

POLYPEPTIDES COMPRISING IMMUNOGLOBULIN SINGLE VARIABLE DOMAINS TARGETING GLYPICAN-3 AND T CELL RECEPTOR

DESCRIPTION

5 1 Field of the invention

The present technology relates to polypeptides targeting Glypican-3 (GPC3) and T cell receptor (TCR). It also relates to nucleic acid molecules encoding the polypeptide and vectors comprising the nucleic acids, and to compositions comprising the polypeptide, nucleic acid or vector. The technology further relates to these products for use in a method
10 of treating a subject suffering from a disease involving abnormal cells, such as cancerous or infected cells. Moreover, the technology relates to methods of producing these products.

2 Technological Background

Cytotoxic T cells (CTL) are T lymphocytes that kill cancer cells, cells that are infected (particularly with viruses), or cells that are damaged in other ways. T lymphocytes (also
15 called T cells) express the T cell receptor (TCR) and the CD3 receptor on the cell surface. The $\alpha\beta$ TCR-CD3 complex (or "TCR complex") is composed of six different type I single-spanning transmembrane proteins: the TCR α and TCR β chains that form the TCR heterodimer responsible for ligand recognition, and the non-covalently associated CD3 γ , CD3 δ , CD3 ϵ and ζ chains, which bear cytoplasmic sequence motifs that are tyrosine phosphorylated upon
20 receptor activation and recruit a large number of signalling components (Call et al. 2004, Molecular Immunology 40: 1295–1305).

Both α and β chains of the heterodimeric T cell receptor (TCR) consist of a constant domain and a variable domain. T cells are activated upon TCR recognition of cognate peptide presented by self-MHC molecules, with signal transduction initiated by tyrosine
25 phosphorylated CD3 complexes, leading to T cell proliferation and differentiation.

Bispecific antibodies have been engineered that have a tumour recognition part on the one arm (target-binding arm) whereas the other arm of the molecule has specificity for a T cell antigen (effector-binding arm), often CD3. These bispecific antibodies, so called T-cell

engagers (TCE), are multitargeting molecules that enhance the patient's immune response to malignant cells. Co-engagement of T-cell and tumour cell by the multispecific antibody leads to the formation of a cytolytic synapse between the T cell and the tumour cell, that induces T-cell activation and results in tumour cell killing.

- 5 While the majority of T cell activating bispecific antibodies target the CD3 complex on the T cell, some bispecific binders that target the constant domain of the $\alpha\beta$ T cell receptor have been described in WO 2016/180969 A1.

Glypican-3 (GPC3) is a GPI anchored cell surface glycoprotein consisting of heparan sulfate GAG chains and a core protein. It has been implicated in embryogenesis and early
10 development, controlling cell growth and differentiation. Whereas the expression of GPC3 is high during development, its expression is nearly absent in normal adult tissue, with moderate/low expression in renal proximal tubules and bronchial cells. Consistent with its role in early development, high level of GPC3 is also expressed in placenta.

GPC3 likely regulates early development via multiple signalling cascades, including the Wnt,
15 Hh and YAP pathways. In addition, GPC3 is able to interact with basic growth factors, such as FGF2 to regulate cell growth. In an experimental setting, overexpression of GPC3 can inhibit FGF2-induced cell proliferation. By contrast, GPC3 also negatively regulates BMP7, an inhibitory growth factor. Altogether, the activity of GPC3 can be highly contextual, as it is able to not only inhibit cell proliferation, but also can promote carcinogenesis in liver and
20 other forms of cancer.

GPC3 expression is especially prevalent in tumour tissue from hepatocellular carcinoma (HCC), a disease with significant unmet needs. Soluble GPC3 can also be detected in serum of HCC patients and has been used to differentiate from liver diseases with different aetiologies.

- 25 Bispecific antibody constructs have been proposed in multiple formats. For example, bispecific antibody formats may involve the chemical conjugation of two antibodies or fragments thereof (Brennan, M, et al., Science, 1985. 229(4708): p. 81-83; Glennie, M. J., et al., J Immunol, 1987. 139(7): p. 2367-2375).

Disadvantages of such bispecific antibody formats include, however, high molecular weight and high viscosity at high concentration, making e.g. subcutaneous administration challenging, and in that each binding unit requires the interaction of two variable domains for specific and high affinity binding, having implications on polypeptide stability and efficiency of production. Such bispecific antibody formats may also potentially lead to CMC issues related to poor production efficiency and low titers and/or mispairing of the light chains or mispairing of the heavy chains.

Therefore, there is a need for antibody constructs that bind both to a target cell and a T cell with sufficient affinity to induce a cytotoxic response. At the same time, such constructs should not induce a cytotoxic response to non-target cells, i.e. cells that do not express the target antigen or only express it at low levels. Thereby, a balance can be struck between efficacy and safety. It is further desirable that such constructs can be efficiently produced, e.g. in microbial hosts. Such constructs should ideally also exhibit a half-life in the subject to be treated that is long enough such that consecutive treatments can be conveniently spaced apart. Furthermore, it is desirable to limit the reactivity of such constructs to pre-existing antibodies in the subject to be treated (i.e. antibodies present in the subject before the first treatment with the antibody construct). Moreover, the polypeptides should exert no or only minimal undesired side effects, e.g. provoked by cytotoxic activity on non-target cells.

3 Summary of the invention

The present inventors found that a polypeptide targeting specifically GPC3 and TCR at the same time leads to efficient T cell-mediated killing of GPC3 expressing cells *in vitro*. Said polypeptides could be efficiently produced (e.g. in microbial hosts). Furthermore, such polypeptides could be shown to exhibit limited reactivity to pre-existing antibodies in the subject to be treated (i.e., antibodies present in the subject before the first treatment with the antibody construct). In preferred embodiments such polypeptides exhibit a half-life in the subject to be treated that is long enough such that consecutive treatments can be conveniently spaced apart. Moreover, such polypeptides showed only limited activity against cells expressing no or low levels of GPC3. This suggests the possibility of inducing a highly specific T cell-mediated cytotoxic response against GPC3 positive cancer target cells, while exhibiting a favourable safety profile.

In one aspect, the polypeptide comprises or consists of at least three immunoglobulin single variable domains (ISVDs), wherein at least two ISVDs specifically bind to GPC3 and one ISVD specifically binds to the constant domain of a TCR on a T cell. Preferably, the at least two ISVDs specifically binding to GPC3 specifically bind to human GPC3 and the ISVD specifically binding to TCR specifically binds to human TCR. More preferably, the at least two ISVDs specifically binding to GPC3 are distinct ISVDs. In another aspect, the polypeptide comprising or consisting of at least three ISVDs preferably further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. For example, the binding unit can be an ISVD that binds to a serum protein, preferably to a human serum protein such as human serum albumin.

In one aspect the present technology provides a polypeptide comprising or consisting of at least one immunoglobulin single variable domain (ISVD) that specifically binds to GPC3. In a further embodiment, the polypeptide of the present technology comprises or consists of at least two ISVDs that specifically bind to GPC3, wherein the two ISVDs are optionally linked via a peptidic linker. Preferably, the two ISVDs specifically binding to GPC3 are distinct ISVDs. Moreover, the polypeptide preferably further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. For example, the binding unit can be an ISVD that binds to a serum protein, preferably to a human serum protein such as human serum albumin.

In another aspect, the polypeptide of the present technology comprises or consists of an ISVD that specifically binds to constant domain of a TCR on a T cell and at least one ISVD that specifically binds to GPC3, wherein the two ISVDs are optionally linked via a peptidic linker. Such a polypeptide can be used to redirect T cells for killing of cells expressing GPC3. Ideally, the ISVD binding to TCR is the only binding moiety comprised in said polypeptide that

specifically binds to a, e.g. human, T cell. Moreover, the examples show that when the ISVD that specifically binds to TCR is located at the N-terminus of such a polypeptide, better T-cell mediated cytotoxicity is achieved compared to when the same anti-TCR ISVD not located at the N-terminus. The ISVD that specifically binds to TCR is thus preferably located N-terminally from the at least one ISVD that specifically binds to GPC3. Most preferably, the ISVD that specifically binds to TCR is located at the N-terminus of a polypeptide comprising the same. Moreover, the polypeptide preferably further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. For example, the binding unit can be an ISVD that binds to a serum protein, preferably to a human serum protein such as human serum albumin.

Also provided is a nucleic acid molecule capable of expressing the polypeptide, a nucleic acid or vector comprising the nucleic acid, and a composition comprising the polypeptide, the nucleic acid or the vector. The composition is preferably a pharmaceutical composition.

Also provided is a host cell or (non-human) host comprising the nucleic acid or vector that encodes the polypeptide as disclosed herein.

Further provided is a method for producing the polypeptide as disclosed herein, said method at least comprising the steps of:

- a. expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid sequence encoding the polypeptide; optionally followed by:
- b. isolating and/or purifying the polypeptide.

Moreover, the present technology provides the polypeptide, the composition comprising the polypeptide, or the composition comprising the nucleic acid or vector comprising the nucleotide sequence that encodes the polypeptide, for use as a medicament. Preferably, the polypeptide or composition is for use in the treatment of cancer, such as liver cancer or lung cancer.

In addition, provided is a method of treating cancer, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of the polypeptide or a composition according to the present disclosure. The cancer is preferably selected from liver cancer or lung cancer. In some embodiments, the method further
5 comprises administering one or more additional therapeutic agents.

Further provided is the use of the polypeptide or composition in the preparation of a pharmaceutical composition for treating cancer, preferably liver cancer or lung cancer.

In particular, the present technology provides the following embodiments:

- Embodiment 1. A polypeptide that comprises or consists of at least three
10 immunoglobulin single variable domains (ISVDs), wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least three ISVDs are optionally linked via one or more peptidic linkers, and wherein:
- a) a first ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid
15 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14;
 - 20 b) a second ISVD comprises
 - iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - 25 vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; and
 - c) a third ISVD comprises
 - vii. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;

- viii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- ix. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
- 5 wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide, wherein the first ISVDs is optionally located at the N-terminus of said polypeptide.

Embodiment 2. The polypeptide according to embodiment 1, wherein:

- a) said first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a
10 CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
- b) said second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15; and
- 15 c) said third ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16.

Embodiment 3. The polypeptide according to any of embodiments 1 or 2, wherein:

- a) the amino acid sequence of said first ISVD exhibits a sequence identity of more than
20 90% with SEQ ID NO: 2;
- b) the amino acid sequence of said second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3; and
- c) the amino acid sequence of said third ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4.

25 Embodiment 4. The polypeptide according to any of embodiments 1 to 3, wherein:

- a) said first ISVD consists of the amino acid sequence of SEQ ID NO: 2;
- b) said second ISVD consists of the amino acid sequence of SEQ ID NO: 3; and
- c) said third ISVD consists of the amino acid sequence of SEQ ID NO: 4.

- Embodiment 5. The polypeptide according to any of embodiments 1 to 4, wherein the first ISVD and the second ISVD are linked to each other via a linker consisting of less than 10 amino acids, preferably less than 6 amino acids, wherein the linker most preferably is a 5GS linker.
- 5 Embodiment 6. The polypeptide according to any of embodiments 1 to 5, wherein said polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups,
10 residues, moieties or binding units.
- Embodiment 7. The polypeptide according to embodiment 6, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc
15 portion, and small proteins or peptides that can bind to serum proteins.
- Embodiment 8. The polypeptide according to any one of embodiments 6 or 7, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).
- 20 Embodiment 9. The polypeptide according to embodiment 8, in which said binding unit that provides the polypeptide with increased half-life is an ISVD that binds to human serum albumin.
- Embodiment 10. The polypeptide according to embodiment 9, wherein the ISVD binding to human serum albumin comprises
- 25 i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.

Embodiment 11. The polypeptide according to any of embodiments 9 or 10, wherein the ISVD binding to human serum albumin comprises a CDR1 being the amino acid
5 sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.

Embodiment 12. The polypeptide according to any of embodiments 9 to 11, wherein the amino acid sequence of said ISVD binding to human serum albumin exhibits a sequence identity of more than 90% with SEQ ID NO: 5.

10 Embodiment 13. The polypeptide according to any of embodiments 9 to 12, wherein said ISVD binding to human serum albumin consists of the amino acid sequence of SEQ ID NO: 5.

Embodiment 14. The polypeptide according to any of embodiments 1 to 13, wherein the polypeptide comprises or consists of an amino acid sequence exhibiting a sequence
15 identity of more than 90% with SEQ ID NO: 1.

Embodiment 15. The polypeptide according to any of embodiments 1 to 14, wherein the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.

Embodiment 16. A polypeptide that comprises or consists of at least one immunoglobulin single variable domain (ISVD), wherein said ISVD comprises three
20 complementarity determining regions (CDR1 to CDR3, respectively), and wherein the at least one ISVD comprises:

- a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid
25 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, or

- b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
5 a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16.

Embodiment 17. The polypeptide according to embodiment 16, wherein the at least one ISVD comprises:

- a) a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,
10 or
b) a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16.

Embodiment 18. The polypeptide according to any of embodiments 16 or 17, wherein
15 the amino acid sequence of the at least one ISVD comprises:

- a) a sequence identity of more than 90% with SEQ ID NO: 3, or
b) a sequence identity of more than 90% identity with SEQ ID NO: 4.

Embodiment 19. The polypeptide according to any of embodiments 16 to 18, wherein said at least one ISVD comprises or consists of:

- 20 a) the amino acid sequence of SEQ ID NO: 3, or
b) the amino acid sequence of SEQ ID NO: 4.

Embodiment 20. A polypeptide that comprises or consists of at least two ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least two ISVDs are optionally linked via one or more peptidic
25 linkers, and wherein:

- a) a first and a second ISVD comprise
i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;

- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,
- 5 b) a first and a second ISVD comprise
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
- c) a first ISVD comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - 15 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, and
- a second ISVD comprises
- 20 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid
 - 25 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
- d) a first ISVD comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid
 - 30 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a second ISVD comprises
- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,
- e) a first ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a second ISVD comprises
 - iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,
- f) a first ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, and a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - 5 vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,
- g) a first ISVD comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and
- a second ISVD comprises
- 15 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid
 - 20 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, or
- h) a first ISVD comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid
 - 25 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and
- a second ISVD comprises
- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid
 - 30 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;

- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,
- 5 wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide.

Embodiment 21. The polypeptide according to embodiment 20, wherein:

- a) the first and the second ISVD comprise a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,
- 10 b) the first and the second ISVD comprise a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16,
- c) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16,
- 15 d) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,
- 20 e) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid
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sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,

- 5 f) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14,
- 10 g) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, or
- 15 h) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14.
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Embodiment 22. The polypeptide according to any of embodiments 20 or 21, wherein:

- a) the amino acid sequence of the first and the second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3,
- 25 b) the amino acid sequence of the first and the second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 4,
- c) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4,

- d) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 4, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 3,
- e) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 3,
- f) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 2
- g) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4, or
- h) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 4, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 2.

Embodiment 23. The polypeptide according to any of embodiments 20 to 22, wherein:

- a) the first and the second ISVD consist of the amino acid sequence of SEQ ID NO: 3,
- b) the first and the second ISVD consist of the amino acid sequence of SEQ ID NO: 4,
- c) the first ISVD consists of the amino acid sequence of SEQ ID NO: 3, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 4,
- d) the first ISVD consists of the amino acid sequence of SEQ ID NO: 4, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 3,
- e) the first ISVD consists of the amino acid sequence of SEQ ID NO: 2, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 3,
- f) the first ISVD consists of the amino acid sequence of SEQ ID NO: 3, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 2,
- g) the first ISVD consists of the amino acid sequence of SEQ ID NO: 2, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 4, or
- h) the first ISVD consists of the amino acid sequence of SEQ ID NO: 4, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 2.

Embodiment 24. The polypeptide according to any of embodiments 20 to 23, wherein the polypeptide comprises or consists of an amino acid sequence selected from SEQ ID NOs: 1, 49-72 and 78-81.

Embodiment 25. The polypeptide according to any of embodiments 16 to 24, wherein
5 said polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.

10 Embodiment 26. The polypeptide according to embodiment 25, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

15 Embodiment 27. The polypeptide according to any one of embodiments 25 to 26, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Embodiment 28. The polypeptide according to embodiment 27, in which said binding
20 unit that provides the polypeptide with increased half-life is an ISVD that binds to human serum albumin.

Embodiment 29. The polypeptide according to embodiment 28, wherein the ISVD binding to human serum albumin comprises

- 25 i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.

Embodiment 30. The polypeptide according to any of embodiments 28 to 29, wherein the ISVD binding to human serum albumin comprises a CDR1 being is the amino acid
5 sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.

Embodiment 31. The polypeptide according to any of embodiments 28 to 30, wherein the amino acid sequence of said ISVD binding to human serum albumin exhibits a sequence identity of more than 90% with SEQ ID NO: 5.

10 Embodiment 32. The polypeptide according to any of embodiments 28 to 31, wherein said ISVD binding to human serum albumin consists of the amino acid sequence of SEQ ID NO: 5.

Embodiment 33. A nucleic acid comprising a nucleotide sequence that encodes a polypeptide according to any of embodiments 1 to 32, preferably according to any of
15 embodiments 1 to 15.

Embodiment 34. A host or host cell comprising a nucleic acid according to embodiment 33.

Embodiment 35. A method for producing a polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15, said method
20 at least comprising the steps of:

- a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid according to embodiment 33; optionally followed by:
- b) isolating and/or purifying the polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15.

25 Embodiment 36. A composition comprising at least one polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15, or a nucleic acid according to embodiment 33.

Embodiment 37. The composition according to embodiment 36, which is a pharmaceutical composition which further comprises at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

- 5 Embodiment 38. A polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15, or a composition according to embodiment 36 or 37, for use as a medicament.

Embodiment 39. A polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15, or a composition according to embodiment 36
10 or 37, for use in the treatment of cancer, preferably liver cancer or lung cancer.

Embodiment 40. The polypeptide or composition for use according to embodiment 39, wherein the liver cancer is hepatocellular carcinoma (HCC).

Embodiment 41. The polypeptide or composition for use according to embodiment 39, wherein the lung cancer is non-small cell lung cancer (NSCLC), preferably squamous cell
15 carcinoma (SCC).

Embodiment 42. A method of treating cancer, preferably liver cancer or lung cancer, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15, or a composition according to
20 embodiment 36 or 37.

Embodiment 43. The method according to embodiment 42, wherein the liver cancer is hepatocellular carcinoma.

Embodiment 44. The method according to embodiment 42, wherein the lung cancer is non-small cell lung cancer (NSCLC), preferably squamous cell carcinoma (SCC).

Embodiment 45. Use of a polypeptide according to any of embodiments 1 to 32, preferably according to any of embodiments 1 to 15, or a composition according to embodiment 36 or 37, in the preparation of a medicament.

Embodiment 46. Use of a polypeptide according to any of embodiments 1 to 32,
5 preferably according to embodiments 1 to 15, or a composition according to embodiment 36 or 37, in the preparation of a pharmaceutical composition for treating cancer, preferably liver cancer or lung cancer.

Embodiment 47. Use of the polypeptide or a composition according to embodiment 46, wherein the liver cancer is hepatocellular carcinoma.

10 Embodiment 48. Use of the polypeptide or the composition according to embodiment 46, wherein the lung cancer is non-small cell lung cancer (NSCLC), preferably squamous cell carcinoma (SCC).

4 Brief description of the drawings

15 **FIGURE 1:** Dose-dependent killing of the trispecific GPC3 T-cell engagers A022600027 (Figure 1A), A022600031 (Figure 1B solid line) and a construct with the reference Ab1 (Figure 1B dotted line) in the Incucyte based human TDC (T-cell dependent cytotoxicity) HepG2-Nuclight green assay using an effector to target ratio of 15:1, analyzed at 60h after seeding. Controls (left) are: (solid square) No compound and (solid triangle) Brefeldin A 1 μ M for
20 100% killing.

FIGURE 2: Dose-dependent killing of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay using an effector to target ratio of 15:1, analyzed at 72h after seeding. The figure represents Step 1 of format optimization where trispecific trivalent T-cell engager formats differ in linker length between anti-TCR ISVD and
25 anti-GPC3 ISVD. Controls (left) are: No compound (open square) and reference for 100% killing (open circle).

FIGURE 3: Dose-dependent killing of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay using an effector to target ratio of 15:1,

analyzed at 60h after seeding. The Figure represents Step 2 of format optimization where trispecific tetravalent T-cell engager formats differ in the orientation of and the linker length between the biparatopic GPC3 binding ISVDs. Controls (left) are: No compound (open square) and reference for 100% killing (open circle).

5 **FIGURE 4:** Step 3 of GPC3 T-cell engager format optimization: the anti-TCR ISVD T017000624 was substituted by the sequenced optimized variant TCE01 in the trivalent and tetravalent formats. Dose-dependent killing of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay (FIGURE 4A) and in the xCELLigence based human TDC Huh7 assay (FIGURE 4B), using an effector to target ratio of 15:1, analyzed at
10 60h after seeding. Controls (left) are: No compound (open square) and reference for 100% killing (open circle).

