

POLYPEPTIDES COMPRISING IMMUNOGLOBULIN SINGLE VARIABLE DOMAINS
TARGETING IL-13 AND TSLP

DESCRIPTION

5 **1 Field of the present technology**

The present technology relates to polypeptides targeting IL-13 and TSLP. It also relates to nucleic acid molecules encoding the polypeptide and vectors comprising the nucleic acids, and to compositions comprising the polypeptide, nucleic acid or vector. The present technology further relates to these products for use in a method of treating a subject
10 suffering from an inflammatory disease. Moreover, the present technology relates to method of producing these products.

2 Technological Background

While necessary for host-defense, unrestrained immune responses can lead to a range of inflammatory diseases such as asthma, atopic dermatitis and rheumatoid arthritis. A cascade
15 of immune responses mediated by the innate and adaptive arms of the immune system (e.g., antigen recognition, antigen processing, antigen presentation, cytokine production, antibody production, target cell killing) drive the initiation and propagation of a range of immunological diseases. Inflammatory diseases are often chronic and can even be life-threatening. Allergic and atopic diseases such as asthma and atopic dermatitis are driven
20 predominantly by type 2 immune responses and characterized by salient features of type 2 immunity such as high IgE production and eosinophilia.

Thymic stromal lymphopoietin (TSLP) and Interleukin-13 (IL-13) are soluble cytokine targets produced by stromal and/or immune cells (Ziegler & Artis, Nat Rev Immunol (2010) 11:289, Gieseck III et al., Nat Rev Immunol (2018) 18:62). Human TSLP and IL-13 drive distinct,
25 overlapping and synergistic aspects of type 2 immunity, type 2 inflammatory diseases such as asthma and atopic dermatitis as well as a broad array of immunological diseases.

The signalling of TSLP begins through a heterodimeric receptor complex composed of the thymic stromal lymphopoietin receptor (TSLPR) and the IL-7R alpha chain (IL-7R α). Similarly,

IL-13 signalling starts by binding to a heterodimeric receptor complex consisting of alpha IL-4 receptor (IL-4R α) and alpha Interleukin-13 receptor (IL-13R1 α). The high affinity of IL-13 to the IL-13R1 leads to their complex formation which further increase the probability of a heterodimer formation to IL-4R α .

5 TSLP drives the maturation of dendritic cells, development and proliferation of mast cells, as well as activating other immune cells such as basophils and innate lymphoid cells (ILC2). Similarly, IL-13 exerts a range of immunopathologies such as epithelial barrier disruption, mucus production from mucosal-epithelial surfaces, airway remodeling as well as the induction of eosinophil recruiting chemokines such as eotaxin. These mechanisms are
10 central to the initiation and propagation of the type 2 inflammatory response and are central to the development of a range of immunopathologies in diseases such as atopic dermatitis and asthma.

Currently, patients with moderate/severe asthma are inadequately responding to presently available standard of care treatments, including biological such as anti-IL4R α monoclonal
15 antibody Dupixent (dupilumab; marketed), anti-IL5s (marketed), anti-IgE monoclonal antibody Xolair (omalizumab; marketed), in particular in asthma patients with a low-eosinophilic phenotype. Although antagonistic monoclonal antibodies against TSLP (tezepelumab) and IL-13 (lebrikizumab) are presently undergoing clinical trials, there are no active clinical development programs that target both TSLP and IL-13. Dual targeting of TSLP
20 and IL-13 with a single agent has the potential to confer efficacy in both low-type 2 and high-type 2 asthma as well as atopic dermatitis, with the potential to confer efficacy in subpopulations within these indications where a single monospecific agent therapy may not be fully efficacious. Accordingly, there is still an unmet medical need for the treatment of type 2 inflammatory diseases such as asthma and atopic dermatitis that is not only more efficacious
25 but also conveniently applicable to the patient.

Such therapy may comprise targeting multiple disease factors, such as IL-13 and TSLP.

Targeting multiple disease factors may be achieved for example by co-administration or combinatorial use of two separate biologicals, e.g. antibodies binding to different therapeutic targets. However, co-administration or combinatorial use of separate biologicals
30 can be challenging, both from a practical and a commercial point of view. For example, two

injections of separate products result in a more inconvenient and more painful treatment regime to the patients which may negatively affect compliance. With regard to a single injection of two separate products, it can be difficult or impossible to provide formulations that allow for acceptable viscosity at the required concentrations and suitable stability of both products. Additionally, co-administration and co-formulation requires production of two separate drugs which can increase overall costs.

Bispecific antibodies that are able to bind to two different antigens have been suggested as one strategy for addressing such limitations associated with co-administration or combinatorial use of separate biologicals, such as antibodies.

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

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

3 Summary of the present technology

In some embodiments, the present technology relates to a polypeptide targeting specifically IL-13 and TSLP at the same time leading to an increased efficiency of modulating a type 2 inflammatory response as compared to monospecific anti-IL-13 or anti-TSLP polypeptides *in vitro*. In some embodiments, the polypeptides are efficiently produced (e.g. in microbial hosts). Furthermore, in some embodiments, such polypeptides have limited reactivity to pre-existing antibodies in the subject to be treated (i.e., antibodies present in the subject before the first treatment with the antibody construct). In other embodiments such

polypeptides exhibit a half-life in the subject to be treated that is long enough such that consecutive treatments can be conveniently spaced apart.

In one embodiment, the present technology provides a polypeptide comprising or consisting of at least one immunoglobulin single variable domain (ISVD) that specifically binds to IL-13.

