PBST 3 times. 2x Read Buffer T (from 4X, dilute to 1/2 with water) was prepared and 150uL were added to each well. Plate was read immediately.

IgG concentration per tissue were calculated upon extrapolation from the standard curve, considering the dilutions of the loaded brain homogenate.

Quantification of monovalent anti-TfR1 binders' exposure in the brain indicate that while reference Ab1 monovalent IgG is enriched over time in the brain upon single IV dose, reference Ab2 high-affinity binder fails to do so (see Figure 9).

These data indicate that in the absence of pH-dependent binding to TfR1, high-affinity binding to TfR1 is detrimental for achieving brain enrichment of the IgG, while reference Ab1 medium affinity to TfR1 is favourable for transcytosis and enhanced brain penetrance (Figure 7A). On the contrary, when binding to TfR1 is pH-dependent, high-affinity TfR1 binders such as anti-TfR1 ISVDs successfully transcytose across the BBB and get enriched in the brain (Figure 7B). Thus, in addition to affinity to TfR1, pH-dependency is a factor that drives anti-TfR1 binder penetration to the brain.

6.21 Example 21: Binding interaction between ISVD of the invention and human TfR1 as determined from cryoEM

To determine the localization of the human TfR1 epitopes recognized by the pH sensitive nanobodies using cryogenic electron microscopy (cryo-EM).

In this study, purified hTfR1 (positions 89 to 760 of SEQ ID NO:1) was mixed with the ISVDs at a molar ratio of 1:1.2 and further purified using a Superdex200 3.3/300 column pre-equilibrated with PBS buffer. UltraAufoil® R 0.6/1 on 300 gold mesh grids were glow discharged at 22 mA

for 45 s and 3 µl of each sample at the concentration of less than 1 mg/ml were added to the grids and plunge frozen in liquid ethane. The raw micrographs from the grids were collected on either a Glacios or a Krios G4 microscopes (ThermoFisher) equipped with a Falcon4i camera at 200 and 300 keV, respectively. Images in the EER format were recorded with EPU at 240,000X and 165,000X nominal magnification at a pixel size of 0.58Å or 0.74Å and a range of defocus from -0.8 to -2.2. Dose on camera during an exposure time of 4.72 s was 60 and 40 e-/Å2 respectively for each microscope.

More than 4000 images were taken in total for each grid. The data analysis was conducted using cryosparc v3 and v4. The resolution of the final reconstructions was estimated using the value at which the FSC curve fell below 0.143. The two cryo-EM maps were sharpened using Phenix Autosharpen and then used to fit the atomic coordinates of the hTfR1 and the ISVD. The atomic coordinates underwent several rounds of manual (on Coot) and real space refinement (in Phenix).

The data show that the ISVD of the invention bind amino acids on both monomers of the TfR1 dimers denoted as first TfR1 monomer and second TfR1 monomer in the following. Epitope is defined as being within 4.5 Å of the ISVD. Numbering is according to SEQ ID NO: 1.

T02810023B05 binds to the following epitope:
(i) amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and
(ii) amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer.

T0281001D02 binds to the following epitope:
(i) amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and
(ii) amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer.

T0281002F11 binds to the following epitope:
(i) amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and

(ii) amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer.

T0281007D02 binds to the following epitope:
(i) amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and
(ii) amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer.

T0281007D02 binds to the following epitope:
(i) amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and
(ii) amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer.

6.22 Example 22: Epitope of the invention at different pH

Structures were determined by Cryo-EM as described in Example 21.

As can be seen from Fig. 9, the loop within amino acid residues 314-329 of TfR1 is shifted during pH change from 6 (dark grey) to 7.4 (light grey). The movement is mediated by the histidine 318 deprotonation at pH 6.0 that creates a repulsion with the Ser644 and thus pushes the loop out of the way.

In sum, the epitope of the invention can be seen as not being present at both pH 6.0 and 7.4, leading to a pH dependent binding to the epitope of the invention.

6.23 Example 23: Fab fragments are not predicted to be able to bind to the epitope of the invention

Structures were determined by Cryo-EM as described in Example 21.