FIGURE 5: Dose-dependent killing of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay (FIGURE 5A) and in the xCELLigence based human TDC Huh7 assay (FIGURE 5B), using an effector to target ratio of 15:1, analyzed at
15 60h after seeding. Step 4 of GPC3 T-cell engager format optimization is represented where the orientation of the anti-TCR ISVD was changed with the anti-GPC3 ISVD or the orientation of anti-GPC3 ISVD was changed with anti-Albumin ISVD. Controls (left) are: (open square) No compound and (open circle) reference for 100% killing.

FIGURE 6: Dose-dependent killing of the trispecific GPC3 ISVD T-cell engagers in the Incucyte
20 based human TDC HepG2-Nuclight green assay (FIGURE 6A) and in the xCELLigence based human TDC Huh7 assay (FIGURE 6B), using an effector to target ratio of 15:1, analyzed at 60h after seeding. Step 5 of GPC3 T-cell engager format optimization is represented where the linker lengths are varied. Controls (left) are: (open square) No compound and (open circle) reference for 100% killing.

25 **FIGURE 7:** Assessment of impact of soluble GPC3 (sGPC3) on cytotoxicity of the trispecific GPC3 ISVD T-cell engagers in the xCELLigence based human TDC Huh7 assay using an effector to target ratio of 15:1, analyzed at 60h after seeding (FIGURE 7A), and on T-cell activation by the trispecific GPC3 ISVD T-cell engagers in the presence (FIGURE 7B) and absence (FIGURE 7C) of target cells (Huh7). Controls (left) are: No compound (open circle,
30 FIGURE 7A) and isotype control (open circle, FIGURE 7B and C).

FIGURE 8: Time-course of T-cell engager internalization (FIGURE 8A) and GPC3 expression (FIGURE 8B) measured at 37 °C at time points 0.5h, 3h, 24h and 48h. Controls (left) are measurements at 0.5h for each compound at 4 °C.

FIGURE 9: Dose-dependent killing of the trispecific GPC3 T-cell engagers in the xCELLigence based human TDC assay on different tumor cell lines expressing decreasing expression levels of GPC3 using an effector to target ratio of 15:1: HepG2 analysed at 60h (FIGURE 9A), NCI-H661 analysed at 75h (FIGURE 9B), Huh-7 analysed at 60h (FIGURE 9C), MKN-45 analysed at 65h (FIGURE 9D), BxPC-3 analysed at 65h (FIGURE 9E), NCI-H292 analysed at 60h (FIGURE 9F). Controls (left) are: (open square) No compound (effector and T cells only).

FIGURE 10: Dose-dependent killing of the five selected trispecific GPC3 ISVD based T-cell engagers in the xCELLigence based human TDC assay on two tumor cell lines using an effector to target ratio of 15:1 and analyzed at 60h, NCI-H661 (FIGURE 10A) and BxPC-3 (FIGURE 10B). Controls (left) are: No compound (open square, effector and T cells only) and T017000698 at 30 nM (open diamond).

FIGURE 11: Median and interquartile range of the pre-existing antibody reactivity from 96 normal human serum samples on tetravalent (FIGURE 11A) and trivalent (FIGURE 11B) selected ISVD based GPC3 T-cell engager formats.

FIGURE 12: Study design for efficacy model. Huh-7 tumor cells were subcutaneously injected in NOG mice. The tumors grew until the mean tumor volume of approximately 150 mm³ was reached. At this point, in vitro expanded T cells were injected into each mouse intraperitoneally (D0). The treatment with A022600424 injected intravenously started on D0, 3 h after T cell injection and continued on D3, D6, D9 and D12 (q3d). Four dose levels of A022600424 were tested (0.1 mg/kg, 0.2 mg/kg, 0.7 mg/kg and 2 mg/kg). The control T017000698 was injected in a control group at 2 mg/kg on D0, D3, D6, D9 and D12 (q3d). Survival blood sampling was done on D6 and D12 prior to administration of test compounds. All mice were sacrificed on D15, blood and tumor samples were collected.

FIGURE 13: Results of the efficacy model. Four dose levels of A022600424 were tested (0.1 mg/kg, 0.2 mg/kg, 0.7 mg/kg and 2 mg/kg). The control T017000698 was injected in a control group at 2 mg/kg.

5 Detailed description of the invention

The present technology aims at providing a novel type of drug for treating cancer, such as liver cancer or lung cancer.

The present inventors found that a polypeptide targeting specifically GPC3 and TCR at the same time leads to efficient T cell-mediated killing of GPC3 expressing cells *in vitro*. Said polypeptides could be efficiently produced (e.g. in microbial hosts). Furthermore, such polypeptides could be shown to exhibit limited reactivity to pre-existing antibodies in the subject to be treated (i.e., antibodies present in the subject before the first treatment with the antibody construct). In preferred embodiments such polypeptides exhibit a half-life in the subject to be treated that is long enough such that consecutive treatments can be conveniently spaced apart. Moreover, such polypeptides showed only limited activity against cells expressing no or low levels of GPC3. This suggests the possibility of inducing a highly specific T cell-mediated cytotoxic response against GPC3 positive target cells.

Apart from the above, the GPC3-binding ISVDs disclosed herein provide high affinity-binding to human and cyno GPC3 and can thus be readily used in monovalent or multivalent form for other applications in which binding to GPC3 is required.

5.1 Polypeptides

Monospecific-monovalent polypeptides

In one aspect, the polypeptide is monospecific and monovalent.

The term “*monospecific*” refers to the binding to one (specific) type of target molecule(s). A monospecific polypeptide thus specifically binds to GPC3.

The term “*monovalent*” indicates the presence of only one binding units/building block that (specifically) targets a molecule, such as an ISVDs.

Accordingly, in one aspect the present technology provides a monospecific-monovalent polypeptide comprising or consisting of one ISVD that specifically binds to GPC3, preferably human GPC3, which comprises three complementarity determining regions (CDR1 to CDR3, respectively). The ISVD can be selected from an ISVD comprising:

- 5 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, and a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; or
- 10 b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16.

Preferably, the ISVD specifically binding to GPC3 is selected from an ISVD comprising:

- 15 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11 and a CDR3 that is the amino acid sequence of SEQ ID NO: 15; or
- b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 16.
- 20 In a further embodiment of this aspect of the technology, the ISVD specifically binding to GPC3 is selected from an ISVD comprising:
- a) an amino acid sequence with a sequence identity of more than 90% with SEQ ID NO: 3, preferably wherein the ISVD comprises or consists of the amino acid sequence of SEQ ID NO: 3; or
- 25 b) an amino acid sequence with a sequence identity of more than 90% with SEQ ID NO: 4, preferably wherein the ISVD comprises or consists of the amino acid sequence of SEQ ID NO: 4.

In another aspect the present technology provides a monospecific-monovalent polypeptide comprising or consisting of one ISVD that specifically binds to the constant domain of a TCR

on a T cell, preferably human TCR, which comprises three complementarity determining regions (CDR1 to CDR3, respectively). The ISVD can be an ISVD comprising a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6, a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10, and a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14.

Preferably, the ISVD specifically binding to TCR is an ISVD comprising a CDR1 that is the amino acid sequence of SEQ ID NO: 6, a CDR2 that is the amino acid sequence of SEQ ID NO: 10 and a CDR3 that is the amino acid sequence of SEQ ID NO: 14.

In a further embodiment of this aspect of the technology, the ISVD specifically binding to TCR is an ISVD comprising an amino acid sequence with a sequence identity of more than 90% with SEQ ID NO: 2, preferably wherein the ISVD comprises or consists of the amino acid sequence of SEQ ID NO: 2.

These monovalent compounds can also serve as building blocks for multivalent and/or multispecific polypeptides.

An ISVD located at the N-terminus of a (monovalent or multivalent) polypeptide comprising the same preferably does not exhibit glutamic acid (E) at its N-terminal end. Therefore, glutamic acid (E) at position 1 is typically substituted by aspartic acid (D) in an ISVD located at the N-terminus of the polypeptide. Thus, for example, if SEQ ID NOs: 3, 4 or 5 are located at the N-terminus of the polypeptide, these sequences will typically exhibit a E1D substitution. Conversely, if SEQ ID NO: 2 is not located at the N-terminus, this sequence will typically exhibit a D1E mutation. Thus, generally, the first position of SEQ ID NOs: 2-5 can be E or D, depending on whether these sequences are located at the N-terminus or not. In a preferred embodiment, the first amino acid of the first ISVD comprised in the polypeptide of the present technology is an aspartic acid (D).

Monospecific-multivalent polypeptides

In another aspect, the polypeptide is monospecific and at least bivalent, but can also be e.g., trivalent, tetravalent, pentavalent, hexavalent, etc.

The terms “bivalent”, “trivalent”, “tetravalent”, “pentavalent”, or “hexavalent” all fall under the term “multivalent” and indicate the presence of two, three, four, five or six binding units/building blocks, respectively, such as ISVDs.

Accordingly, in one aspect the present technology provides a monospecific-bivalent
5 polypeptide comprising or consisting of two ISVDs that specifically bind to GPC3, preferably human GPC3, wherein each of the two ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the two ISVDs are preferably linked via one or more peptidic linkers, and wherein:

- 10 a) a first and a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, and a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15;
- 15 b) a first and a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid
20 difference(s) with SEQ ID NO: 16;
- c) a first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, and a CDR3 that is the amino acid
25 sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, and
a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or

- 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16; or
- 5 d) a first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and
- 10 a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, and a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15.
- 15

Preferably, the monospecific-bivalent polypeptide comprises or consists of two ISVDs that specifically bind to GPC3, wherein:

- a) the first and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being
- 20 the amino acid sequence of SEQ ID NO: 15;
- b) the first and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16;
- c) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a
- 25 CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16; or

- d) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15.

In a further embodiment of this aspect of the technology, the monospecific-bivalent polypeptide comprises or consists of two ISVDs that specifically bind to GPC3, wherein:

- a) the amino acid sequence of the first and the second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3, wherein the first and the second ISVD preferably consists of the amino acid sequence of SEQ ID NO: 3;
- b) the amino acid sequence of the first and the second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 4, wherein the first and the second ISVD preferably consists of the amino acid sequence of SEQ ID NO: 4;
- c) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3 and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4, preferably wherein the first ISVD consists of the amino acid sequence of SEQ ID NO: 3 and the second ISVD consists of the amino acid sequence of SEQ ID NO: 4; or
- d) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 4 and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 3, preferably wherein the first ISVD consists of the amino acid sequence of SEQ ID NO: 4 and the second ISVD consists of the amino acid sequence of SEQ ID NO: 3.

The terms "first ISVD" and "second ISVD" in this regard only indicate the relative position of the specifically recited ISVDs binding to GPC3 to each other, wherein the numbering is started from the N-terminus of the polypeptide. The "first ISVD" is thus closer to the N-terminus than the "second ISVD". Accordingly, the "second ISVD" is thus closer to the C-terminus than the "first ISVD". Since the numbering is not absolute and only indicates the relative position of the two ISVDs it does not exclude the possibility that additional binding

units/building blocks such as ISVDs binding to GPC3, TCR or serum albumin, respectively, can be present in the polypeptide. Moreover, it does not exclude the possibility that other binding units/building blocks such as ISVDs can be placed in between. For instance, as described further below (see in particular, section “multispecific-multivalent polypeptides” and 5.4 “(In vivo) half-life extension”), the polypeptide can further comprise another ISVD binding to human serum albumin that can even be located between the “first ISVD” and “second ISVD” (such a construct is then referred to as multispecific as described in the subsequent section).

In a preferred embodiment, the (at least two) ISVDs of the monospecific-multivalent polypeptides, in particular of the above described monospecific-bivalent polypeptides, are linked via peptidic linkers. The use of peptidic linkers to connect two or more (poly)peptides is well known in the art. Exemplary peptidic linkers that can be used with the monospecific-multivalent polypeptides, in particular with the above described monospecific-bivalent polypeptides, are shown in Table A-5. One often used class of peptidic linkers is known as the “Gly-Ser” or “GS” linkers. These are linkers that essentially consist of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif such as the GGGGS (SEQ ID NO: 100) motif (for example, exhibiting the formula (Gly-Gly-Gly-Gly-Ser)_n in which n may be 1, 2, 3, 4, 5, 6, 7 or more). Some often used examples of such GS linkers are 9GS linkers (GGGGSGGGGS, SEQ ID NO: 103) 15GS linkers (n=3) and 35GS linkers (n=7). Reference is for example made to Chen et al., Adv. Drug Deliv. Rev. 2013 Oct 15; 65(10): 1357–1369; and Klein et al., Protein Eng. Des. Sel. (2014) 27 (10): 325-330. In one embodiment, the ISVDs of the monospecific-multivalent polypeptides, in particular the monospecific-bivalent polypeptides are linked via a linker set forth in Table A-5. In one embodiment, the (at least) two ISVDs are linked via a 35GS or a 9GS linker. In a preferred embodiment, the (at least) two ISVDs are linked via a 9GS linker(s).

Multispecific-multivalent polypeptides

In a further aspect, the polypeptide is at least bispecific, but can also be e.g., trispecific, tetraspecific, pentaspecific, etc. Moreover, the polypeptide is at least bivalent, but can also be e.g., trivalent, tetravalent, pentavalent, hexavalent, etc.

The terms “*bispecific*”, “*trispecific*”, “*tetraspecific*”, “*pentaspecific*”, etc., all fall under the term “*multispecific*” and refer to binding to two, three, four, five, etc., different target molecules, respectively.

The terms “*bivalent*”, “*trivalent*”, “*tetravalent*”, “*pentavalent*”, “*hexavalent*”, etc. all fall
5 under the term “*multivalent*” and indicate the presence of two, three, four, five, six, etc., binding units/building blocks, respectively, such as ISVDs.

For example, the polypeptide may be bispecific-bivalent, such as a polypeptide comprising or consisting of two ISVDs, wherein one ISVD specifically binds to GPC3 and one ISVD specifically binds to the constant domain of a TCR on a T cell, wherein the GPC3 and TCR are
10 preferably human GPC3 and human TCR. The polypeptide may also be bispecific-trivalent, such as a polypeptide comprising or consisting of three ISVDs, wherein two ISVD specifically bind to GPC3 and one ISVD specifically binds to the constant domain of a TCR on a T cell, wherein the GPC3 and TCR are preferably human GPC3 and human TCR. In another example, the polypeptide may be trispecific-tetravalent, such as a polypeptide comprising or
15 consisting of four ISVDs, wherein two ISVDs specifically bind to human GPC3, one ISVD specifically binds to the constant domain of a human TCR on a T cell and one ISVD binds to human serum albumin. Such a polypeptide may at the same time be biparatopic, for example if two ISVDs bind two different epitopes on human GPC3. The term “biparatopic” refers to binding to two different parts (e.g., epitopes) of the same target molecule. A
20 preferred trispecific-tetravalent polypeptide is e.g., ISVD construct A022600424, comprising two ISVDs specifically binding to human GPC3, one ISVD specifically binding to the constant domain of a human TCR on a T cell, one ISVD binding to human serum albumin, and which is biparatopic for binding to GPC3.

In one embodiment, the present technology provides a bispecific-bivalent polypeptide
25 comprising or consisting of at least two ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least two ISVDs are optionally linked via one or more peptidic linkers, and wherein:

a) a first ISVD comprises

i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid
30 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;

- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and

5 a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- 10 vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,

b) a first ISVD comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- 15 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, and

a second ISVD comprises

- 20 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid
- 25 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,

c) a first ISVD comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
- 30 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and
a second ISVD comprises
- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, or
- d) a first ISVD comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and
a second ISVD comprises
- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14.

In a preferred embodiment, such a polypeptide comprises or consists of at least two ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least two ISVDs are optionally linked via one or more peptidic linkers, and wherein

- a) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid

sequence of SEQ ID NO: 14, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,

5 b) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14,

10 c) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, or

15 d) the first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, and the second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14.

Thus, such a polypeptide can be a polypeptide, wherein:

20 a) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 3,

b) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3, and the second ISVD exhibits a sequence identity of more than
25 90% identity with SEQ ID NO: 2

c) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4, or

d) the amino acid sequence of the first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 4, and the second ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 2.

Preferably, in such a polypeptide:

- 5 a) the first ISVD consists of the amino acid sequence of SEQ ID NO: 2, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 3,
- b) the first ISVD consists of the amino acid sequence of SEQ ID NO: 3, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 2,
- c) the first ISVD consists of the amino acid sequence of SEQ ID NO: 2, and the second
10 ISVD consists of the amino acid sequence of SEQ ID NO: 4, or
- d) the first ISVD consists of the amino acid sequence of SEQ ID NO: 4, and the second ISVD consists of the amino acid sequence of SEQ ID NO: 2.

In another aspect, the present technology provides a trispecific-trivalent polypeptide comprising the bispecific-bivalent polypeptides described above and a third ISVD binding to
15 human serum albumin as described in detail below (section 5.4; “(In vivo) half-life extension”).

In one embodiment, the present technology provides a bispecific-trivalent polypeptide comprising or consisting of at least three ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least
20 three ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- a) a first ISVD specifically binds the constant domain of a TCR on a T cell and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6, a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ
25 ID NO: 10, and a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14;
- b) a second ISVD specifically binds GPC3 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s)

with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, and a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; and

- 5 c) a third ISVD specifically binds GPC3 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
- 10

wherein the TCR and GPC3 bound by said polypeptide is preferably human TCR and human GPC3, respectively.

In a preferred embodiment of the multispecific-multivalent polypeptide:

- a) said first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
- 15
- b) said second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15; and
- c) said third ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16.
- 20

In a further aspect of the multispecific-multivalent polypeptide:

- a) the amino acid sequence of said first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 2, wherein preferably said first ISVD consists of the amino acid sequence of SEQ ID NO: 2;
- 25

b) the amino acid sequence of said second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3, wherein preferably said second ISVD consists of the amino acid sequence of SEQ ID NO: 3; and

5 c) the amino acid sequence of said third ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4, wherein preferably said third ISVD consists of the amino acid sequence of SEQ ID NO: 4.

The terms "first ISVD", "second ISVD", "third ISVD", etc., in this regard only indicate the relative position of the ISVDs to each other, wherein the numbering is started from the N-terminus of the polypeptide. The "first ISVD" is thus closer to the N-terminus than the
10 "second ISVD", whereas the "second ISVD" is closer to the N-terminus than the "third ISVD". Accordingly, the ISVD arrangement is inverse when considered from the C-terminus. Since the numbering is not absolute and only indicates the relative position of the at least four ISVDs it is not excluded that other binding units/building blocks such as additional ISVDs binding to GPC3, or ISVDs binding to another target may be present in the polypeptide.
15 Moreover, it does not exclude the possibility that other binding units/building blocks such as ISVDs can be placed in between. For instance, as described further below (see in particular, section 5.4 "(In vivo) half-life extension" below), the polypeptide can further comprise another ISVD binding to human serum albumin that can even be located between e.g. the "third ISVD" and "fourth ISVD".

20 In a further aspect the present technology thus provides a trispecific-tetravalent polypeptide comprising the bispecific-trivalent polypeptides described above and a fourth ISVD binding to human serum albumin as described in detail below (section 5.4; "(In vivo) half-life extension").

In a preferred embodiment, the first ISVD binding to TCR is positioned at the N-terminus of
25 the polypeptide.

In another aspect the present technology provides a bispecific-bivalent polypeptide comprising an ISVD that specifically binds to GPC3 as described in detail for the monospecific-monovalent polypeptides above (section 5.1; "Monospecific-monovalent

polypeptides”) and an ISVD binding to human serum albumin as described in detail below (section 5.4; “(In vivo) half-life extension”).

In another aspect the present technology provides a bispecific-trivalent polypeptide comprising the monospecific-bivalent polypeptides above (section 5.1; “monospecific-bivalent polypeptides”) and an ISVD binding to human serum albumin as described in detail below (section 5.4; “(In vivo) half-life extension”).

The components, preferably ISVDs, of said multispecific-multivalent polypeptides described herein may be linked to each other by one or more suitable linkers, such as peptidic linkers.

The use of linkers to connect two or more (poly)peptides is well known in the art. Exemplary peptidic linkers are shown in Table A-5. One often used class of peptidic linker are known as the “Gly-Ser” or “GS” linkers. These are linkers that essentially consist of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif such as the GGGGS (SEQ ID NO: 100) motif (for example, exhibiting the formula (Gly-Gly-Gly-Gly-Ser)_n in which n may be 1, 2, 3, 4, 5, 6, 7 or more). Some often used examples of such GS linkers are 9GS linkers (GGGGSGGGGS, SEQ ID NO: 103) 15GS linkers (n=3) and 35GS linkers (n=7). Reference is for example made to Chen et al., Adv. Drug Deliv. Rev. 2013 Oct 15; 65(10): 1357–1369; and Klein et al., Protein Eng. Des. Sel. (2014) 27 (10): 325-330. In the polypeptide(s) disclosed herein, the use of 5GS and 9GS linkers to link the components of the polypeptide to each other is preferred. Preferably, a linker of less than 10 amino acids, such as less than 6 amino acids, in particular a 5GS linker, is used to link a first ISVD specifically binding to TCR to a second ISVD specifically binding to GPC3.

