

by malignant cells. By binding to TAAs with high affinity, immunotherapies can restrict their immunomodulatory activity to immunological synapses between tumor cells and immune effector cells to a degree.

[0003] A common class of TAA-binding immunotherapeutics are mAbs that elicit anti-tumor immunity by opsonizing tumor-cells and triggering antibody-dependent cell-mediated cytotoxicity (ADCC) by Fcγ receptor (FcγR)-expressing cells, primarily natural killer (NK) cells. Other TAA-binding immunotherapeutics leverage cytotoxic T lymphocytes (CTLs) to induce targeted depletion of malignant cells, such as CAR-T cells as well as bsAbs that simultaneously engage the T cell antigen CD3 (TAA/CD3 bsAbs).

[0004] While the therapeutic utility of TAA-(re)directed CTLs and conventional TAA/CD3 bsAbs have been clinically validated, dose-limiting toxicities (DLTs) often preclude administration at maximally effective doses (MEDs) or lead to discontinuation of treatment, resulting in limited efficacy.

[0005] One reason for the DLTs is that conventional TAA/CD3 bsAbs are also commonly associated with cytokine release syndrome (CRS), putatively due to excessive activity of anti-CD3 domains. Extratumoral activity of immunotherapies results in the secretion of pro-inflammatory cytokines in healthy tissues, which can result in undesirable safety profiles. Furthermore, while TAA/CD3 bsAbs potentially deplete TAA-overexpressing cells, they do so by recruiting and stimulating CTLs regardless of whether such cells express a T cell receptor (TCR) that recognizes a tumor-antigen(s) (*i. e.*, tumor-reactive T cell). Therefore, rather than stimulating or reactivating the host's native anti-tumor immunity, TAA/CD3 bsAbs somewhat indiscriminately stimulate CTLs, potentially posing safety risks.

[0006] Although the exact pathways by which such DLTs arise can vary, the risk of immunotherapy-related toxicities can typically be minimized or eliminated by enhancing the tumor-localization of pharmacological activity.

[0007] TAAs that are almost exclusively expressed on cancer cells, such as oncofetal tumor antigens, are referred to as clean TAAs. TAA that are also expressed on normal, non-cancer cells – typically at lower levels compared to cancer cells – are considered non-clean TAAs. Due to the very high potency of TAA/CD3 bsAbs approaches, non-clean TAAs are a challenge as they damage non-tumor cells that also express the TAA. Mesothelin (MSLN) is an example of a non-clean TAA; it

is not only expressed on tumor cells but also in various other tissues, albeit at a lower level. Therefore, when targeting non-clean TAAs, novel therapies that improve the selectivity of TAA/CD3 bsAb approaches for tumor tissues and minimize off tumor/on target effects are needed. This particularly applies to MSLN/CD3 bsAb approaches.

[0008] Mesothelin (MSLN) has been proposed as a tumor-associated antigen (TAA) that can be targeted to treat MSLN<sup>+</sup> solid cancers such as mesothelioma. Many other types of cancers are also MSLN<sup>+</sup>, including certain forms of ovarian cancer and pancreatic cancer, as well as triple negative breast cancer. The current standard of care for mesothelioma includes tumor resection, chemotherapy, and radiation therapy, as well as palliative measures such as fluid reduction and pain management. Immunotherapies for tumors that continue growing include the use of PD-1/PD-L1 blockers such as pembrolizumab and nivolumab, or anti-CTLA4 antibodies such as ipilimumab to stimulate the immune system, as well as VEGF inhibitors such as bevacizumab to block blood vessel angiogenesis. While these therapies have achieved clinical success, they come with a higher risk of systemic side effects. Therefore, a need exists for a MSLN-targeted, specific approach.

[0009] A number of preclinical and early stage clinical studies have been performed or are underway to assess the feasibility of targeting MSLN via a few different approaches, which include antibody-based drugs and CAR-T cells. Antibody-based drugs include targeting MSLN-expressing cells with: the anti-MSLN fragment SS1P immunotoxin together with chemotherapeutics such as pemetrexed and cisplatin or coupled with PE38; amatuximab, a chimeric monoclonal antibody to induce ADCC; antibody drug conjugates such as anetumab ravtansine (BAY 94-9343: anti-MSLN + tubulin inhibitor DM4) or DMOT4039A (anti-MSLN + anti-mitotic monomethyl auristatin E) to inhibit tumor growth. HPN536, a multispecific engager (anti-MSLN + anti-CD3 + anti-albumin) with an improved half-life appears to redirect T cells to kill MSLN-expressing targets *in vitro* and *in vivo* and seems to be well tolerated by cynomolgous monkeys. Several anti-MSLN chimeric antigen receptor (CAR)-T therapies have also been well tolerated, including transient mRNA-transfected CAR-T (RNA CARTmeso) and CAR-T that have been engineered with a suicide gene (iCasp9m28z). Responses to most anti-MSLN therapies thus far have been modest, indicating the challenges associated with treating solid tumors.

[0010] For anti-MSLN therapies, this problem is further compounded by the fact that MSLN is shed into the serum of cancer patients, where it is referred to as soluble mesothelin-related protein (SMRP). Antibodies with a high affinity to MSLN also strongly bind to SMRP, which significantly counteracts their activity reducing the effective dose on cancer cells.

[0011] Therefore, there is a need for novel molecules that are able to effectively localize to tumors and promote T cell responses in the presence of SMRP.

[0012] Multispecific antibodies having at least three binding domains, of which two specifically bind to mesothelin (MSLN-BD) and one specifically binds to CD3 (CD3-BD), wherein the binding affinity of the two MSLN-BD is in a well-balanced range, could theoretically address many of the foregoing limitations with respect to safety and efficacy. Such multispecific antibodies are theoretically capable of eliciting a high tumor localization and improved selectivity, which could provide safer and more effective therapies for a variety of cancers. Additionally, such molecules would further limit the need for co-administration of additional immunotherapies to boost patient responses, supporting ease-of-development and minimizing treatment costs. However, implementation of multispecific antibodies for therapeutic use has been complicated due to issues with their molecular architecture, the properties of their component antigen-binding domains, their producibility and/or poor biophysical properties. In summary, there remains a clear need for novel multi-specific antibodies that exhibit increased tumor cell localization and elicit effective T cell activation with a tolerable toxicological profile and that have biophysical properties rendering them suitable for pharmaceutical development.

[0013] In addition, despite the fact that numerous antibodies already exist that are specific for MSLN and/or CD3, the complex and specific requirements of such multispecific antibodies require the development of novel antibody domains with tailor-made properties.

[0014] Thus, in spite of numerous treatment options for patients suffering from cancer, there remains a need for effective and safe therapeutic agents and a need for their preferential use in a more targeted manner. Immune-modulating biologics offer promising approaches in treatment of cancers due to their modes of action, however global immunostimulation and lack of any restriction of this immunomodulation to pathologically relevant cells and sites cause numerous side

effects and significant toxicities, which potentially may lead to increased morbidity and mortality of patients. It is therefore an object of the present invention to provide a medicament to improve treatment of a proliferative disease, particularly a cancer.

## **SUMMARY OF THE INVENTION**

[0015] It is an object of the present ~~invention~~disclosure to provide a medicament to improve treatment of a proliferative disease, particularly a cancer. In particular, it was an object of the present ~~invention~~disclosure to provide a medicament having increased on target efficacy thereby improving the toxicological profile.

[0016] In a first aspect, the present ~~invention~~disclosure relates to a multispecific antibody comprising:

- a) two antibody-based binding domains, which specifically bind to mesothelin (MSLN-BD); and
- b) at least one antibody-based binding domain, which specifically binds to CD3 (CD3-BD);

wherein said multispecific antibody does not comprise an immunoglobulin Fc region polypeptide, and wherein each of said MSLN-BD binds to mesothelin (MSLN) with a monovalent dissociation constant ( $K_D$ ) in the range of from 0.5 to 20 nM, when measured by SPR.

[0017] In a second aspect, the present ~~invention~~disclosure relates to a specific MSLN-binding domain.

[0018] In a third aspect, the present ~~invention~~disclosure relates to a nucleic acid sequence or two nucleic acid sequences encoding the multispecific antibody or the specific MSLN-binding domain of the present ~~invention~~disclosure.

[0019] In a fourth aspect, the present ~~invention~~disclosure relates to a vector or two vectors comprising the nucleic acid sequence or the two nucleic acid sequences of the present ~~invention~~disclosure.

[0020] In a fifth aspect, the present ~~invention~~disclosure relates to a host cell or host cells comprising the vector or the two vectors of the present ~~invention~~disclosure.

[0021] In a sixth aspect, the present ~~invention~~disclosure relates to a method for producing the multispecific antibody or a specific binding domain of the present ~~invention~~disclosure,

comprising (i) providing the nucleic acid sequence or the two nucleic acid sequences of the present inventiondisclosure, or the vector or the two vectors of the present inventiondisclosure, expressing said nucleic acid sequence or nucleic acid sequences, or said vector or vectors, and collecting said multispecific antibody or said specific binding domain from the expression system, or (ii) providing a host cell or host cells of the present inventiondisclosure, culturing said host cell or said host cells; and collecting said multispecific antibody or said specific binding domain, from the cell culture.

[0022] In a seventh aspect, the present inventiondisclosure relates to a pharmaceutical composition comprising the multispecific antibody of the present inventiondisclosure and a pharmaceutically acceptable carrier.

[0023] In an eighth aspect, the present inventiondisclosure relates to a multispecific antibody of the present invention for use in the manufacture of a medicament for treating in the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, wherein said multispecific antibody is a single-chain protein comprising three or four binding domains.

[0024] In a ninth aspectone example, the present inventiondisclosure relates to a multispecific antibody of the present inventiondisclosure for use in the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, wherein said multispecific antibody is a hetero-dimeric protein comprising three or four binding domains.

[0025] In a tenth aspectanother example, the present inventiondisclosure relates to a method for the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, comprising the step of administering the above defined single-chain multispecific antibody of the present inventiondisclosure, said single-chain multispecific antibody comprising three or four binding domains.

[0026] In an eleventh aspectyet another example, the present inventiondisclosure relates to a method for the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, comprising the step of administering the above defined hetero-dimeric multispecific antibody of the present inventiondisclosure, said hetero-dimeric multispecific antibody comprising three or four binding domains.

[0027] The aspects, advantageous features and preferred embodiments of the present invention summarized in the following items, respectively alone or in combination, further contribute to solving the object of the invention:

1. A multispecific antibody comprising:
  - a) two antibody-based binding domains, which specifically bind to mesothelin (MSLN-BDs); and
  - b) at least one antibody-based binding domain, which specifically binds to CD3 (CD3-BD);wherein said multispecific antibody does not comprise an immunoglobulin Fc region polypeptide, and wherein each of said MSLN-BDs binds to mesothelin (MSLN) with a monovalent dissociation constant ( $K_D$ ) in the range of from 0.5 to 20 nM, when measured by SPR.
2. The multispecific antibody of item 1, wherein the  $EC_{50}$  of said multispecific antibody for killing target cells, which have a 6 to 8-fold higher MSLN expression level than MeT-5A cells (ATCC CRL-9444) as determined by flow cytometry, does not increase by more than 25-fold in the presence of at least 200 ng/ml, particularly of at least 300 ng/ml, particularly of at least 400 ng/ml, particularly of at least 500 ng/ml soluble mesothelin, as determined in a T-cell driven cytotoxicity assay against said target cells.
3. The multispecific antibody of item 1, wherein said multispecific antibody is capable of killing target cells, which have a 6 to 8-fold higher MSLN expression level than MeT-5A cells (ATCC CRL-9444) as determined by flow cytometry, with an  $EC_{50}$  that is at least 10 fold, particularly at least 20 fold, particularly at least 25 fold, smaller than the  $EC_{50}$  for killing said MeT-5A cells, as determined in a T-cell driven cytotoxicity assay against said target cells and said MeT-5A cells.
4. The multispecific antibody of any one of items 1 to 3, wherein each of said MSLN-BDs binds to mesothelin (MSLN) with a monovalent dissociation constant

( $K_D$ ) in the range of from 0.5 to 15 nM, particularly in the range of from 0.6 to 10 nM, particularly in the range of from 0.7 to 5 nM, when measured by SPR.

5. A multispecific antibody comprising:

- a) two antibody-based binding domains, which specifically bind to mesothelin (MSLN-BDs); and
- b) at least one antibody-based binding domain, which specifically binds to CD3 (CD3-BD);

wherein said multispecific antibody does not comprise an immunoglobulin Fc region polypeptide, and wherein each of said MSLN-BDs binds to mesothelin (MSLN) with a monovalent dissociation constant ( $K_D$ ) in the range of from 0.1 to 5 nM, when measured by SPR.

6. The multispecific antibody of item 5, wherein the  $EC_{50}$  of said multispecific antibody for killing target cells, which have a 6 to 8-fold higher MSLN expression level than MeT-5A cells (ATCC CRL-9444) as determined by flow cytometry, does not increase by more than 50-fold in the presence of at least 200 ng/ml, particularly of at least 300 ng/ml, particularly of at least 400 ng/ml, particularly of at least 500 ng/ml soluble mesothelin, as determined in a T-cell driven cytotoxicity assay against said target cells.
7. The multispecific antibody of any one of items 5 or 6, wherein each of said MSLN-BDs binds to mesothelin (MSLN) with a monovalent dissociation constant ( $K_D$ ) in the range of from 0.1 to 3 nM, particularly in the range of from 0.15 to 2 nM, particularly in the range of from 0.2 to 1 nM, when measured by SPR.
8. The multispecific antibody of any one of the preceding items, wherein each of said MSLN-BDs specifically binds to human mesothelin.
9. The multispecific antibody of any one of the preceding items, wherein each of said MSLN-BDs binds to Region I, Region II and/or Region III of MSLN, preferably to Region I and/or Region II of MSLN, in particular to Region I of MSLN.

a.2) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 1, 2 (or 10) and 3, respectively,

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b.2) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 4, 5 and 6, respectively,

c.2) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 8, and

d.2) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 9;

or

a.3) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,

b.3) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,

c.3) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 17, and

d.3) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 18;

or

a.4) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,

b.4) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,

c.4) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 19, and

d.4) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 21;

or

a.5) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,

b.5) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,

c.5) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 20, and

d.5) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 21;

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or

a.6) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,

b.6) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,

c.6) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 22, and

d.6) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 24;

or

a.7) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,

b.7) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,

c.7) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 23, and

d.7) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 24.