5 In a further embodiment, the polypeptide of the present technology comprises or consists of at least two ISVDs that specifically bind to IL-13, wherein the two ISVDs are optionally linked via a peptidic linker. In one embodiment, the two ISVDs specifically binding to IL-13 are distinct ISVDs. Moreover, in one embodiment the polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more
10 peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. For example, the binding unit can be an ISVD that binds to a (human) serum protein, such as human serum albumin.

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

In another embodiment, the polypeptide of the present technology comprises or consists of at least four ISVDs, wherein at least two ISVDs specifically bind to IL-13 and at least two ISVDs specifically bind to TSLP. In one embodiment, the at least two ISVDs specifically binding to IL-13 specifically bind to human IL-13 and the at least two ISVDs specifically
30 binding to TSLP specifically bind to human TSLP. In one embodiment, the at least two ISVDs

specifically binding to IL-13 are distinct ISVDs and the at least two ISVDs binding to TSLP are distinct ISVDs. In another embodiment, the polypeptide comprising or consisting of at least four ISVDs further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. For example, the binding unit can be an ISVD that binds to a (human) serum protein, such as human serum albumin.

Also provided is a nucleic acid molecule capable of expressing the polypeptide of the present technology, a nucleic acid or vector comprising the nucleic acid, and a composition comprising the polypeptide, the nucleic acid or the vector. In one embodiment, the composition is a pharmaceutical composition.

Also provided is a host or host cell comprising the nucleic acid or vector that encodes the polypeptide according to the present technology.

Further provided is a method for producing the polypeptide according to present technology, said method at least comprising the steps of:

- a. expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid comprising a nucleotide sequence that encodes a polypeptide of the present technology; optionally followed by:
- b. isolating and/or purifying the polypeptide according to the present technology.

Moreover, the present technology provides the polypeptide, the composition comprising the polypeptide, or the composition comprising the nucleic acid or vector comprising the nucleotide sequence that encodes the polypeptide, for use as a medicament. In one embodiment, the polypeptide or composition is for use in the treatment of an inflammatory disease, such as a type 2 inflammatory disease. In one embodiment, the type 2 inflammatory disease is selected from atopic dermatitis and asthma.

In addition, provided is a method of treating an inflammatory disease, such as a type 2 inflammatory disease, wherein said method comprises administering, to a subject in need

thereof, a pharmaceutically active amount of the polypeptide or a composition according to the present technology. In one embodiment, the type 2 inflammatory disease is selected from atopic dermatitis and asthma. In one embodiment, the method further comprises administering one or more additional therapeutic agents.

- 5 Further provided is the use of the polypeptide or composition of the present technology in the preparation of a pharmaceutical composition for treating an inflammatory disease, such as a type 2 inflammatory disease. In one embodiment, the type 2 inflammatory disease is selected from atopic dermatitis and asthma.

In particular, the present technology provides the following embodiments:

- 10 Embodiment 1. A polypeptide, a composition comprising the polypeptide, or a composition comprising a nucleic acid comprising a nucleotide sequence that encodes the polypeptide, for use as a medicament, wherein the polypeptide comprises or consists of at least one immunoglobulin single variable domain (ISVD), wherein said ISVD comprises three complementarity determining regions (CDR1 to CDR3, respectively), and wherein
- 15 the at least one ISVD comprises:
- a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12;
20 and
a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17,
 - b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
25 a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13;
and

- a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18,
- c) a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- 5 a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14; and
- a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19,
- 10 or
- d) a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
- a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16;
- 15 and
- a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

Embodiment 2. The polypeptide or composition for use according to embodiment 1, wherein the at least one ISVD comprises:

- 20 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17,
- b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO:
- 25 18,
- c) a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, or

d) a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

Embodiment 3. The polypeptide or composition for use according to any of
5 embodiments 1 or 2, wherein the amino acid sequence of the at least one ISVD comprises:

- a) a sequence identity of more than 90% with SEQ ID NO: 2,
- b) a sequence identity of more than 90% with SEQ ID NO: 3,
- c) a sequence identity of more than 90% identity with SEQ ID NO: 4, or
- d) a sequence identity of more than 90% identity with SEQ ID NO: 6.

10 Embodiment 4. The polypeptide or composition for use according to any of embodiments 1 to 3, wherein said at least one ISVD comprises:

- a) the amino acid sequence of SEQ ID NO: 2,
- b) the amino acid sequence of SEQ ID NO: 3,
- c) the amino acid sequence of SEQ ID NO: 4, or
- 15 d) the amino acid sequence of SEQ ID NO: 6.

Embodiment 5. The polypeptide or composition for use according to embodiment 1, wherein the polypeptide comprises or consists of at least two ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least two ISVDs are optionally linked via one or more peptidic linkers, and
20 wherein:

- a) a first and a second ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12;
 - 25 and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17,
- b) a first and a second ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18,
- c) a first ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17, and
- a second ISVD comprises
 - iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18,
- d) a first ISVD comprises

- 5
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13;
 - and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18, and

a second ISVD comprises

- 10
- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12;
 - and
 - 15 vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17,

e) a first ISVD comprises

- 20
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16;
 - and
 - 25 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21, and

a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;

- 5
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, or

f) a first ISVD comprises

- 10
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14; and
 - 15 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, and/or

a second ISVD comprises

- 20
- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16; and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21,

25 wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide.