In one aspect of the multispecific-multivalent polypeptide, the polypeptide comprising or consisting of at least three ISVDs, comprises the at least two ISVDs specifically binding to GPC3 and one ISVD specifically binding to TCR. In this aspect of the technology, the ISVD binding to TCR is linked to one of the at least two ISVDs binding to GPC3 via a 5GS linker, whereas the at least two ISVDs specifically binding to GPC3 are linked to each other via a 9GS linker. In further aspect, the multispecific-multivalent polypeptide further comprises an ISVD binding to albumin, which is further linked via a 9 GS linker to the ISVD binding to GPC3 that is not linked to the ISVD binding to TCR (as described in section 5.4 “(In vivo) half-life

extension" below). The inventors surprisingly found that such a configuration can increase the efficiency of the polypeptide in eliciting a T cell-mediated cytotoxic response.

Accordingly, it is preferred that the polypeptide comprises or consists of the following, in the order starting from the N-terminus of the polypeptide: a first ISVD specifically binding to
5 TCR, a second ISVD specifically binding to GPC3, a third ISVD specifically binding to GPC3, and an optional binding unit providing the polypeptide with increased half-life as defined herein. The binding unit providing the polypeptide with increased half-life is preferably an ISVD, that preferably binds to serum albumin.

It is even more preferred that the polypeptide comprises or consists of the following, in the
10 order starting from the N-terminus of the polypeptide: an ISVD specifically binding to TCR, a linker, a second ISVD specifically binding to GPC3, a linker, a third ISVD specifically binding to GPC3, a linker, and an ISVD binding to human serum albumin, wherein the linker between the first and second ISVDs is preferably a 5GS linker, whereas the other linkers are preferably 9GS linkers.

15 Such configurations of the polypeptide can provide for strong potencies with regard to treating cancer as well as low binding to pre-existing antibodies.

Preferably, the multispecific-multivalent polypeptide exhibits reduced binding by pre-existing antibodies in human serum. To this end, in one embodiment, the polypeptide exhibits a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89
20 (according to Kabat numbering) in at least one ISVD (and preferably the ISVD at the C-terminal end of the polypeptide), but preferably in each ISVD. In another embodiment, the polypeptide exhibits an extension of 1 to 5 (preferably naturally occurring) amino acids, such as a single alanine (A) extension, at the C-terminus of the C-terminal ISVD. The C-terminus of an ISVD is normally VTVSS (SEQ ID NO: 116). In another embodiment, the polypeptide
25 exhibits a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD. In another embodiment, the ISVD exhibits a lysine (K) or glutamine (Q) at position 112 (according to Kabat numbering) in at least one ISVD. In these embodiments, the C-terminus of the ISVD is VKVSS (SEQ ID NO: 117), VQVSS (SEQ ID NO: 118), VTVKS (SEQ ID NO: 119), VTVQS (SEQ ID NO: 120), VKVKS (SEQ ID NO: 121), VKVQS (SEQ ID NO: 122), VQVKS
30 (SEQ ID NO: 123), or VQVQS (SEQ ID NO: 124) such that after addition of a single alanine the

C-terminus of the polypeptide for example exhibits the sequence VTVSSA (SEQ ID NO: 125), VKVSSA (SEQ ID NO: 126), VQVSSA (SEQ ID NO: 127), VTVKSA (SEQ ID NO: 128), VTVQSA (SEQ ID NO: 129), VKVKSA (SEQ ID NO: 130), VKVQSA (SEQ ID NO: 131), VQVKSA (SEQ ID NO: 132), or VQVQSA (SEQ ID NO: 133), preferably VTVSSA (see Table A-7). In another embodiment, the polypeptide exhibits a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in at least the C-terminal ISVD, optionally a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD, and exhibits an extension of 1 to 5 (preferably naturally occurring) amino acids, such as a single alanine (A) extension, at the C-terminus of the C-terminal ISVD (such that the C-terminus of the polypeptide for example consists of the sequence VTVSSA, VKVSSA or VQVSSA, preferably VTVSSA). See e.g. WO2012/175741 and WO2015/173325 for further information in this regard.

In a preferred embodiment, the multispecific-multivalent polypeptide comprises or consists of an amino acid sequence exhibiting a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 1, wherein more preferably the CDRs of the four ISVDs are as defined in items A to D using the Abm definition (or A' to D' if using the Kabat definition) set forth in sections "5.2 Immunoglobulin single variable domains" and "5.4 (In vivo) half-life extension" below, respectively, wherein in particular:

- the first ISVD specifically binding to the constant domain of a TCR on a T cell comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
- the second ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15;
- the third ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16; and

- the fourth ISVD binding to human serum albumin comprises a CDR1 being the amino acid sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17,

or alternatively if using the Kabat definition:

- 5 • the first ISVD specifically binding to TCR comprises a CDR1 being the amino acid sequence of SEQ ID NO: 31, a CDR2 being the amino acid sequence of SEQ ID NO: 35 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
- the second ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 32, a CDR2 being the amino acid sequence of SEQ ID NO: 36
10 and a CDR3 being the amino acid sequence of SEQ ID NO: 15;
- the third ISVD specifically binding to GPC3 comprises a CDR1 being the amino acid sequence of SEQ ID NO: 33, a CDR2 being the amino acid sequence of SEQ ID NO: 37 and a CDR3 being the amino acid sequence of SEQ ID NO: 16; and
- the fourth ISVD binding to human serum albumin comprises a CDR1 being the amino
15 acid sequence of SEQ ID NO: 34, a CDR2 being the amino acid sequence of SEQ ID NO: 38 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.

In some aspects, the polypeptide comprises or consists of an amino acid sequence selected from SEQ ID NOs: 1, 49-72 and 78-81.

- Preferably, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1 (see Table A-3). In a most preferred embodiment, the polypeptide consists of the amino
20 acid sequence of SEQ ID NO: 1.

- The polypeptide preferably exhibits at least half the binding affinity, more preferably at least the same binding affinity, to human TCR and to human GPC3 as compared to a polypeptide consisting of the amino acid of SEQ ID NO: 1 wherein the binding affinity is measured using
25 the same method, such as Surface Plasmon Resonance (SPR).

5.2 Immunoglobulin single variable domains

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}. Preferably, it 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) ; other single variable domains, or any suitable fragment of any one thereof.

- 5 In particular, the immunoglobulin single variable domain may be an immunoglobulin single variable domain (such as a V_{HH} , including a humanized V_{HH} or camelized V_H) or a suitable fragment thereof. Nanobody®, Nanobodies® and Nanoclone® are registered trademarks of Ablynx N.V.

- 10 "V_{HH} domains", also known as V_{HHS}, 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
15 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
20 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 has been described extensively in various publications, among which WO 94/04678, Hamers-Casterman *et al.* 1993 and Muyldermans et al. 2001 can be exemplified. In these methods, camelids are immunized with the target
25 antigen in order to induce an immune response against said target antigen. The repertoire of VHHs obtained from said immunization is further screened for VHHs 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
5 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 technology comprises camelid immunoglobulin sequences and humanized camelid immunoglobulin
10 sequences, or camelized domain antibodies, e.g. camelized dAb as described by Ward et al (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996)). Moreover, the technology 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.
15 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
20 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
25 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 are preferably 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*). Preferably, the V_H sequence that is used as a starting material or starting point for generating or designing the camelized V_H is preferably a V_H sequence from a mammal, more preferably 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, 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.

In the present application, unless indicated otherwise, CDR sequences were determined according to the AbM definition as described in Kontermann and Dübél (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, 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.

- 5 The framework sequences are preferably (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).

- 15 In particular, the framework sequences present in the ISVD sequence used in the technology may contain one or more of hallmark residues (as defined herein), such that the ISVD sequence is 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.

- 20 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).

- 25 However, it should be noted that the technology 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
30 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
5 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.

10 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
15 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 above, an ISVD may be a Nanobody® or a suitable fragment thereof. For a
20 general description of Nanobodies, 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 Nanobodies of the so-called “V_H3 class” (i.e. Nanobodies 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 technology in
25 its broadest sense can generally use any type of Nanobody, and for example also uses the Nanobodies belonging to the so-called “V_H4 class” (i.e. Nanobodies 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, Nanobodies (in particular V_{HH} sequences, including (partially) humanized V_{HH}
30 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 Nanobody can be defined as an immunoglobulin sequence with the (general) structure

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

- 5 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, a Nanobody can be an immunoglobulin sequence with the (general) structure

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

- 10 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, a Nanobody can be an immunoglobulin sequence with the (general) structure

- 15 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 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

- 20 Table A-0 below.

Table A-0: Hallmark Residues in Nanobodies

Position	Human V _H 3	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:

- (1) In particular, but not exclusively, in combination with KERE or KQRE at positions 43-46.
- (2) Usually as GLEW at positions 44-47.
- (3) Usually as KERE or KQRE at positions 43-46, e.g. as KEREL, KEREV, KQREL, KQREV, KEREH, KQREH or KQREG at positions 43-47. Alternatively, also sequences such as TERE (for example TEREL), TQRE (for example TQREL), KECE (for example KECER), KQCE (for example KQCEL), RERE (for example REREG), RQRE (for example RQREL, RQREV or RQREH), QERE (for example QEREG), QQRE, (for example QQREH, QQREL or QQREV), KGRE (for example KGREG), KDRE (for example KDREV) are possible.

Some other possible, but less preferred sequences include for example DECKL and NVCEL.

- (4) With both GLEW at positions 44-47 and KERE or KQRE at positions 43-46.
- (5) Often as KP or EP at positions 83-84 of naturally occurring V_{HH} domains.
- (6) In particular, but not exclusively, in combination with GLEW at positions 44-47.
- (7) With the proviso that when positions 44-47 are GLEW, position 108 is always Q in (non-humanized) V_{HH} sequences that also contain a W at 103.
- (8) The GLEW group also contains GLEW-like sequences at positions 44-47, such as for example GVEW, EPEW, GLER, DQEW, DLEW, GIEW, ELEW, GPEW, EWLP, GPER, GLER and ELEW.

The technology *inter alia* uses ISVDs that can bind to the constant domain of a TCR or GPC3. 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
5 antigens.

The multispecific-multivalent polypeptide may comprise one or more ISVDs specifically binding to GPC3. For example, the polypeptide may comprise two ISVDs that specifically bind to GPC3 and an ISVD that specifically binds to TCR.

The ISVDs used in the technology can form part of a polypeptide, which comprises or
10 consists of at least two ISVDs, such that the polypeptide can specifically bind to GPC3 and TCR.

Accordingly, the target molecules of the ISVDs used in the technology are GPC3 and the constant domain of the TCR, respectively. Binding to TCR can be achieved, for example, by binding to the TCRalpha subunit and/or the TCR beta subunit. Examples are mammalian
15 GPC3 and TCR. While human GPC3 (Uniprot accession P51654, see Table A-8) and human TCR (see Table A-8) are preferred, the versions from other species are also amenable to the present technology, for example GPC3 and TCR from mice, rats, rabbits, cats, dogs, goats,

sheep, horses, pigs, non-human primates, such as cynomolgus monkeys (also referred to herein as "*cyno*"), or camelids, such as llama or alpaca.

Specific examples of ISVDs specifically binding to the constant domain of a TCR on a T cell that can be used in the technology are as described in the following item A:

- 5 A. An ISVD that specifically binds to human TCR and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,
- preferably a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14.
- 15 In a preferred embodiment, the ISVD binds to the constant domain of a human TCR- α of SEQ ID NO: 135 and/or of a TCR- β of SEQ ID NO: 136, or polymorphic variants or isoforms thereof.

Preferred examples of such an ISVD that specifically binds to human TCR comprise one or more (and preferably all) framework regions as indicated for ISVD TCE01 in Table A-2 (in addition to the CDRs as defined in the preceding item A), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD TCE01 (SEQ ID NO: 2; see Table A-1 and A-2).

Also, in a preferred embodiment, the amino acid sequence of the ISVD specifically binding to human TCR may exhibit a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 2, wherein the CDRs are as defined in the preceding item A.

25 In particular, the ISVD specifically binding to TCR most preferably is the amino acid sequence of SEQ ID NO: 2.

When such an ISVD binding to TCR exhibits 2 or 1 amino acid difference in at least one CDR relative to a corresponding reference CDR sequence (item A above), the ISVD preferably

exhibits at least half the binding affinity, more preferably at least the same binding affinity to human TCR as the construct TCE01 set forth in SEQ ID NO: 2, wherein the binding affinity is measured using the same method, such as SPR.

Specific examples of ISVDs specifically binding to GPC3 that can be used in the technology
5 are as described in the following items B and C:

B. An ISVD that specifically binds to human GPC3 and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,

preferably a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of
15 SEQ ID NO: 15.

C. An ISVD that specifically binds to human GPC3 and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- 20 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,

preferably a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of
25 SEQ ID NO: 16.

In a preferred embodiment, the ISVD binds to human GPC3 of SEQ ID NO: 134.

Preferred examples of such an ISVD that specifically binds to human GPC3 comprise one or more (and preferably all) framework regions as indicated for ISVD A022600351 and A022600314, respectively, in Table A-2 (in addition to the CDRs as defined in the preceding items B and C, respectively), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD A022600351 or A022600314 (SEQ ID NOs: 3 or 4, see Table A-1 and A-2).

Also, in a preferred embodiment, the amino acid sequence of an ISVD(s) specifically binding to human GPC3 may exhibit a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 3 or 4, respectively, wherein the CDRs are as defined in the preceding item B or C, respectively. In particular, the ISVD binding to human GPC3 most preferably is the amino acid sequence of SEQ ID NOs: 3 or 4.

When such an ISVD binding to human GPC3 exhibits 2 or 1 amino acid difference in at least one CDR relative to a corresponding reference CDR sequence (item B or C above), the ISVD preferably exhibits at least half the binding affinity, more preferably at least the same binding affinity to human GPC3 as construct A022600351 or A022600314 set forth in SEQ ID NO: 3 and 4, respectively, wherein the binding affinity is measured using the same method, such as SPR.

Preferably, each of the ISVDs as defined under items A to C above is comprised in the polypeptide.

Such a polypeptide comprising each of the ISVDs as defined under items A to C above preferably exhibits at least half the binding affinity, more preferably at least the same binding affinity, to human TCR and to human GPC3 as a polypeptide consisting of the amino acid of SEQ ID NO: 1, wherein the binding affinity is measured using the same method, such as SPR.

The SEQ ID NOs referred to in the above items A to C and item D below (see section 5.4 “(In vivo) half-life extension”) are based on the CDR definition according to the AbM definition (see Table A-2). It is noted that the SEQ ID NOs defining the same CDRs according to the Kabat definition (see Table A-2-1) can likewise be used in the above items A to C and item D below (see section 5.4 “(In vivo) half-life extension”).

Accordingly, the specific examples of ISVDs specifically binding to the constant domain of a TCR on a T cell or GPC3 that can be used in the technology are as described above using the AbM definition can be also described using the Kabat definition as set forth in items A' to C' below:

- 5 A'. An ISVD that specifically binds to human TCR and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 31 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 31;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 35 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 35; and
 - 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,
- preferably a CDR1 being the amino acid sequence of SEQ ID NO: 31, a CDR2 being the amino acid sequence of SEQ ID NO: 35 and a CDR3 being the amino acid sequence of SEQ ID NO: 14.
- 15 Preferred examples of such an ISVD that specifically binds to human TCR comprise one or more (and preferably all) framework regions as indicated for ISVD TCE01, respectively, in Table A-2-1 (in addition to the CDRs as defined in the preceding item A'), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD TCE01 (SEQ ID NO: 2; see Table A-1 and A-2-1).
- 20 B'. An ISVD that specifically binds to human GPC3 and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 32 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 32;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 36 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 36; and
 - 25 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,

preferably a CDR1 being the amino acid sequence of SEQ ID NO: 32, a CDR2 being the amino acid sequence of SEQ ID NO: 36 and a CDR3 being the amino acid sequence of SEQ ID NO: 15.

C'. An ISVD that specifically binds to human GPC3 and comprises

- 5 i. a CDR1 that is the amino acid sequence of SEQ ID NO: 33 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 33;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 37 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 37; and
- 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,

preferably a CDR1 being the amino acid sequence of SEQ ID NO: 33, a CDR2 being the amino acid sequence of SEQ ID NO: 37 and a CDR3 being the amino acid sequence of SEQ ID NO: 16.

Preferred examples of such an ISVD(s) that specifically binds to human GPC3 comprise one or more (and preferably all) framework regions as indicated for ISVD A022600351 and A022600314, respectively, in Table A-2-1 (in addition to the CDRs as defined in the preceding items B' and C', respectively), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD A022600351 or A022600314 (SEQ ID NOs: 3 or 4, see Table A-1 and A-2-1).

20 The percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence may be calculated 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 (i.e. at a single position).

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.

5 An “amino acid difference” as used herein refers to a deletion, insertion or substitution of a single amino acid residue vis-à-vis a reference sequence, and preferably is a substitution.

Amino acid substitutions are preferably conservative substitutions. Such conservative substitutions preferably are substitutions in which one amino acid within the following groups (a) – (e) is substituted by another amino acid residue within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp.

15 Particularly preferred conservative substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

5.3 Specificity

20 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, preferably specifically bind to their designated targets.

25 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 KD, 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 $(\text{mol/liter})^{-1}$ (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 $10\mu\text{M}$) 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 TCR and/or GPC3 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. Examples of related targets for GPC3 are GPC1, GPC2, GPC4, GPC5 or GPC6. Thus, in an embodiment of the technology, the ISVDs comprised in the polypeptide that bind to GPC3, bind to GPC3 with a K_D value of 10^{-5} to 10^{-12} moles/liter or less and bind to GPC1, GPC2, GPC4, GPC5 and GPC6 of the same species with a K_D value greater than 10^{-4} moles/liter.

Thus, the polypeptide preferably exhibits at least half the binding affinity, more preferably at least the same binding affinity, to human TCR and to human GPC3 as compared to a polypeptide consisting of the amino acid of SEQ ID NO: 1, wherein the binding affinity is measured using the same method, such as SPR.

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 TCR does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to TCR from cynomolgus monkeys. Likewise, for example, specific binding to human GPC3 does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to GPC3 from cynomolgus monkeys ("cyno").

The ISVD with SEQ ID NO: 2 that binds to human TCR and that is comprised in the polypeptides of the current technology, exhibits improved binding characteristics compared to ISVD T0170056G05, which is described in WO 2016/180969 A1. More specifically, the ISVD with SEQ ID NO:2 is derived from T017056G05 and comprises specific mutations in CDR1 and CDR3 which result in improved cross-reactivity for binding to human and non-human primate (such as cynomolgus monkey) TCR compared to ISVD T0170056G05.

When an ISVD is said to exhibit "improved cross-reactivity for binding to human and non-human primate TCR" 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 TCR and for non-human primate TCR 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 TCR allows for the assessment of toxicity of a multispecific T cell engaging polypeptide in preclinical studies conducted on non-human primates.

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: 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 Johnson 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
5 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).

5.4 (In vivo) half-life extension

The polypeptide may further comprise one or more other groups, residues, moieties or
10 binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased (*in vivo*) half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. *In vivo* half-life extension means, for example, that the polypeptide exhibits an increased half-life in a mammal, such as a human subject,
15 after administration. Half-life can be expressed for example as $t_{1/2\beta}$.

The type of groups, residues, moieties or binding units is not generally restricted and may for example be chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

20 More specifically, said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life can be chosen from the group consisting of binding units that can bind to serum albumin, such as human serum albumin, or a serum immunoglobulin, such as IgG, and preferably is a binding unit that can bind to human serum albumin. The binding unit is preferably an ISVD.

25 For example, WO 04/041865 describes Nanobodies® binding to serum albumin (and in particular against human serum albumin) that can be linked to other proteins (such as one or more other Nanobodies binding to a desired target) in order to increase the half-life of said protein.

The international application WO 06/122787 describes a number of Nanobodies® against (human) serum albumin. These Nanobodies® include the Nanobody® called Alb-1 (SEQ ID NO: 52 in WO 06/122787) and humanized variants thereof, such as Alb-8 (SEQ ID NO: 62 in WO 06/122787). Again, these can be used to extend the half-life of therapeutic proteins and polypeptide and other therapeutic entities or moieties.

Moreover, WO2012/175400 describes a further improved version of Alb-1, called Alb-23.