13. The multispecific antibody of any one of the items 5 to 10, wherein said MSLN-BD comprises

(i) the HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 25, 26 and 27, respectively, and the LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 28, 29 and 30, respectively; and

(ii) VH3 or VH4 domain framework sequences FR1 to FR4; particularly VH3 domain framework sequences FR1 to FR4; and

(iii) a VL domain comprising a VL framework comprising Vk frameworks FR1, FR2 and FR3, particularly Vk1 or Vk3 FR1 to FR3, particularly Vk1 FR1 to FR3, and a framework FR4, which is selected from a Vk FR4, and a Vλ FR4, particularly a Vλ FR4 comprising an amino acid sequence having at least 70, 80, or 90 percent identity to any of SEQ ID NO: 132 to SEQ ID NO: 139,

more particularly V $\lambda$  FR4 selected from any of SEQ ID NO: 132 to SEQ ID NO: 139, particularly V $\lambda$  FR4 according to SEQ ID NO: 132 or 139.

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14. The multispecific antibody of any one of the items 5 to 10 and 13, wherein said MSLN-BD comprises
- a.1) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 25, 26 and 27, respectively,
  - b.1) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 28, 29 and 30, respectively,
  - c.1) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 31, and
  - d.1) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 33;
- or
- a.2) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 25, 26 and 27, respectively,
  - b.2) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 28, 29 and 30, respectively,
  - c.2) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 32, and
  - d.2) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 33;
- or
- a.3) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 25, 26 and 27, respectively,
  - b.3) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 28, 29 and 30, respectively,
  - c.3) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 34, and
  - d.3) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 36;
- or

- a.4) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 25, 26 and 27, respectively,
- b.4) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 28, 29 and 30, respectively,
- c.4) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 35, and
- d.4) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 36.
15. The multispecific antibody of any one of items 11 to 14, wherein said VH domain comprises a C51 amino acid residue (Aho numbering) and said VL domain comprises a C141 amino acid residue (Aho numbering).
16. The multispecific antibody of any one of items 8 to 15, wherein each of said MSLN-BDs is cross reactive with *Macaca fascicularis* (Cynomolgus) MSLN, in particular binds to Cynomolgus MSLN with a monovalent  $K_D$  in the range of 0.2 to 75 nM, particularly in the range of 0.3 to 60 nM, particularly of 0.4 to 50 nM, particularly of 0.5 to 40 nM, as measured by SPR.
17. The multispecific antibody of any one of the preceding items, wherein said CD3-BD is binding to CD3 $\epsilon$ .
18. The multispecific antibody of any one of the preceding items, wherein said antibody comprises one or two CD3-BD, particularly one CD3-BD.
19. The multispecific antibody of any one of the preceding items, wherein said CD3-BD binds CD3 $\epsilon$  with a monovalent  $K_D$  of less than 50 nM, particularly with a monovalent  $K_D$  of 0.5 to 50 nM, particularly of 1 to 40 nM, particularly of 2 to 30 nM, as measured by SPR.
20. The multispecific antibody of any one of the preceding items, wherein said CD3-BD comprises

- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 45, 46 and 47, respectively in a human antibody VH framework, particularly a VH3 framework, and
  - (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 48, 49 and 50, respectively in a human antibody VL framework, wherein the VL framework comprises Vk frameworks FR1, FR2 and FR3, particularly Vk1 frameworks, and a framework FR4, which is selected from a Vk FR4, and a Vλ framework 4.
21. The multispecific antibody of item 20, wherein said CD3-BD comprises
- (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 51, 140, 141 or 142; and
  - (ii) a VL domain comprising the amino acid sequence of SEQ ID NO: 52.
22. The multispecific antibody of any one of the preceding items, wherein said antibody further comprises at least one human serum albumin binding domain (hSA-BD), particularly one hSA-BD.
23. The multispecific antibody of item 22, wherein said hSA-BD comprises
- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 53, 54 and 55, respectively in a human antibody VH framework, particularly a VH3 framework, and
  - (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 56, 57 and 58, respectively in a human antibody VL framework, wherein the VL framework comprises Vk frameworks FR1, FR2 and FR3, particularly Vk1 frameworks, and a framework FR4, which is selected from a Vk FR4, particularly Vk1 FR4, and a Vλ framework 4;
- or
- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 63, 64 and 65, respectively in a human antibody VH framework, particularly a VH3 framework, and
  - (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 66, 67 and 68, respectively in a human antibody VL framework, wherein the VL framework

comprises  $V_k$  frameworks FR1, FR2 and FR3, particularly  $V_{k1}$  frameworks, and a framework FR4, which is selected from a  $V_k$  FR4, particularly  $V_{k1}$  FR4, and a  $V_\lambda$  framework 4;

or

- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 63, 64 and 65, respectively in a human antibody VH framework, particularly a VH3 framework, and
- (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 66, 67 and 68, respectively in a human antibody VL framework, wherein the VL framework comprises  $V_k$  frameworks FR1, FR2 and FR3, particularly  $V_{k1}$  frameworks, and a framework FR4, which is selected from a  $V_k$  FR4, particularly  $V_{k1}$  FR4, and a  $V_\lambda$  framework 4.

24. The multispecific antibody of items 22 or 23, wherein said hSA-BD comprises

- (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 59 and a VL domain comprising the amino acid sequence of SEQ ID NO: 60; or
- (ii) a VH domain comprising the amino acid sequence of SEQ ID NO: 61 and a VL domain comprising the amino acid sequence of SEQ ID NO: 62; or
- (iii) a VH domain comprising the amino acid sequence of SEQ ID NO: 69 and a VL domain comprising the amino acid sequence of SEQ ID NO: 70; or
- (iv) a VH domain comprising the amino acid sequence of SEQ ID NO: 71 and a VL domain comprising the amino acid sequence of SEQ ID NO: 72; or
- (v) a VH domain comprising the amino acid sequence of SEQ ID NO: 79 or 143; and a VL domain comprising the amino acid sequence of SEQ ID NO: 80 or 144; or
- (vi) a VH domain comprising the amino acid sequence of SEQ ID NO: 81 or 145; and a VL domain comprising the amino acid sequence of SEQ ID NO: 82 or 146.

25. The multispecific antibody of item 24, wherein said VH domain comprises a C51 amino acid residue (AHO numbering) and said VL domain comprises a C141 amino acid residue (AHO numbering).

26. The multispecific antibody of any one of the preceding items, wherein said binding domains are independently selected from the group consisting of a Fab, an Fv, an scFv, dsFv, a scAb, and a STAB.
27. The multispecific antibody of item 26, wherein each of said binding domains is independently selected from
- (a) a cognate pair of a VL domain and a VH domain (Fv fragment); or
  - (b) a cognate pair of a VL domain and a VH domain linked by an oligo- or polypeptide linker (scFv fragment).
28. The multispecific antibody of any one of the preceding items, wherein said multispecific antibody is in a format selected from the group consisting of: a tandem scDb (Tandab), a linear dimeric scDb (LD-scDb), a circular dimeric scDb (CD-scDb), a tandem tri-scFv, a tribody (Fab-(scFv)<sub>2</sub>), Fab-Fv<sub>2</sub>, triabody, scDb-scFv, tetrabody, di-diabody, CODV, tandem-di-scFv, tandem tri-scFv, Fab-(scFv)<sub>2</sub>, Fab-Fv<sub>2</sub>, or CODV fused to the N- and/or the C-terminus of a heterodimerization domain other than heterodimeric Fc domains, and MATCH.
29. The multispecific antibody of any one of items 1 to 28, wherein said antibody does not comprise CH1 and/or CL regions.
30. The multispecific antibody of any one of the preceding items, wherein said antibody is a scDb-scFv, tribody, MATCH, in particular wherein said multispecific antibody is in a MATCH or tribody format, more particularly wherein said multispecific antibody is in a MATCH format, more particularly wherein said multispecific antibody is a MATCH3 or a MATCH4.
31. The multispecific antibody of items 1 to 30, wherein said multispecific antibody is a single-chain protein.
32. The multispecific antibody of item 31, wherein said single-chain protein comprises an amino acid sequence consisting of:

- (i) a first VL domain,
  - (ii) a first polypeptide linker,
  - (iii) a first VH domain,
  - (iv) a second polypeptide linker,
  - (v) a second VL domain,
  - (vi) a third polypeptide linker, and
  - (vii) a second VH domain,
- arranged one after another in the stated order,

wherein said first VL domain associates with said second VH domain to form a first binding domain, and said second VL domain associates with said first VH domain to form a second binding domain,

and wherein said single-chain protein further comprises

- (viii) a third binding domain, which is formed by a third VL domain and a third VH domain that are connected via a fourth polypeptide linker, where said third binding domain is fused C-terminally or N-terminally via a fifth polypeptide linker to said amino acid sequence,

wherein said three binding domains have the following specificities:

- a) the first binding domain specifically binds to human CD3 (CD3-BD); and
- b) the second and third binding domains specifically bind to mesothelin (MSLN-BD).

33. The multispecific antibody of item 32, wherein said single-chain protein further comprises a hSA-BD, which is formed by a fourth VL domain and a fourth VH domain that are connected via a sixth polypeptide linker, where said hSA-BD is fused C-terminally or N-terminally via a seventh polypeptide linker to said amino acid sequence.
34. The multispecific antibody of items 1 to 30, wherein said multispecific antibody is a hetero-dimeric protein comprising a first and a second single-chain protein,

wherein said first single-chain protein comprises a first amino acid sequence consisting of (from the N- to the C-terminus):

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- (ia) a first VL domain,
- (iia) a first polypeptide linker, and
- (iia) a second VL domain, and

wherein said second single-chain protein comprises a second amino acid sequence consisting of (from the N- to the C-terminus):

- (ib) a first VH domain,
- (iib) a second polypeptide linker, and
- (iib) a second VH domain, and

wherein said first VL domain associates with either said first or said second VH domain to form a first binding domain, and said second VL domain associates with the other of said VH domains to form a second binding domain,

and wherein at least one of said first and said second single-chain proteins further comprises

- (iv) a third binding domain, which is formed by a third VL domain and a third VH domain that are connected via a third polypeptide linker, where said third binding domain is fused via a fourth polypeptide linker to said first or said second amino acid sequence,

and wherein optionally at least one of said first and said second single-chain proteins further comprises

- (v) a fourth binding domain, which is formed by a fourth VL domain and a fourth VH domain that are connected via a fifth polypeptide linker, where said fourth binding domain is fused via a sixth polypeptide linker to said first or said second amino acid sequence,

wherein said three, or optionally four, binding domains have the following specificities:

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- a) one antibody-based binding domain specifically binds to human CD3 (CD3-BD);
- b) another two antibody-based binding domains specifically bind to mesothelin (MSLN-BD), and, when the optional fourth binding domain is present,
- c) the remaining binding domain specifically binds to human serum albumin (hSA-BD).

35. The multispecific antibody of item 34, wherein the optional fourth domain is present and wherein one of said first and second binding domains is a CD3-BD and the other one of said first and second binding domains is a hSA-BD.
36. The multispecific antibody of item 34 or 35, wherein the optional fourth domain is present and wherein the third binding domain is fused to either the first or the second amino acid sequence, and the fourth binding domain is fused to the other one of the two said amino acid sequences.
37. The multispecific antibody of any one of the items 34 to 36, wherein any binding domains comprised in said hetero-dimeric protein exclusively consist of immunoglobulin variable domains, arranged in said first and second single-chain protein.
38. The multispecific antibody of any one of the items 34 to 37, wherein said hetero-dimeric protein does not comprise a cognate pair of a first and a second proteinaceous interaction domain, other than said first and second VL and VH domains, wherein said first proteinaceous interaction domain is comprised in said first single-chain protein and wherein said second proteinaceous interaction domain is comprised in said second single-chain protein.
39. The multispecific antibody of any one of items 34 to 38, wherein said first single-chain protein and said second single-chain protein hetero-dimerize in a parallel

orientation, *i. e.* said first VL domain associates with said first VH domain and said second VL domain associates with said second VH domain.