Embodiment 6. The polypeptide or composition for use according to embodiment 5, wherein:

- a) the first and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17,
- b) the first and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18,
- c) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18,
- d) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17,
- e) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, or
- f) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

Embodiment 7. The polypeptide or composition for use according to any of embodiments 5 or 6, wherein:

- a) the amino acid sequence of the first and the second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2,
- b) the amino acid sequence of the first and the second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3,
- 5 c) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2, and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 3,
- d) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3, and the second ISVD comprises a sequence identity of more than
10 90% identity with SEQ ID NO: 2,
- e) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 6, and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 4,
- f) the amino acid sequence of the first ISVD comprises a sequence identity of more than
15 90% with SEQ ID NO: 4, and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 6.

Embodiment 8. The polypeptide or composition for use according to any of embodiments 5 to 7, wherein:

- a) the first and the second ISVD comprises the amino acid sequence of SEQ ID NO: 2,
- 20 b) the first and the second ISVD comprises the amino acid sequence of SEQ ID NO: 3,
- c) the first ISVD comprises the amino acid sequence of SEQ ID NO: 2, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 3,
- d) the first ISVD comprises the amino acid sequence of SEQ ID NO: 3, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 2,
- 25 e) the first ISVD comprises the amino acid sequence of SEQ ID NO: 6, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 4, or

f) the first ISVD comprises the amino acid sequence of SEQ ID NO: 4, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 6.

Embodiment 9. The polypeptide according to any of embodiments 5 to 8, wherein the polypeptide comprises or consists of:

- 5 a) the amino acid sequence of SEQ ID NO: 148,
- b) the amino acid sequence of SEQ ID NO: 149,
- c) the amino acid sequence of SEQ ID NO: 150,
- d) the amino acid sequence of SEQ ID NO: 151,
- e) the amino acid sequence of SEQ ID NO: 152,
- 10 f) the amino acid sequence of SEQ ID NO: 153,
- g) the amino acid sequence of SEQ ID NO: 154,
- h) the amino acid sequence of SEQ ID NO: 155,
- i) the amino acid sequence of SEQ ID NO: 156,
- j) the amino acid sequence of SEQ ID NO: 157,
- 15 k) the amino acid sequence of SEQ ID NO: 158, or
- l) the amino acid sequence of SEQ ID NO: 159.

Embodiment 10. The polypeptide or composition for use according to any of embodiments 1 or 5, wherein the polypeptide comprises or consists of at least four ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least four ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- a) a first ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - 25 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17;

b) a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13;
- and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18;

c) a third ISVD comprises

- vii. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- viii. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14;
- and
- ix. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19;
- and

d) a fourth ISVD comprises

- x. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
- xi. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16;
- and
- xii. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21,

wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide.

Embodiment 11. The composition for use according to any one of embodiments 1 to 10, which is a pharmaceutical composition which further comprises at least one

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

Embodiment 12. The polypeptide or composition for use according to embodiment 10 or 11, wherein:

- a) said first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17;
- b) said second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18;
- c) said third ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19; and
- d) said fourth ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

Embodiment 13. The polypeptide or composition for use according to any of embodiments 10 to 12, wherein:

- a) the amino acid sequence of said first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2;
- b) the amino acid sequence of said second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3;
- c) the amino acid sequence of said third ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 4; and
- d) the amino acid sequence of said fourth ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 6.

Embodiment 14. The polypeptide or composition for use according to any of embodiments 10 to 13, wherein:

- a) said first ISVD comprises the amino acid sequence of SEQ ID NO: 2;
- b) said second ISVD comprises the amino acid sequence of SEQ ID NO: 3;
- 5 c) said third ISVD comprises the amino acid sequence of SEQ ID NO: 4; and
- d) said fourth ISVD comprises the amino acid sequence of SEQ ID NO: 6.

Embodiment 15. The polypeptide or composition for use according to any of embodiments 1 to 14, wherein said polypeptide further comprises one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic
10 linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.

Embodiment 16. The polypeptide or composition for use according to embodiment 15,
15 in which said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule, serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

Embodiment 17. The polypeptide or composition for use according to any one of
20 embodiments 15 to 16, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Embodiment 18. The polypeptide or composition for use according to embodiment 17,
25 in which said binding unit that provides the polypeptide with increased half-life is an ISVD that can bind to human serum albumin.

Embodiment 19. The polypeptide or composition for use according to embodiment 18, wherein the ISVD binding to human serum albumin comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10;
- 5 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 20 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 20.

10 Embodiment 20. The polypeptide or composition for use according to any of embodiments 18 to 19, wherein the ISVD binding to human serum albumin comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 10, a CDR2 that is the amino acid sequence of SEQ ID NO: 15 and a CDR3 that is the amino acid sequence of SEQ ID NO: 20.

15 Embodiment 21. The polypeptide or composition for use according to any of embodiments 18 to 20, wherein the amino acid sequence of said ISVD binding to human serum albumin comprises a sequence identity of more than 90% with SEQ ID NO: 5.

Embodiment 22. The polypeptide or composition for use according to any of embodiments 18 to 21, wherein said ISVD binding to human serum albumin comprises the amino acid sequence of SEQ ID NO: 5.

20 Embodiment 23. The polypeptide or composition for use according to any of embodiments 10 to 22, wherein the amino acid sequence of the polypeptide comprises a sequence identity of more than 90% with SEQ ID NO: 1.

25 Embodiment 24. The polypeptide or composition for use according to any of embodiments 10 to 23, wherein the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.

Embodiment 25. The polypeptide or composition for use according to any of embodiments 1 to 24, for use in the treatment of an inflammatory disease, such as a type 2 inflammatory disease.