In a preferred embodiment, the polypeptide comprises a serum albumin binding moiety selected from Alb-1, Alb-3, Alb-4, Alb-5, Alb-6, Alb-7, Alb-8, Alb-9, Alb-10 and Alb-23, preferably Alb-8 or Alb-23 or its variants, as shown in pages 7-9 of WO2012/175400 and the albumin binders described in WO 2012/175741, WO2015/173325, WO2017/080850, WO2017/085172, WO2018/104444, WO2018/134235, WO2018/134234. Some preferred serum albumin binders are also shown in Table A-4. A particularly preferred further component of the polypeptide is as described in item D:

D. An ISVD that binds to human serum albumin and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17;
- preferably a CDR1 being the amino acid sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.

Preferred examples of such an ISVD that binds to human serum albumin comprise one or more (and preferably all) framework regions as indicated for ISVD ALB23002 in Table A-2 (in addition to the CDRs as defined in the preceding item D), and most preferred is an ISVD that consists of the full amino acid sequence of ISVD ALB23002 (SEQ ID NO: 5, see Table A-1 and A-2).

Item D can be also described using the Kabat definition as:

D'. An ISVD that binds to human serum albumin and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 34 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 34;
 - 5 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 38 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 38; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17;
- preferably a CDR1 being the amino acid sequence of SEQ ID NO: 34, a CDR2 being the amino acid sequence of SEQ ID NO: 38 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.

Preferred examples of such an ISVD that binds to human serum albumin comprise one or more (and preferably all) framework regions as indicated for ISVD ALB23002 in Table A-2-1 (in addition to the CDRs as defined in the preceding item D'), and most preferred is an ISVD consists of the full amino acid sequence of ISVD ALB23002 (SEQ ID NO: 5, see Table A-1 and A-2-1).

Also, in a preferred embodiment, the amino acid sequence of an ISVD binding to human serum albumin may exhibit a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 5, wherein the CDRs are as defined in the preceding item D or D'. In particular, the ISVD binding to human serum albumin preferably is the amino acid sequence of SEQ ID NO: 5.

When such an ISVD binding to human serum albumin exhibits 2 or 1 amino acid difference in at least one CDR relative to a corresponding reference CDR sequence (item D or D' above), the ISVD exhibits at least half the binding affinity, preferably at least the same binding affinity to human serum albumin as construct ALB23002 set forth in SEQ ID NO: 5, wherein the binding affinity is measured using the same method, such as SPR.

When such an ISVD binding to human serum albumin is at a C-terminal position it can exhibit a C-terminal alanine (A) or glycine (G) extension (preferably A) and is preferably selected

from SEQ ID NOs: 83, 85, 87, 89, 91, 93, 95, 96 and 98, most preferably SEQ ID NO: 96, which represents SEQ ID NO: 5 with a single alanine extension (see table A-4 below). In some embodiments, the ISVD binding to human serum albumin is at another position than the C-terminal position (i.e. is not the C-terminal ISVD of the polypeptide) and is selected from SEQ ID NOs: 5, 82, 84, 86, 88, 90, 92, 94 and 97 (see table A-4 below).

5.5 Nucleic acid molecules

Also provided is a nucleic acid molecule encoding the polypeptide as disclosed herein.

A “nucleic acid molecule” (used interchangeably with “nucleic acid”) is a chain of nucleotide monomers linked to each other via a phosphate backbone to form a nucleotide sequence. A nucleic acid may be used to transform/transfect a host cell or host organism, e.g. for expression and/or production of a polypeptide. Suitable hosts or host cells for production purposes 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. A host or host cell comprising a nucleic acid encoding the polypeptide is also encompassed by the technology.

A nucleic acid may be for example DNA, RNA, or a hybrid thereof, and may also comprise (e.g. chemically) modified nucleotides, like PNA. It can be single- or double-stranded, and is preferably in the form of double-stranded DNA. For example, the nucleotide sequences may be genomic DNA or cDNA.

The nucleic acids can be prepared or obtained in a manner known per se, and/or can be isolated from a suitable natural source. Nucleotide sequences encoding naturally occurring (poly)peptides can for example be subjected to site-directed mutagenesis, so as to provide a nucleic acid molecule encoding polypeptide with sequence variation. Also, as will be clear to the skilled person, to prepare a nucleic acid, also several nucleotide sequences, such as at least one nucleotide sequence encoding a targeting moiety and for example nucleic acids encoding one or more linkers can be linked together in a suitable manner.

Techniques for generating nucleic acids 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
5 more “mismatched” primers.

5.6 Vectors

Also provided is a vector comprising the nucleic acid molecule encoding the polypeptide as disclosed herein. A vector as used herein is a vehicle suitable for carrying genetic material into a cell. A vector includes naked nucleic acids, such as plasmids or mRNAs, or nucleic acids
10 embedded into a bigger structure, such as liposomes or viral vectors.

Vectors generally comprise at least one nucleic acid that is optionally linked to one or more regulatory elements, such as for example one or more suitable promoter(s), enhancer(s), terminator(s), etc.). The vector preferably is an expression vector, i.e. a vector suitable for expressing an encoded polypeptide or construct under suitable conditions, e.g. when the
15 vector is introduced into a (e.g. human) cell. For DNA-based vectors, this usually includes the presence of elements for transcription (e.g. a promoter and a polyA signal) and translation (e.g. Kozak sequence).

Preferably, in the vector, said at least one nucleic acid and said regulatory elements are “operably linked” to each other, by which is generally meant that they are in a functional
20 relationship with each other. For instance, a promoter is considered “operably linked” to a coding sequence if said promoter is able to 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
25 also in the same reading frame. They will usually also be essentially contiguous, although this may also not be required.

Preferably, any regulatory elements of the vector are such that they are capable of providing their intended biological function in the intended host cell or host organism.

For instance, a promoter, enhancer or terminator should be “operable” in the intended host cell or host organism, by which is meant that for example said promoter should be capable of initiating or otherwise controlling/regulating the transcription and/or the expression of a nucleotide sequence - e.g. a coding sequence - to which it is operably linked.

5 5.7 Compositions

The technology also provides a composition comprising at least one polypeptide as disclosed herein, at least one nucleic acid molecule encoding a polypeptide as disclosed herein or at least one vector comprising such a nucleic acid molecule. The composition may be a pharmaceutical composition. The composition may further comprise at least one
10 pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally comprise one or more further pharmaceutically active polypeptides and/or compounds.

5.8 Host organisms

The technology also pertains to host cells or host organisms comprising the polypeptide as disclosed herein, the nucleic acid encoding the polypeptide as disclosed herein, and/or the
15 vector comprising the nucleic acid molecule encoding the polypeptide as disclosed herein.

Suitable host cells or host organisms are clear to the skilled person, and are for example any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism. Specific examples include HEK293 cells, CHO cells, *Escherichia coli* or *Pichia pastoris*. The most preferred host is *Pichia pastoris*.

20 5.9 Methods and uses of the polypeptide

The technology also provides a method for producing the polypeptide as disclosed herein. The method may comprise transforming/transfecting a host cell or host organism with a nucleic acid encoding the polypeptide, expressing the polypeptide in the host, optionally followed by one or more isolation and/or purification steps. Specifically, the method may
25 comprise:

- a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid sequence encoding the polypeptide; optionally followed by:
- b) isolating and/or purifying the polypeptide.

Suitable host cells or host organisms for production purposes 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. Specific examples include HEK293 cells, CHO cells, *Escherichia coli* or *Pichia pastoris*. The most preferred host is *Pichia pastoris*.

- 5 The polypeptide, a nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector -preferably the polypeptide or a composition comprising the same- are useful as a medicament.

Accordingly, the technology provides the polypeptide, nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector for
10 use as a medicament.

Also provided is the polypeptide, nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector for use in the treatment of cancer.

Further provided is a method of treating cancer, wherein said method comprises
15 administering, to a subject in need thereof, a pharmaceutically active amount of the polypeptide, nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector.

Further provided is the use of the polypeptide, nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector in the
20 preparation of a medicament.

Further provided is the use of the polypeptide, nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector in the preparation of a pharmaceutical composition, preferably for treating cancer.

The cancer can be any type of GPC3 expressing cancer. The cancer preferably is liver cancer
25 or lung cancer, preferably liver cancer. Liver cancer is preferably hepatocellular carcinoma. Lung cancer is preferably non-small cell lung cancer (NSCLC), most preferably squamous cell carcinoma (SCC).

Preferably, the GPC3 expressing cancer cells express (on average) at least half the amount of GPC3 protein, preferably at least the same amount of GPC3 protein as Huh7 cells (on

average). Huh7 cells are publicly available from e.g. the National Institutes of Biomedical Innovation, Health and Nutrition, JCRB Cell Bank, under accession number JCRB0403.

The expressed GPC3 preferably refers to cell surface-exposed GPC3. The expression of (cell surface-exposed) GPC3 on a cell (and the amount thereof) can be readily determined by
5 routine methods commonly known in the art, such as flow cytometry, immunohistochemistry or as described in the examples.

A “subject” as referred to in the context of the technology can be any animal, preferably a mammal. Among mammals, a distinction can be made between humans and non-human mammals. Non-human animals may be for example companion animals (e.g. dogs, cats),
10 livestock (e.g. bovine, equine, ovine, caprine, or porcine animals), or animals used generally for research purposes and/or for producing antibodies (e.g. mice, rats, rabbits, cats, dogs, goats, sheep, horses, pigs, non-human primates, such as cynomolgus monkeys, or camelids, such as llama or alpaca).

In the context of prophylactic and/or therapeutic purposes, the subject can be any animal,
15 and more specifically any mammal, but preferably is a human subject.

Substances (including polypeptides, nucleic acid molecules and vectors) or compositions may be administered to a subject by any suitable route of administration, for example by enteral (such as oral or rectal) or parenteral (such as epicutaneous, sublingual, buccal, nasal, intra-articular, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous,
20 transdermal, or transmucosal) administration. Parenteral administration, such as intramuscular, subcutaneous or intradermal, administration is preferred. Most preferred is subcutaneous administration.

An effective amount of a polypeptide, a nucleic acid molecule or vector as described, or a composition comprising the polypeptide, nucleic acid molecule or vector can be
25 administered to a subject in order to provide the intended treatment results.

One or more doses can be administered. If more than one dose is administered, the doses can be administered in suitable intervals in order to maximize the effect of the polypeptide, composition, nucleic acid molecule or vector.

Table A-1: Amino acid sequences of the different monovalent ISVD building blocks identified within the pentavalent polypeptide A022600424 ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
TCE01	2	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVAH ISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRIW PYDYWGQGTLVTVSS
A022600351*	3	EVQLVESGGGVVQPGGSLRLSCAASGFTFSSFAMTWVRRPPGKGLEWVAT ITNKGVTSYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYICANARRTG PRAPTDIGSYRGQGTLVTVSS
A022600314°	4	EVQLVESGGGVVQPGGSLRLSCAASGSIFRSVFSSTMEWYRQAPGKKREL VARIAPGEGTYYGALYADSVKGRFTISRDNKNTVYLQMNSLRPEDTALYYC ASGVAWGQGTLVTVSS
ALB23002	5	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVS SISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLS RSSQGTLVTVSS

* Sequence optimized variant of A02260018C08 (SEQ ID NO: 48)

5 ° Sequence optimized variant of A02260015A08 (SEQ ID NO: 47)

Table A-2: Sequences for CDRs and frameworks ("ID" refers to the given SEQ ID NO)

ID	ISVD	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
2	TCE01	18	DVQLVESGGG VVQPGGSLRL SCVAS	6	GVVHKL NFYG	20	WYRQAPG KEREKVA	10	HISIGD QTD	24	YADSAKGRFTISRDESKN TVYLQMNSLRPEDTAAY YCRAY	14	LSRIWPDY	28	WGQGT LVTVSS
3	A022600 351*	19	EVQLVESGGG VVQPGGSLRL SCAAS	7	GFTFSF AMT	21	WVRRPPGK GLEWVA	11	TITNKG VTS	25	YADSVKGRFTISRDNK NTLYLQMNSLRPEDTAL YICAN	15	ARRTGPRAP TDIGSY	29	RGQGT LVTVSS
4	A022600 314°	19	EVQLVESGGG VVQPGGSLRL SCAAS	8	GSIFRSV FSSSTM E	22	WYRQAPG KKRELVA	12	RIAPGE GTYYG AL	26	YADSVKGRFTISRDNK NTVYLQMNSLRPEDTAL YYCAS	16	GVA	28	WGQGT LVTVSS
5	ALB23002	19	EVQLVESGGG VVQPGGSLRL SCAAS	9	GFTFRSF GMS	23	WVRRQAPG KGPEWVS	13	SISGSG SDTL	27	YADSVKGRFTISRDNK NTLYLQMNSLRPEDTAL YYCTI	17	GGSLSR	30	SSQGT LVTVSS

* Sequence optimized variant of A02260018C08 (SEQ ID NO: 48)

° Sequence optimized variant of A02260015A08 (SEQ ID NO: 47)

Table A-2-1: Sequences for CDRs and frameworks ("ID" refers to the given SEQ ID NO)

ID	ISVD	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
2	TCE01	39	DVQLVESGGG												
			VVQPGGSLRL	31	INFYG	20	WYRQAPG KEREKVA	35	HISIGD QTDYA DSAKG	43	RFTISRDESKNTVYLQM NSLRPEDTAAYYCRA	14	LSRIWPDY	28	WGQGT LVTSS
3	A022600 351*	40	EVQLVESGGG												
			VVQPGGSLRL	32	SFAMT	21	WVRRPPGK GLEWVA	36	TITNKG VTSYA DSVKG	44	RFTISRDNNAKNTLYLQM NSLRPEDTALYICAN	15	ARRTGPRAP TDIGSY	29	RGQGT VTVSS
4	A022600 314°	41	EVQLVESGGG												
			VVQPGGSLRL	33	SVFSSST ME	22	WYRQAPG KKRELVA	37	RIAPGE GTYYG ALYADS VKG	45	RFTISRDNNAKNTVYLQM NSLRPEDTALYICAS	16	GVA	28	WGQGT LVTSS
5	ALB2300242		EVQLVESGGG												
			VVQPGGSLRL	34	SFGMS	23	WVRRQAPG KGPEWVS	38	SISGSG SDTLYA DSVKG	46	RFTISRDNNAKNTLYLQM NSLRPEDTALYICTI	17	GGSLR	30	SSQGT VTVSS

* Sequence optimized variant of A02260018C08 (SEQ ID NO: 48)

° Sequence optimized variant of A02260015A08 (SEQ ID NO: 47)

Table A-3: Amino acid sequences of selected multivalent ISVD

Name	ID	Amino acid sequence
A022600424	1	DVQLVESGGGVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVAHISIGDQTDYADSAKGRF TISRDESKNTVYLQMNSLRPEDTAAYYCRALSRIWPYDYWGQGLTVTSSSGGGSEVQLVESGGGW QPGGSLRLSCAASGFTSSFAMTWVRRPPGKLEWVAITITNKGVTSYADSVKGRFTISRDNAKNTLYL QMNSLRPEDTALYICANARRTGPRPTDIGSYRGQGLTVTSSSGGGSEVQLVESGGGVVQPG GSLRLSCAASGSIFRSVFSSSTMEWYRQAPGKKRELVARAPGEGTYYGALYADSVKGRFTISRDNKNT VYLQMNSLRPEDTALYICASGVAWGQGLTVTSSSGGGSEVQLVESGGGVVQPGGSLRLSCA ASGFTFRSFGMSWVRQAPGKPEWVSSISGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDT ALYYCTIGGSLRSRSQGLTVTVSSA

Table A-4: Serum albumin binding ISVD sequences ("ID" refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
Alb8	82	EVQLVESGGGLVQPGNLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLLTVSS
Alb8-A	83	EVQLVESGGGLVQPGNLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLLTVSSA
Alb23	84	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSI SGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLLTVSS
Alb23-A	85	EVQLLESGGGLVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSI SGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLLTVSSA
Alb83	86	EVQLVESGGGVVQPGNLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLLTVSS
Alb83-A	87	EVQLVESGGGVVQPGNLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLLTVSSA
Alb132	88	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLLTVSS
Alb132-A	89	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTATYYCTIGGSLSR SSQGTLLTVSSA
Alb73	90	EVQLVESGGGLVQPGNLRLSCAASGFTFSSFGMSWVRQAPGKGLEWVSSI SGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTAVYYCTIGGSLSR

		SSQGTLVKVSS
Alb73-A	91	EVQLVESGGGLVQPGLSLRLSCAASGFTFSFGMSWVRQAPGKGLEWVSSI ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTAVYYCTIGGSLSR SSQGTLVKVSSA
Alb82	92	EVQLVESGGGVVQPGLSLRLSCAASGFTFSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSS
Alb82-A	93	EVQLVESGGGVVQPGLSLRLSCAASGFTFSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSSA
Alb199	94	EVQLVESGGGVVQPGLSLRLSCAASGFTFSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSS
Alb199-A	95	EVQLVESGGGVVQPGLSLRLSCAASGFTFSFGMSWVRQAPGKGLEWVSS ISGSGSDTLYADSVKGRFTISRDNKTTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSSA
Alb23002	5	EVQLVESGGGVVQPGGLSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSS
Alb223	96	EVQLVESGGGVVQPGGLSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVTVSSA
Alb216	97	EVQLVESGGGVVQPGGLSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSS
Alb216-A	98	EVQLVESGGGVVQPGGLSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLVKVSSA

Table A-5: Linker sequences (“ID” refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
3A linker	99	AAA
5GS linker	100	GGGGS
7GS linker	101	SGGSGGS
8GS linker	102	GGGGS
9GS linker	103	GGGGS
10GS linker	104	GGGGS
15GS linker	105	GGGGS
18GS linker	106	GGGGS
20GS linker	107	GGGGS
25GS linker	108	GGGGS
30GS linker	109	GGGGS
35GS linker	110	GGGGS
40GS linker	111	GGGGS
G1 hinge	112	EPKSCDKTHTCPPCP
9GS-G1 hinge	113	GGGGS
Llama upper long hinge region	114	EPKTPKPQAAA
G3 hinge	115	ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRC

Table A-6: Amino acid sequences of selected multivalent polypeptides ("ID" refers to the given SEQ ID NO)

SEQ	ID	Sequence
49	A022600027	DVQLVESGGGLVQPGGSLRLSCVASGDVHKINFLGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNMVYLQMNSLKPEDTAVYFCRAFSR IYPYDYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS GGGGSEVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKG LEWVATITNGGVTSYRDSVKGRFTISRDNKNTLYLEMTSLNPEDTAVYIC ANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGLTFSSYA MGWFRQAPGKERERVVISRGGGYTYADSVKGRFTISRDNSENTVYLQ MNSLRPEDTALYYCAAARYWATGSEYEFDYWGQGTTLTVSS
50	A022600031	DVQLVESGGGLVQPGGSLRLSCVASGDVHKINFLGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNMVYLQMNSLKPEDTAVYFCRAFSR IYPYDYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS GGGGSEVQLVESGGGLVQAGGSLRLSCVASGSIFRSVFSSTMEWYRQPP GKKRELVARIPAGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLKP EDTGVIYFCASGVAWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGG GSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGLTFSSYAMGW FRQAPGKERERVVISRGGGYTYADSVKGRFTISRDNSENTVYLQMNSLR PEDTALYYCAAARYWATGSEYEFDYWGQGTTLTVSS
51	A022600096	DVQLVESGGGLVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS GGGGSEVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKG LEWVATITNGGVTSYRDSVKGRFTISRDNKNTLYLEMTSLNPEDTAVYIC ANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGSGGGGSGGGGSGGGGS GGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFG MSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA

52	A022600102	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSAAAEVQLVESGGGLVQPGGSLRLSCVASGFTFS SFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANTLYL EMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGSGG GGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVVQPGGSLR LSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKGRFT ISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA
53	A022600103	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGG GGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVVQPGG SLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVK RFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA
54	A022600104	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSCV ASGFTFSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDN AKNTLYLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSG GGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVV QPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYA DSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTV SSA
55	A022600105	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSGGGSGGGSGGGSGGGSEVQLVESGGGL VQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKGLEWVATITNGGVTSYR DSVKGRFTISRDNANTLYLEMTSLNPEDTAVYICANARRTGPRAPTDIGS YRGQGTTLTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGG EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWV SSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGG SLRSSQGTTLTVSSA

56	A022600122	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNKNTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGG GGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGG SLRLSCVASGSIFRSVFSSSTMEWYRQPPGKKRELVARIAPGDGTNYGALY ADSVKGRFTISRDDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGTTLTV VSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGG GVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPWVSSISGSGSDT LYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTL VTVSSA
57	A022600131	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNKNTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGG GGGGSGGGSGGGSEVQLVESGGGLVQPGGSLRLSCVASGSIFRSVFSS STMEWYRQPPGKKRELVARIAPGDGTNYGALYADSVKGRFTISRDDAKK VDLQMNSLKPEDTGVYFCASGVAWGQGTTLTVSSGGGGSGGGSGGG GSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASG FTFRSFGMSWVRQAPGKGPWVSSISGSGSDTLYADSVKGRFTISRDNK NTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTLTVSSA
58	A022600132	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNKNTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGG GGGSEVQLVESGGGLVQPGGSLRLSCVASGSIFRSVFSSSTMEWYRQPPG KKRELVARIAPGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLKPE DTGVYFCASGVAWGQGTTLTVSSGGGGSGGGSGGGSGGGSGGGSGGG GSGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSW VRQAPGKGPWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSL RPEDTALYYCTIGGSLRSSQGTLTVSSA