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40. The multispecific antibody of any one of items 34 to 38, wherein said first single-chain protein and said second single-chain protein hetero-dimerize in an anti-parallel orientation, *i. e.* said first VL domain associates with said second VH domain and said second VL domain associates with said first VH domain.
41. The multispecific antibody of any one of items 34 to 40, wherein  
said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 83, more particularly comprises the amino acid sequence of SEQ ID NO: 83, especially consists of the amino acid sequence SEQ ID NO: 83 and  
said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 84, more particularly comprises the amino acid sequence of SEQ ID NO: 84, especially consists of the amino acid sequence SEQ ID NO: 84; or  
said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 85, more particularly comprises the amino acid sequence of SEQ ID NO: 85, especially consists of the amino acid sequence SEQ ID NO: 85 and  
said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 86, more particularly comprises the amino acid sequence of SEQ ID NO: 86, especially consists of the amino acid sequence SEQ ID NO: 86; or  
said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 87, more particularly comprises the amino acid sequence of SEQ ID NO: 87, especially consists of the amino acid sequence SEQ ID NO: 87 and  
said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 88, more particularly comprises the amino acid sequence of SEQ ID NO: 88, especially consists of the amino acid sequence SEQ ID NO: 88; or

said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 89, more particularly comprises the amino acid sequence of SEQ ID NO: 89, especially consists of the amino acid sequence SEQ ID NO: 89 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 90, more particularly comprises the amino acid sequence of SEQ ID NO: 90, especially consists of the amino acid sequence SEQ ID NO: 90; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 91, more particularly comprises the amino acid sequence of SEQ ID NO: 91, especially consists of the amino acid sequence SEQ ID NO: 91 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 92, more particularly comprises the amino acid sequence of SEQ ID NO: 92, especially consists of the amino acid sequence SEQ ID NO: 92; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 93, more particularly comprises the amino acid sequence of SEQ ID NO: 93, especially consists of the amino acid sequence SEQ ID NO: 93 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 94, more particularly comprises the amino acid sequence of SEQ ID NO: 94, especially consists of the amino acid sequence SEQ ID NO: 94; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 95, more particularly comprises the amino acid sequence of SEQ ID NO: 95, especially consists of the amino acid sequence SEQ ID NO: 95 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 96, more particularly comprises the amino acid sequence of SEQ ID NO: 96, especially consists of the amino acid sequence SEQ ID NO: 96; or

said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 97, more particularly comprises the amino acid sequence of SEQ ID NO: 97, especially consists of the amino acid sequence SEQ ID NO: 97 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 98, more particularly comprises the amino acid sequence of SEQ ID NO: 98, especially consists of the amino acid sequence SEQ ID NO: 98; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 99, more particularly comprises the amino acid sequence of SEQ ID NO: 99, especially consists of the amino acid sequence SEQ ID NO: 99 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 100, more particularly comprises the amino acid sequence of SEQ ID NO: 100, especially consists of the amino acid sequence SEQ ID NO: 100; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 101, more particularly comprises the amino acid sequence of SEQ ID NO: 101, especially consists of the amino acid sequence SEQ ID NO: 101 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 102, more particularly comprises the amino acid sequence of SEQ ID NO: 102, especially consists of the amino acid sequence SEQ ID NO: 102; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 103, more particularly comprises the amino acid sequence of SEQ ID NO: 103, especially consists of the amino acid sequence SEQ ID NO: 103 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 104, more particularly comprises the amino acid sequence of SEQ ID NO: 104, especially consists of the amino acid sequence SEQ ID NO: 104; or

said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 105, more particularly comprises the amino acid sequence of SEQ ID NO: 105, especially consists of the amino acid sequence SEQ ID NO: 105 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 106, more particularly comprises the amino acid sequence of SEQ ID NO: 106, especially consists of the amino acid sequence SEQ ID NO: 106; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 107, more particularly comprises the amino acid sequence of SEQ ID NO: 107, especially consists of the amino acid sequence SEQ ID NO: 107 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 108, more particularly comprises the amino acid sequence of SEQ ID NO: 108, especially consists of the amino acid sequence SEQ ID NO: 108; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 109, more particularly comprises the amino acid sequence of SEQ ID NO: 109, especially consists of the amino acid sequence SEQ ID NO: 109 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 110, more particularly comprises the amino acid sequence of SEQ ID NO: 110, especially consists of the amino acid sequence SEQ ID NO: 110; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 111, more particularly comprises the amino acid sequence of SEQ ID NO: 111, especially consists of the amino acid sequence SEQ ID NO: 111 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 112, more particularly comprises the amino acid sequence of SEQ ID NO: 112, especially consists of the amino acid sequence SEQ ID NO: 112; or

said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 113, more particularly comprises the amino acid sequence of SEQ ID NO: 113, especially consists of the amino acid sequence SEQ ID NO: 113 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 114, more particularly comprises the amino acid sequence of SEQ ID NO: 114, especially consists of the amino acid sequence SEQ ID NO: 114; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 115, more particularly comprises the amino acid sequence of SEQ ID NO: 115, especially consists of the amino acid sequence SEQ ID NO: 115 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 116, more particularly comprises the amino acid sequence of SEQ ID NO: 116, especially consists of the amino acid sequence SEQ ID NO: 116; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 117, more particularly comprises the amino acid sequence of SEQ ID NO: 117, especially consists of the amino acid sequence SEQ ID NO: 117 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 118, more particularly comprises the amino acid sequence of SEQ ID NO: 118, especially consists of the amino acid sequence SEQ ID NO: 118; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 119, more particularly comprises the amino acid sequence of SEQ ID NO: 119, especially consists of the amino acid sequence SEQ ID NO: 119 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 120, more particularly comprises the amino acid sequence of SEQ ID NO: 120, especially consists of the amino acid sequence SEQ ID NO: 120; or

said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 121, more particularly comprises the amino acid sequence of SEQ ID NO: 121, especially consists of the amino acid sequence SEQ ID NO: 121 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 122, more particularly comprises the amino acid sequence of SEQ ID NO: 122, especially consists of the amino acid sequence SEQ ID NO: 122; or said first single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 123, more particularly comprises the amino acid sequence of SEQ ID NO: 123, especially consists of the amino acid sequence SEQ ID NO: 123 and said second single-chain protein comprises an amino acid sequence having at least 90, 95, 96, 97, 98 or 99 percent identity to the amino acid sequence of SEQ ID NO: 124, more particularly comprises the amino acid sequence of SEQ ID NO: 124, especially consists of the amino acid sequence SEQ ID NO: 124.

42. The multispecific antibody of any one of the preceding items, wherein at least one of said antibody variable domains comprises CDR regions derived from a parental rabbit antibody.
43. The multispecific antibody of any one of the preceding items, wherein at least one of the MSLN-BDs and the CD3-BD are capable of binding to their respective antigens simultaneously, particularly wherein both MSLN-BDs and the CD3-BD are capable of binding to their respective antigens simultaneously.
44. A MSLN-binding domain as defined in any one of items 11 to 15.
45. A nucleic acid sequence or two nucleic acid sequences encoding the multispecific antibody of any one of items 1 to 43 or the MSLN-binding domain of item 44.

46. A vector or two vectors comprising the nucleic acid sequence or the two nucleic acid sequences of item 45.
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47. A host cell or host cells comprising the vector or the two vectors of item 46.
48. A method for producing the multispecific antibody of any one of items 1 to 43, or the MSLN-binding domain of item 44, comprising (i) providing the nucleic acid sequence or the two nucleic acid sequences of item 45, or the vector or the two vectors of item 46, expressing said nucleic acid sequence or nucleic acid sequences, or said vector or vectors, and collecting said multispecific antibody or said MSLN-binding domain from the expression system, or (ii) providing a host cell or host cells according to item 47, culturing said host cell or said host cells; and collecting said multispecific antibody or said MSLN-binding domain from the cell culture.
49. A pharmaceutical composition comprising the multispecific antibody of any one of items 1 to 43 and a pharmaceutically acceptable carrier.
50. The multispecific antibody of any one of items 1 to 43 for use in the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease.
51. The multispecific antibody of any one of items 1 to 43 for use in the treatment of a disease according to item 50, wherein said disease is a cancer, particularly a cancer selected from mesothelioma, pancreatic cancer, and ovarian cancer.
52. A method for the treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, an inflammatory and an autoimmune disease, comprising the step of administering the multispecific antibody of any one of items 1 to 43.
53. The method of item 52, wherein said disease is a cancer, particularly a cancer selected from mesothelioma, pancreatic cancer, and ovarian cancer.

( $IC_{50} = 0.014$  nM). PRO1925 has an  $IC_{50}$  similar to PRO1783, while the  $IC_{50}$  of PRO1922 is closer to the  $IC_{50}$  of Amatumimab.

[0031] **FIG. 4** shows chimeric variants of hMSLN/mMSLN. Domain highlighted in dark grey is the segment of the human MSLN sequence replaced by the corresponding mouse sequence. Segment VI corresponds to the C-terminal part of MSLN extracellular domain being the closest to the plasma membrane. Segments I and II correspond to Region I of MSLN; Segments III and IV correspond to Region II of MSLN; and Segments V and VI correspond to Region III of MSLN.

[0032] **FIG. 5** shows different MATCH formats. (left) Architecture of the anti-parallel MATCH4 format in which the split, heterodimer-forming variable domains on each chain are organized in the opposite N-terminus-to-C-terminus order as their cognate variable domains on the complementary MATCH chain. (right) Exemplary architecture of the scMATCH3 format in which split variable domains are located on a single peptide chain, which assembles into trispecific molecules. Alternative arrangements such as VL2-VL1-VH1-VH2-scFv are also within the scope of the scMATCH3 format concept. Gly-Ser linkers which were used to connect the domains are indicated by lines. Table 17 describes the domains comprised in the different molecules produced and their positioning within the molecules (Domains 1 to 4). The domain numbering does not correlate with the binding-domain numbering, as defined in the claims.

[0033] **FIG. 6** shows the cytotoxic activity and effect on CD8+ T cell activation of PRO2000 and PRO1872 in the presence of human serum albumin. (A) Specific killing of high MSLN expressing cancer cells (H226 cells). On cancer cells expressing high levels of mesothelin, the target cell killing potency observed for PRO2000 is 75-fold better than for PRO1872. (B) Specific killing of low MSLN expressing mesothelial cells (MeT-5A cells). On cells derived from healthy mesothelial tissue (MeT-5A; ATCC CRL-9444) expressing low mesothelin levels the monovalent mesothelin binding protein PRO1872 shows the best killing potency. (C) CD8+ T cell activation in presence of high MSLN expressing cancer cells (H226 cells), and (D) CD8+ T cell activation in presence of low MSLN expressing mesothelial cells (MeT-5A cells). Similar data were observed for CD8+ T cell activation. PBMCs from donor #1 were used. Target cells and CD8+ T cells were

analyzed by flow cytometry 40 h after the beginning of their incubation with the respective molecules and data were fitted using sigmoidal 4PL fit (GraphPad Prism). [0034] **FIG. 7** shows the cytotoxic activity and effect on CD8+ T cell activation of PRO2000 and PRO1872 in the presence of human serum albumin. (A) Specific killing of low MSLN expressing cancer cells (H292 cells). On cancer cells expressing low levels of mesothelin, the target cell killing potency observed for PRO1872 is 7-fold better than for PRO2000. (B) Specific killing of intermediate MSLN expressing cancer cells (HPAC cells). On cancer cells expressing intermediate mesothelin levels both monovalent and bivalent mesothelin binders PRO1872 and PRO2000 show similar killing potency. (C) CD8+ T cell activation in presence of low MSLN expressing cancer cells (H292 cells), and (D) CD8+ T cell activation in presence of intermediate MSLN expression cancer cells (HPAC cells). Similar data were observed for CD8+ T cells activation, except that PRO2000 was clearly more potent than PRO1872 in presence of HPAC cells (4-fold). PBMCs from donor #1 were used. Target cells and CD8+ T cells were analyzed by flow cytometry 40 h after the beginning of their incubation with the respective molecules and data were fitted using sigmoidal 4PL fit (GraphPad Prism).

[0035] **FIG. 8** shows the cytotoxic activity and effect on CD8+ T cell activation of PRO2000 and PRO1872 in absence or presence of sMSLN. (A to C) Cytotoxic activity of PRO2000 and PRO1872 on H226 target cells. Specific killing of H226 cells in absence of sMSLN (A), in presence of 50 ng/ml sMSLN (B), or in presence of 500 ng/ml sMSLN (C). PRO2000 killing potency is less affected by increasing concentrations of sMSLN as compared to PRO1872 (D to F) Similar data are observed for CD8+ T cell activation in the corresponding conditions. CD8+ T cell activation in presence of H226 cells without sMSLN (D), in presence of 50 ng/ml sMSLN (E), or in presence of 500 ng/ml sMSLN (F). PBMCs from donor #2 were used. Target cells and CD8+ T cells were analyzed by flow cytometry 40 h after the beginning of their incubation with the respective molecules and data were fitted using sigmoidal 4PL fit (GraphPad Prism).

[0036] **FIG. 9** shows the cytotoxic activity of PRO2000, PRO2100 and PRO1872 in absence or presence of 100 ng/ml sMSLN: specific killing of H226 cells in absence of sMSLN (A) or in presence of 100 ng/ml sMSLN (B); specific killing of Met-5A cells (ATCC CRL-9444) in absence of sMSLN (C) or in presence of 100 ng/ml sMSLN (D).

PBMCs from donor #3 were used. Target cells were analyzed by flow cytometry 40 h after the beginning of their incubation with the respective molecules and data were fitted using sigmoidal 4PL fit (GraphPad Prism).

[0037] **FIG. 10** shows the cytotoxic activity of PRO2562, PRO2566, PRO2567 and PRO2660 against high MSLN expressing cancer cells (H226 cells), intermediate MSLN expressing cancer cells (OVCAR-3 cells) and low MSLN expressing cancer cells (Met-5A cells). Palivizumab was used as the negative control ("Control").

[0038] **FIG. 11** shows a summary of the EC<sub>50</sub> values of PRO2562, PRO2566, PRO2567, PRO2660 and of the monovalent reference antibody PRO1872 for the specific killing of H226 cells (A), the specific killing of OVCAR3 cells (B, left side) and the specific killing of Met-5A cells (B, right side).

[0039] **FIG. 12** shows the cytotoxic activity of PRO2562, PRO2566, PRO2567, PRO2660 and of the monovalent reference antibody PRO1872 against H226 cells in the absence or presence of 50 ng/ml sMSLN or 500 ng/ml sMSLN.

[0040] **FIG. 13** shows a summary of the EC<sub>50</sub> values of PRO2562, PRO2566, PRO2567, PRO2660 and of the monovalent reference antibody PRO1872 for the specific killing of H226 cells in the absence or presence of 50 ng/ml sMSLN or 500 ng/ml sMSLN.

[0041] **FIG. 14** shows binding of MATCH molecules to target cell lines expressing different mesothelin cell surface levels. Binding of PRO2000, PRO2100 and PRO1872 to (A) high mesothelin expressing H226 cells, (C) intermediate mesothelin expressing HPAC cells, (B) low mesothelin expressing H292 cancer cells and (D) low mesothelin expressing mesothelial cells, MeT-5A (ATCC CRL-9444) and of PRO2562, PRO2566, PRO2567 and PRO2660 to (E) high mesothelin expressing H226 cells, (F) intermediate mesothelin expressing OVCAR-3 cells and (G) low mesothelin expressing mesothelial cells, MeT-5A (ATCC CRL-9444) was assessed by flow cytometry and data were fitted using sigmoidal 4PL fit (GraphPad Prism).