Fig. 10 A shows in dark grey the TfR1 dimer with the ISVD 30F12 in ribbon diagram and in light grey a Fab molecule aligned in the same orientation as the ISVD in the structure. The heavy chain or the light chain of the Fab, if in the same orientation an ISVD of the invention, clashes with the TfR1 molecule and the binding in the same position and orientation would not be possible. Other

orientations are possible (see Fig. 10B), but the curved surface of the specific epitope where the pH sensitive loop is found (see Example 22), would make the binding of the antibody or Fab fragment very weak, due to poor interacting regions.

In sum, compact molecules such as ISVDs are important for binding to the epitope of the invention.

6.24 Example 24: Epitope mapping

The localization of the human TfRC epitopes recognized by the pH sensitive nanobodies using cryogenic electron microscopy (cryo-EM) was determined.

In this study, purified hTfR1 (SEQ ID NO:1) was mixed with the nanobodies at a molar ratio of 1:1.2 and further purified using a Superdex200 3.3/300 column pre-equilibrated with PBS buffer. UltraAufoil® R 0.6/1 on 300 gold mesh grids were glow discharged at 22 mA for 45 s and 3 µl of each sample at the concentration of less than 1 mg/ml were added to the grids and plunge frozen in liquid ethane. The raw micrographs from the grids were collected on either a Glacios or a Krios G4 microscopes (Thermo Fisher) equipped with a Falcon4i camera at 200 and 300 keV respectively. Images in the EER format were recorded with EPU at 240,000X and 165,000X nominal magnification at a pixel size of 0.58Å or 0.74Å and a range of defocus from -0.8 to -2.2. Dose on camera during an exposure time of 4.72 s was 60 and 40 e-/Å2 respectively for each microscope.

More than 4000 images were taken in total for each grid. The data analysis was carried out using cryosparc v3 and v4. The resolution of the final reconstructions was estimated using the value at which the FSC curve fell below 0.143. The two cryo-EM maps were sharpened using Phenix Autosharpen and then used to fit the atomic coordinates of the hTfRC and the nanobodies. The atomic coordinates underwent several rounds of manual (on Coot) and real space refinement (in Phenix). The amino acid residues of hTfR forming hydrogen bonds or salt bridges with the nanobodies are listed in Tables 17-21 and were obtained using PISA from the ccp4 suite. ChimeraX was used to determine the contacting residues between epitope and paratope at 4 Å distance also in tables 17-21.

Table 17 - ISVD 30F12

Hydrogen Bonds		
Paratope residue	Distance (Å)	Epitope residue
TYR 32 [OH]	2.4	PHE 321 [O]
Salt bridges		
Paratope residue	Distance (Å)	Epitope residue
ARG 56 [NH1]	2.9	GLU 383 [OE1]
ARG 56 [NH1]	2.8	GLU 383 [OE2]
ARG 56 [NH2]	3.2	GLU 383 [OE2]
ASP 30 [OD1]	3.7	ARG 325 [NH1]

Interfacing residues within 4.0Å	
Paratope residue	Epitope residue
Asp30	Arg325
Tyr32	Phe321, Pro322, Pro323, Arg732
Leu33	Arg732
Arg35	Met635, Glu728
Tyr37	Glu634
Leu47	Glu634
His53	Tyr309, Leu381
Gly54	Glu383
Arg56	Lys189, Asn317, Phe321, Glu383
Asn58	Gly636
Leu98	Glu728
Glu99	Thr729
Phe100	Thr729 Pro323 Ser324 Arg325
Gly102	Thr729
Gln104	Glu728

Interfacing residues within 4.0Å of the sugars	
Paratope residue	Sugar residue
Arg56, Thr57	NAG760
Ser101, Gly102, Ser103	NAG761