59	A022600133	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGSI FRSVFSSSTMEWYRQPPGKKRELVARIAPGDGTNYGALYADSVKGRFTISR DDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGLTVTVSSGGGGSGGG GSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRL SCVASGFTFSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTIS RDNANTLYLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGLTVT VSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVESGG GVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPWVSSISGSGSDT LYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGLT VTVSSA
60	A022600134	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGSI FRSVFSSSTMEWYRQPPGKKRELVARIAPGDGTNYGALYADSVKGRFTISR DDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGLTVTVSSGGGGSGGG GSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWV RRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNANTLYLEMTSLNPE DTAVYICANARRTGPRAPTDIGSYRGQGLTVTVSSGGGGSGGGGSGGGG SGGGGSGGGGSGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGF TFRSFGMSWVRQAPGKGPWVSSISGSGSDTLYADSVKGRFTISRDNKNT LYLQMNSLRPEDTALYYCTIGGSLRSSQGLTVTVSSA
61	A022600135	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTALYYCRALSRI WPYDYWGQGLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGSI FRSVFSSSTMEWYRQPPGKKRELVARIAPGDGTNYGALYADSVKGRFTISR DDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGLTVTVSSGGGGSGGG SEVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKLEWV ATITNGGVTSYRDSVKGRFTISRDNANTLYLEMTSLNPEDTAVYICANAR RTGPRAPTDIGSYRGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGG SGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWV RQAPGKGPWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLR PEDTALYYCTIGGSLRSSQGLTVTVSSA

62	A022600167	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGG GGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVVQPGG SLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVKG RFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA
63	A022600168	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGG GGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGG SLRLSCVASGSIFRSVFSSTMEWYRQPPGKKRELVARIPGDGTNYGALY ADSVKGRFTISRDDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGTTLT VSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGG GVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDT LYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT TLTVSSA
64	A022600169	DVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKGLEWVA TITNGGVTSYRDSVKGRFTISRDNANTLYLEMTSLNPEDTAVYICANARRT GPRAPTDIGSYRGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCV ASGYVHKINFYGWYRQAPGKEREKVAHISIGDQTDYADSAKGRFTISRDES KNTVYLQMNSLRPEDTAAYYCRALSRIWPYDYWGQGTTLTVSSGGGGSE VQLVESGGGLVQPGGSLRLSCVASGSIFRSVFSSTMEWYRQPPGKKREL VARIPGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLKPEDTGVY FCASGVAWGQGTTLTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGG GSGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAP GKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTA LYYCTIGGSLRSSQGTTLTVSSA

65	A022600170	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNKNTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVTVSSGGGG GGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGG SLRLSCVASGSIFRSVSSSTMEWYRQPPGKKRELVARIPGDGTNYGALY ADSVKGRFTISRDDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGTTLTV TVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSW VRQAPGKGPEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSL RPEDTALYYCTIGGSLRSSQGTTLTVTVSSA
66	A022600172	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNKNTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVTVSSGGGG GGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKG PEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYY CTIGGSLRSSQGTTLTVTVSSGGGGSGGGSEVQLVESGGGLVQPGGSLRLSC VASGSIFRSVSSSTMEWYRQPPGKKRELVARIPGDGTNYGALYADSVK GRFTISRDDAKKTVDLQMNSLKPEDTGVYFCASGVAWGQGTTLTVTVSSA
67	A022600174	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKLEWVATITNGGVTSYRDSVKGRFTISRDNKNTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVTVSSGGGG GGGSEVQLVESGGGLVQPGGSLRLSCVASGSIFRSVSSSTMEWYRQPPG KKRELVARIPGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLKP DTGVYFCASGVAWGQGTTLTVTVSSGGGGSGGGSEVQLVESGGGVVQPG GSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSDTLYADSVK GRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVTVSSA

68	A022600175	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVTVSSGGGG GGGSEVQLVESGGGLVQPGGSLRLSCVASGSIFRSVFSSSTMEWYRQPPG KKRELVARIAPGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLKPE DTGVYFCASGVAWGQGTTLTVTVSSGGGGSGGGSGGGSGGGSGGG GSGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSW VRQAPGKGPWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSL RPEDTALYYCTIGGSLRSSQGTTLTVVSSA
69	A022600178	DVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKGLEWVA TITNGGVTSYRDSVKGRFTISRDNANTLYLEMTSLNPEDTAVYICANARRT GPRAPTDIGSYRGQGTTLTVTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCV ASGYVHKINFYGWYRQAPGKEREKVAHISIGDQTDYADSAKGRFTISRDES KNTVYLQMNSLRPEDTAAYYCRALSRIWPYDYWGQGTTLTVTVSSGGGGSG GGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGVVQPGGSL RLSCAASGFTFRSFGMSWVRQAPGKGPWVSSISGSGSDTLYADSVKGRF TISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVVSSA
70	A022600179	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVTVSSGGGGSEVQLVESGGGLVQPGGSLRLSCVASGFT FSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANTL YLEMTSLNPEDTAVYICANARRTGPRAPTDIGSYRGQGTTLTVTVSSGGGG GGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGK PEWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYY CTIGGSLRSSQGTTLTVVSSA
71	A022600370	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCVASGF TFSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNANT LYLEMTSLRPEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVTVSSGGGG GGGGSGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFG MSWVRQAPGKGPWVSSISGSGSDTLYADSVKGRFTISRDNKNTLYLQ MNSLRPEDTALYYCTIGGSLRSSQGTTLTVVSSGGC

72	A022600372	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCVASGF TFSSFAMTWVRRPPGKGLEWVATITNGGVTSYRDSVKGRFTISRDNNAKNT LYLEMTSLRPEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGG GGGSEVQLVESGGGVVQPGGSLRLSCVASGSIFRSVFSSSTMEWYRQPPG KKRELVARIAPGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLRPE DTGLYFCASGVAWGQGTTLTVSSGGGGSGGGSEVQLVESGGGVVQPGG SLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDTLYADSVKG RFTISRDN SKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSGGC
73	A022600373	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSGGGSGGGSGGGGSEVQLVESGGG VVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDTL YADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLV TVSSGGC
74	T017000698	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCA ASGFTFRSFGMSWVRQAPGKGP EWVSSISGSGSDTLYADSVKGRFTISR DN SKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA
78	A022600412	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGF TFSSFAMTWVRRPPGKGLEWVATITNAGVTSYADSVKGRFTISRDNNAKNT LYLQMNSLRPEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGG SGGGSGGGGGSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFRSFG MSWVRQAPGKGP EWVSSISGSGSDTLYADSVKGRFTISRDN SKNTLYLQ MNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA

1	A022600424	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGF TFSSFAMTWVRRPPGKGLEWVATITNKGVTSYADSVKGRFTISRDNNAKNT LYLQMNSLRPEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGG SGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFRSVFSSTMEWYRQAP GKKRELVARIAPGEGTYYGALYADSVKGRFTISRDNNAKNTVYLQMNSLRPE DTALYICASGVAWGQGTTLTVSSGGGGSGGGSEVQLVESGGGVVQPGG SLRLSCAASGFTFRSFGMSWVRQAPGKGPWVSSISGSGSDTLYADSVKG RFTISRDNNAKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA
79	A022600425	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGSI FRSVFSSTMEWYRQAPGKKRELVARIAPGEGTYYGALYADSVKGRFTISR DNAKNTVYLQMNSLRPEDTALYICASGVAWGQGTTLTVSSGGGGSGGG GSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFSSFAMTW VRRPPGKGLEWVATITNAGVTSYADSVKGRFTISRDNNAKNTLYLQMNSLR PEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGSGGGSEVQL VESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPWVSSISG SGSDTLYADSVKGRFTISRDNNAKNTLYLQMNSLRPEDTALYYCTIGGSLRS SQGTTLTVSSA
80	A022600426	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGSI FRSVFSSTMEWYRQAPGKKRELVARIAPGEGTYYGALYADSVKGRFTISR DNAKNTVYLQMNSLRPEDTALYICASGVAWGQGTTLTVSSGGGGSGGG GSGGGGSGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFSSFAMTW VRRPPGKGLEWVATITNKGVTSYADSVKGRFTISRDNNAKNTLYLQMNSLR PEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGGSGGGSEVQL VESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPWVSSISG SGSDTLYADSVKGRFTISRDNNAKNTLYLQMNSLRPEDTALYYCTIGGSLRS SQGTTLTVSSA

81	A022600427	DVQLVESGGGVVQPGGSLRLSCVASGYVHKINFYGWYRQAPGKEREKVA HISIGDQTDYADSAKGRFTISRDESKNTVYLQMNSLRPEDTAAYYCRALSRI WPYDYWGQGTTLTVSSGGGGSEVQLVESGGGVVQPGGSLRLSCAASGF TFSSFAMTWVRRPPGKGLEWVATITNAGVTSYADSVKGRFTISRDNANKNT LYLQMNSLRPEDTALYICANARRTGPRAPTDIGSYRGQGTTLTVSSGGGG SGGGSEVQLVESGGGVVQPGGSLRLSCAASGSIFRSVFSSSTMEWYRQAP GKKRELVARIAPGEGTYYGALYADSVKGRFTISRDNANKNTVYLQMNSLRPE DTALYICASGVAVWGQGTTLTVSSGGGGSGGGSEVQLVESGGGVVQPGG SLRLSCAASGFTFRSFGMSWVRQAPGKGPVWSSISGSGSDTLYADSVKG RFTISRDNANKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTTLTVSSA
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Table A-7: C-termini with or without C-terminal extensions (“ID” refers to the given SEQ

5 **ID NO as used herein)**

ID	Amino acid sequence
116	VTVSS
117	VKVSS
118	VQVSS
119	VTVKS
120	VTVQS
121	VKVKS
122	VKVQS
123	VQVKS
124	VQVQS
125	VTVSSA
126	VKVSSA
127	VQVSSA
128	VTVKSA
129	VTVQSA
130	VKVKSA
131	VKVQSA
132	VQVKSA
133	VQVQSA

Table A-8: Amino acid sequences related to GPC3 and TCR

ID	description	Amino acid sequence
134	Human GPC3 (P51654)	MAGTVRTACLVVAMLLSLDFPGQAQPPPPPDATCHQVRSFFQRLQPGL KWVPETPVPGSDLQVCLPKGPTCCSRKMEEKYQLTARLNMEQLQSASM ELKFLIIQNAAVFQEAFAEIVVRHAKNYTNAMFKNNYPSLTPQAFEFVGEFFT DVSLYILGSDINVDDMVNELFDSLFPVIYTQLMNPGLPDSALDINECLRGA RRDLKVFGNFPKLIMTQVSKSLQVTRIFLQALNLGIEVINTTDHLKFSKDCG RMLTRMWYCSYCQGLMMVKPCGGYCNVVMQGC MAGVVEIDKYWRE YILSLEELVNGMYRIYDMENVLLGLFSTIHDSIQYVQKNAGKLTITIGKLCA HSQQRQYRSAYYPEDLFIDKKVLKVAHVEHEETLSSRRRELIQKLKSFISFYS ALPGYICSHSPVAENDTLCWNGQELVERYSQKAARNGMKNQFNLHELK MKGPEPVVSQIIDKLKHINQLLRTMSMPKGRVLDKNLDEEGFESGDCGDD EDECIGGSGDGMIVKNQLRFLAELAYDLVDVDDAPGNSQQATPKDNEIST FHNLGNVHSPLKLLTSMASVVCFFFLVH
135	Human TCR alpha constant domain (derived from P01848)	PNIQNPDPVAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKTVL DMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPSSC
136	Human TCR beta constant domain (derived from P01850)	EDLNKVPPEVAVFEPSEAEISHTQKATLVCLATGFFPDHVELSWWVNGK EVHSGVSTDPQPLKEQPALNDSRYALSSRLRVSATFWQNPRNHFRQCQVQ FYGLSENDEWTQDRAKPVTQIVSAEAWGRADC

6 Examples

5 6.1 Example 1: Discovery of ISVDs specifically binding to GPC3

2 Llamas and 1 alpaca were immunized with DNA double gene vector containing the sequence encoding the human glypican-3 isoform 2 precursor [NP_004475; 580 AA; Homo sapiens]. Later, the animals were boosted with recombinant human Glypican-3 (R&D Systems, cat nr. 2119-GP).

- 10 Following the final immunogen injection, blood samples were collected and peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll-Hypaque according to the

manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, US) and total RNA was extracted and stored.

Total RNA was used as starting material for RT-PCR to amplify ISVD encoding gene fragments. These fragments were cloned into phagemid vector pAX212. Phage were
5 prepared according to standard protocols (Antibody Phage Display: Methods and Protocols (First Edition, 2002, O'Brian and Aitken eds., Humana Press, Totowa, NJ) and stored after filter sterilization at 4°C until further use. From each immunized animal a phage library was constructed, yielding library sizes of 3×10^8 , 6×10^8 and 8×10^8 cfu.

The phage display libraries were probed using recombinant protein. Briefly, the phage
10 particles were added to the biotinylated antigen (human GPC3 R&D Systems, cat nr. 2119-GP; cyno GPC3 DGPI DHS-HIS, in-house produced (accession number P51654; Q3-R358; S359-H559, S495A, S509A); all biotinylated using standard protocols) at 50 nM (in PBS supplemented with 2% Marvel and 0.05% Tween 20). The biotinylated antigen was captured on streptavidin or anti-biotin coated magnetic beads (Invitrogen). Unbound
15 phage were washed away (with PBS supplemented with 0.05% Tween 20); bound phage were eluted by addition of trypsin (1 mg/mL in PBS). Eluted phage were allowed to infect exponentially growing *E. coli* TG-1 cells to use for either a subsequent selection round (after rescue with helper phage), and/or for screening of individual clones after plating out on agar plates. For this, individual colonies were picked into 96-deep-well plates
20 containing 0.5 mL medium and grown overnight. 80 µL of the overnight culture of each clone were mixed with 40 µL of 60% glycerol in 2xTY and stored at -80°C.

For small-scale production of ISVDs, 96 deep well plates (1 mL volume) were inoculated with the overnight cultures. ISVD expression was induced by adding IPTG to a final concentration of 1 mM. Periplasmic extracts were prepared by freezing the cell pellets
25 and dissolving them in 100 µL PBS. Cell debris was removed by centrifugation.

Periplasmic extracts were screened in an ELISA for binding to human and cyno GPC3. 384-well high binding SpectraPlates (PerkinElmer, 6007509) were coated overnight at 4 °C with 1 µg/mL of protein (in PBS). The plates were then blocked for at least one hour (PBS, 1 % casein) at RT. 1:10 dilutions (in PBS, 0.1 % casein, 0.05 % Tween 20) of periplasmic

extracts were added for one hour at RT. Unbound periplasmic extracts were washed away (PBS supplemented with 0.05 % Tween 20) and bound ISVDs were detected using mouse anti-FLAG-HRP (Sigma-Aldrich, cat nr A8592) and a subsequent enzymatic reaction in the presence of the substrate esTMB (3,3',5,5'-tetramethylbenzidine; SDT).

- 5 Positive hits in ELISA were DNA-sequenced and non-redundant clones were further analyzed for off-rates on human and cyno GPC3 as well as binding to cells expressing human and cyno GPC3.

Off-rates of ISVDs were determined on a ProteOn XPR36 instrument (Bio-Rad Laboratories, Inc.). ProteOn GLC Sensor Chips were coated with cyno Glypican-3 ΔGPI-HIS
10 (in-house produced; accession number P51654; Q3-R358; S359-H559) and human GPC3 (R&D Systems, cat nr. 2119-GP). Periplasmic extracts were diluted 1:10 in ProteOn PBS Tween buffer (PBS, pH 7.4, 0.005% Tween 20 (167-2720, BioRad)). Experiments were carried out at 25 °C. Data obtained was double referenced by reference lane subtraction as well as subtraction of a blank buffer injection. Processed curves were used for off-rate
15 analysis based on the Langmuir dissociation (off-rate analysis) model.

Periplasmic extracts were screened for human and cyno GPC3 binding in flow cytometry using CHO Flp-In cells (Invitrogen cat nr. K6010) expressing either human GPC3 (accession number P51654; Q25-R358, S359-H580) or cyno GPC3 (accession number P51654; Q3-R358; S359-H559), respectively. In brief, 1×10^5 cells were incubated in 1:5 diluted
20 periplasmic extracts for 30 min at 4°C, and then washed 3 times. As a control, the parental CHO Flp-In cell line (Invitrogen cat nr. K6010) was included. Next, cells were incubated with 1 µg/mL monoclonal anti-FLAG® M2 antibody (Sigma-Aldrich, cat nr F1804) for 30 min at 4°C, washed again, and incubated for 30 min at 4°C with goat anti-mouse PE labelled antibody (1:100; Jackson ImmunoResearch, cat nr. 115-115-164).
25 Samples were washed and resuspended in FACS Buffer (D-PBS (Gibco) with 10% FBS (Sigma) and 0.05% sodium azide (Merck)) supplemented with 5 nM TOPRO3 (Molecular Probes, cat nr T3605). Cell suspensions were then analyzed on a FACS Array. Gating was set on live, intact cells using forward/side scatter and TOPRO3 channel fluorescence

parameters. Mean PE channel fluorescence values higher than those obtained for control conditions including a non-binding ISVD indicated a hit.

Based on off-rate analysis and binding to human and cyno GPC3-expressing CHO cells (Table 2), two ISVDs were selected (Table 1).

5 **Table 1: Amino acid sequences of anti-GPC3 ISVDs**

ISVD ID	SEQUENCE
A0226015A08 (SEQ ID NO: 47)	EVQLVESGGGLVQAGGSLRLSCVASGSIFRSVFSSTMEWYRQPPGKKRELV ARIAPGDGTNYGALYADSVKGRFTISRDDAKKTVDLQMNSLKPEDTGVYFC ASGVAWGQGTLTVSS
A0226018C08 (SEQ ID NO: 48)	EVQLVESGGGLVQPGGSLRLSCVASGFTFSSFAMTWVRRPPGKGLEWVATI TNGGVTSYRDSVKGRFTISRDNKNTLYLEMTSLNPEDTAVYICANARRTGP RAPTDIGSYRGQGTTLTVSS

Table 2: Summary of the screening results of anti-GPC3 ISVDs A0226015A08 and A0226018C08

ISVD ID	k_{off} hGPC3 (1/s)	k_{off} cyGPC3 (1/s)	Ratio MFI hGPC3 CHO / CHO	Ratio MFI cyGPC3 CHO / CHO
A0226015A08	3.3E-04	4.0E-04	58.2	13.7
A0226018C08	9.7E-04	1.1E-03	158.9	19.9

10 **6.2 Example 2: Generation of trisppecific GPC3 ISVD based T-cell engager**

The selected anti-GPC3 ISVDs (sequences in Table 1) were formatted in a trisppecific construct with a fixed T-cell engager ISVD which binds to the constant domain of the TCR (T0170056G05, anti-TCR) at the N-terminus and a fixed albumin binding ISVD (ALBX00001) at the C-terminus. The building blocks in the construct are genetically linked
15 by a flexible 35GS (GlySer linker), resulting in the format anti-TCR-35GS-anti-GPC3-35GS-ALBX00001 (Table 3). Amino acid sequences are shown in Table A-6.

Multivalent ISVDs were expressed in *Pichia pastoris*. *P. pastoris* NRRL Y-11430 cells containing ISVD constructs of interest were grown in BGCM medium. Subsequently, medium was switched to BMCM and the constructs were further grown and induced by
20 stepwise addition of methanol. The cells were spun down and the supernatant (containing the secreted ISVD) was collected.

Multivalent ISVDs were purified on protein A resin followed by a desalting step and if necessary, preparative SEC in D-PBS.