[0042] **FIG. 15** shows that treatment with molecule PRO2000 (biMSLN.CD3) results in tumor growth inhibition of an H292 xenograft model relative to control conditions. (A) Longitudinal analysis of tumor growth in the presence or absence of biMSLN.CD3 treatment. The lines depict the median. Animals were subcutaneously co-implanted with  $1 \times 10^7$  H292 tumor cells and  $1 \times 10^7$  PBMCs, and treatment was administered intravenously starting on day 5 and repeated every 5 days until the end

of the experiment. (B) Day 40 data displayed as a scatter plot. Each point corresponds to one animal, and the data are displayed with the mean and standard deviation. After a two-way repeated measures ANOVA, the Tukey's multiple comparisons test was performed and the significance of each data set is depicted relative to palivizumab control (Ctrl, lower line), or no treatment (upper line). ns = not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ . The spark line in gray indicates 0 on the y-axis.

[0043] **FIG. 16** shows that treatment with molecule PRO2000 (biMSLN.CD3) results in tumor growth inhibition of a human pancreatic cancer (HPAC) xenograft model relative to control conditions. (A) Longitudinal analysis of tumor growth in the presence of increasing concentrations of PRO2000 (biMSLN.CD3). The lines depict the median. Animals were subcutaneously co-implanted with  $1 \times 10^7$  HPAC tumor cells and  $2.5 \times 10^6$  PBMCs, and treatment was administered intravenously starting on day 5 and repeated every 5 days until the end of the experiment. (B) Corresponding longitudinal analysis of tumor growth in the presence increasing concentrations of PRO2000 (biMSLN.CD3) and, as a comparison, in the presence of increasing concentrations of PRO1872 (MSLN.CD3). The lines depict the median. Palivizumab was used as the negative control. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.0001$ ; \*\*\*\*,  $p < 0.00001$ . (C) Subsection of the graph shown in (B) showing only the lowest doses over time.

## DETAILED DESCRIPTION OF THE INVENTION

[0044] Known MSLN/CD3 bsAbs-based immunotherapies typically suffer from dose-limiting toxicities and limited *in vivo* efficacy. There is thus a need in the medical field for novel MSLN/CD3 bsAbs-based immunotherapies, which have lower or no dose-limiting toxicities and higher efficacy than the currently available approaches.

[0045] The present invention provides a multispecific antibody comprising a combination of two mesothelin binding domains (MSLN-BD) and at least one binding domain for CD3 (CD3-BD), wherein the binding affinity of the MSLN-BD to MSLN is tuned such that it allows efficient localization on high MSLN expressing target cells

while low MSLN expressing healthy mesothelial cells are much less affected. Due to the presence of two MSLN-BD, which are embedded in well-defined and compact multi-domain antibody architecture that is devoid of immunoglobulin Fc region polypeptides, in combination with well balanced MSLN and CD3 binding affinities, these multispecific antibodies show high on-target potency while exhibiting low off-tumor side effects. The compact bivalent design of multispecific antibodies of the present invention, which cannot be achieved for bivalent multispecific antibodies that are based on classical IgG-architecture, as well as their well tuned MSLN and CD3 binding affinities are crucial features for achieving the desired selectivity and efficacy profile.

[0046] The multispecific antibodies of the present invention are capable of binding to target cells via the two MSLN-BD in a highly antigen-density dependent manner by taking advantage of avidity effects. Simultaneously, the multispecific antibodies of the present invention are capable of inducing T-cell activation and tumor cell killing by binding to CD3 via the CD3-BD. Due to their enhanced selectivity for high MSLN expressing cells that leads to efficient tumor localization, the multispecific antibodies of the present invention enable treatments without dose-limiting toxicities caused by non-specific activation of T cells.

[0047] In addition, it has surprisingly been found that the potency of killing high MSLN expressing target cells is not significantly reduced in the presence of high levels of soluble mesothelin that is often observed in patient sera. Furthermore, the multispecific antibodies of the present invention comprising (a) two MSLN binding domains, and (b) at least one CD3-BD and having the above defined design and antigen-binding affinities demonstrated further beneficial properties as shown in the Examples and accompanying figures. Furthermore, the optional addition of a half-life-extending anti-hSA domain not only enables convenient dosing, but should also promote delivery of the molecule to tumor microenvironments.

[0048] The multispecific antibodies of the present invention thus provide distinct therapeutic advantages over conventional compositions and therapies.

[0049] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains.

[0050] The terms “comprising” and “including” are used herein in their open-ended and non-limiting sense unless otherwise noted. With respect to such latter embodiments, the term “comprising” thus includes the narrower term “consisting of”.

[0051] The terms “a” and “an” and “the” and similar references in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. For example, the term “a cell” includes a plurality of cells, including mixtures thereof. Where the plural form is used for compounds, salts, and the like, this is taken to mean also a single compound, salt, or the like.

[0052] In one aspect, the present invention relates to a multispecific antibody comprising:

- a) two antibody-based binding domains, which specifically bind to mesothelin (MSLN-BDs); and
- b) at least one antibody-based binding domain, which specifically binds to CD3 (CD3-BD);

wherein said multispecific antibody does not comprise an immunoglobulin Fc region polypeptide, and wherein each of said MSLN-BD binds to mesothelin (MSLN) with a monovalent dissociation constant ( $K_D$ ) in the range of from 0.1 to 20 nM, when measured by SPR.

[0053] The term “antibody” and the like, as used herein, includes whole antibodies or single chains thereof; and any antigen-binding fragment (*i. e.*, “antigen-binding portion”) or single chains thereof; and molecules comprising antibody CDRs, VH regions or VL regions (including without limitation multispecific antibodies). A naturally occurring “whole antibody” is a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FRs). Each VH and VL is composed of three CDRs and

four FRs arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e. g., effector cells) and the first component (C1q) of the classical complement system.

[0054] The term “immunoglobulin Fc region”, as used herein, refers to the CH2 and CH3 domains of the heavy chain constant regions.

[0055] The terms “binding domain”, “antigen-binding fragment thereof”, “antigen-binding portion” of an antibody, and the like, as used herein, refer to one or more fragments of an intact antibody that retain the ability to specifically bind to a given antigen (e. g., MSLN, CD3, hSA). Antigen-binding functions of an antibody can be performed by fragments of an intact antibody. In some embodiments, a binding domain of a multispecific antibody of the present invention is selected from the group consisting of a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; a F(ab)<sub>2</sub> fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; an Fd fragment consisting of the VH and CH1 domains; an Fv fragment consisting of the VL and VH domains of a single arm of an antibody; a single domain antibody (dAb) fragment (Ward et al., 1989 Nature 341:544-546), which consists of a VH domain; an isolated complementarity determining region (CDR), a single-chain Fv, a dsFv, a scAb, STAB, a single domain antibody (sdAb or dAb), a single domain heavy chain antibody, and a single domain light chain antibody, a VHH, a VNAR, single domain antibodies based on the VNAR structure from shark, and binding domains based on alternative scaffolds including but limited to ankyrin-based domains, fynomers, avimers, anticalins, fibronectins, and binding sites being built into constant regions of antibodies (e. g. f-star technology (F-star’s Modular Antibody Technology™)). Suitably, a binding domain of the present invention is a single-chain Fv fragment (scFv) or a single antibody variable domain. In a preferred embodiment, a binding domain of the present invention is a single-chain Fv fragment (scFv). In particular embodiments, the two variable domains of an antigen-binding fragment, as in an Fv or an scFv fragment, are stabilized by an interdomain disulfide bond, in particular wherein said VH domain comprises a single cysteine residue in position 51 (AHo

numbering) and said VL domain comprises a single cysteine residue in position 141 (AHo numbering).

[0056] The term “Complementarity Determining Regions” (“CDRs”) refers to amino acid sequences with boundaries determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), “Sequences of Proteins of Immunological Interest,” 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (“Kabat” numbering scheme); Al-Lazikani et al., (1997) JMB 273, 927-948 (“Chothia” numbering scheme); ImMunoGenTics (IMGT) numbering (Lefranc, M.-P., The Immunologist, 7, 132-136 (1999); Lefranc, M.-P. et al., Dev. Comp. Immunol., 27, 55-77 (2003)) (“IMGT” numbering scheme); and the numbering scheme described in Honegger & Plückthun, J. Mol. Biol. 309 (2001) 657-670 (“AHo” numbering). For example, for classic formats, under Kabat, the CDR amino acid residues in the heavy chain variable domain (VH) are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the light chain variable domain (VL) are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under Chothia the CDR amino acids in the VH are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the amino acid residues in VL are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). By combining the CDR definitions of both Kabat and Chothia, the CDRs consist of amino acid residues 26-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3) in human VH and amino acid residues 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3) in human VL. Under IMGT the CDR amino acid residues in the VH are numbered approximately 26-35 (HCDR1), 51-57 (HCDR2) and 93-102 (HCDR3), and the CDR amino acid residues in the VL are numbered approximately 27-32 (LCDR1), 50-52 (LCDR2), and 89-97 (LCDR3) (numbering according to “Kabat”). Under IMGT, the CDRs of an antibody can be determined using the program IMGT/DomainGap Align.

[0057] In the context of the present invention, the numbering system suggested by Honegger & Plückthun (“AHo”) is used (Honegger & Plückthun, J. Mol. Biol. 309 (2001) 657-670), unless specifically mentioned otherwise. In particular, the following residues are defined as CDRs according to AHo numbering scheme: LCDR1 (also referred to as CDR-L1): L24-L42; LCDR2 (also referred to as CDR-L2): L58-L72; LCDR3 (also referred to as CDR-L3): L107-L138; HCDR1 (also referred to as CDR-H1): H27-H42; HCDR2 (also referred to as CDR-H2): H57-H76; HCDR3 (also

referred to as CDR-H3): H108-H138. For the sake of clarity, the numbering system according to Honegger & Plückthun takes the length diversity into account that is found in naturally occurring antibodies, both in the different VH and VL subfamilies and, in particular, in the CDRs, and provides for gaps in the sequences. Thus, in a given antibody variable domain usually not all positions 1 to 149 will be occupied by an amino acid residue.

[0058] The term “binding specificity” as used herein refers to the ability of an individual antibody to react with one antigenic determinant and not with a different antigenic determinant. As use herein, the term “specifically binds to” or is “specific for” refers to measurable and reproducible interactions such as binding between a target and an antibody, which is determinative of the presence of the target in the presence of a heterogeneous population of molecules including biological molecules. For example, an antibody that specifically binds to a target (which can be an epitope) is an antibody that binds this target with greater affinity, avidity, more readily, and/or with greater duration than it binds to other targets. In its most general form (and when no defined reference is mentioned), “specific binding” is referring to the ability of the antibody to discriminate between the target of interest and an unrelated molecule, as determined, for example, in accordance with a specificity assay known in the art. Such assays comprise, but are not limited to Western blots, ELISA, RIA, ECL, IRMA, SPR (Surface plasmon resonance) tests and peptide scans. For example, a standard ELISA assay can be carried out. The scoring may be carried out by standard colour development (*e. g.* secondary antibody with horseradish peroxidase and tetramethyl benzidine with hydrogen peroxide). The reaction in certain wells is scored by the optical density, for example, at 450 nm. Typical background (= negative reaction) may be about 0.1 OD; typical positive reaction may be about 1 OD. This means the ratio between a positive and a negative score can be 10-fold or higher. In a further example, an SPR assay can be carried out, wherein at least 10-fold, particularly at least 100-fold difference between a background and signal indicates specific binding. Typically, determination of binding specificity is performed by using not a single reference molecule, but a set of about three to five unrelated molecules, such as milk powder, transferrin or the like.

[0059] Suitably, the antibody of the invention is an isolated antibody. The term “isolated antibody”, as used herein, refers to an antibody that is substantially free of

other antibodies having different antigenic specificities (*e. g.*, an isolated antibody that specifically binds MSLN and CD3 is substantially free of antibodies that specifically bind antigens other than MSLN and CD3 and an isolated antibody that specifically binds MSLN, CD3 and human serum albumin is substantially free of antibodies that specifically bind antigens other than MSLN, CD3 and human serum albumin). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

[0060] Suitably, the antibody of the invention is a monoclonal antibody. The term “monoclonal antibody” or “monoclonal antibody composition” as used herein refers to antibodies that are substantially identical to amino acid sequence or are derived from the same genetic source. A monoclonal antibody composition displays a binding specificity and affinity for a particular epitope, or binding specificities and affinities for specific epitopes.

[0061] Antibodies of the invention include, but are not limited to, chimeric, human and humanized antibodies.

[0062] The term “chimeric antibody” (or antigen-binding fragment thereof) is an antibody molecule (or antigen-binding fragment thereof) in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen-binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e. g.*, an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity. For example, a mouse antibody can be modified by replacing its constant region with the constant region from a human immunoglobulin. Due to the replacement with a human constant region, the chimeric antibody can retain its specificity in recognizing the antigen while having reduced antigenicity in human as compared to the original mouse antibody.

[0063] The term “human antibody” (or antigen-binding fragment thereof), as used herein, is intended to include antibodies (and antigen-binding fragments thereof) having variable regions in which both the framework and CDR regions are derived from sequences of human origin. Furthermore, if the antibody contains a constant region, the constant region also is derived from such human sequences, *e. g.*,

human germline sequences, or mutated versions of human germline sequences. The human antibodies and antigen-binding fragments thereof of the invention may include amino acid residues not encoded by human sequences (e. g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries (Hoogenboom and Winter, J. Mol. Biol, 227:381 (1991); Marks et al, J. Mol. Biol, 222:581 (1991)). Also available for the preparation of human monoclonal antibodies are methods described in Cole et al, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boemer et al, J. Immunol, 147(1):86-95 (1991). See also van Dijk and van de Winkel, Curr. Opin. Pharmacol, 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e. g., immunized xenomice (see, e. g., U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li et al, Proc. Natl. Acad. Sci. USA, 103:3557- 3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology.