Embodiment 26. The polypeptide or composition for use according to embodiment 25,
5 wherein the type 2 inflammatory disease is selected from asthma and atopic dermatitis.

Embodiment 27. A polypeptide that comprises or consists of at least one immunoglobulin single variable domain (ISVD), wherein said ISVD comprises three complementarity determining regions (CDR1 to CDR3, respectively); and wherein the at least one ISVD comprises:

- 10 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12;
and
15 a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17,
b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino
20 acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13;
and
a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18,
c) a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino
25 acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14;
and

a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, or

5 d) a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;

a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16; and

10 a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

Embodiment 28. The polypeptide according to embodiment 27, wherein the at least one ISVD comprises:

15 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17,

b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18,

20 c) a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, or

d) a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

25 Embodiment 29. The polypeptide according to any of embodiments 27 or 28, wherein the amino acid sequence of the at least one ISVD comprises:

a) a sequence identity of more than 90% with SEQ ID NO: 2,

- b) a sequence identity of more than 90% with SEQ ID NO: 3,
- c) a sequence identity of more than 90% identity with SEQ ID NO: 4, or
- d) a sequence identity of more than 90% identity with SEQ ID NO: 6.

Embodiment 30. The polypeptide according to any of embodiments 27 to 29, wherein
5 said at least one ISVD comprises:

- a) the amino acid sequence of SEQ ID NO: 2,
- b) the amino acid sequence of SEQ ID NO: 3,
- c) the amino acid sequence of SEQ ID NO: 4, or
- d) the amino acid sequence of SEQ ID NO: 6.

10 Embodiment 31. The polypeptide according to embodiment 1 or 27, wherein the polypeptide comprises or consists of at least two ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least two ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- 15 a) a first and a second ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12;
 - 20 and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17,
- b) a first and a second ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - 25

- 5 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18,

c) a first ISVD comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- 10 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17, and

15 a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13;
- 20 and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18,

d) a first ISVD comprises

- 25 i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and

- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18, and

a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17,

e) a first ISVD comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21, and

a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, or

f) a first ISVD comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, and

a second ISVD comprises

- iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
- v. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16; and
- vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21,

wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide.

Embodiment 32. The polypeptide according to embodiment 31, wherein:

- a) the first and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17,
- b) the first and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18,
- c) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17, and the second ISVD comprises a CDR1 that is the amino acid

sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18,

5 d) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17,

10 e) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, or

15 f) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

Embodiment 33. The polypeptide according to any of embodiments 31 or 32, wherein:

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

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

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

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

5 e) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 6, and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 4,

f) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 4, and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 6.

10 Embodiment 34. The polypeptide according to any of embodiments 31 to 33, wherein:

a) the first and the second ISVD comprises the amino acid sequence of SEQ ID NO: 2,

b) the first and the second ISVD comprises the amino acid sequence of SEQ ID NO: 3,

c) the first ISVD comprises the amino acid sequence of SEQ ID NO: 2, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 3,

15 d) the first ISVD comprises the amino acid sequence of SEQ ID NO: 3, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 2,

e) the first ISVD comprises the amino acid sequence of SEQ ID NO: 6, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 4, or

20 f) the first ISVD comprises the amino acid sequence of SEQ ID NO: 4, and the second ISVD comprises the amino acid sequence of SEQ ID NO: 6.

Embodiment 35. The polypeptide according to any of embodiments 31 to 34, wherein the polypeptide comprises or consists of:

a) the amino acid sequence of SEQ ID NO: 148,

b) the amino acid sequence of SEQ ID NO: 149,

25 c) the amino acid sequence of SEQ ID NO: 150,

d) the amino acid sequence of SEQ ID NO: 151,

e) the amino acid sequence of SEQ ID NO: 152,

f) the amino acid sequence of SEQ ID NO: 153,

- g) the amino acid sequence of SEQ ID NO: 154,
- h) the amino acid sequence of SEQ ID NO: 155,
- i) the amino acid sequence of SEQ ID NO: 156,
- j) the amino acid sequence of SEQ ID NO: 157,
- 5 k) the amino acid sequence of SEQ ID NO: 158, or
- l) the amino acid sequence of SEQ ID NO: 159.

Embodiment 36. The polypeptide according to any of embodiments 27 or 31, wherein the polypeptide comprises or consists of at least four ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively),
10 wherein the at least four ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- a) a first ISVD comprises
 - i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
 - 15 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17;
- 20 b) a second ISVD comprises
 - iv. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
 - v. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13;
 - 25 and
 - vi. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18;
- c) a third ISVD comprises

- vii. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- viii. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14;
- 5 and
- ix. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19;
- and
- d) a fourth ISVD comprises
- 10 x. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
- xi. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16;
- and
- 15 xii. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21,

wherein the order of the ISVDs indicates their relative position to each other considered from the N-terminus to the C-terminus of said polypeptide.

Embodiment 37. The polypeptide according to embodiment 36, wherein:

- 20 a) said first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17;
- b) said second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid
- 25 sequence of SEQ ID NO: 18;
- c) said third ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19; and

d) said fourth ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

Embodiment 38. The polypeptide according to any of embodiments 36 or 37, wherein:

- 5 a) the amino acid sequence of said first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2;
- b) the amino acid sequence of said second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3;
- c) the amino acid sequence of said third ISVD comprises a sequence identity of more
10 than 90% identity with SEQ ID NO: 4; and
- d) the amino acid sequence of said fourth ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 6.