Table 3: Sample ID and description of trispecific ISVDs constructs

Sample ID	SEQ ID NO	Description
A022600027	49	T0170056G05-35GS-A0226018C08-35GS-ALBX00001
A022600031	50	T0170056G05-35GS-A0226015A08-35GS-ALBX00001

5 **6.3 Example 3: T-cell dependent cytotoxicity of trispecific GPC3 ISVD T-cell engagers**

The trispecific T-cell engagers containing the anti-GPC3 ISVDs (Table 3) were characterized in a T-cell dependent cytotoxicity assay (TDC). HepG2 (ATCC, clone HB8065), a liver cancer cell line with high expression of GPC3, was labelled with Nuclight Green (Essen Bioscience, cat no. 4624) and used as target for T-cell killing in the presence of the trispecific T-cell engager constructs comprising A0226015A08 or A0226018C08 (i.e. constructs A022600031 or A022600027, respectively) or in the presence of a construct with a reference GPC3 binding single domain antibody (Ab1) in the format anti-TCR-35GS-anti-GPC3-35GS-HLE (HLE = half-life extender). To this end, plates (96-well F-bottom, Greiner, cat no 655180) were pre-blocked with 200 µL/well assay medium (2 h, 37°C). Simultaneous addition of each assay component was performed in a total volume of 200 µL/well: (1) 50 µL of diluted/titrated compounds (Nbs; Brefeldin A (Sigma-Aldrich, cat no B7651); (2) 25 µL of diluted HSA (Sigma-Aldrich, cat no A8763-10G) (final concentration: 30 µM); (3) 25 µL of diluted Cytotox Red (Essen Bioscience, cat no 4632) (final concentration: 250 nM) (4) 50 µL of human T-cells (T cells were isolated from buffy coats (Red Cross) using the RosetteSep T cell enrichment cocktail (StemCell, cat no. 15061) and 50 µL of HepG2 Nuclight green (fresh in DNEM, High Glucose, GlutaMAX, Pyruvate, Life Technologies-Gibco, cat no 31966) in a 15:1 ratio. Plates were placed in an IncuCyte ZOOM for readout in all three channels (phase-contrast, green and red) with intervals of 4 or 6 hours, for a total of 72h.

The tested trispecific GPC3 T-cell engagers induced human T-cell mediated killing of HepG2 Nuclight green in a dose-dependent manner as shown in Figure 1. The IC₅₀ values and maximum percentage of killing are shown in Table 4.

Table 4: IC₅₀ (M) and maximum percentage of killing (%) of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay using an effector to target ratio of 15:1, analyzed at 60h after seeding

Sample ID	IC ₅₀ (M)	Max % of killing
A022600027	8.6E-09	80
A022600031	1.5E-08	75
Ab1	1.3E-08	40

5 6.4 Example 4: Epitope binning of GPC3 binders

Epitope binning of the anti-GPC3 ISVDs was done via flow cytometry allowing periplasmic extracts of the monovalent ISVDs A0226015A08 or A0226018C08 to compete with the ISVDs in the purified trispecific format. To this end, 4×10^4 human GPC3 CHO Flp-In cells (reference, see example 1) were incubated with 1/15 and 1/150 dilutions of periplasmic
 10 extracts, 150 nM competitor (trispecific ISVD format; oversaturating concentration) and 0.2 µg/mL of monoclonal anti-FLAG® M2 antibody (Sigma-Aldrich, cat nr F1804) and rat anti-mouse APC (BD-Pharmingen, cat no 550874), for 5 h at RT and 300 rpm. The samples were read in the iQue Screener. No competition was observed between A0226015A08 and A0226018C08 revealing that they bind to different, non-overlapping epitopes.

15 6.5 Example 5: Optimization of format for GPC3 ISVD T-cell engagers

The GPC3 ISVD T-cell engagers in the format described in Table 3, do not reach full efficacy in the T-cell dependent cytotoxicity (TDC) HepG2 Nuclight green assay as indicated by the maximum percentage of killing (Example 3). To increase potency and efficacy, trispecific constructs were generated in which the anti-GPC3 ISVDs were
 20 combined with a sequence optimized variant of the anti-TCR ISVD T0170056G05, i.e. T017000624, at the N-terminus, an albumin binding ISVD, ALB23002-A (SEQ ID NO: 5 with C-terminal single alanine extension), at the C-terminus and the GPC3 binding ISVD(s) in the central position of the construct (Tables 5 and 6). The optimization encompassed two steps: Step 1 – optimization of the linker length between the anti-TCR ISVD and an anti-
 25 GPC3 ISVD (trispecific trivalent format); Step 2 – generation of biparatopic GPC3 ISVD T-

cell engagers (trispecific tetravalent format) and optimization of the linker length between the two GPC3 binding ISVDs. Amino acid sequences are shown in Table A-6.

The generated formats were tested in TDC HepG2 Nuclight green assay, as described in Example 3. Analysis for the Step 1 and Step 2 formats was performed 72h or 60h after seeding, respectively.

Tables 5 and 6 show the IC50 values and the maximum % killing for the different formats in Step 1 and Step 2, respectively, of format optimization in the TDC HepG2 Nuclight green assay.

In Step 1, the trispecific GPC3 ISVD T-cell engagers showed cell tumor killing with increased efficacy (increased maximum killing) with decreasing length of the linker between the anti-TCR ISVD and anti-GPC3 ISVD, from 72% (35GS linker) to 94% (AAA linker) (FIGURE 2). In Step 2, GPC3 biparatopic T-cell engagers were tested for which A0226018C08 and A0226015A08 were combined in the same construct and placed in different orientations and with different linker lengths between the two building blocks. Here, no impact of the tested variables on efficacy was observed (FIGURE 3).

Table 5: IC50 (M) and maximum percentage of killing (%) of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay using an effector to target ratio of 15:1, analyzed at 72h after seeding

Step	Sample ID	SEQ ID NO	Description	IC50 (M)	Max % Killing
1	A022600096	51	T017000624-35GS-A0226018C08-35GS-ALB23002-A	1.4E-09	72
	A022600105	55	T017000624-20GS-A0226018C08-35GS-ALB23002-A	2.14E-09	85
	A022600104	54	T017000624-9GS-A0226018C08-35GS-ALB23002-A	2.13E-09	90
	A022600103	53	T017000624-5GS-A0226018C08-35GS-ALB23002-A	2.04E-09	92
	A022600102	52	T017000624-AAA-A0226018C08-35GS-ALB23002-A	1.72E-09	94

Table 6: IC50 (M) and maximum percentage of killing (%) of the trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay using an effector to target ratio of 15:1, analyzed at 72h after seeding

Step	Sample ID	SEQ ID NO	Description	IC50 (M)	Max % Killing
2	A022600103	53	T017000624-5GS-A0226018C08-35GS-ALB23002-A	5.43E-10	90
	A022600122	56	T017000624-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-A	4.45E-10	92
	A022600131	57	T017000624-5GS-A0226018C08-20GS-A0226015A08-35GS-ALB23002-A	4.44E-10	94
	A022600132	58	T017000624-5GS-A0226018C08-9GS-A0226015A08-35GS-ALB23002-A	7.02E-10	91
	A022600133	59	T017000624-5GS-A0226015A08-35GS-A0226018C08-35GS-ALB23002-A	4.08E-10	92
	A022600134	60	T017000624-5GS-A0226015A08-20GS-A0226018C08-35GS-ALB23002-A	5.28E-10	92
	A022600135	61	T017000624-5GS-A0226015A08-9GS-A0226018C08-35GS-ALB23002-A	6.11E-10	91

- 5 To evaluate the ability to kill a liver cancer cell line expressing medium levels of GPC3 compared to the high levels of HepG2, a TDC Huh7 assay was performed. To this end, the xCELLigence® (Acea) system was used. Firstly, 96-well E-plates (Acea, Cat no 5232368001) containing 50 µL of assay medium with a 4x concentration (120 µM) of Alburex 20 Human serum albumin (CSL Behring, Cat no 2160-979) (final assay concentration 30 µM) were
- 10 placed inside the xCELLigence® for background measurement. After background measurement, simultaneous addition of each assay component was performed to a total volume of 200 µL/well: (1) 50 µL of diluted/titrated compounds; (2) 50 µL of single cell suspensions of Huh7 (HSRRB, clone JCRB0403); (3) 50 µL of single suspensions of effector cells (human T-cells, obtained as described in Example 3) to match an effector:target ratio
- 15 of 15:1. Plates were placed in the xCELLigence® with 400 sweeps at 15-minute intervals. At the appropriate timepoint (ca. 60h), cell indexes (CI) were analyzed, where a CI of 0 represented 100% killing.

In a 3rd Step of GPC3 T-cell engager format optimization the anti-TCR ISVD T017000624 was substituted by the sequenced optimized variant T017000680 (TCE01, SEQ ID NO: 2) in the trivalent and tetravalent formats. Potency and efficacy was assessed in the HepG2 and Huh7 TDC assay, as described above. The formats, their description and functionality is summarized in Table 7. Data is depicted in Figure 4. The third step of format optimization resulted in a further small increase in efficacy across constructs. From this exercise GPC3 T-cell engager formats A022600167 and A022600168 were taken forward.

Step 4 of GPC3 T-cell engager format optimization consisted in changing the orientation of the anti-TCR ISVD with the anti-GPC3 ISVD, but this had an influence on potency on efficacy (Table 8, Figure 5). For the trivalent format the killing efficacy was lost. For the tetravalent formats functionality could still be observed, be it with a decrease in potency and efficacy; the killing efficacy decreased by 20% on HepG2 Nuclight green and by 40% on Huh7. Moreover, changing orientation of anti-GPC3 ISVD with anti-Albumin ISVD also had an influence on efficacy of killing. From this exercise no changes were made to GPC3 T-cell engager formats A022600167 and A022600168.

Step 5 of GPC3 T-cell engager format optimization consisted in varying the linker lengths (Table 9, Figure 6). Decreasing the linker length before the anti-Albumin ISVD in the trivalent format resulted in decreased efficacy. This is not seen in the tetravalent format, so in this case a choice can be made between 9GS and 35GS linker lengths.

From the set of GPC3 T-cell engager formats A022600167 and A022600168 represent the trispecific trivalent and tetravalent formats with the highest potency and efficacy in the TDC assays. While efficacies are comparable, the trivalent format A022600167 and the tetravalent format A022600168 differ in potency. In the HepG2 TDC assay, A022600167 is 5-fold less potent than A022600168 while in the Huh7 TDC assay the difference increases to 50-fold.

5 **Table 7: Step 3 of GPC3 ISVD T-cell engager format optimization. IC₅₀ (M) and maximum percentage of killing (%) of trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay and in the xCELLigence based human TDC Huh7 assay, using an effector to target ratio of 15:1, analyzed at 60h after seeding**

Sample ID	SEQ ID NO	Description	HepG2 on Incucyte IC ₅₀ (M)	HepG2 on Incucyte Max % killing	Huh7 on xCELLigence IC ₅₀ (M)	Huh7 on xCELLigence Max % killing
A022600103	53	T017000624-5GS-A0226018C08-35GS-ALB23002-A	1.23E-09	96	7.11E-09	71
A022600167	62	TCE01-5GS-A0226018C08-35GS-ALB23002-A	1.92E-09	98	8.61E-09	91
A022600122	56	T017000624-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-A	5.3E-10	93	2.61E-10	101
A022600168	63	TCE01-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-A	3.67E-10	103	1.98E-10	107
A022600132	58	T017000624-5GS-A0226018C08-9GS-A0226015A08-35GS-ALB23002-A	6.83E-10	83	2.9E-10	95
A022600175	68	TCE01-5GS-A0226018C08-9GS-A0226015A08-35GS-ALB23002-A	5.27E-10	96	5.86E-11	99

5 **Table 8: Step 4 of GPC3 ISVD T-cell engager format optimization. IC50 (M) and maximum percentage of killing (%) of trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay and in the xCELLigence based human TDC Huh7 assay, using an effector to target ratio of 15:1, analyzed at 60h after seeding**

Sample ID	SEQ ID NO	Description	HepG2 on Incucyte IC50 (M)	HepG2 on Incucyte Max % killing	Huh7 on xCELLigence IC50 (M)	Huh7 on xCELLigence Max % killing
A022600167	62	TCE01-5GS-A0226018C08-35GS-ALB23002-A	1.92E-09	98	8.61E-09	91
A022600178	69	A0226018C08-5GS-TCE01-35GS-ALB23002-A	no effect		no effect	
A022600168	63	TCE01-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-A	3.67E-10	103	1.98E-10	107
A022600169	64	A0226018C08-5GS-TCE01-5GS-A0226015A08-35GS-ALB23002-A	6.41E-10	81	1.02E-09	61
A022600174	67	TCE01-5GS-A0226018C08-9GS-A0226015A08-9GS-ALB23002-A	6.73E-10	96	6.26E-11	99
A022600172	66	TCE01-5GS-A0226018C08-9GS-ALB23002-9GS-A0226015A08-A	6.01E-10	81	6.70E-11	90

Table 9: Step 5 of GPC3 T-cell engager format optimization. IC50 (M) and maximum percentage of killing (%) of trispecific GPC3 ISVD T-cell engagers in the Incucyte based human TDC HepG2-Nuclight green assay and in the xCELLigence based human TDC Huh7 assay, using an effector to target ratio of 15:1, analyzed at 60h after seeding

Sample ID	SEQ ID NO	Description	HepG2 on Incucyte IC50 (M)	HepG2 on Incucyte Max % killing	Huh7 on xCELLigence IC50 (M)	Huh7 on xCELLigence Max % killing
A022600167	62	TCE01-5GS-A0226018C08-35GS-ALB23002-A	1.92E-09	98	8.61E-09	91
A022600179	70	TCE01-5GS-A0226018C08-9GS-ALB23002-A	2.92E-09	76	6.13E-09	66
A022600168	63	TCE01-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-A	3.67E-10	103	1.98E-10	107
A022600170	65	TCE01-5GS-A0226018C08-35GS-A0226015A08-9GS-ALB23002-A	3.19E-10	97	1.86E-10	103
A022600175	68	TCE01-5GS-A0226018C08-9GS-A0226015A08-35GS-ALB23002-A	5.27E-10	96	5.86E-11	99
A022600174	67	TCE01-5GS-A0226018C08-9GS-A0226015A08-9GS-ALB23002-A	6.73E-10	96	6.26E-11	99

6.6 Example 6: Assessment of T-cell activation induction in the presence of soluble GPC3

GPC3 can be released into circulation in a soluble form, with levels increased in up to 50% of HCC patients. Soluble GPC3 antibody aggregates in circulation could lead to immune complex deposition and related toxicities. Thus, it is important to assess the effect on T cell activation of soluble GPC3 in presence of the GPC3 T-cell engager.

Reported serum levels of GPC3 can vary between 10 to 300 ng/mL, which corresponds to 0.1 to 10 nM. The assessment was done using 1, 10 and 100 nM of soluble GPC3, in the presence of the trispecific GPC3 ISVD T-cell engagers A022600167 and 168, in three ways:

- (1) Cytotoxicity in the presence of target cells (Huh-7) and soluble GPC3 (Figure 7A): no additional killing was observed compared to in the absence of soluble GPC3;
- (2) CD69 upregulation in presence of target cells and soluble GPC3 (Figure 7B): no additional T-cell activation was observed compared to in the absence of soluble GPC3;
- (3) CD69 upregulation in absence of target cells and presence of soluble GPC3 (Figure 7C): no T-cell activation was seen. The risk of toxicity effects due to increased serum levels of soluble GPC3 is therefore considered low.

The cytotoxicity assay on Huh-7 was performed as described in Example 5. CD69 expression on T-cells was determined by flow cytometry using an anti-CD69 antibody (BD Pharmingen, cat no. 557050) and anti-mouse IgG1 antibody (BD Pharmingen, cat no. 556650).

6.7 Example 7: GPC3 mediated T-cell engager internalization

GPC3 is known to internalize and its internalization rate may have an impact on the efficacy of the compound. The internalization rate and the GPC3 receptor density in the presence of the trispecific GPC3 ISVD T-cell engagers equivalent to A022600167 and A022600168 and of a reference CD3-GPC3 bispecific T-cell engager antibody (Ab2) (Table 10) were assessed in Huh-7 cells over a time course of 48h.

Internalization was determined by labeling A022600167 and A022600168 and Ab2 with pHAb (Promega, cat no. G9841), a pH sensor dye with low fluorescence at pH > 7 and a strong increase in fluorescence as the pH of the solution becomes acidic. For this labeling,

formats with an extra -GGC at the C-terminus were generated in order to have a single site incorporation of the label (Table 10): A022600167-GGC corresponds to A022600370, A022600168-GGC corresponds to A022600372 and as a control a format without a GPC3 ISVD, A022600373 was generated. The assay was read on a BD FACSArray with a yellow laser (pHAb: excitation maximum at 532 nm, emission maximum at 560 nm). The internalization rate was determined by quantifying the internalization at 37°C at different timepoints (0.5 h, 3 h, 24 h and 48 h) compared to 4°C at 0.5 h (Table 10, Figure 8A).

The receptor expression was determined using a fixed concentration of a 3xFLAG-His6-tagged ISVD (20 nM) that binds to a different GPC3 epitope than A02260018C08 and A02260015A08 in combination with APC labelled anti-FLAG for detection on a BD FACSArray with a red laser (Table 10, Figure 8B).

The internalization rate was calculated as the slope of the kinetic curve with arbitrary units. The trispecific GPC3 ISVD T-cell engagers show a slower internalization rate than the reference bispecific T-cell engager Ab2. Internalization of the GPC3 ISVD T-cell engagers is GPC3 mediated as the control format without a GPC3 binding ISVD (A022600373) does not show internalization. No decrease of GPC3 cell surface expression within 48 h was observed (Table 10, Figure 8).

Table 10: Internalization rate of pHAb labelled trispecific GPC3 T-cell engagers

Sample ID	SEQ ID NO	Description	pHAb degree of labeling	Internalization rate (normalized slope n=2)
A022600370	71	TCE01-5GS-A0226018C08-35GS-ALB23002-GGC	1	194
A022600372	72	TCE01-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-GGC	1	297
A022600373	73	TCE01-20GS-ALB23002-GGC	1	2
Ab2	-	-	1.4	597

6.8 Example 8: GPC3 expression profiling of cancer cell lines and correlation with functionality of GPC3 T-cell engagers

GPC3 protein expression levels for a panel of cancer cell lines was determined both by immunocytochemistry (ICC) and using the QIFIKIT® (Dako, Cat no K0078) according to the manufacturer's instructions (Table 11). Additionally, immunohistochemistry (IHC) was performed on hepatocellular carcinoma and normal kidney samples.

ICC and IHC was performed using the Ventana discovery XT robot (Ventana medical system, Roche). The cell lines and the tissue samples were first fixed on 4% formalin and subsequently paraffin embedded. After deparaffinization, cells were conditioned with buffer CC1 standard (Ventana, Cat no 950-124) at a temperature of 95°C for 48 minutes, followed by a blocking step of 4 minutes with each of Blocker A and B (Ventana, Cat no 760-104). Mouse monoclonal IgG2a anti-GPC3 antibody (Ventana, Cat no 790-4564) was applied for 60 minutes at room temperature, followed by 4 minutes of fixation with 0.05% Glutaraldehyde in 5M NaCl (Prolabo, Cat no 20879-238). Biotinylated goat anti-mouse IgG2a antibody (Southern Biotech, Cat no 1080-080) at 1/200 dilution in antibody diluent (Ventana; Cat no 760-108) was applied for 32 minutes at room temperature. Detection was performed with the DABMap Kit (Ventana; Cat no 760-124). Sections were counterstained for 4 minutes with Hematoxylin II (Ventana, Cat no 790-2208) and post-counterstained for 4 minutes with bluing agent (Ventana, Cat no 760-2037), followed by deshydration and mounting with Cytoseal XYL (Richard-Allan Scientific, Cat no 8312-4). Immunohistochemical staining was evaluated by a semi-quantitative assessment of both the intensity of staining of the cells (graded as 0: no staining, 1 (or +): weak, 2 (or ++): moderate; 3 (or +++): strong) and percentage of positive cells in every intensity categories. Histoscore (H-score) was calculated according to following formula:

$$\text{H-score} = 3 \times (\text{cell \% with grade 3}) + 2 \times (\text{cell \% with grade 2}) + 1 \times (\text{cell \% with grade 1}).$$

The range of the possible score was from 0 to 300, as described in literature (Detre et al., J Clin Pathol 1995; 48:876-878 and Lui et al., Journal of Latex Class filed, august 2015, vol 14, No.8). Determination of H-score in HCC was based on evaluation of membranous expression of GPC3.

Within the panel of cell lines tested, as determined with the QIFIKIT®, Hep-G2 (ATCC, clone HB-8065; 5.2E5 receptors/cell) showed the highest level of expression of GPC3 followed by NCI-H661 (ATCC, clone HTB-183; 3.4E5 receptors/cell) and Huh-7 (HSRRB, clone JCRB0403; 6.8E4 receptors/cell), the latter considered as medium expressing cell line. These cell lines originated from liver and lung cancers which are relevant GPC3 expressing solid tumors. The low or very low GPC3 expressing cell lines, which did not show any staining in ICC, were MKN-45 (DSMZ, clone ACC409; 1.5E4 receptors/cell), NCI-H23 (ATCC, clone CRL-5800; 2.6E3 receptors/cell), BxPC-3 (ATCC, clone CRL-1687; 1.5E3 receptors/cell) and NCI-H292 (ATCC, clone CRL-1848; 6E2 receptors/cell).