[0064] A “humanized” antibody (or antigen-binding fragment thereof), as used herein, is an antibody (or antigen-binding fragment thereof) that retains the reactivity of a non-human antibody while being less immunogenic in humans. This can be achieved, for instance, by retaining the non-human CDR regions and replacing the remaining parts of the antibody with their human counterparts (*i. e.*, the constant region as well as the framework portions of the variable region). Additional framework region modifications may be made within the human framework sequences as well as within the CDR sequences derived from the germline of another mammalian species. The humanized antibodies of the invention may include amino acid residues not encoded by human sequences (e. g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*, or a conservative substitution to promote stability or manufacturing). See, e. g., Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855, 1984; Morrison and Oi, Adv. Immunol., 44:65-92, 1988; Verhoeyen et al., Science, 239: 1534-1536, 1988; Padlan, Molec. Immun., 28:489-498, 1991; and Padlan, Molec. Immun., 31: 169-217,

1994. Other examples of human engineering technology include, but are not limited to the Xoma technology disclosed in U.S. Pat. No. 5,766,886.

[0065] The term “recombinant humanized antibody” as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell transformed to express the humanized antibody, *e. g.*, from a transfectoma, and antibodies prepared, expressed, created or isolated by any other means that involve splicing of all or a portion of a human immunoglobulin gene, sequences to other DNA sequences.

[0066] Suitably, the antibody of the invention or antigen-binding fragment thereof is humanized. Suitably, the antibody of the invention or antigen-binding fragment thereof is humanized and comprises rabbit-derived CDRs.

[0067] The term “multispecific antibody” as used herein, refers to an antibody that binds to two or more different epitopes on at least two or more different targets (*e. g.*, MSLN and CD3). The term “multispecific antibody” includes bispecific, trispecific, tetraspecific, pentaspecific and hexaspecific. The term “bispecific antibody” as used herein, refers to an antibody that binds to two different epitopes on two different targets (*e. g.*, MSLN and CD3). The term “trispecific antibody” as used herein, refers to an antibody that binds to three different epitopes on three different targets (*e. g.*, MSLN, CD3 and hSA).

[0068] The term “epitope” means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics. “Conformational” and “linear” epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

[0069] The term “conformational epitope” as used herein refers to amino acid residues of an antigen that come together on the surface when the polypeptide chain folds to form the native protein.

[0070] The term “linear epitope” refers to an epitope with all of the points of interaction between the protein and the interacting molecule (such as an antibody) occurring linearly along the primary amino acid sequence of the protein (continuous).

[0071] The term “recognize” as used herein refers to an antibody antigen-binding fragment thereof that finds and interacts (*e. g.*, binds) with its conformational epitope.

[0072] As used herein, the term “affinity” refers to the strength of interaction between antibody and antigen at single antigenic sites. Within each antigenic site, the variable region of the antibody “arm” interacts through weak non-covalent forces with antigen at numerous sites; the more interactions, the stronger the affinity.

[0073] “Binding affinity” generally refers to the strength of the total sum of non-covalent interactions between a single binding site of a molecule (e. g., of an antibody) and its binding partner (e. g., an antigen). Unless indicated otherwise, as used herein, “binding affinity”, “bind to”, “binds to” or “binding to” refers to intrinsic binding affinity that reflects a 1:1 interaction between members of a binding pair (e. g., an antibody fragment and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant ( $K_D$ ). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative and exemplary embodiments for measuring binding affinity, i. e. binding strength are described in the following.

[0074] The term “K<sub>assoc</sub>”, “K<sub>a</sub>” or “K<sub>on</sub>”, as used herein, is intended to refer to the association rate of a particular antibody-antigen interaction, whereas the term “K<sub>dis</sub>”, “K<sub>d</sub>” or “K<sub>off</sub>”, as used herein, is intended to refer to the dissociation rate of a particular antibody- antigen interaction. In one embodiment, the term “K<sub>D</sub>”, as used herein, is intended to refer to the dissociation constant, which is obtained from the ratio of K<sub>d</sub> to K<sub>a</sub> (i. e. K<sub>d</sub>/K<sub>a</sub>) and is expressed as a molar concentration (M). The “K<sub>D</sub>” or “K<sub>D</sub> value” or “K<sub>D</sub>” or “K<sub>D</sub> value” according to this invention is in one embodiment measured by using surface-plasmon resonance assays. Affinity to recombinant human mesothelin (human MSLN) and recombinant *Cynomolgus* MSLN (*Cynomolgus* MSLN) was determined by surface plasmon resonance (SPR) measurements as described in section [0168]. Affinity to recombinant human CD3 was measured by SPR as described in section [0196].

[0075] Suitably, the multispecific antibody of the present invention is bivalent for MSLN specificity.

[0076] Suitably, the multispecific antibody of the present invention is monovalent, bivalent or multivalent for CD3 specificity. In one embodiment, the multispecific antibody of the present invention is bivalent for CD3 specificity. In a preferred embodiment, the multispecific antibody of the present invention is monovalent for CD3 specificity.

[0077] The term “multivalent antibody” refers to a single binding molecule with more than one valency, where “valency” is described as the number of antigen-binding moieties that binds to epitopes on identical target molecules. As such, the single binding molecule can bind to more than one binding site on a target molecule.

Examples of multivalent antibodies include, but are not limited to bivalent antibodies, trivalent antibodies, tetravalent antibodies, pentavalent antibodies, and the like.

[0078] The term “monovalent antibody”, as used herein, refers to an antibody that binds to a single epitope on a target molecule, such as CD3. Also, the term “binding domain” or “monovalent binding domain”, as used herein, refers to a binding domain that binds to a single epitope on a target molecule such as CD3.

[0079] The term “bivalent antibody” as used herein, refers to an antibody that binds to two epitopes on two identical target molecules, such as MSLN target molecules.

[0080] The two MSLN-BDs of the multispecific antibodies of the present invention bind to any region of the extracellular part of MSLN, e.g. to Region I, Region II and/or Region III of MSLN. Preferably, the two MSLN-BDs of the multispecific antibodies of the present invention bind to Region I and/or Region II of MSLN, in particular to Region I of MSLN. Region I is the part of MSLN that is most distal from the cell surface, where MSLN is attached to.

[0081] The two MSLN-BDs of the multispecific antibodies of the present invention either bind the same or different epitopes on the MSLN target molecules. Preferably, the two MSLN-BDs of the multispecific antibodies of the present invention bind the same epitopes on the MSLN target molecules. The term “same epitope”, as used herein, refers to individual protein determinants on the same protein capable of specific binding to an antibody, where these individual protein determinants are identical, i.e. consist of identical chemically active surface groupings of molecules such as amino acids or sugar side chains having identical three-dimensional structural characteristics, as well as identical charge characteristics. The term “different epitope”, as used herein in connection with a specific protein target, refers

to individual protein determinants on the same protein capable of specific binding to an antibody, where these individual protein determinants are not identical, i.e. consist of non-identical chemically active surface groupings of molecules such as amino acids or sugar side chains having different three-dimensional structural characteristics, as well as different charge characteristics. These different epitopes can be overlapping or non-overlapping.

[0082] The inventors of the present invention have now surprisingly found that for example the tri-specific molecules (biMSLN<sub>high</sub> KDXCD3xhSA) PRO2000, PRO2562, PRO2565, PRO2566 and PRO2567 are capable of killing target cells, which have an approximately 7-fold higher MSLN expression level than healthy MeT-5A cells (ATCC CRL-9444), as determined by flow cytometry, with high efficiency and with an EC<sub>50</sub> that is at least 25-fold lower than the EC<sub>50</sub> for killing said MeT-5A cells, as determined in a T-cell driven cytotoxicity assay against said target cells and said MeT-5A cells (see for example Table 31). Thus, although PRO2000, PRO2562, PRO2566 and PRO2567 exhibit a very high killing potency for high MSLN expressing target cells, their killing potency towards healthy cells is much lower, indicating a potentially large therapeutic window for treatments using PRO2000, PRO2562, PRO2566 and PRO2567. In contrast thereto, the potencies of a tri-specific reference molecule PRO1872 (MSLN<sub>low</sub> KDXCD3xhSA), which comprises one MSLN-BD having a more than 5-fold better binding affinity (K<sub>D</sub>) than the MSLN-BDs of PRO2000, PRO2562, PRO2566 and PRO2567, for killing said high MSLN expressing target cells and said healthy Met-5A cells do not differ significantly. This finding indicates that the therapeutic window for the use of the multispecific antibodies of the present invention in the treatment of cancer patients is significantly increased. In addition, the inventors of the present invention have surprisingly found that the EC<sub>50</sub> values of the tri-specific molecules PRO2000, PRO2562, PRO2566 and PRO2567 (biMSLN<sub>high</sub> KDXCD3xhSA) for killing target cells, which have an approximately 7-fold higher MSLN expression level than said healthy MeT-5A cells, as determined by flow cytometry, do not increase by more than 6-fold in the presence of 50 ng/ml soluble mesothelin (sMSLN), and by not more than 40-fold in the presence of 500 ng/ml soluble mesothelin (sMSLN), as determined in a T-cell driven cytotoxicity assay against said target cells. On the other hand, the EC<sub>50</sub> value of the tri-specific reference molecule PRO1872 (MSLN<sub>low</sub> KDXCD3xhSA) for killing

said target cells increased by almost 8-fold in the presence of 50 ng/ml sMSLN and by more than 75-fold in the presence of 500 ng/ml sMSLN. Thus, the high killing potency of the tri-specific molecules PRO2000, PRO2562, PRO2566 and PRO2567 for high MSLN expressing target cells is only marginally affected by high concentrations of sMSLN. On the other hand, the killing potency of the tri-specific molecules PRO2000, PRO2562, PRO2566 and PRO2567 for healthy cells is further decreased in the presence of high concentration of sMSLN (data not shown), indicating that the therapeutic window for their use in the clinic is even increased by the presence of sMSLN. This is an important finding, since high plasma-levels of soluble mesothelin-related protein (SMRP) are often observed in patients. The inventors obtained similar advantageous results for the CD8+ T cell activation potency of PRO2000. The above findings are even more surprising as it could not *a priori* be expected that all four binding domains remain functional without sterically or otherwise inhibiting each other in a complex multi-target, multi-cell situation.

[0083] Suitable MSLN-BDs for use in the multispecific antibody of the invention are binding domains provided in the present disclosure. The mesothelin-BDs of the invention include, but are not limited to, the humanized MSLN-binding domains whose sequences are listed in Table 1.

[0084] Suitable CD3-BDs for use in the multispecific antibody of the invention are binding domains provided in the present disclosure. The CD3-BDs of the invention include, but are not limited to, the humanized CD3-binding domains whose sequences are listed in Table 3.

[0085] Suitably, the multispecific antibody of the invention has two different specificities (MSLN and CD3). Suitably, the multispecific antibody of the invention is a bispecific antibody, which is bivalent for MSLN. The multispecific antibody of the present invention may comprise a further specificity (trispecific antibody) or specificities (tetraspecific or pentaspecific or hexaspecific antibody). In one embodiment, the multispecific antibody is bispecific (MSLN and CD3). In another embodiment, the multispecific antibody is trispecific (MSLN, CD3 and hSA).

[0086] Suitably, the antibody of the invention does not comprise an immunoglobulin Fc region polypeptide.

[0087] In order to increase the number of specificities/functionalities at the same or lower molecular weight, it is advantageous to use antibodies comprising antibody

fragments, such as Fv, Fab, Fab' and F(ab')<sub>2</sub> fragments and other antibody fragments. These smaller molecules retain the antigen-binding activity of the whole antibody and can also exhibit improved tissue penetration and pharmacokinetic properties in comparison to the whole immunoglobulin molecules. Whilst such fragments appear to exhibit a number of advantages over whole immunoglobulins, they also suffer from an increased rate of clearance from serum since they lack the Fc domain that imparts a long half-life *in vivo* (Medasan et al., 1997, J. Immunol. 158:2211-2217). Molecules with lower molecular weights penetrate more efficiently into target tissues (e. g. solid cancers) and thus hold the promise for improved efficacy at the same or lower dose.

[0088] The inventors have surprisingly found that an addition of human serum albumin binding domain (hSA-BD) to the multispecific antibody of the invention does not interfere with the ability of the other binding domains to bind to their respective targets. This finding is insofar surprising as it cannot *a priori* be expected that all four binding domains remain functional without sterically or otherwise inhibiting each other in a complex multi-target, multi-cell *in vivo* situation.

[0089] Suitably, the multispecific antibody of the present invention may comprise a further binding domain having specificity to human serum albumin. In one embodiment, the multispecific antibody comprises: (i) two MSLN-BD; (ii) at least one CD3-BD; and (iii) at least one hSA-BD.

[0090] The term "hSA" refers in particular to human serum albumin with UniProt ID number P02768. Human Serum Albumin (hSA) is 66.4 kDa abundant protein in human serum (50 % of total protein) composed of 585 amino acids (Sugio, Protein Eng, Vol. 12, 1999, 439-446). Multifunctional hSA protein is associated with its structure that allowed binding and transporting a number of metabolites such as fatty acids, metal ions, bilirubin and some drugs (Fanali, Molecular Aspects of Medicine, Vol. 33, 2012, 209-290). HSA concentration in serum is around 3.5-5 g/dL. Albumin binding antibodies and fragments thereof may be used, for example, for extending the *in vivo* serum half-life of drugs or proteins conjugated thereto.

[0091] In some embodiments, the hSA-BD is derived from a monoclonal antibody or antibody fragment.

[0092] Suitable hSA-BDs for use in the multispecific antibody of the invention are binding domains provided in the present disclosure. The hSA-BDs of the invention

include, but are not limited to, the humanized hSA-binding domains whose sequences are listed in Table 4.

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[0093] In particular, the hSA-BDs of the invention specifically bind to human serum albumin.

[0094] Other suitable hSA-BD for use in the multispecific antibody of the invention comprises or is derived from an antibody selected from the group consisting of: (i) polypeptides that bind serum albumin (see, for example, Smith et al., 2001, Bioconjugate Chem. 12:750-756; EP0486525; US6267964; WO 2004/001064; WO 2002/076489; and WO 2001/45746); (ii) anti-serum albumin binding single variable domains described in Holt et al., Protein Engineering, Design & Selection, vol 21, 5, pp283-288, WO 2004/003019, WO 2008/096158, WO 2005/118642, WO 2006/0591056 and WO 2011/006915; (iii) anti-serum albumin antibodies described in WO 2009/040562, WO 2010/035012 and WO 2011/086091.