Embodiment 39. The polypeptide according to any of embodiments 36 to 38, wherein:

- a) said first ISVD comprises the amino acid sequence of SEQ ID NO: 2;
- 15 b) said second ISVD comprises the amino acid sequence of SEQ ID NO: 3;
- c) said third ISVD comprises the amino acid sequence of SEQ ID NO: 4; and
- d) said fourth ISVD comprises the amino acid sequence of SEQ ID NO: 6.

Embodiment 40. The polypeptide according to any of embodiments 27 to 39, wherein said polypeptide further comprises one or more other groups, residues, moieties or
20 binding units, optionally linked via one or more peptidic linkers, in which said one or more other groups, residues, moieties or binding units provide the polypeptide with increased half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units.

Embodiment 41. The polypeptide according to embodiment 40, in which said one or
25 more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life is chosen from the group consisting of a polyethylene glycol molecule,

serum proteins or fragments thereof, binding units that can bind to serum proteins, an Fc portion, and small proteins or peptides that can bind to serum proteins.

Embodiment 42. The polypeptide according to any one of embodiments 40 to 41, in which said one or more other binding units that provide the polypeptide with increased half-life is chosen from the group consisting of binding units that can bind to serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

Embodiment 43. The polypeptide according to embodiment 42, in which said binding unit that provides the polypeptide with increased half-life is an ISVD that can bind to human serum albumin.

Embodiment 44. The polypeptide according to embodiment 43, wherein the ISVD binding to human serum albumin comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 20 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 20.

Embodiment 45. The polypeptide according to any of embodiments 43 to 44, wherein the ISVD binding to human serum albumin comprises a CDR1 that is the amino acid sequence of SEQ ID NO:10, a CDR2 that is the amino acid sequence of SEQ ID NO: 15 and a CDR3 that is the amino acid sequence of SEQ ID NO: 20.

Embodiment 46. The polypeptide according to any of embodiments 43 to 45, wherein the amino acid sequence of said ISVD binding to human serum albumin comprises a sequence identity of more than 90% with SEQ ID NO: 5.

Embodiment 47. The polypeptide according to any of embodiments 43 to 46, wherein said ISVD binding to human serum albumin comprises the amino acid sequence of SEQ ID NO: 5.

5 Embodiment 48. The polypeptide according to any of embodiments 36 to 47, wherein the amino acid sequence of the polypeptide comprises a sequence identity of more than 90% with SEQ ID NO: 1.

Embodiment 49. The polypeptide according to any of embodiments 36 to 48, wherein the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1.

10 Embodiment 50. A nucleic acid comprising a nucleotide sequence that encodes a polypeptide according to any of embodiments 27 to 49, or a polypeptide according to embodiments 36 to 49.

Embodiment 51. A host or host cell comprising a nucleic acid according to embodiment 50.

15 Embodiment 52. A method for producing a polypeptide according to any of embodiments 27 to 49, or a polypeptide according to embodiments 36 to 49, said method at least comprising the steps of:

- a) expressing, in a suitable host cell or host organism or in another suitable expression system, a nucleic acid according to embodiment 50; optionally followed by:
 - b) isolating and/or purifying the polypeptide according to any of embodiments 27 to 49, or the polypeptide according to embodiments 36 to 49.
- 20

Embodiment 53. A composition comprising at least one polypeptide according to any of embodiments 27 to 49, or at least one polypeptide according to embodiments 36 to 49, or a nucleic acid according to embodiment 50.

25 Embodiment 54. The composition according to embodiment 53, which is a pharmaceutical composition which further comprises at least one pharmaceutically

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

Embodiment 55. A method of treating an inflammatory disease, such as a type 2 inflammatory disease, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide according to any of
5 embodiments 27 to 49, or according to embodiments 36 to 49, or a composition according to any of embodiments 53 to 54.

Embodiment 56. The method according to embodiment 55, wherein the type 2 inflammatory disease is selected from asthma and atopic dermatitis.

10 Embodiment 57. Use of a polypeptide according to any of embodiments 27 to 49, or according to embodiments 36 to 49, or a composition according to any of embodiments 53 to 54, in the preparation of a pharmaceutical composition for treating an inflammatory disease, such as a type 2 inflammatory disease.

Embodiment 58. Use of the polypeptide or a composition according to embodiments
15 57, wherein the type 2 inflammatory disease is selected from asthma and atopic dermatitis.

4 Brief description of the drawings

Figure 1: Sensorgram showing simultaneous binding of IL13 and HSA to F027400161 captured via TSLP.

20 **Figure 2:** Inhibition of human, cyno and rhesus IL13 in the eotaxin release assay by V_{HH} F027400161 and the reference compounds anti-hIL-13 mAb1 and anti-hIL-13 mAb2.

Figure 3: Inhibition of human and cyno IL13 in the SEAP reporter assay by F027400161 and the reference compounds anti-hIL-13 mAb1 and anti-hIL-13 mAb2.

Figure 4: Inhibition of human and cyno TSLP induced BaF3 proliferation by F027400161 and
25 the reference compound anti-hTSLP mAb1. IRR00096 is a negative control Nb.

Figure 5: Box plot showing the binding of pre-existing antibodies present in 96 human serum samples to F027400161, 163 and 164 compared to control F027301186.