Table 18 - ISVD 23B05

Hydrogen Bonds (PISA) – in blue are the residues in the other monomer		
Paratope residue	Distance (Å)	Epitope residue
THR 33 [OG1]	3.5	THR 729 [OG1]
TYR 35 [OH]	2.7	THR 729 [OG1]
TYR 37 [OH]	2.7	GLU 728 [OE2]
ASP 98 [O]	3.4	GLU 728 [N]
ASN 56 [ND2]	3.8	SER 324 [O]
THR 57 [O]	3.8	LYS 189 [NZ]
ASN 56 [OD1]	2.5	LYS 189 [NZ]
Salt bridges		
Paratope residue	Distance (Å)	Epitope residue
LYS 96 [NZ]	2.4	GLU 728 [OE1]
Interfacing residues within 4.0Å (ChimeraX)		
Paratope residue	Epitope residue	
SER29	NAG762	
SER30	NAG761	
ILE31	NAG762	
ASN32	THR729, NAG761	
THR33	PRO323, THR729	
TYR35	PRO323, GLU728, THR729	
TYR37	GLU728	
GLN44	GLU634	
ARG45	GLU634	
GLU46	GLU634	
TRP47	GLU634, MET635	
TRP50	PHE321, PRO323, ARG732	
THR52	ARG325	
ARG53	NAG762	
GLY54	ARG325	

ASP55	LEU381
ASN56	LYS189, TYR309
LYS96	GLU728
ASP98	PHE726, ASN727, GLU728, NAG761
VAL99	GLY724, PHE726, ASN727, NAG761
TRP101	ASN722

Table 19 - ISVD 7D02

Hydrogen Bonds (PISA)		
Paratope residue	Distance (Å)	Epitope residue
THR 61 [OG1]	3.0	GLU 634 [O]
ASN 62 [N]	3.8	LYS 633 [O]
ARG 47 [NH2]	3.8	MET 635 [O]
LYS 65 [NZ]	2.8	GLY 636 [O]
ARG 103 [NH1]	3.5	ASN 723 [O]
ASP 101 [O]	3.7	THR 729 [OG1]
PRO 105 [O]	3.6	ASN 723 [ND2]
TYR 59 [OH]	2.2	PHE 321 [O]
ALA 56 [O]	2.7	LYS 189 [NZ]
ASP 101 [OD1]	3.2	NAG 759 [N2]
Salt bridges (PISA)		
Paratope residue	Distance (Å)	Epitope residue
ARG 47 [NH2]	3.9	GLU 728 [OE2]
ASP 54 [OD1]	2.6	LYS 224 [NZ]
ASP 54 [OD2]	3.9	ARG 325 [NE]
Interfacing residues within 4.0Å (CHIMERAX)		
Paratope residue	Epitope residues	
TRP33	ASN723	
ARG47	MET635, GLU728	

ASP54	LYS 224, TYR309, ARG 325, LEU381
ALA56	LYS189, LEU381
PHE57	LYS189, TYR309 PRO322, PRO323, SER324
TYR59	ARG732, PHE321, PRO322, PRO323
TYR60	MET635, GLY636
THR61	GLY636, GLU634
ASN62	LYS633, GLY636, LEU637
LYS65	GLY636
ASP101	NAG759, THR729
LEU102	THR729, GLU728
ARG103	GLU728, ASN723, PHE726
TRP104	ARG719, GLU634, ASN723
PRO105	ASN723

Table 20 - ISVD 1D02

Hydrogen Bonds (PISA)		
Paratope residue	Distance (Å)	Epitope residue
GLN 44 [NE2]	3.6	GLU 634 [OE2]
TYR 37 [OH]	2.7	GLU 728 [OE1]
TYR 35 [OH]	2.6	THR 729 [OG1]
ASP 98 [O]	3.1	GLU 728 [N]
ASP 98 [OD2]	2.4	THR 729 [OG1]
TYR 37 [OH]	3.9	ARG 732 [NH2]
ASN 56 [ND2]	3.5	SER 324 [O]