[0095] Other variable domains of the invention include amino acid sequences that have been mutated, yet have at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 percent identity in the CDR regions with the CDR regions depicted in the sequences described in Tables 1, 3 and 4. Other variable domains of the invention include mutant amino acid sequences wherein no more than 1, 2, 3, 4 or 5 amino acids have been mutated in the CDR regions when compared with the CDR regions depicted in the sequence described in Tables 1, 3 and 4.

[0096] Suitably, the VH domains of the binding domains of the invention belong to a VH3 or VH4 family. In one embodiment, a binding domain of the invention comprises a VH domain belonging to the VH3 family. In the context of the present invention, the term "belonging to VHx family (or VLx family)" means that the framework sequences FR1 to FR3 show the highest degree of homology to said VHx family (or VLx, respectively). Examples of VH and VL families are given in Knappik et al., J. Mol. Biol. 296 (2000) 57-86, or in WO 2019/057787. A specific example of a VH domain belonging to VH3 family is represented by SEQ ID NO: 129, and a specific example of a VH domain belonging to VH4 family is represented by SEQ ID NO: 130. In particular, framework regions FR1 to FR3 taken from SEQ ID NO: 129 belong to VH3 family (Table 7, regions marked in non-bold). Suitably, a VH belonging to VH3 family, as used herein, is a VH comprising FR1 to FR3 having at least 85 %, particularly at least 90 %, more particularly at least 95 % sequence identity to FR1 to

FR3 of SEQ ID NO: 129. Alternative examples of VH3 sequences, and examples of VH4 sequences, may be found in Knappik et al., J. Mol. Biol. 296 (2000) 57-86 or in WO 2019/057787. Suitably, the hSA-BD of the invention comprises: Vk frameworks FR1, FR2 and FR3, particularly Vk1 or Vk3 frameworks, particularly Vk1 frameworks FR1 to 3, and a framework FR4, which is selected from a Vk FR4, and a Vλ FR4, particularly a Vλ FR4. Suitable Vk1 frameworks FR1 to 3 as well as an exemplary Vλ FR4 are set forth in SEQ ID NO: 131 (Table 7, FR regions are marked in non-bold). Alternative examples of Vk1 sequences, and examples of Vk2, Vk3 or Vk4 sequences, may be found in Knappik et al., J. Mol. Biol. 296 (2000) 57-86. Suitable Vk1 frameworks FR1 to 3 comprise the amino acid sequences having at least 70, 80, 90, 95 percent identity to amino acid sequences corresponding to FR1 to 3 and taken from SEQ ID NO: 131 (Table 7, FR regions are marked in non-bold). Suitable Vλ FR4 are as set forth in SEQ ID NO: 132 to SEQ ID NO: 138 and in SEQ ID NO: 139 comprising a single cysteine residue, particular in a case where a second single cysteine is present in the corresponding VH chain, particularly in position 51 (AHO numbering) of VH, for the formation of an inter-domain disulfide bond. In one embodiment, the VL domains of the present invention comprises Vλ FR4 having at least 70, 80, or 90 percent identity to an amino acid sequence selected from any of SEQ ID NO: 132 to SEQ ID NO: 139, particularly to SEQ ID NO: 132 or 139.

[0097] The binding domains of the invention comprises a VH domain listed in Tables 1, 3 and 4. Suitably, a binding domain of the invention comprises a VH amino acid sequence listed in one of Tables 1, 3 and 4, wherein no more than 20 amino acids in a framework sequence (for example, a sequence which is not a CDR) have been mutated (wherein a mutation is, as various non-limiting examples, an addition, substitution or deletion). Suitably, a binding domain of the present invention comprises a VH amino acid sequence listed in one of Tables 1, 3 and 4, wherein no more than 15 amino acids, particularly not more than 10 amino acids, particularly not more than 5 amino acids in a framework sequence (for example, a sequence which is not a CDR) have been mutated (wherein a mutation is, as various non-limiting examples, an addition, substitution or deletion). Other binding domains of the invention include amino acids that have been mutated, yet have at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 percent identity in the VH regions with the VH regions depicted in the corresponding sequences described in one of Tables 1, 3

and 4, including VH domains comprising at least positions 5 to 140 (AHo numbering), particularly at least positions 3 to 145 of one of the sequences shown in Tables 1, 3 and 4.

[0098] In particular, a binding domain of the invention comprises a VL domain listed in one of Tables 1, 3 and 4. Suitably, a binding domain of the invention comprises a VL amino acid sequence listed in one of Tables 1, 3 and 4, wherein no more than 20 amino acids in a framework sequence (for example, a sequence which is not a CDR) have been mutated (wherein a mutation is, as various non-limiting examples, an addition, substitution or deletion). Suitably, a binding domain of the invention comprises a VL amino acid sequence listed in one of Tables 1, 3 and 4, wherein no more than 15 amino acids, particularly not more than 10 amino acids, particularly not more than 5 amino acids in a framework sequence (for example, a sequence which is not a CDR) have been mutated (wherein a mutation is, as various non-limiting examples, an addition, substitution or deletion). Other binding domains of the invention include amino acids that have been mutated, yet have at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99 percent identity in the VL regions with a VL region depicted in the sequences described in Tables 1, 3 and 4, including VL domains comprising at least positions 5 to 140 (AHo numbering), particularly at least positions 3 to 145 of one of the sequences shown in Tables 1, 3 and 4.

[0099] In the context of the present invention, the term “binding domain of the present invention” relates both to a binding domain as such, *i. e.* independent of a multispecific context, and, in particular, to a binding domain comprised in a multispecific construct, *e. g.* one of the binding domains comprised in a bispecific, trispecific or tetraspecific construct.

[0100] Suitably, a binding domain of the invention is selected from the group consisting of: a Fab, an Fv, an scFv, dsFv, a scAb, and STAB.

[0101] Suitably, a binding domain of the invention is an scFv antibody fragment.

[0102] The multispecific antibody of the invention may be in any suitable format.

[0103] Suitably, the binding domains of the multispecific antibody are operably linked. The binding domains of the multispecific antibody of the invention are capable of binding to their respective antigens or receptors simultaneously. The term “simultaneously”, as used in this connection, refers to the simultaneous binding of at least one of the MSLN-BDs and the CD3-BD. In specific cases, *e.g.* in cases of

target cells having a high density of MSLN on the cell surface, it might also be possible that three binding domains, *i. e.* both MSLN-BD and the CD3-BD, bind simultaneously.

[0104] The multispecific antibody of the invention comprises two MSLN-BD, and at least one CD3-BD, wherein said MSLN-BDs, and said CD3-BD are operably linked to each other.

[0105] The term “operably linked”, as used herein, indicates that two molecules (*e. g.*, polypeptides, domains, binding domains) are attached so as to each retain functional activity. Two molecules can be “operably linked” whether they are attached directly or indirectly (*e. g.*, via a linker, via a moiety, via a linker to a moiety). The term “linker” refers to a peptide or other moiety that is optionally located between binding domains or antibody fragments of the invention. A number of strategies may be used to covalently link molecules together. These include but are not limited to polypeptide linkages between N- and C-termini of proteins or protein domains, linkage via disulfide bonds, and linkage via chemical cross-linking reagents. In one aspect of this embodiment, the linker is a peptide bond, generated by recombinant techniques or peptide synthesis. Choosing a suitable linker for a specific case where two polypeptide chains are to be connected depends on various parameters, including but not limited to the nature of the two polypeptide chains (*e. g.*, whether they naturally oligomerize), the distance between the N- and the C-termini to be connected if known, and/or the stability of the linker towards proteolysis and oxidation. Furthermore, the linker may contain amino acid residues that provide flexibility.

[0106] In the context of the present invention, the term “polypeptide linker” refers to a linker consisting of a chain of amino acid residues linked by peptide bonds that is connecting two domains, each being attached to one end of the linker. The polypeptide linker should have a length that is adequate to link two molecules in such a way that they assume the correct conformation relative to one another so that they retain the desired activity. In particular embodiments, the polypeptide linker has a continuous chain of between 2 and 30 amino acid residues (*e. g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acid residues). In addition, the amino acid residues selected for inclusion in the polypeptide linker should exhibit properties that do not interfere significantly with

the activity of the polypeptide. Thus, the linker peptide on the whole should not exhibit a charge that would be inconsistent with the activity of the polypeptide, or interfere with internal folding, or form bonds or other interactions with amino acid residues in one or more of the monomers that would seriously impede the binding of receptor monomer domains. In particular embodiments, the polypeptide linker is non-structured polypeptide. Useful linkers include glycine-serine, or GS linkers. By "Gly-Ser" or "GS" linkers is meant a polymer of glycines and serines in series (including, for example, (Gly-Ser)<sub>n</sub>, (GSGGS)<sub>n</sub>, (GGGGS)<sub>n</sub> and (GGGS)<sub>n</sub>, where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers such as the tether for the shaker potassium channel, and a large variety of other flexible linkers, as will be appreciated by those in the art. Glycine-serine polymers are preferred since both of these amino acids are relatively unstructured, and therefore may be able to serve as a neutral tether between components. Secondly, serine is hydrophilic and therefore able to solubilize what could be a globular glycine chain. Third, similar chains have been shown to be effective in joining subunits of recombinant proteins such as single-chain antibodies.

[0107] Suitably, the multispecific antibody is in a format selected from any suitable multispecific, *e. g.* at least bispecific, format known in the art, which do not comprise immunoglobulin Fc region(s), including, by way of non-limiting example, formats based on a tandem scDb (Tandab), a linear dimeric scDb (LD-scDb), a circular dimeric scDb (CD-scDb), a tandem tri-scFv, a tribody (Fab-(scFv)<sub>2</sub>), Fab-Fv<sub>2</sub>, triabody, scDb-scFv, tetrabody, di-diabody, CODV, tandem-di-scFv, tandem tri-scFv, Fab-(scFv)<sub>2</sub>, Fab-Fv<sub>2</sub>, or CODV fused to the N- and/or the C-terminus of a heterodimerization domain other than heterodimeric Fc domains, and MATCH (described in WO 2016/0202457; Egan T. et al., MABS 9 (2017) 68-84) and DuoBodies (bispecific IgGs prepared by the Duobody technology) (MAbs. 2017 Feb/Mar;9(2):182-212. doi: 10.1080/19420862.2016.1268307). Particularly suitable, the multispecific antibody is a single-chain diabody (scDb)-scFv or a MATCH.

[0108] In one embodiment, the multispecific antibody of the invention does not comprise CH1 and/or CL regions.

[0109] In another embodiment, the multispecific antibody of the invention is in a format selected from the list consisting of scDb-scFv, triabody, and tribody.

Particularly suitable for use herein is a scDb-scFv, in particular wherein one of said

MSLN-BDs and said CD3-BD are in the form of a scDb and the second MSLN-BD is an scFv operably linked to said scDb.

[0110] The term “diabodies” refers to antibody fragments with two antigen-binding sites, which fragments comprise a VH connected to VL in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain to create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404 097, WO 93/01161, Hudson et al., Nat. Med. 9:129-134 (2003), and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., Nat. Med. 9:129-134 (2003).

[0111] The bispecific scDb, in particular the bispecific monomeric scDb, particularly comprises two variable heavy chain domains (VH) or fragments thereof and two variable light chain domains (VL) or fragments thereof connected by linkers L1, L2 and L3 in the order VHA-L1-VLB-L2-VHB-L3-VLA, VHA-L1-VHB-L2-VLB-L3-VLA, VLA-L1-VLB-L2-VHB-L3-VHA, VLA-L1-VHB-L2-VLB-L3-VHA, VHB-L1-VLA-L2-VHA-L3-VLB, VHB-L1-VHA-L2-VLA-L3-VLB, VLB-L1-VLA-L2-VHA-L3-VHB or VLB-L1-VHA-L2-VLA-L3-VHB, wherein the VLA and VHA domains jointly form the antigen-binding site for the first antigen, and VLB and VHB jointly form the antigen-binding site for the second antigen.

[0112] The linker L1 particularly is a peptide of 2-10 amino acids, more particularly 3-7 amino acids, and most particularly 5 amino acids, and linker L3 particularly is a peptide of 1-10 amino acids, more particularly 2-7 amino acids, and most particularly 5 amino acids. In particular embodiments, the linker L1 and/or L3 comprises one or two units of four (4) glycine amino acid residues and one (1) serine amino acid residue (GGGGS)<sub>n</sub>, wherein n=1 or 2, particularly n=1.

[0113] The middle linker L2 particularly is a peptide of 10-40 amino acids, more particularly 15-30 amino acids, and most particularly 20-25 amino acids. In particular embodiments, said linker L2 comprises one or more units of four (4) glycine amino acid residues and one (1) serine amino acid residue (GGGGS)<sub>n</sub>, wherein n=1, 2, 3, 4, 5, 6, 7 or 8, particularly n=4.

[0114] In one embodiment, the multispecific antibody of the invention is a scDb-scFv. The term “scDb-scFv” refers to an antibody format, wherein a single-chain Fv

(scFv) fragment is fused by a flexible Gly-Ser linker to a single-chain diabody (scDb).

In one embodiment, said flexible Gly-Ser linker is a peptide of 2-40 amino acids, e. g., 2-35, 2-30, 2-25, 2-20, 2-15, 2-10 amino acids, particularly 10 amino acids. In particular embodiments, said linker comprises one or more units of four (4) glycine amino acid residues and one (1) serine amino acid residue (GGGGS)<sub>n</sub>, wherein n=1, 2, 3, 4, 5, 6, 7 or 8, particularly n=2.

[0115] In one embodiment of the present invention, the multispecific antibody of the invention is in a MATCH format described in WO 2016/0202457; Egan T., et al., MABS 9 (2017) 68-84. In particular, in this embodiment, the multispecific antibody of the invention is in a MATCH3 or a MATCH4 format.