Figure 6: Dose-response inhibition profiles of F-27400161 (also referred to as F027400161) and comparator antibody anti-hTSLP reference mAb1 on TSLP-induced CCL17 response in human DCs. Enriched human DCs were treated with 4 ng/mL of TSLP and incubated with 8 concentrations of the ISVD construct and anti-hTSLP reference mAb1 for 36 hours. CCL17 concentration in freshly collected supernatant was measured by ELISA. IC₅₀ values were calculated by nonlinear regression (log inhibitor vs responses – variable slope four parameters least squares fit) in Graphpad Prism 8.0 with no constraints. Data are represented as mean ± standard error of mean (SEM) of all 8 donors combined from 8 individual experiments.

Figure 7: Dose inhibition responses of F-27400161 (also referred to as F027400161) and comparators Anti-hIL-13 reference mAb1 and anti-hTSLP reference mAb1 on 0.5 ng/mL IL-13 and TSLP-induced synergistic production of CCL17 in human PBMCs. Healthy donor PBMCs were stimulated with 0.5 ng/mL of recombinant IL-13+TSLP and incubated with 10 doses of the ISVD construct (Nb) and comparators for 20 hours. CCL17 concentration in the cell culture supernatant was measured by MSD V-Plex kit. IC₅₀ values were calculated by nonlinear regression (log inhibitor vs responses – variable slope four parameters least squares fit) in Graphpad Prism 8.0 with no constraints. Data are represented as mean ± standard error of mean (SEM) of all donors combined from 8 individual experiments.

Figure 8: Dose inhibition responses of F-27400161 (also referred to as F027400161) and comparator antibodies anti-hIL-13 reference mAb1 and anti-hTSLP reference mAb1 on 5 ng/mL IL-13 and TSLP-induced synergistic production of CCL17 in human PBMCs. Healthy donor PBMCs were stimulated with 5 ng/mL of recombinant IL-13+TSLP and incubated with 10 doses of the ISVD construct (Nb) and comparator antibodies for 20 hours. CCL17 concentration in freshly collected supernatant was measured by MSD V-Plex kit. IC₅₀ values were calculated by nonlinear regression (log inhibitor vs responses – variable slope four parameters least squares fit) in Graphpad Prism 8.0 with no constraints. Data are represented as mean ± standard error of mean (SEM) of all donors combined from 8 individual experiments.

Figure 9: Inhibition profiles of F-27400161 (also referred to as F027400161) and comparator antibodies anti-hIL-13 reference mAb1 and anti-hTSLP reference mAb1 on allergen Der P-

induced IL-5, CCL17, and CCL26 production by human PBMCs in a triculture assay. Normal donor PBMCs cocultured with MRC5 fibroblasts and A549 epithelial cells were stimulated with 3mg/mL of Der P, and incubated with 11.1 nM of the ISVD, anti-hIL-13 reference mAb1, or anti-hTSLP reference mAb1 in a 24-well plate for 6 days in a 37°C cell-culture incubator.

5 IL-5, CCL17, and CCL26 concentration in freshly collected supernatant was measured by Human Magnetic Luminex Assays. Percentage of inhibition were calculated relative to unstimulated (min) and stimulated (max) control samples which did not receive either ISVD polypeptides or antibodies. All calculations were performed using GraphPad Prism 8.0. Data are represented as mean $\pm$ standard error of mean (SEM) of all donors combined from 3

10 independent experiments.

Figure 10: F027400161 significantly reduced detectable levels of human TSLP in the plasma of NSG-SGM3 mice. When compared to vehicle treated mice (376.166 pg/ml), human plasma TSLP levels were reduced with the 0.1 mg/kg F027400161 dose (45.772 pg/ml) and the 10 mg/kg F027400161 dose (0.072 pg/ml), demonstrating that the TSLP arm of

15 F027400161 can bind to human TSLP in the plasma of humanized NSG-SGM3 mice.

Figure 11: F027400161 significantly reduced detectable levels of human IL-13 in the plasma of NSG-SGM3 mice. When compared to vehicle treated mice (274.052 pg/ml), human IL-13 levels were reduced with the 0.01 mg/kg F027400161 dose (201.286 pg/ml), 0.05 mg/kg F027400161 dose (22.028 pg/ml), 0.1 mg/kg F027400161 dose (40.740 pg/ml), and

20 with the 10 mg/kg F027400161 dose (1.777 pg/ml), demonstrating that the IL-13 arm of F027400161 can bind to human IL-13 in the plasma of humanized NSG-SGM3 mice.

Figures 12 and 13: F027400161 Significantly reduced mouse Retnla and Clca1 transcript expression in the lungs of NSG-SGM3 mice that received hydrodynamic delivery of hTSLP and hIL-4. Overexpression of human TSLP and IL-4 in the NSG-SGM3 mice induces the

25 production of hIL-13 from human immune cells derived from the precursor CD34⁺. Neutralization of the human IL-13 by F027400161 resulted in the inhibition of the mouse Retnla and Clca1 marker genes at the 10mg/Kg dose.

Figure 14: Schematic presentation of ISVD construct F027400161 showing from the N-terminus to the C-terminus the monovalent building blocks/ISVDs 4B02, 4B06, 501A02,

529F10, as well as the albumin binder ALB23002. Whereas 4B02 and 4B06 are connected via a 35GS linker, the remaining building block are linked via 9GS linkers.

5 Detailed description of the present technology

The present technology aims at providing a novel type of drug for treating inflammatory diseases, such as atopic dermatitis and asthma.