ASN 56 [OD1]	2.3	LYS 189 [NZ]
THR 57 [O]	2.9	LYS 189 [NZ]
ASP 55 [OD1]	2.6	TYR 309 [OH]
Salt bridges (PISA)		
Paratope residue	Distance (Å)	Epitope residue
LYS 96 [NZ]	3.7	GLU 728 [OE1]
ASP 55 [OD1]	3.8	ARG 325 [NE]
ASP 55 [OD2]	3.8	ARG 325 [NE]
ASP 55 [OD1]	3.7	ARG 325 [NH2]
Interfacing residues within 4.0Å (CHIMERAX)		
Paratope residue	Epitope residue	
SER 29	NAG 760	
ILE 31	NAG 760	
ASN 32	NAG 759, NAG 760, THR 729	
THR 33	THR 729, PRO 323	
TYR 35	LYS 189, PRO 323, GLU 728, THR 729, ARG 732	
TYR 37	LYS 189, GLU 634, MET 635, PHE 726, GLU 728, THR 729, ARG 732	
GLN 44	GLU 634	
ARG 45	GLU 634	
GLU 46	GLU 634	
TRP 47	GLU 634, MET 635, ARG 732, NAG 759	
TRP 50	PHE 321, PRO 322, PRO 323,	
THR 52	PRO 323	
GLY 53	ARG 325	
ASP 55	TYR 309, ARG 325, LEU 381, GLY 724, THR 729	
ASN 56	LYS 189, TYR 309, SER 324, LEU 381	
THR 57	LYS 189	
LYS 96	GLU 728	

ASP 98	PHE726, ASN 727, GLU 728 ,THR 729, NAG 759, ARG 325
THR 99	ASN 723, GLY 724, PHE 726, ASN 727, NAG 759
TRP 101	ASN 723

Table 21 - ISVD 2F11

Hydrogen Bonds (PISA)		
Paratope residue	Distance (Å)	Epitope residue
TYR 32[OH]	2.4	PHE 321 [O]
TYR 32 [OH]	3.8	ARG 732 [NH1]
ARG 35 [NH1]	3.1	MET 635 [O]
TYR 37 [OH]	2.6	GLU 634 [O]
LEU 47 [N]	3.5	GLU 634 [OE1]
GLY 54 [O]	3.4	GLU 383 [N]
ARG 56 [NH2]	3.7	PHE 321 [O]
ASN 58 [ND2]	3.6	GLY 636 [O]
THR 74 [OG1]	3.7	ASP 194 [OD2]
GLU 99 [O]	3.6	THR 729 [OG1]
TYR 100 [O]	3.3	THR 729 [OG1]
GLN 104 [NE2]	2.6	GLU 728 [OE1]
SER 101 [O]	3.2	NAG 759 [N2]
Salt bridges (PISA)		
Paratope residue	Distance (Å)	Epitope residue
ASP 30 [OD1]	3.6	ARG 325 [NH1]
ARG 56 [NH1]	3.4	GLU 383 [OE2]
ARG 35 [NH1]	2.6	GLU 728 [OE2]
Interfacing residues within 4.0Å (CHIMERAX)		
Paratope residue	Epitope residue	
ASP30	ARG325	

TYR32	PHE 321, PRO323, ARG732
LEU33	ARG732
ARG35	MET635, GLU728, ARG732
TYR37	GLU634
LEU47	LYS633, GLU634
HIS53	LEU381
GLY54	LYS189, LEU381, LYS382, GLU383
ARG56	LYS 189, PHE321, GLU383, NAG760
ASN58	GLY636
ASN73	VAL380
LEU98	GLU728
GLU99	THR729
TYR100	TYR309, PRO323, ARG325, THR729
SER101	NAG759
GLY102	THR729
GLN104	GLU728

CLAIMS

1. An immunoglobulin single variable domain (ISVD),
wherein the ISVD specifically binds to an epitope of a human transferrin receptor 1 (TfR1) homodimer,
wherein the TfR1 homodimer comprises (A) a first TfR1 monomer and (B) a second TfR1 monomer,
wherein the ISVD is characterized in that the epitope comprises:
(i) at least one of amino acid residues F187, K189, D194, K224, A225, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, K382, and E383 of the first TfR1 monomer, and
(ii) at least one of amino acid residues K633, E634, M635, G363, L637, R719, N722, N723, G724, A725, F726, N727, E728, T729, and R732 of the second TfR1 monomer,
wherein amino acid residues of the first and the second TfR1 monomer are numbered according to SEQ ID NO: 1.