For comparison between the cancer cell lines and patient tumour samples, the H-scores were determined for both. GPC3 positive cancer cell lines in ICC, i.e. Huh-7, NCI-H661 and HepG2, showed H-scores of superior to 80 (Table 11), which corresponds to the average H-score determined for the GPC3 positive hepatocellular carcinoma (HCC) samples in IHC of 80,75 (Table 12). Normal kidney GPC3 positive samples show an average H-score of 0,75 (Table 12) while the cancer cell lines MKN-45, NCI-H23, BxPC-3 and NCI-H292 were negative for GPC3 staining (Table 11). These cell lines were taken as representatives of GPC3 normal-like expression level cells.

To assess the functionality of the trispecific GPC3 T-cell engagers with the same panel of cancer cell lines, TDC assays were performed using the xCELLigence system, as described in Example 5; results are shown in Table 13 and Figure 9. For the GPC3 high expressing cell lines, Hep-G2 and NCI-H661, the trispecific GPC3 ISVD T-cell engagers A022600167 and A022600168 show similar potency (NCI-H661) and 10-fold lower potency (Hep-G2), compared to the bispecific T-cell engager Ab2. For the medium expressing cell line, Huh-7, A022600168, the tetravalent format with biparatopic binding to GPC3, shows the same potency as Ab2, while the potency of the trivalent format A022600167 is 10-fold lower. For the GPC3 low expressing cell lines, MKN-45 and BxPC-3, Ab2 shows a potency in the nM range, while the trispecific GPC3 ISVD T-cell engagers show no killing effect. For the very low GPC3 expressing cell line, NCI-H292, none of the compounds show an effect. The

T-cell engager lacking a GPC3 binding ISVD, T017000698, does not show any killing effect on any cell line, confirming the GPC3 specific effect of the trispecific GPC3 T-cell engagers.

In conclusion, Ab2 is a potent T-cell engager able to kill cancer cell lines with GPC3 expression levels as low as a thousand receptors per cell and H-score 0. In comparison, the trispecific T-cell engager formats potentially kill high and medium GPC3 expressing cancer cell lines with H-scores similar to HCC and large cell lung cancer samples while sparing cell lines expressing GPC3 levels below ten thousand receptors per cell and with H-scores below the average of normal kidney samples.

Table 11: GPC3 expression levels of different cancer cell lines.

Cell line	Cancer tissue	Expression RNA FPKM	Expression protein #GPC3 / cell	Expression protein ICC (H-score)
Hep-G2	Liver	2253	619006	70%+++, 25%++ (260)
NCI-H661	Lung	237	346756	40%++ (80)
Huh-7	Liver	549	78027	20%++, 40%+ (80)
MKN-45	Stomach	20.9	7453	0
NCI-H23	Lung	3.17	2255	0
BxPC3	Pancreas	5.3	1332	0
NCI-H292	Lung	0.04	452	0

10

Table 12: GPC3 H-score determined by immunohistochemistry on Hepatocellular carcinoma and normal kidney samples (based on evaluation of membranous expression of GPC3).

	Total cases	H-score	Total GPC3+ cases	H-score
HCC	288	52,22	187	80,75
Normal kidney	35	0,17	8	0,75

15

Table 13: IC₅₀ (M) of the GPC3 T-cell engagers in the xCELLigence based human TDC assay on different tumor cell lines expressing decreasing expression levels of GPC3 using an effector to target ratio of 15:1: HepG2 analysed at 60h, NCI-H661 analysed at 75h, Huh-7 analysed at 60h, MKN-45 analysed at 65h, BxPC-3 analysed at 65h, NCI-H292 analysed at 60h. n.a. = not available

Sample ID	SEQ ID NO	Description	Hep-G2	NCI-H661	Huh-7	MKN-45	BxPC-3	NCI-H292
A022600167	62	TCE01-5GS-A0226018C08-35GS-ALB23002-A	9.2E-11	1.8E-10	4.7E-10	n.a.	n.a.	n.a.
A022600168	63	TCE01-5GS-A0226018C08-35GS-A0226015A08-35GS-ALB23002-A	7.3E-11	1.4E-10	2.4E-11	n.a.	n.a.	n.a.
T017000698	74	TCE01-9GS-ALB23002-A	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ab2	-	Bispecific CD3-GPC3	1.1E-11	2.8E-11	3.0E-11	2.0E-09	3.6E-09	n.a.

6.9 Example 9: Sequence optimization of A0226015A08 and A0226018C08

The ISVDs A0226015A08 and A0226018C08 were further sequence optimized.

Sequence optimization involves replacing one or more specific amino acid residues in the sequence in order to improve one or more (desired) properties of the ISVDs.

Some examples of such sequence optimization are mentioned in the further description herein and for example include, without limitation:

- 1) Substitutions in parental wild type ISVD sequences to yield ISVD sequences that are more identical to the human VH3-JH germline consensus sequences, a process called humanization. To this end, specific amino acids, with the exception of the so-called hallmark residues, in the FRs that differ between the ISVD and the human VH3-JH

germline consensus are altered to the human counterpart in such a way that the protein structure, activity and stability are kept intact.

- 2) Substitutions towards the llama germline to increase the stability of the ISVD, which is defined as camelisation. To this end, the parental wild type ISVD amino acid sequence is aligned to the llama IGHV germline amino acid sequence of the ISVD (identified as the top hit from a BlastP analysis of the ISVD against the llama IGHV germlines).
- 3) Substitutions that improve long-term stability or properties under storage, substitutions that increase expression levels in a desired host cell or host organism, and/or substitutions that remove or reduce (undesired) post-translational modification(s) (such as glycosylation or phosphorylation), again depending on the desired host cell or host organism.
- 4) Mutations on position 11 towards Val and on position 89 towards Leu (according to Kabat) to minimize the binding of any naturally occurring pre-existing antibodies.

Sequence optimization of A0226015A08 yielded the final sequence optimized variant A022600314, which comprises 13 amino acid substitutions (i.e. L11V, A14P, V23A, P40A, D52cE, N54Y, D73N, K76N, D79Y, K83R, G88A, V89L, F91Y) (Table 14) compared to the parental ISVD clone A0226015A08.

Sequence optimization of A0226018C08 yielded two sequence optimized variants, A022600345, which comprises 8 amino acid substitutions (i.e. L11V, V23A, G54A, R60A, E81Q, T82aN, N83R, V89L) and A022600351, which comprises 8 amino acid substitutions (i.e. L11V, V23A, G54K, R60A, E81Q, T82aN, N83R, V89L) (Table 14), compared to the parental ISVD clone A0226018C08.

Table 14: Amino acid sequences of the sequence optimized versions of A0226015A08 and A0226018C08.

Sample ID	SEQ ID NO	Description	Sequence
A022600314	75 (same as 4)	A0226015A08(L11V, A14P, V23A, P40A, D52cE, N54Y, D73N, K76N, D79Y, K83R, G88A, V89L, F91Y)	EVQLVESGGGVVQPGGSLRLSCAASGSIFR SVFSSSTMEWYRQAPGKKRELVARIAPGE GTYYGALYADSVKGRFTISRDNANTVYLQ MNSLRPEDTALYYCASGVAWGQGTLVTVSS
A022600345	76	A0226018C08(L11V, V23A, G54A, R60A, E81Q, T82aN, N83R, V89L)	EVQLVESGGGVVQPGGSLRLSCAASGFTFS SFAMTWVRRPPGKLEWVATITNAGVTS YADSVKGRFTISRDNANTLYLQMNSLRPE DTALYICANARRTGPRAPTDIGSYRGQGTL VTVSS
A022600351	77 (same as 3)	A0226018C08(L11V, V23A, G54K, R60A, E81Q, T82aN, N83R, V89L)	EVQLVESGGGVVQPGGSLRLSCAASGFTFS SFAMTWVRRPPGKLEWVATITNKGVTSY ADSVKGRFTISRDNANTLYLQMNSLRPED TALYICANARRTGPRAPTDIGSYRGQGTLV TVSS

Properties of the sequence optimized variants in comparison to the parental ISVD were
5 assessed as follows:

Variants were evaluated for binding to HepG2, Huh-7 and CHO Flip-In cyno-GPC3 cells by
flow cytometry, as described in Example 1.

Thermal stability of the variants was tested in a thermal shift assay (TSA) using a
Lightcycler (Roche). In this assay, the parental ISVDs and their variants are incubated at
10 different pH in the presence of SYPRO™ orange and a temperature gradient is applied.
When an ISVD starts denaturing, SYPRO™ orange binds leading to an increase in
fluorescence, allowing determination of the melting temperature (T_m) for a certain pH.

Results are summarized in Table 15 and Table 16.

Table 15: Results of the analysis of the sequence optimization variant A022600314 of the parental ISVD A0226015A08.

Sample ID	Mutations	EC50 (M) HepG2	EC50 (M) Huh-7	EC50 (M) CHO- cGPC3	Tm (°C) at pH 7 TSA
A0226015A08	WT	7.5E-09	6.6E-09	7.8E-09	70
A022600314	L11V, A14P, V23A, P40A, D52cE, N54Y, D73N, K76N, D79Y, K83R, G88A, V89L, F91Y	1.6E-08	9.3E-09	1.2E-08	65

Table 16: Results of the analysis of the sequence optimization variants A022600345 and A0226000351 of the parental ISVD A0226018C08.

Sample ID	Mutations	EC50 (M) HepG2	EC50 (M) Huh-7	EC50 (M) CHO- cGPC3	Tm (°C) at pH 7 TSA
A0226018C08	WT	9.15E-10	6.96E-10	4.9E-10	67
A022600345	L11V, V23A, G54A, R60A, E81Q, T82aN, N83R, V89L	3.91E-10	2.76E-10	1.5E-10	75
A022600351	L11V, V23A, G54K, R60A, E81Q, T82aN, N83R, V89L	4.31E-10	4.77E-10	2.1E-10	74

Compared with the parental ISVD A0226015A08, A022600314 exhibited a decrease in binding potency for the different cell lines expressing human GPC3 or cyno GPC3 of less than 2-fold. Tm decreased slightly by 5°C and within acceptable values. The % framework identity in the framework regions compared to the reference hIGHV3-23SO/IGHJ4 for A022600314 is 88.8% based on the AbM definition (see Antibody Engineering, Vol2 by Kontermann & Dübel (Eds), Springer Verlag Heidelberg Berlin, 2010) and 85.1% based on the Kabat definition.

Compared with the parental ISVD A0226018C08, the binding potency of variants A022600345 and A022600351 for the different cell lines expressing human GPC3 or cyno GPC3 is increased 2-fold. The Tm for variants A022600345 and A022600351 increased to

75 and 74°C, respectively. The % framework identity in the framework regions compared to the reference HIGHV3-23SO/IGHJ4 for both, A022600345 and A022600351, is 89.9% based on the AbM definition and 89.7% based on the Kabat definition.

6.10 Example 10: Generation of trispecific sequence optimized GPC3 ISVD T-cell engager

The sequence optimized GPC3 ISVDs A022600314 (optimized variant of A0226015A08), A022600345 and A022600351 (optimized variants of A0226018C08) were used for the generation of five trispecific GPC3 T-cell engager formats for assessment of optimal combination of building blocks and linker length as described in Table 17.

10 **Table 17: Selection of different trispecific GPC3 ISVD T-cell engager formats evaluated**

Sample ID	SEQ ID NO	Description
A022600427	81	TCE01-5GS-A022600345-9GS-A022600314-9GS-ALB23002-A
A022600424	1	TCE01-5GS-A022600351-9GS-A022600314-9GS-ALB23002-A
A022600425	79	TCE01-5GS-A022600314-20GS-A022600345-9GS-ALB23002-A
A022600426	80	TCE01-5GS-A022600314-20GS-A022600351-9GS-ALB23002-A
A022600412	78	TCE01-5GS-A022600345-20GS-ALB23002-A

The five selected formats were tested in the xCELLingence based TDC assay with different cancer cell lines for functionality, as described in Example 8. Results are depicted in Table 18. For high and medium GPC3 expressing cell lines HepG2, NCI-H661 and Huh-7, higher potencies were obtained for the tetravalent formats compared to the trivalent format. For high GPC3 expressing cell line NCI-H661, tetravalent formats A022600424 and A022600427 are more potent than A022600425 and A022600426. For the GPC3 low expressing cell lines, NCI-H23 and BxPC-3, the lack of a killing effect was confirmed for all trispecific GPC3 ISVD T-cell engager formats.

20 Figure 10 shows the dose-dependent killing curves for the xCELLingence based TDC assay, exemplified by cell lines NCI-H661 and BxPC-3, using three different T-cell donors for the five selected ISVD formats.

Table 18: IC₅₀ (M) of GPC3 T-cell engagers in the xCELLigence based human TDC assay on different tumor cell lines using an effector to target ratio of 15:1 and analyzed at 60h. na = not available; no fit = no curve fit obtained, IC₅₀ estimated as >1E-7M

Sample ID	IC ₅₀ (M) HepG2	IC ₅₀ (M) NCI-H661	IC ₅₀ (M) Huh-7	IC ₅₀ (M) NCI-H23	IC ₅₀ (M) BxPC-3
A022600427	1.03E-10	1.00E-10	1.37E-10	no fit	no fit
A022600424	1.15E-10	1.14E-10	1.16E-10	no fit	no fit
A022600425	1.11E-10	2.59E-10	1.58E-10	no fit	no fit
A022600426	1.25E-10	2.64E-10	1.09E-10	no fit	no fit
A022600412	3.82E-10	6.18E-10	1.53E-09	na	no fit
Ab2	1.49E-11	4.60E-11	8.41E-11	1.28E-09	1.09E-09

- 5 Binding of pre-existing antibodies to the 5 selected formats was assessed using a SPR-based setup (Example 14). Figure 11 shows that for all formats only low levels of pre-existing antibody binding were observed, compatible with developability requirements.

Performance in *Pichia pastoris* was assessed for the 5 formats, focusing on product titer and purity during upstream processing (USP) at 5 L fermentor scale, as well as on yield, after downstream processing (DSP) (Table 19). A022600424 and A022600426 were identified as preferred ISVD development candidates combining low percentage of high molecular weight (HMW) species, low percentage of variants on RPC with a high titer and best overall DSP yield.

15 **Table 19: Performance of the 5 selected ISVD based GPC3 T-cell engager formats in *Pichia*.**

Sample ID	Titer (g/L)	Analytical SEC (% HMW)	RPC (% Post-peaks)	DSP yield (total %)
A022600412	3.9	5.4	3.3	26
A022600424	4.9	1.8	1.7	45
A022600425	5.6	6.3	2.7	24
A022600426	5.4	2.4	3.3	32
A022600427	4.7	5.1	1.4	32

Based on the best combination of GPC3 driven killing potency, reduced binding to pre-existing antibodies, and performance in *Pichia*, A022600424 was selected as development candidate.

6.11 Example 11: Binding and affinity of A022600424 to GPC3, TCRab and serum albumin

The affinity, expressed as the equilibrium dissociation constant (K_D), of A022600424 towards human and cyno GPC3 (R&D Systems, cat no 2119-GP and in house produced (accession number P51654, Q3-R358, S359-H559), respectively), human and cyno TCRab (both in house produced, where alpha and beta chains extracellular domains are fused to a zipper peptide for dimerization; accession numbers: human alpha chain P01848, human beta chain P01850; predicted sequence of cyno alpha chain is identical to human, predicted sequence of cyno beta chain differs in 4 aa: A125V, E136V, V167M, S177F) and human, cyno and mouse serum albumin (Sigma cat no A8763, in house produced from animal tissue, DivBioScience cat no IMSA, respectively) was quantified by surface plasmon resonance (SPR) using a ProteOn XPR36.

Recombinant GPC3 proteins were captured on a GLC Sensor Chip (Biorad) immobilized with THE anti-His antibody (Genscript, cat no ABIN387699) via amine coupling, using EDC and NHS chemistry (running buffer: HBS-EP+, pH7.4). Purified ISVDs were injected for 2 minutes (flow rate 45 μ L/min) at different concentrations (between 0.3 nM and 1000 nM) and dissociation was followed for 900s. Regeneration was performed by injecting 10mM Glycine-HCl (pH 1.5) for 1 minute (flow rate 45 μ L/min). Data was double referenced by subtracting a reference ligand lane and a blank buffer injection. Processed sensorgrams were analyzed based on the 1:1 interaction model (Langmuir binding model) using ProteOn Manager 3.1.0 (Version 3.1.0.6) software.

Recombinant TCR proteins were immobilized on a GLC Sensor Chip (Biorad) via amine coupling, using EDC and NHS chemistry (running buffer: HBS-EP+, pH 7.4). Purified ISVDs were injected for 2 minutes (flow rate 45 μ L/min) at different concentrations (between 0.2 nM and 200 nM) and dissociation was followed for 900 s. Regeneration was performed by injecting 3 M $MgCl_2$ for 3 minutes (flow rate 90 μ L/min). Data was double

referenced by subtracting a reference ligand lane and a blank buffer injection. Processed sensorgrams were analyzed based on the 1:1 interaction model (Langmuir binding model) using ProteOn Manager 3.1.0 (Version 3.1.0.6) software.

Serum albumin proteins were immobilized on a GLC Sensor Chip (Biorad) via amine coupling, using EDC and NHS chemistry (running buffer: HBS-EP+, pH 7.4). Purified ISVDs were injected for 2 minutes (flow rate 45 μ L/min) at different concentrations (between 0.24 nM and 500 nM) and dissociation was followed for 900s. Regeneration was performed by injecting 10mM Glycine-HCl (pH 1.5) for 47 seconds (flow rate 100 μ L/min). Data was double referenced by subtracting a reference ligand lane and a blank buffer injection. Processed sensorgrams were analyzed based on the 1:1 interaction model (Langmuir binding model) using ProteOn Manager 3.1.0 (Version 3.1.0.6) software.

The results (Table 20) demonstrate that A022600424 binds human and cyno GPC3 with high affinity.

Table 20: Binding affinities to human and cyno GPC3, human and cyno TCRab and human, cyno and mouse serum albumin. n.a. = not available

Antigen	Sample ID	KD (M) human	KD (M) cynomolgus	KD mouse
GPC3	A022600424	< 5.6E-12	< 5.9E-12	n.a.
	Ab2	1.8E-09	1.7E-09	n.a.
TCRab	A022600424	6.3E-09	5.4E-09	n.a.
	T017000698	8.5E-09	1.1E-08	n.a.
Serum albumin	A022600424	8.3E-10	3.3E-10	6.9E-09
	ALB223	8.8E-10	5.7E-10	5.3E-09

Binding of A022600424 to cell expressed human and cyno GPC3 was assessed by flow cytometry for CHO-Flp-In cells overexpressing human and cyno GPC3, as well as Huh-7 cells, yielding EC50 values between 1 and 2 nM (Table 21).

A022600424 was evaluated for binding to human and cyno T cells in competition with TCRab binding monovalent ISVDs T017000624 and T017000623 (T0170056G05 variants) respectively, at EC30 concentrations. T cells (human T cells obtained as described in Example 3 and cyno T cells purchased from LPT laboratory, Germany) were thawed and

counted on the day of the assay and diluted to a concentration of 1E+06 cells/mL, before adding 75 μ L to the wells of a V-bottom 96-well plate (Greiner, cat no 651180). Cells were washed once with cold FACS buffer, before adding 25 μ L Nb and 25 μ L competitor to the wells. T017000624 was diluted to a 2x concentration of 4E-08 M (2E-08 M in well),

5 T017000623 was diluted to a 2x concentration of 1E-07 M (5E-08 M in well) and A022600424 was diluted to final concentrations in the wells ranging between 8 μ M and 7.8 nM. Cells were resuspended and plates were incubated at 4°C for 90 minutes, after which plates were washed twice in cold FACS buffer. Cells were resuspended in 50 μ L 1/1000 diluted Monoclonal ANTI-FLAG® M2 (Sigma Aldrich, cat no F1804) in FACS buffer

10 and incubated at 4°C for 30 minutes. Plates were washed twice in cold FACS buffer. Cells were resuspended in 50 μ L 1/100 diluted Allophycocyanin-conjugated AffiniPure Goat Anti-Mouse IgG (subclasses 1+2a+2b+3), Fc Fragment Specific (Jackson ImmunoResearch, cat no 115-135-164) in FACS buffer and incubated at 4°C for 30 minutes. Plates were washed twice in cold FACS buffer. Cells were resuspended in 55 μ L 1/1000 diluted

15 Propidium Iodide (Sigma-Aldrich, cat no P4170) in FACS buffer before acquiring data on the MACSQuant X (Miltenyi biotec).

The results are shown in Table 21. A022600424 binds with approx. 200nM affinity to both, human and cyno T-cells.