In some embodiments, the present technology relates to a polypeptide targeting IL-13 and TSLP at the same time leading to an increased efficiency of modulating a type 2 inflammatory response as compared to monospecific anti-IL-13 or anti-TSLP polypeptides *in vitro* and/or *in vivo*. In some embodiments, the polypeptides are efficiently produced (e.g. in microbial hosts). Furthermore, in some embodiments, such polypeptides could be shown to have limited reactivity to pre-existing antibodies in the subject to be treated (i.e. antibodies present in the subject before the first treatment with the antibody construct). In other embodiments such polypeptides exhibit a half-life in the subject to be treated that is long enough such that consecutive treatments can be conveniently spaced apart.

5.1 The polypeptides of the present technology

Monospecific-monovalent polypeptides

In one embodiment, the polypeptide of the present technology is monospecific and monovalent.

The term “*monospecific*” refers to the binding to one (specific) type of target molecule(s). A monospecific polypeptide of the present technology thus specifically binds to IL-13. Another monospecific polypeptide of the present technology specifically binds to TSLP.

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

Accordingly, in one aspect, the present technology provides a monospecific-monovalent polypeptide comprising or consisting of one ISVD that specifically binds to IL-13, which comprises three complementarity determining regions (CDR1 to CDR3, respectively). The ISVD can be selected from an ISVD comprising:

- 5 a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17; or
- 10 b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13, and a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18.

In one embodiment of this aspect of the present technology, the ISVD specifically binds to human IL-13.

- 15 In a further embodiment, the ISVD specifically binding to IL-13 is selected from an ISVD comprising:
- a) a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17; or
- 20 b) a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18.

In a further embodiment of this aspect of the present technology, the ISVD specifically binding to IL-13 is selected from an ISVD comprising:

- 25 a) a sequence identity of more than 90% with SEQ ID NO: 2; or
- b) a sequence identity of more than 90% with SEQ ID NO: 3.

In one embodiment, the ISVD specifically binding to IL-13 is selected from an ISVD comprising the amino acid sequence of SEQ ID NO: 2; or the amino acid sequence of SEQ ID NO: 3.

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

a) a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19; or

b) a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

In one embodiment of this aspect of the present technology, the ISVD specifically binds to human TSLP.

In a further embodiment, the ISVD specifically binding to TSLP is selected from an ISVD comprising:

a) a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19; or

b) a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

In a further embodiment of this aspect of the present technology, the ISVD specifically binding to TSLP is selected from an ISVD comprising:

- a) a sequence identity of more than 90% with SEQ ID NO: 4; or
- b) a sequence identity of more than 90% with SEQ ID NO: 6.

- 5 In one embodiment, the ISVD specifically binding to TSLP is selected from an ISVD comprising the amino acid sequence of SEQ ID NO: 4; or the amino acid sequence of SEQ ID NO: 6.

Monospecific-multivalent polypeptides

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

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

Accordingly, in one aspect the present technology provides a monospecific-bivalent
15 polypeptide comprising or consisting of two ISVDs that specifically bind to IL-13, wherein each of the two ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the two ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- a) a first and a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID
20 NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17;
- 25 b) a first and a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13, and a CDR3 that is the amino

acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18;

5 c) a first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17, and

10 a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13, and a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18; or

15 d) a first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13, and a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18, and

20 a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.

25

In one embodiment of this aspect of the present technology, the ISVDs are linked via one or more peptidic linkers. In one embodiment, the two ISVDs specifically bind human IL13.

In a further embodiment, the monospecific-bivalent polypeptide comprises or consists of two ISVDs that specifically bind to IL-13, wherein:

- 5 a) the first and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17;
- b) the first and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18;
- 10 c) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18; or
- 15 d) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17.

20 In a further embodiment of this aspect of the present technology, the monospecific-bivalent polypeptide comprises or consists of two ISVDs that specifically bind to IL-13, wherein:

- a) the amino acid sequence of the first and the second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2;
- b) the amino acid sequence of the first and the second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3;
- 25 c) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2 and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 3; or

d) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3 and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 2.

In one embodiment, the monospecific-bivalent polypeptide comprises or consists of two
5 ISVDs that specifically bind to IL-13, wherein:

- a) the first and the second ISVD comprise the amino acid sequence of SEQ ID NO: 2;
- b) the first and the second ISVD comprise the amino acid sequence of SEQ ID NO: 3;
- c) the first ISVD comprises the amino acid sequence of SEQ ID NO: 2 and the second ISVD comprises the amino acid sequence of SEQ ID NO: 3; or
- 10 d) the first ISVD comprises the amino acid sequence of SEQ ID NO: 3 and the second ISVD comprises the amino acid sequence of SEQ ID NO: 2.

In another aspect the present technology provides a monospecific-bivalent polypeptide comprising or consisting of two ISVDs that specifically bind to TSLP, wherein each of the two ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively),
15 wherein the two ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- a) a first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a CDR3 that is the amino acid
20 sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21, and
- a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence
25 with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, or

- 5 b) a first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, and
- 10 a second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

In one embodiment of this aspect of the present technology, the ISVDs are linked via one or more peptidic linkers. In one embodiment, the two ISVDs specifically bind human TSLP.

- 15 In a further embodiment, the monospecific-bivalent polypeptide comprises or consists of two ISVDs that specifically bind to TSLP, wherein:

- 20 a) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, or
- 25 b) the first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19, and the second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

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

- a) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 6 and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 4, or
- b) the amino acid sequence of the first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 4 and the second ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 6.

In one embodiment, the monospecific-bivalent polypeptide comprises or consists of two ISVDs that specifically bind to TSLP, wherein:

- a) the first ISVD comprises the amino acid sequence of SEQ ID NO: 6 and the second ISVD comprises the amino acid sequence of SEQ ID NO: 4; or
- b) the first ISVD comprises the amino acid sequence of SEQ ID NO: 4 and the second ISVD comprises the amino acid sequence of SEQ ID NO: 6.