2. The ISVD of claim 1, wherein the amino acid residues of the epitope form a conformation that differs between pH 7.4 (neutral conformation) and pH 6.0 (acidic conformation), wherein the ISVD has a reduced affinity to the TfR1 homodimer at pH 6.0 compared to pH 7.4.

3. The ISVD of claim 2, wherein binding of the ISVD to the TfR1 homodimer has a dissociation rate constant (k_{off}) that is at least 10 times lower for the neutral conformation compared to the acidic conformation.

4. The ISVD of any one of the preceding claims, wherein the epitope comprises:
(i) amino acid residues F321, P322, P323, R325, and L381 of the first TfR1 monomer, and
(ii) amino acid residues E634, M635, E728, T729, and R732 of the second TfR1 monomer.

5. The ISVD of any one of claims 1-4, wherein the epitope comprises:
(i) at least one of amino acid residues K189, K224, A225, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and
(ii) at least one of amino acid residues E634, M635, R719, N722, G724, A725, F726, N727, E728, T729, R732 of the second TfR1 monomer.

6. The ISVD of any one of claims 1-4, wherein the epitope comprises:
(i) at least one of amino acid residues K189, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and
(ii) at least one of amino acid residues E634, M635, N723, G724, F726, N727, E728, T729, and R732 of the second TfR1 monomer;

7. The ISVD of any one of claims 1-4, wherein the epitope comprises:
(i) at least one of amino acid residues D194, Y309, F321, P322, P323, R325, V380, L381, K382, and 383E of the first TfR1 monomer, and
(ii) at least one of amino acid residues E634, M635, G636, N727, E728, T729, and R732 of the second TfR1 monomer;

8. The ISVD of any one of claims 1-4, wherein the epitope comprises:
(i) at least one of amino acid residues K189, K224, Y309, F321, P322, P323, S324, R325, and L381 of the first TfR1 monomer, and
(ii) at least one of amino acid residues K633, E634, M635, G636, L637, R719, N723, F726, E728, T729, and R732 of the second TfR1 monomer; or

9. The ISVD of any one of claims 1-4, wherein the epitope comprises:
(i) at least one of amino acid residues F187, K189, Y309, N317, Q320, F321, P322, P323, S324, R325, V380, L381, and E383 of the first TfR1 monomer, and
(ii) at least one of amino acid residues K633, E634, M635, G636, R719, F726, N727, E728, T729, and R732 of the second TfR1 monomer.

10. The ISVD of any one of the preceding claims, wherein the ISVD specifically binds to TfR1 with a k_{off} rate of less than 10×10^{-3} s^{-1} at a neutral pH, such as pH 7.4.

11. The ISVD of any one of the preceding claims, wherein the ISVD binds to TfR1 with a k_{off} rate of more than 5×10^{-2} s^{-1} at an acidic pH, such as pH 6.0.

12. The ISVD of any one of the preceding claims, wherein the first TfR1 monomer, the second TfR1 monomer, or the first and the second TfR1 monomer comprises or consists of an

amino acid sequence as depicted in SEQ ID NO: 1, or polymorphic variants or isoforms thereof.

13. The ISVD of any one of the preceding claims, wherein the ISVD consists of 4 framework regions (FR1 to FR4 respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively) and is characterized in that:

(i) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 7 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 7; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 8 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 8; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 9, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 9;

(ii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 10 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 10; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 11 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 11; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 12, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 12;

(iii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 13 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 13; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 14 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 14; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 15, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 15;

(iv) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 16 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 16; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 17 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 17; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 18, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 18;

(v) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 19 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 19; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 20 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 20; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 21, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 21;

(vi) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 28 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 28; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 30 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 30; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 32, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 32;

(vii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 39 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 39; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 41 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 41; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 43, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 43;

(viii) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 50 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 50; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 52 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 52; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 54, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 54;

(ix) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 61 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 61; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 63 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 63; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 65, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 65;

or

(x) a) CDR1 (according to IMGT) has the amino acid sequence of SEQ ID NO: 68 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 68; and

b) CDR2 (according to IMGT) has the amino acid sequence of SEQ ID NO: 70 and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 70; and

c) CDR3 (according to IMGT) has the amino acid sequence of SEQ ID NO: 72, and optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NO: 72.