20 **Table 21: Binding assessment of A022600424 to cell expressed human and cyno GPC3 and human and cyno TCRab.**

Antigen	Sample ID	CHO huGPC3 EC50 (M)	CHO cyGPC3 EC50 (M)	Huh-7 EC50 (M)
GPC3 (binding FACS)	A022600424	1.82E-09	1.11E-09	1.25E-09
	Ab2	1.69E-08	5.4E-09	5.2E-09
		Primary hu T cells IC50 (M)	Primary cy T cells IC50 (M)	
TCRab (competition FACS)	A022600424	1.7E-07	2.5E-07	
	T017000698	1.9E-07	2.5E-07	

To assess A022600424 functionality with cyno T-cells, a xCELLigence based TDC assay on Huh-7 was performed using cyno T-cells (LPT laboratory, Germany), as described in Example 8. IC50 values for cyno and human T cells were found to be comparable (Table 22).

5 **Table 22: Functionality of A022600424 using cynomolgus T-cells in the xCELLigence based TDC assay on Huh-7, with an effector to target ratio of 15:1 and analyzed at 60h.**

Sample ID	Primary hu T cells IC50 (M)	Primary cy T cells IC50 (M)
A022600424	7.59E-11	2.29E-10
Ab2	7.88E-11	3.57E-11

6.12 Example 12: Selectivity of A022600424 for binding to GPC3

Absence of A022600424 binding to family members of GPC3, namely GPC1, GPC2, GPC5
10 and GPC6, was assessed by ELISA and to GPC4 by SPR (Proteon XPR36).

Human GPC1 (R&D systems, cat no 4519-GP), human GPC2 (R&D systems, cat no 2304-GP), human GPC3 (R&D systems, cat no 2119-GP), human Glypican-5 (R&D systems, cat no 2607-G5) and human Glypican-6 (R&D systems, cat no 2845-GP) were directly coated overnight at 4°C (2 µg/mL, 1xPBS buffer) on a 384-well HB SpectraPlate (PerkinElmer).
15 The following day the plate was washed 6 times (AquaMax microplate washer, Molecular devices) and blocked with 1xPBS + 1% Casein for 2 hours at room temperature. After an additional 6 wash steps A022600424 (1xPBS, 0.1% casein, 0.05% TWEEN 20) was added to the plate and incubated at room temperature for 1 hour. Next, the samples were removed, followed by 6 wash steps and anti-ISVD mAb ABH0077 was added at 17 nM
20 final concentration (1xPBS, 0.1% casein, 0.05% TWEEN 20) during 1 hour at room temperature. The plate was washed again 6 times and goat anti-mouse IgG polyclonal antibody conjugated to HRP (Abcam, cat no ab97040) (1/1250 dilution; 1xPBS, 0.1% casein, 0.05% TWEEN 20) was applied to the plate for 1 hour at room temperature. After 6 final wash steps the es(HS)TMB substrate (SDT) was added and the reaction stopped by
25 addition of 1 M HCl after 10 minutes. The absorbance of the plate was measured at

wavelengths 450 and 620 nm on the Clariostar instrument (BMG LABTECH) and the OD₄₅₀-OD₆₂₀ calculated and plotted for data analysis.

Recombinant human GPC4/hFc (R&D Systems, cat no 9195-GP) was captured on a GLC Sensor Chip (Biorad) immobilized with mouse anti-human IgG1 (GE Healthcare, cat no BR-1008-39) via amine coupling, using EDC and NHS chemistry (running buffer used: HBS-EP+, pH7.4). Purified ISVDs were injected for 2 minutes (flow rate 45 µL/min) at different concentrations (between 4nM and 1000 nM) and dissociation was followed for 900s. Regeneration was performed by injecting 10mM Glycine-HCl (pH1.5) for 1 minute (flow rate 45 µL/min). Data was double referenced by subtracting a reference ligand lane and a blank buffer injection. Processed sensorgrams were analysed based on the 1:1 interaction model (Langmuir binding model) using ProteOn Manager 3.1.0 (Version 3.1.0.6) software. No binding was detected of A022600424 to any of the GPC3 family members tested.

6.13 Example 13: Reactivity of human pre-existing antibodies for A022600424

Binding of pre-existing antibodies to A022600424 was assessed for normal human serum (n=96) using the ProteOn XPR36 (Bio-Rad Laboratories, Inc.). PBS/Tween (phosphate buffered saline, pH 7.4, 0.005% Tween20) was used as running buffer and the experiments were performed at 25°C.

A022600424 was captured on the sensor chip via binding of the ALB23002 building block to HSA immobilized on the chip. To immobilize HSA, the ligand lanes of a ProteOn GLC Sensor Chip were activated with EDC/NHS (flow rate 30 µL/min) and HSA was injected at 100 µL/mL in ProteOn Acetate buffer pH4.5 to render immobilization levels of approximately 2900 RU. After immobilization, surfaces were deactivated with ethanolamine HCl (flow rate 30µL/min).

Subsequently, A022600424 was injected for 2 min at 45 µl/min over the HSA surface to render an ISVD capture level of approximately 800 RU. The samples containing pre-existing antibodies were centrifuged for 2 minutes at 14,000 rpm and supernatants were diluted 1:10 in PBS-Tween20 (0.005%) before being injected for 2 minutes at 45 µl/min followed by a subsequent 400 seconds dissociation step. After each cycle (i.e. before a

new ISVD capture and blood sample injection step) the HSA surfaces were regenerated via a 2 minute injection of HCl (100 mM) at 45 μ L/min. Sensorgrams showing pre-existing antibody binding were obtained after double referencing by subtracting 1) ISVD-HSA dissociation and 2) non-specific binding to reference ligand lane. Binding levels of pre-existing antibodies were determined by setting report points at 125 seconds (5 seconds after end of association). Percentage reduction in pre-existing antibody binding is calculated relative to the binding levels at 125 seconds of a reference ISVD.

Tetravalent ISVD format A022600424, optimized for reduced pre-existing antibody binding by introduction of mutations L11V and V89A for the anti-TCR building block, mutations L11V and V89L in each GPC3 and the serum albumin binding building block and a C-terminal alanine, shows substantially less binding to pre-existing antibodies compared to a control non-optimized tetravalent ISVD format F027301099 (Figure 11A).

6.14 Example 14: Assessment of T-cell activation induction by A022600424 in the presence of soluble GPC3

T-cell activation induction by A022600424 in the presence of soluble GPC3 was assessed as described in Example 6. A022600424 showed the same behaviour as its wild-type variant A022600168 (Figure 7).

6.15 Example 15: *In vivo* proof-of-concept for A022600424 in Huh-7 tumor bearing NOG mice engrafted with *in vitro* expanded T cells

In an *in vivo* efficacy study in tumor bearing NOG mouse, Hepatocellular Carcinoma Huh-7 tumor cells were injected subcutaneously, and tumors were allowed to grow until a mean tumor volume of ~ 150 mm³ was reached. At this point, *in vitro* expanded T cells were injected intraperitoneally into the mice. Tumor cell killing by ISVD-mediated recruitment of T cells was evaluated by measuring the tumor volume and analyzing the tumor growth kinetics. The *in vivo* efficacy of A022600424 on tumor cell killing was evaluated and compared with the control T-cell engager T017000698 (SEQ ID NO: 74, Table A-6) lacking the GPC3 specificity.

In detail, 2×10^6 Huh-7 tumor cells resuspended in 100 μL of HBSS were subcutaneously injected in NOG mice. The tumors grew until the mean tumor volume of approximately 150 mm^3 was reached. At this point, 10^7 in vitro expanded T cells resuspended in 200 μL of PBS were injected into each mouse intraperitoneally (D0). This injection of T cells took place 24 hours after mice were randomized into different groups. The treatment with A022600424 injected intravenously started on D0, 3 h after T cell injection and continued D3, D6, D9 and D12 (q3d; Figure 12). Four dose levels of the TCR/GPC3 binding polypeptides were tested (0.1 mg/kg, 0.2 mg/kg, 0.7 mg/kg and 2 mg/kg). T017000698 was injected in a control group at 2 mg/kg on D0, D3, D6, D9 and D12 (q3d). Survival blood sampling in heparin containing tubes was done on D6 and D12 to measure antibody exposure. Mice were sacrificed on D15, and blood and tumor tissue were collected. Blood was used for the antibody exposure measurements and tumor tissue was used for analysis of target expression (GPC3) and T cell infiltration analysis.

Results for tumor growth kinetics are shown in Figure 13. Mice treated with T017000698 were used as control group for analyses on D24, when all control mice were alive as they did not reach end point criteria (2000 mm^3 tumor volume). A dose response pattern was seen in tumor growth profile for A022600424 versus the control T017000698 for inducing tumor stasis. The 0.7 mg/kg (**, $p = 0.0016$) and 2 mg/kg (*, $p = 0.0415$) dose levels for the A022600424 were significantly different from the control T017000698. The 0.1 mg/kg, 0.2 mg/kg doses had lower effects on controlling tumor growth and were not significantly different to the control group. Statistical analysis has been performed with one-way ANOVA, using the Dunnett's multiple comparisons test for analysis.

In conclusion, the results demonstrate that A022600424 can dose-dependently induce statistically significant tumor stasis in this model. This confirms the concept of polypeptide-induced T cell-mediated killing via a GPC3 ISVD T-cell engager by cross-linking T cells to GPC3 on Huh-7 tumor cells.

7 Industrial applicability

The polypeptides, nucleic acid molecules encoding the same, vectors comprising the nucleic acids and compositions described herein may be used for example in the treatment of subjects suffering from cancer.

CLAIMS

1. A polypeptide that comprises or consists of at least three immunoglobulin single variable domains (ISVDs), wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least three ISVDs are optionally linked via one or more peptidic linkers, and wherein:
- 5 a) a first ISVD specifically binds to the constant domain of the TCR on a T cell and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
- 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14;
- b) a second ISVD specifically binds to GPC3 and comprises
- 15 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- 20 vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; and
- c) a third ISVD specifically binds to GPC3 and comprises
- vii. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- 25 viii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- ix. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
- wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide, wherein the first ISVDs is optionally located at the N-terminus of said polypeptide.
- 30

2. The polypeptide according to claim 1, wherein:
- a) said first ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14;
 - 5 b) said second ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15; and
 - c) said third ISVD comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16.
- 10
3. The polypeptide according to any of claims 1 or 2, wherein:
- a) the amino acid sequence of said first ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 2;
 - b) the amino acid sequence of said second ISVD exhibits a sequence identity of more than 90% with SEQ ID NO: 3; and
 - 15 c) the amino acid sequence of said third ISVD exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4.
4. The polypeptide according to any of claims 1 to 3, wherein:
- a) said first ISVD consists of the amino acid sequence of SEQ ID NO: 2;
 - 20 b) said second ISVD consists of the amino acid sequence of SEQ ID NO: 3; and
 - c) said third ISVD consists of the amino acid sequence of SEQ ID NO: 4.
5. The polypeptide according to any of claims 1 to 4, wherein the first ISVD and the second ISVD are linked to each other via a linker consisting of less than 10 amino acids, preferably less than 6 amino acids, wherein the linker most preferably is a 5GS linker.
- 25 6. The polypeptide according to any of claims 1 to 5, wherein said polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased

half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.

7. The polypeptide according to claim 6, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.
5
8. The polypeptide according to any one of claims 6 or 7, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).
10
9. The polypeptide according to claim 8, in which said binding unit that provides the polypeptide with increased half-life is an ISVD that binds to human serum albumin.
10. The polypeptide according to claim 9, wherein the ISVD binding to human serum albumin comprises
15
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and
 - 20 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.
11. The polypeptide according to any of claims 9 or 10, wherein the ISVD binding to human serum albumin comprises a CDR1 being the amino acid sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.
25

12. The polypeptide according to any of claims 9 to 11, wherein the amino acid sequence of said ISVD binding to human serum albumin exhibits a sequence identity of more than 90% with SEQ ID NO: 5.
13. The polypeptide according to any of claims 9 to 12, wherein said ISVD binding to human serum albumin consists of the amino acid sequence of SEQ ID NO: 5.
14. The polypeptide according to any of claims 1 to 13, wherein the polypeptide comprises or consists of an amino acid sequence exhibiting a sequence identity of more than 90% with SEQ ID NO: 1.
15. The polypeptide according to any of claims 1 to 14, wherein the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.
16. A polypeptide that comprises or consists of at least one immunoglobulin single variable domain (ISVD) that specifically binds GPC3, wherein said ISVD comprises three complementarity determining regions (CDR1 to CDR3, respectively), and wherein the at least one ISVD comprises:
- 15 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid
20 sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, or
- b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
25 a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16.
17. The polypeptide according to claim 16, wherein the at least one ISVD comprises:

- a) a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, or
- b) a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16.
18. The polypeptide according to any of claims 16 or 17, wherein the amino acid sequence of the at least one ISVD comprises or consists of:
- a) an amino acid sequence with a sequence identity of more than 90% with SEQ ID NO: 3, or
- b) an amino acid sequence with a sequence identity of more than 90% identity with SEQ ID NO: 4.
19. The polypeptide according to any of claims 16 to 18, wherein said at least one ISVD comprises or consists of:
- a) the amino acid sequence of SEQ ID NO: 3, or
- b) the amino acid sequence of SEQ ID NO: 4.
20. A polypeptide that comprises or consists of at least two ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least two ISVDs are optionally linked via one or more peptidic linkers, and wherein:
- a) a first and a second ISVD specifically binds to GPC3 and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,
- b) a first and a second ISVD specifically binds to GPC3 and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - 5 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
- c) a first ISVD specifically binds to GPC3 and comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, and a second ISVD specifically binds to GPC3 and comprises
 - 15 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16,
 - 20 d) a first ISVD specifically binds to GPC3 and comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - 25 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a second ISVD specifically binds to GPC3 and comprises
 - iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - 30

- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,
- 5 e) a first ISVD specifically binds to the constant domain of the T cell receptor (TCR) on a T cell and comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a second ISVD specifically binds to GPC3 and comprises
 - 15 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15,
- 20 f) a first ISVD specifically binds to GPC3 and comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - 25 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15, and a second ISVD specifically binds to the constant domain of the TCR on a T cell and comprises
 - 30 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;

- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,
- 5 g) a first ISVD specifically binds to the constant domain of the TCR on a T cell and comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;
 - 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a second ISVD specifically binds to GPC3 and comprises
 - 15 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, or
- 20 h) a first ISVD specifically binds to GPC3 and comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - 25 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a second ISVD specifically binds to the constant domain of the TCR on a T cell and comprises
 - 30 iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 6 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 6;

- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14,
- 5 wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide.
21. The polypeptide according to claim 20, wherein:
- a) the first and the second ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid
10 sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,
 - b) the first and the second ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ
15 ID NO: 16,
 - c) the first ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, and the second ISVD specifically binds to GPC3 and comprises a CDR1 being
20 the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16,
 - d) the first ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, and the second ISVD specifically binds to GPC3 and comprises a CDR1 being
25 the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,

- 5 e) the first ISVD specifically binds to the constant domain of the TCR on a T cell and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14, and the second ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15,
- 10 f) the first ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 7, a CDR2 being the amino acid sequence of SEQ ID NO: 11 and a CDR3 being the amino acid sequence of SEQ ID NO: 15, and the second ISVD specifically binds to the constant domain of the TCR on a T cell and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14,
- 15 g) the first ISVD specifically binds to the constant domain of the TCR on a T cell and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14, and the second ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, or
- 20 h) the first ISVD specifically binds to GPC3 and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 8, a CDR2 being the amino acid sequence of SEQ ID NO: 12 and a CDR3 being the amino acid sequence of SEQ ID NO: 16, and the second ISVD specifically binds to the constant domain of the TCR on a T cell and comprises a CDR1 being the amino acid sequence of SEQ ID NO: 6, a CDR2 being the amino acid sequence of SEQ ID NO: 10 and a CDR3 being the amino acid sequence of SEQ ID NO: 14.
- 25

22. The polypeptide according to any of claims 20 or 21, wherein:

- a) the amino acid sequence of the first and the second ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% with SEQ ID NO: 3,
- 5 b) the amino acid sequence of the first and the second ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% with SEQ ID NO: 4,
- c) the amino acid sequence of the first ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% with SEQ ID NO: 3, and the second ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4,
- 10 d) the amino acid sequence of the first ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% with SEQ ID NO: 4, and the second ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% identity with SEQ ID NO: 3,
- 15 e) the amino acid sequence of the first ISVD that specifically binds to the constant domain of the TCR on a T cell exhibits a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% identity with SEQ ID NO: 3,
- 20 f) the amino acid sequence of the first ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% with SEQ ID NO: 3, and the second ISVD that specifically binds to the constant domain of the TCR on a T cell exhibits a sequence identity of more than 90% identity with SEQ ID NO: 2
- g) the amino acid sequence of the first ISVD that specifically binds to the constant domain of the TCR on a T cell exhibits a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% identity with SEQ ID NO: 4, or
- 25 h) the amino acid sequence of the first ISVD that specifically binds to GPC3 exhibits a sequence identity of more than 90% with SEQ ID NO: 4, and the
- 30

second ISVD that specifically binds to the constant domain of the TCR on a T cell exhibits a sequence identity of more than 90% identity with SEQ ID NO: 2.

23. The polypeptide according to any of claims 20 to 22, wherein:

- 5 a) the first and the second ISVD that specifically binds to GPC3 consist of the amino acid sequence of SEQ ID NO: 3,
- b) the first and the second ISVD that specifically binds to GPC3 consist of the amino acid sequence of SEQ ID NO: 4,
- 10 c) the first ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 3, and the second ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 4,
- d) the first ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 4, and the second ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 3,
- 15 e) the first ISVD that specifically binds to the constant domain of the TCR on a T cell consists of the amino acid sequence of SEQ ID NO: 2, and the second ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 3,
- 20 f) the first ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 3, and the second ISVD that specifically binds to the constant domain of the TCR on a T cell consists of the amino acid sequence of SEQ ID NO: 2,
- g) the first ISVD that specifically binds to the constant domain of the TCR on a T cell consists of the amino acid sequence of SEQ ID NO: 2, and the second ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 4, or
- 25 h) the first ISVD that specifically binds to GPC3 consists of the amino acid sequence of SEQ ID NO: 4, and the second ISVD that specifically binds to the constant domain of the TCR on a T cell consists of the amino acid sequence of SEQ ID NO: 2.

24. The polypeptide according to any of claims 20 to 23, wherein the polypeptide comprises or consists of an amino acid sequence selected from SEQ ID NOs: 1, 49-72 and 78-81.
25. The polypeptide according to any of claims 16 to 24, wherein said polypeptide
5 further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.
- 10 26. The polypeptide according to claim 25, in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.
- 15 27. The polypeptide according to any one of claims 25 to 26, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).
28. The polypeptide according to claim 27, in which said binding unit that provides the
20 polypeptide with increased half-life is an ISVD that binds to human serum albumin.
29. The polypeptide according to claim 28, wherein the ISVD binding to human serum albumin comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
 - 25 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.
30. The polypeptide according to any of claims 28 to 29, wherein the ISVD binding to human serum albumin comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 being the amino acid sequence of SEQ ID NO: 13 and a CDR3 being the amino acid sequence of SEQ ID NO: 17.
31. The polypeptide according to any of claims 28 to 30, wherein the amino acid sequence of said ISVD binding to human serum albumin exhibits a sequence identity of more than 90% with SEQ ID NO: 5.
32. The polypeptide according to any of claims 28 to 31, wherein said ISVD binding to human serum albumin consists of the amino acid sequence of SEQ ID NO: 5.
33. A nucleic acid comprising a nucleotide sequence that encodes a polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15.
34. A host or host cell comprising a nucleic acid according to claim 33.
35. A method for producing a polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15, said method at least comprising the steps of:
- expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid according to claim 33; optionally followed by:
 - isolating and/or purifying the polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15.
36. A composition comprising at least one polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15, or a nucleic acid according to claim 33.
37. The composition according to claim 36, which is a pharmaceutical composition which further comprises at least one pharmaceutically acceptable carrier, diluent or

excipient and/or adjuvant, and optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

38. A polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15, or a composition according to claim 36 or 37, for use as a medicament.

5 39. A polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15, or a composition according to claim 36 or 37, for use in the treatment of cancer, preferably liver cancer or lung cancer.

40. The polypeptide or composition for use according to claim 39, wherein the liver cancer is hepatocellular carcinoma.

10 41. A method of treating cancer, preferably liver cancer or lung cancer, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15, or a composition according to claim 36 or 37.

42. The method according to claim 41, wherein the liver cancer is hepatocellular carcinoma.
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43. Use of a polypeptide according to any of claims 1 to 32, preferably according to any of claims 1 to 15, or a composition according to claim 36 or 37, in the preparation of a medicament.

44. Use of a polypeptide according to any of claims 1 to 32, preferably according to claims 1 to 15, or a composition according to claim 36 or 37, in the preparation of a pharmaceutical composition for treating cancer, preferably liver cancer or lung cancer.
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45. Use of the polypeptide or a composition according to claims 44, wherein the liver cancer is hepatocellular carcinoma.