[0116] The multispecific antibody of the invention can be produced using any convenient antibody manufacturing method known in the art (see, e. g., Fischer, N. & Leger, O., Pathobiology 74 (2007) 3-14 with regard to the production of bispecific constructs; Hornig, N. & Färber-Schwarz, A., Methods Mol. Biol. 907 (2012) 713-727, and WO 99/57150 with regard to bispecific diabodies and tandem scFvs). Specific examples of suitable methods for the preparation of the bispecific construct of the invention further include, inter alia, the Genmab (see Labrijn et al., Proc. Natl. Acad. Sci. USA 110 (2013) 5145-5150) and Merus (see de Kruif et al., Biotechnol. Bioeng. 106 (2010) 741-750) technologies. Methods for production of bispecific antibodies comprising a functional antibody Fc part are also known in the art (see, e. g., Zhu et al., Cancer Lett. 86 (1994) 127-134); and Suresh et al., Methods Enzymol. 121 (1986) 210-228).

[0117] These methods typically involve the generation of monoclonal antibodies, for example by means of fusing myeloma cells with the spleen cells from a mouse that has been immunized with the desired antigen using the hybridoma technology (see, e. g., Yokoyama et al., Curr. Protoc. Immunol. Chapter 2, Unit 2.5, 2006) or by means of recombinant antibody engineering (repertoire cloning or phage display/yeast display) (see, e. g., Chames & Baty, FEMS Microbiol. Letters 189 (2000) 1-8), and the combination of the antigen-binding domains or fragments or parts thereof of two or more different monoclonal antibodies to give a bispecific or multispecific construct using known molecular cloning techniques.

[0118] The multispecific molecules of the invention can be prepared by conjugating the constituent binding specificities, using methods known in the art. For example,

each binding specificity of the bispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-5-acetyl-thioacetate (SATA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) (see *e. g.*, Karpovsky et al., 1984 J. Exp. Med. 160: 1686; Liu, M A et al., 1985 Proc. Natl. Acad. Sci. USA 82:8648). Other methods include those described in Paulus, 1985 Behring Ins. Mitt. No. 78, 118-132; Brennan et al., 1985 Science 229:81-83), and Glennie et al., 1987 J. Immunol. 139: 2367-2375). Conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, 111).

[0119] When the binding specificities are antibodies, they can be conjugated by sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particular embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, for example one, prior to conjugation.

[0120] Alternatively, two or more binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific molecule is a mAb X mAb, mAb X Fab, Fab X F (ab')<sub>2</sub> or ligand X Fab fusion protein. A multispecific antibody of the invention can be a single-chain molecule comprising one single-chain antibody and a binding determinant, or a single-chain multispecific antibody comprising two binding determinants. Multispecific antibody may comprise at least two single-chain molecules. Methods for preparing multispecific antibodies and molecules are described for example in U.S. Pat. No. 5,260,203; U.S. Pat. No. 5,455,030; U.S. Pat. No. 4,881,175; U.S. Pat. No. 5,132,405; U.S. Pat. No. 5,091,513; U.S. Pat. No. 5,476,786; U.S. Pat. No. 5,013,653; U.S. Pat. No. 5,258,498; and U.S. Pat. No. 5,482,858.

[0121] Binding of the multispecific antibodies to their specific targets can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (REA), FACS analysis, bioassay (*e. g.*, growth inhibition), or Western Blot assay. Each of these assays generally detects the presence of protein-

antibody complexes of particular interest by employing a labeled reagent (*e. g.*, an antibody) specific for the complex of interest.

[0122] In a further aspect, the invention provides a nucleic acid encoding the multispecific antibody of the invention or fragments thereof or binding domains thereof. Such nucleic acid sequences can be optimized for expression in mammalian cells.

[0123] The term “nucleic acid” is used herein interchangeably with the term “polynucleotide(s)” and refers to one or more deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphorates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs). Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e. g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, as detailed below, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucleic Acid Res.* 19:5081, 1991; Ohtsuka et al., *J. Biol. Chem.* 260:2605-2608, 1985; and Rossolini et al., *Mol. Cell. Probes* 8:91-98, 1994).

[0124] The invention provides substantially purified nucleic acid molecules which encode polypeptides comprising segments or domains of the multispecific antibody described above. When expressed from appropriate expression vectors, polypeptides encoded by these nucleic acid molecules are capable of exhibiting antigen-binding capacity or capacities of the multispecific antibody of the present invention.

[0125] Also provided in the invention are polynucleotides which encode at least one CDR region and usually all three CDR regions of the binding domains of the multispecific antibody of the present invention set forth in Tables 1, 3 and 4. Because

of the degeneracy of the code, a variety of nucleic acid sequences will encode each of the immunoglobulin amino acid sequences.

[0126] The polynucleotide sequences can be produced by de novo solid-phase DNA synthesis or by PCR mutagenesis of an existing sequence (*e. g.*, sequences as described in the Examples below) encoding the multispecific antibody of the invention or fragments thereof or binding domains thereof. Direct chemical synthesis of nucleic acids can be accomplished by methods known in the art, such as the phosphotriester method of Narang et al., 1979, *Meth. Enzymol.* 68:90; the phosphodiester method of Brown et al., *Meth. Enzymol.* 68: 109, 1979; the diethylphosphoramidite method of Beaucage et al., *Tetra. Lett.*, 22: 1859, 1981; and the solid support method of U.S. Pat. No. 4,458,066. Introducing mutations to a polynucleotide sequence by PCR can be performed as described in, *e. g.*, *PCR Technology: Principles and Applications for DNA Amplification*, H. A. Erlich (Ed.), Freeman Press, NY, N.Y., 1992; *PCR Protocols: A Guide to Methods and Applications*, Innis et al. (Ed.), Academic Press, San Diego, Calif, 1990; Mattila et al., *Nucleic Acids Res.* 19:967, 1991; and Eckert et al., *PCR Methods and Applications* 1:17, 1991.

[0127] Also provided in the invention are expression vectors and host cells for producing the multispecific antibody of the invention or fragments thereof or binding domains thereof.

[0128] The term “vector” is intended to refer to a polynucleotide molecule capable of transporting another polynucleotide to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e. g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e. g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome.

[0129] Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as “recombinant expression vectors” (or simply, “expression vectors”). In general,

expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e. g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions. In this particular context, the term "operably linked" refers to a functional relationship between two or more polynucleotide (*e. g.*, DNA) segments. Typically, it refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, a promoter or enhancer sequence is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, *i. e.*, they are *cis*-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

[0130] Various expression vectors can be employed to express the polynucleotides encoding the multispecific antibody chains or binding fragments. Both viral-based and nonviral expression vectors can be used to produce the antibodies in a mammalian host cell. Nonviral vectors and systems include plasmids, episomal vectors, typically with an expression cassette for expressing a protein or RNA, and human artificial chromosomes (see, *e. g.*, Harrington et al., *Nat Genet.* 15:345, 1997). For example, nonviral vectors useful for expression of the MSLN-binding polynucleotides and polypeptides in mammalian (*e. g.*, human) cells include pThioHis A, B and C, pcDNA3.1/His, pEBVHis A, B and C, (Invitrogen, San Diego, Calif.), MPS V vectors, and numerous other vectors known in the art for expressing other proteins. Useful viral vectors include vectors based on retroviruses, adenoviruses, adenoassociated viruses, herpes viruses, vectors based on SV40, papilloma virus, HBP Epstein Barr virus, vaccinia virus vectors and Semliki Forest virus (SFV). See, Brent et al., *supra*; Smith, *Annu. Rev. Microbiol.* 49:807, 1995; and Rosenfeld et al., *Cell* 68: 143, 1992.

[0131] The choice of expression vector depends on the intended host cells in which the vector is to be expressed. Typically, the expression vectors contain a promoter and other regulatory sequences (e. g., enhancers) that are operably linked to the polynucleotides encoding a multispecific antibody chain or a fragment. In one embodiment, an inducible promoter is employed to prevent expression of inserted sequences except under inducing conditions. Inducible promoters include, e. g., arabinose, lacZ, metallothionein promoter or a heat shock promoter. Cultures of transformed organisms can be expanded under noninducing conditions without biasing the population for coding sequences whose expression products are better tolerated by the host cells. In addition to promoters, other regulatory elements may also be required or desired for efficient expression of a multispecific antibody chain or a fragment. These elements typically include an ATG initiation codon and adjacent ribosome binding site or other sequences. In addition, the efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (see, e. g., Scharf et al., *Results Probl. Cell Differ.* 20: 125, 1994; and Bittner et al., *Meth. Enzymol.*, 153:516, 1987). For example, the SV40 enhancer or CMV enhancer may be used to increase expression in mammalian host cells.

[0132] The expression vectors may also provide a secretion signal sequence position to form a fusion protein with polypeptides encoded by inserted multispecific antibody of the invention or fragments thereof or binding domains thereof sequences. More often, the inserted multispecific antibody of the invention or fragments thereof or binding domains thereof sequences are linked to signal sequences before inclusion in the vector. Vectors to be used to receive sequences encoding binding domains of the multispecific antibody light and heavy chain variable domains sometimes also encode constant regions or parts thereof.

[0133] The term "recombinant host cell" (or simply "host cell") refers to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

[0134] The host cells for harboring and expressing the multispecific antibody of the invention or fragments thereof or binding domains thereof can be either prokaryotic or eukaryotic. *E. coli* is one prokaryotic host useful for cloning and expressing the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which typically contain expression control sequences compatible with the host cell (e. g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation. Other microbes, such as yeast, can also be employed to express MSLN-binding polypeptides of the invention. Insect cells in combination with baculovirus vectors can also be used.

[0135] In one embodiment, mammalian host cells are used to express and produce the multispecific antibody of the invention or fragments thereof or binding domains thereof. For example, they can be either a hybridoma cell line expressing endogenous immunoglobulin genes or a mammalian cell line harboring an exogenous expression vector. These include any normal mortal or normal or abnormal immortal animal or human cell. For example, a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed including the CHO cell lines, various Cos cell lines, HeLa cells, myeloma cell lines, transformed B-cells and hybridomas. The use of mammalian tissue cell culture to express polypeptides is discussed generally in, e. g., Winnacker, FROM GENES TO CLONES, VCH Publishers, N.Y., N.Y., 1987. Expression vectors for mammalian host cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer (see, e. g., Queen, et al., Immunol. Rev. 89:49-68, 1986), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. These expression vectors usually contain promoters derived from mammalian genes or from mammalian viruses. Suitable promoters may be constitutive, cell type-

specific, stage-specific, and/or modulatable or regulatable. Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPS V promoter, the tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), the constitutive CMV promoter, and promoter-enhancer combinations known in the art.

[0136] Methods for introducing expression vectors containing the polynucleotide sequences of interest vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See generally Sambrook, et al., *supra*). Other methods include, *e. g.*, electroporation, calcium phosphate treatment, liposome-mediated transformation, injection and microinjection, ballistic methods, virosomes, immunoliposomes, polycationic nucleic acid conjugates, naked DNA, artificial virions, fusion to the herpes virus structural protein VP22 (Elliot and O'Hare, *Cell* 88:223, 1997), agent-enhanced uptake of DNA, and *ex vivo* transduction. For long-term, high-yield production of recombinant proteins, stable expression will often be desired. For example, cell lines which stably express the multispecific antibody of the invention or fragments thereof or binding domains thereof can be prepared using expression vectors of the invention which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth of cells which successfully express the introduced sequences in selective media. Resistant, stably transfected cells can be proliferated using tissue culture techniques appropriate to the cell type. The present invention thus provides a method of producing the antibody of the invention or antigen-binding fragment thereof, wherein said method comprises the step of culturing a host cell comprising a nucleic acid or a vector encoding the antibody of the invention or antigen-binding fragment thereof, whereby said antibody of the disclosure or a fragment thereof is expressed.

[0137] In one aspect, the present invention relates to a method of producing the multispecific antibody of the invention or a binding domain thereof or a fragment

thereof, the method comprising the step of culturing a host cell expressing a nucleic acid encoding the multispecific antibody of the invention or a binding domain thereof or a fragment thereof. In particular, the present invention relates to a method of producing the multispecific antibody of the invention or a binding domain thereof or a fragment thereof, the method comprising (i) providing a nucleic acid sequence or two nucleic acid sequences encoding the multispecific antibody of the invention or a binding domain thereof, or a vector or two vectors encoding the multispecific antibody of the invention or a binding domain thereof, expressing said nucleic acid sequence or nucleic acid sequences, or said vector or vectors, and collecting said multispecific antibody or said binding domain from the expression system, or (ii) providing a host cell or host cells expressing a nucleic acid encoding the multispecific antibody of the invention or a binding domain thereof, culturing said host cell or said host cells; and collecting said multispecific antibody or said binding domain from the cell culture.

[0138] In a further aspect, the present invention relates to a pharmaceutical composition comprising the multispecific antibody of the invention, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers enhance or stabilize the composition, or facilitate preparation of the composition.

Pharmaceutically acceptable carriers include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible.

[0139] A pharmaceutical composition of the invention can be administered by a variety of methods known in the art. The route and/or mode of administration vary depending upon the desired results. Administration can be intravenous, intramuscular, intraperitoneal, or subcutaneous, or administered proximal to the site of the target. The pharmaceutically acceptable carrier should be suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e. g.*, by injection or infusion). Depending on the route of administration, the active compound, *i. e.*, the multispecific antibody of the invention, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

[0140] Pharmaceutical compositions of the invention can be prepared in accordance with methods well known and routinely practiced in the art. See, *e. g.*,

Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000; and Sustained and Controlled Release Drug Delivery Systems, J. R.

Robinson, ed., Marcel Dekker, Inc., New York, 1978. Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically, a therapeutically effective dose or efficacious dose of the multispecific antibody of the invention is employed in the pharmaceutical compositions of the invention. The multispecific antibodies of the invention are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e. g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[0141] Actual dosage levels of the active ingredients in the pharmaceutical compositions of the invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors.