14. The ISVD of any one of the preceding claims, wherein the ISVD comprises or consists of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26, and

optionally has an amino acid sequence having 4, 3, 2 or 1 amino acid difference(s) with the sequence of SEQ ID NOs: 2-6 and 22-26, respectively.

15. The ISVD of any one of the preceding claims, wherein the ISVD comprises or consists of an amino acid sequence as depicted in any one of SEQ ID NOs: 2-6 and 22-26.

16. The ISVD of any one of the preceding claims, wherein the net charge of the CDRs or the paratope of the ISVD is not altered by changing the pH from pH 6.0 to 7.4 (or from pH 7.4 to 6).

17. The ISVD of any one of the preceding claims, wherein the ISVD is cross-reactive to cynomolgus TfR1, mouse TfR1, or both.

18. The ISVD of any one of the preceding claims, wherein two ISVDs can bind to the same TfR1 homodimer.

19. A polypeptide comprising the ISVD of any one of the preceding claims.

20. A nucleic acid encoding the ISVD of any one of claims 1-18 or the polypeptide of claim 19.

21. A vector comprising the nucleic acid of claim 20.

22. A host cell comprising the nucleic acid of claim 20 or the vector of claim 21

23. A conjugate comprising the ISVD of any one of claims 1-18 or the polypeptide of claim 19, and an agent, wherein the agent optionally is covalently attached to the ISVD or the polypeptide.

24. The conjugate of claim 23, wherein the agent is a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, or a radioactive isotope.

25. A pharmaceutical composition comprising the ISVD of any one of claims 1-18, the polypeptide of claim 19, or the conjugate of claims 23 or 24, optionally comprising a pharmaceutically acceptable carrier.

26. The ISVD of any one of claims 1-18, the polypeptide of claim 19, the conjugate of claim 23 or 24, or the pharmaceutical composition of claim 25 for use in a method of delivering an agent across the blood brain barrier (BBB) in a subject, wherein optionally the agent is a small molecule, a nucleotide (such as an oligonucleotide), a peptide, a protein, an enzyme, or a radioactive isotope.

27. The ISVD of any one of claims 1-18, the polypeptide of claim 19, the conjugate of claims 23 or 24, or the pharmaceutical composition of claim 25 for use in a method of delivering an agent into a cell in a subject.

28. The ISVD, the polypeptide, the conjugate, or the pharmaceutical composition for use of any one of claims 26 or 27, wherein the ISVD, the polypeptide, the conjugate, or the pharmaceutical composition is administered to the subject.

29. Use of the ISVD of any one of claims 1-18, the polypeptide of claim 19, the conjugate of claim 23 or 24, or the pharmaceutical composition of claim 25 for delivery of an agent into a cell in a subject.

30. Use of the ISVD of any one of claims 1-18, the polypeptide of claim 19, the conjugate of claims 23 or 24, or the pharmaceutical composition of claim 25 for delivery of an agent across the blood-brain-barrier in a subject.

31. The ISVD for use of any one of claims 26-28 or the use of claims 29 or 30, wherein the subject is a human.

32. The ISVD for use of any one of claims 26-28 or the use of any one of claims 29-31, wherein the subject has a disease of the nervous system (such as central nervous system, peripheral nervous system, or sciatic nerve) or of muscle tissue (such as skeletal muscle, heart muscle, smooth muscle).

33. A method for increasing the exposure of the CNS to an agent comprising:

(i) administering the conjugate of claims 23 or 24 to a subject.

34. A method of manufacturing a conjugate as defined in claims 23 or 24, comprising:

(i) providing an ISVD as defined in any one of claims 1-18; and

(ii) conjugating the agent to the ISVD.