[0142] The multispecific antibody of the invention is usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of the

multispecific antibody of the invention in the patient. Alternatively, the multispecific antibody of the invention can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the antibody in the patient. In general, humanized antibodies show longer half-life than that of chimeric antibodies and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

[0143] In one aspect, the present invention relates to the multispecific antibody of the invention or the pharmaceutical composition of the invention for use as a medicament. In a suitable embodiment, the present invention provides the multispecific antibody or the pharmaceutical composition for use in treatment of a proliferative disease, in particular a cancer in a subject in need thereof.

[0144] In another aspect, the present invention provides the multispecific antibody or the pharmaceutical composition for use in a manufacture of a medicament for treatment of a proliferative disease, in particular a cancer.

[0145] In another aspect, the present invention relates to the use of the multispecific antibody or the pharmaceutical composition for treating a proliferative disease, in particular a cancer in a subject in need thereof.

[0146] In a further aspect, the present invention relates to the use of the multispecific antibody or the pharmaceutical composition in the manufacture of a medicament for treatment of a proliferative disease, in particular a cancer, in a subject in need thereof.

[0147] In another aspect, the present invention relates to a method of treating a subject comprising administering to the subject a therapeutically effective amount of the multispecific antibody of the present invention. In a suitable embodiment, the present invention relates to a method of treating a proliferative disease, in particular

a cancer in a subject comprising administering to the subject a therapeutically effective amount of the multispecific antibody of the present invention.

[0148] The term “subject” includes human and non-human animals. Non-human animals include all vertebrates, *e. g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, and reptiles. Except when noted, the terms “patient” or “subject” are used herein interchangeably.

[0149] The terms “treatment”, “treating”, “treat”, “treated”, and the like, as used herein, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease or delaying the disease progression.

“Treatment”, as used herein, covers any treatment of a disease in a mammal, *e. g.*, in a human, and includes: (a) inhibiting the disease, *i. e.*, arresting its development; and (b) relieving the disease, *i. e.*, causing regression of the disease.

[0150] The term “therapeutically effective amount” or “efficacious amount” refers to the amount of an agent that, when administered to a mammal or other subject for treating a disease, is sufficient to effect such treatment for the disease. The “therapeutically effective amount” will vary depending on the agent, the disease and its severity and the age, weight, etc., of the subject to be treated.

[0151] In one embodiment, the proliferative disease is a cancer. The term “cancer” refers to a disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. The terms “tumor” and “cancer” are used interchangeably herein, *e. g.*, both terms encompass solid and liquid, *e. g.*, diffuse or circulating, tumors. As used herein, the term “cancer” or “tumor” includes premalignant, as well as malignant cancers and tumors. The term “cancer” is used herein to mean a broad spectrum of tumors, including all solid and hematological malignancies. Examples of such tumors include, but are not limited to: a benign or especially malignant tumor, solid tumors, brain cancer, kidney cancer, liver cancer, adrenal gland cancer, bladder cancer, breast cancer, stomach cancer (*e. g.*, gastric tumors), esophageal cancer, ovarian cancer, cervical cancer, colon cancer, rectum cancer, prostate cancer, pancreatic cancer, lung cancer (*e. g.* non-small cell lung cancer and small cell lung cancer), vaginal cancer, thyroid cancer, melanoma (*e. g.*, unresectable or metastatic melanoma), renal cell carcinoma, sarcoma, glioblastoma,

multiple myeloma or gastrointestinal cancer, especially colon carcinoma or colorectal adenoma, a tumor of the neck and head, endometrial cancer, Cowden syndrome, Lhermitte-Duclos disease, Bannayan-Zonana syndrome, prostate hyperplasia, a neoplasia, especially of epithelial character, preferably mammary carcinoma or squamous cell carcinoma, chronic lymphocytic leukemia, chronic myelogenous leukemia (*e. g.*, Philadelphia chromosome-positive chronic myelogenous leukemia), acute lymphoblastic leukemia (*e. g.*, Philadelphia chromosome-positive acute lymphoblastic leukemia), non-Hodgkin's lymphoma, plasma cell myeloma, Hodgkin's lymphoma, a leukemia, and any combination thereof. In a preferred embodiment, the cancer is a cancer selected from mesothelioma, pancreatic cancer, and ovarian cancer.

[0152] The multispecific antibody of the present invention, or the composition of the present invention, inhibits the growth of solid tumors, but also liquid tumors. In a further embodiment, the proliferative disease is a solid tumor. The term "solid tumor" especially means a breast cancer, ovarian cancer, colon cancer, rectum cancer, prostate cancer, stomach cancer (especially gastric cancer), cervical cancer, lung cancer (*e. g.*, non-small cell lung cancer and small cell lung cancer), and a tumor of the head and neck. Further, depending on the tumor type and the particular combination used, a decrease of the tumor volume can be obtained. The multispecific antibody of the present invention, or the composition of the present invention, is also suited to prevent the metastatic spread of tumors and the growth or development of micro metastases in a subject having a cancer.

## CLAIMS

1. A multispecific antibody comprising:

- 5 a) two antibody-based binding domains, which specifically bind to mesothelin (MSLN-BDs); and  
b) at least one antibody-based binding domain, which specifically binds to CD3 (CD3-BD);

10 wherein said multispecific antibody does not comprise an immunoglobulin Fc region polypeptide, and wherein each of said MSLN-BDs binds to mesothelin (MSLN) with a monovalent dissociation constant ( $K_D$ ) in the range of from 0.5 to 20 nM, in particular in the range of from 0.6 to 10 nM when measured by SPR.

2. The multispecific antibody of claim 1, wherein said MSLN-BD comprises

- 15 (i) the HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 1, 2 (or 10) and 3, respectively, and the LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 4, 5 and 6, respectively; or the HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively, and the LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively;  
20 and  
(ii) VH3 or VH4 domain framework sequences FR1 to FR4; particularly VH3 domain framework sequences FR1 to FR4; and  
(iii) a VL domain comprising a VL framework comprising V $\kappa$  frameworks FR1, FR2 and FR3, particularly V $\kappa$ 1 or V $\kappa$ 3 FR1 to FR3, particularly V $\kappa$ 1 FR1 to FR3, and a framework FR4, which is selected from a V $\kappa$  FR4, and a V $\lambda$  FR4,  
25 particularly a V $\lambda$  FR4 comprising an amino acid sequence having at least 70, 80, or 90 percent identity to any of SEQ ID NO: 132 to SEQ ID NO: 139, more particularly V $\lambda$  FR4 selected from any of SEQ ID NO: 132 to SEQ ID NO: 139, particularly V $\lambda$  FR4 according to SEQ ID NO: 132 or 139.

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3. The multispecific antibody of claim 1 or 2, wherein said MSLN-BD comprises  
a.1) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 1, 2 (or 10) and 3, respectively,

- b.1) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 4, 5 and 6, respectively,
- c.1) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 7, and
- 5 d.1) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 9;
- or
- a.2) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 1, 2 (or 10) and 3, respectively,
- 10 b.2) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 4, 5 and 6, respectively,
- c.2) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 8, and
- d.2) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 9;
- 15 or
- a.3) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,
- b.3) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,
- 20 c.3) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 17, and
- d.3) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 18;
- 25 or
- a.4) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,
- b.4) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,
- 30 c.4) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 19, and
- d.4) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 21;
- or

- a.5) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,
- b.5) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,
- 5 c.5) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 20, and
- d.5) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 21;
- or
- 10 a.6) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,
- b.6) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,
- c.6) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 22, and
- 15 d.6) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 24;
- or
- a.7) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 11, 12 and 13, respectively,
- 20 b.7) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 14, 15 and 16, respectively,
- c.7) a VH sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 23, and
- 25 d.7) a VL sequence at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent identical to the amino acid sequence SEQ ID NO: 24.
4. The multispecific antibody of any one of claims 1 to 3, wherein said CD3-BD is binding to CD3ε.

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5. The multispecific antibody of any one of the preceding claims, wherein said antibody comprises one CD3-BD, wherein said CD3-BD binds CD3 $\epsilon$  with a monovalent  $K_D$  of 0.5 to 50 nM, particularly of 1 to 40 nM, particularly of 2 to 35 nM, as measured by SPR.

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6. The multispecific antibody of claim 4 or 5, wherein said CD3-BD comprises
- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 45, 46 and 47, respectively in a human antibody VH framework, particularly a VH3 framework, and
  - 10 (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 48, 49 and 50, respectively in a human antibody VL framework, wherein the VL framework comprises Vk frameworks FR1, FR2 and FR3, particularly Vk1 frameworks, and a framework FR4, which is selected from a Vk FR4, and a V $\lambda$  framework 4,
  - 15 particularly, wherein said CD3-BD comprises
  - (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 51, 140, 141 or 142, and
  - (ii) a VL domain comprising the amino acid sequence of SEQ ID NO: 52.

- 20 7. The multispecific antibody of any one of the preceding claims, wherein said antibody further comprises at least one human serum albumin binding domain (hSA-BD), particularly one hSA-BD, particularly wherein said hSA-BD comprises
- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 53, 54 and 55, respectively in a human antibody VH framework, particularly a VH3 framework,
  - 25 and
  - (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 56, 57 and 58, respectively in a human antibody VL framework, wherein the VL framework comprises Vk frameworks FR1, FR2 and FR3, particularly Vk1 frameworks, and a framework FR4, which is selected from a Vk FR4, particularly Vk1 FR4,
  - 30 and a V $\lambda$  framework 4;

or

- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 63, 64 and 65, respectively in a human antibody VH framework, particularly a VH3 framework, and
  - (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 66, 67 and 68, respectively in a human antibody VL framework, wherein the VL framework comprises Vk frameworks FR1, FR2 and FR3, particularly Vk1 frameworks, and a framework FR4, which is selected from a Vk FR4, particularly Vk1 FR4, and a Vλ framework 4;
- or
- (i) HCDR1, HCDR2, and HCDR3 sequences of SEQ ID NOs: 73, 74 and 75, respectively in a human antibody VH framework, particularly a VH3 framework, and
  - (ii) LCDR1, LCDR2, and LCDR3 sequences of SEQ ID NOs: 76, 77 and 78, respectively in a human antibody VL framework, wherein the VL framework comprises Vk frameworks FR1, FR2 and FR3, particularly Vk1 frameworks, and a framework FR4, which is selected from a Vk FR4, particularly Vk1 FR4, and a Vλ framework 4;
- particularly, wherein said hSA-BD comprises
- (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 59, and
  - (ii) a VL domain comprising the amino acid sequence of SEQ ID NO: 60;
- or
- (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 69, and
  - (ii) a VL domain comprising the amino acid sequence of SEQ ID NO: 70;
- or
- (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 79 or 143, and
  - (ii) a VL domain comprising the amino acid sequence of SEQ ID NO: 80 or 144;
- or
- (i) a VH domain comprising the amino acid sequence of SEQ ID NO: 81 or 145, and
  - (ii) a VL domain comprising the amino acid sequence of SEQ ID NO: 82 or 146.

8. The multispecific antibody of any one of the preceding claims, wherein said antibody does not comprise CH1 and/or CL regions.

5 9. The multispecific antibody of any one of the preceding claims, wherein  
said first single-chain protein comprises the amino acid sequence of SEQ ID NO:  
101, especially consists of the amino acid sequence SEQ ID NO: 101 and  
said second single-chain protein comprises the amino acid sequence of SEQ ID  
NO: 102, especially consists of the amino acid sequence SEQ ID NO: 102; or  
10 said first single-chain protein comprises the amino acid sequence of SEQ ID NO:  
109, especially consists of the amino acid sequence SEQ ID NO: 109 and  
said second single-chain protein comprises the amino acid sequence of SEQ ID  
NO: 110, especially consists of the amino acid sequence SEQ ID NO: 110; or  
said first single-chain protein comprises the amino acid sequence of SEQ ID NO:  
15 111, especially consists of the amino acid sequence SEQ ID NO: 111 and  
said second single-chain protein comprises the amino acid sequence of SEQ ID  
NO: 112, especially consists of the amino acid sequence SEQ ID NO: 112; or  
said first single-chain protein comprises the amino acid sequence of SEQ ID NO:  
119, especially consists of the amino acid sequence SEQ ID NO: 119 and  
20 said second single-chain protein comprises the amino acid sequence of SEQ ID  
NO: 120, especially consists of the amino acid sequence SEQ ID NO: 120; or  
said first single-chain protein comprises the amino acid sequence of SEQ ID NO:  
121, especially consists of the amino acid sequence SEQ ID NO: 121 and  
said second single-chain protein comprises the amino acid sequence of SEQ ID  
25 NO: 122, especially consists of the amino acid sequence SEQ ID NO: 122; or  
said first single-chain protein comprises the amino acid sequence of SEQ ID NO:  
123, especially consists of the amino acid sequence SEQ ID NO: 123 and  
said second single-chain protein comprises the amino acid sequence of SEQ ID  
NO: 124, especially consists of the amino acid sequence SEQ ID NO: 124.

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10. A nucleic acid sequence or two nucleic acid sequences encoding the multispecific antibody of any one of claims 1 to 9.
11. A vector or two vectors comprising the nucleic acid sequence or the two nucleic acid sequences of claim 10.
12. A host cell or host cells comprising the vector or the two vectors of claim 11.
13. A method for producing the multispecific antibody of any one of claims 1 to 9, comprising (i) providing the nucleic acid sequence or the two nucleic acid sequences of claim 10, or the vector or the two vectors of claim 11, expressing said nucleic acid sequence or nucleic acid sequences, or said vector or vectors, and collecting said multispecific antibody from the expression system, or (ii) providing a host cell or host cells according to claim 12, culturing said host cell or said host cells; and collecting said multispecific antibody from the cell culture.
14. A pharmaceutical composition comprising the multispecific antibody of any one of claims 1 to 9 and a pharmaceutically acceptable carrier.
15. Use of the The multispecific antibody of any one of claims 1 to 9 ~~for use in the manufacture of a medicament for treating~~ treatment of a disease, particularly a human disease, more particularly a human disease selected from cancer, particularly a cancer selected from mesothelioma, pancreatic cancer, and ovarian cancer, an inflammatory and an autoimmune disease, particularly wherein said multispecific antibody is either a single-chain protein comprising three or four binding domains, or a hetero-dimeric protein comprising three or four binding domains.