The terms “first ISVD” and “second ISVD” in this regard only indicate the relative position of the specifically recited ISVDs binding to IL-13/TSLP to each other, wherein the numbering is started from the N-terminus of the polypeptide of the present technology. The “first ISVD” is thus closer to the N-terminus than the “second ISVD”. Accordingly, the “second ISVD” is thus closer to the C-terminus than the “first ISVD”. Since the numbering is thus not absolute and only indicates the relative position of the two ISVDs it does not exclude the possibility that additional binding units/building blocks such as ISVDs binding to IL-13 and TSLP, respectively, can be present in the polypeptide. Moreover, it does not exclude the possibility that other binding units/building blocks such as ISVDs can be placed in between. For instance, as described further below (see in particular, section “multispecific-multivalent polypeptides” and 5.4 “(In vivo) half-life extension”), the polypeptide can further comprise another ISVD binding to human serum albumin that can even be located between the “first ISVD” and “second ISVD” (such a construct is then referred to as multispecific as described in the subsequent section).

In one embodiment, the (at least two) ISVDs of the monospecific-multivalent polypeptides, in particular of the above described monospecific-bivalent polypeptides, are linked via peptidic linkers. The use of peptidic linkers to connect two or more (poly)peptides is well

known in the art. Exemplary peptidic linkers that can be used with the monospecific-multivalent polypeptides, in particular with the above described monospecific-bivalent polypeptides are shown in Table A-5. One often used class of peptidic linkers is known as the “Gly-Ser” or “GS” linkers. These are linkers that essentially consist of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif such as the GGGGS (SEQ ID NO: 73) motif (for example, comprising the formula (Gly-Gly-Gly-Gly-Ser)_n in which n may be 1, 2, 3, 4, 5, 6, 7 or more). Some often used examples of such GS linkers are 9GS linkers (GGGGSGGGGS, SEQ ID NO: 76) 15GS linkers (n=3) and 35GS linkers (n=7). Reference is for example made to Chen et al., Adv. Drug Deliv. Rev. 2013 Oct 15; 65(10): 1357–1369; and Klein et al., Protein Eng. Des. Sel. (2014) 27 (10): 325-330. In one embodiment of the present technology, the ISVDs of the monospecific-multivalent polypeptides, in particular the monospecific-bivalent polypeptides of the present technology are linked via a linker set forth in Table A-5. In one embodiment, the (at least) two ISVDs are linked via a 35GS linker(s).

Accordingly, in one embodiment, the monospecific-bivalent polypeptide comprises or consists of:

- the amino acid sequence of SEQ ID NO: 148,
- the amino acid sequence of SEQ ID NO: 149,
- the amino acid sequence of SEQ ID NO: 150,
- the amino acid sequence of SEQ ID NO: 151,
- the amino acid sequence of SEQ ID NO: 152,
- the amino acid sequence of SEQ ID NO: 153,
- the amino acid sequence of SEQ ID NO: 154,
- the amino acid sequence of SEQ ID NO: 155,
- the amino acid sequence of SEQ ID NO: 156,
- the amino acid sequence of SEQ ID NO: 157,
- the amino acid sequence of SEQ ID NO: 158, or
- the amino acid sequence of SEQ ID NO: 159.

Multispecific-multivalent polypeptides

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

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

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

For example, the polypeptide may be bispecific-tetravalent, such as a polypeptide comprising or consisting of at least four ISVDs, wherein at least two ISVD specifically bind to IL-13 and at least two ISVDs specifically bind to TSLP. In one embodiment, IL-13 and TSLP are human IL-13 and human TSLP. In another example, the polypeptide may be trispecific-pentavalent, such as a polypeptide comprising or consisting of five ISVDs, wherein two ISVDs specifically bind to human IL-13, two ISVDs specifically bind to human TSLP and one ISVD binds to human serum albumin. Such a polypeptide may at the same time be biparatopic, for example if two ISVDs bind two different epitopes on human IL-13 or human TSLP. The term "biparatopic" refers to binding to two different parts (e.g., epitopes) of the same target molecule. In one embodiment, the trispecific-pentavalent polypeptide of the present technology is e.g., ISVD construct F027400161, comprising two ISVDs specifically binding to human IL-13, two ISVDs specifically binding to human TSLP, one ISVD binding to human serum albumin, and which is biparatopic for both binding to IL-13 and TSLP.

In one embodiment, the multispecific-multivalent polypeptide comprises or consists of at least four ISVDs, wherein each of said ISVDs comprises three complementarity determining regions (CDR1 to CDR3, respectively), wherein the at least four ISVDs are optionally linked via one or more peptidic linkers, and wherein:

- a) a first ISVD that specifically binds IL-13 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino

acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12, and a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17;

5 b) a second ISVD that specifically binds IL-13 and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13, and a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18;

10 c) a third ISVD that specifically binds TSLP and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14, and a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19, and

15 d) a fourth ISVD that specifically binds TSLP and comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16, and a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

In one embodiment, the IL-13 and TSLP bound by said polypeptide is human IL-13 and human TSLP, respectively.

In a further embodiment of the multispecific-multivalent polypeptide:

25 a) said first ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17;

b) said second ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18;

5 c) said third ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19; and

d) said fourth ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

10 In a further aspect of the multispecific-multivalent polypeptide:

a) the amino acid sequence of said first ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 2;

b) the amino acid sequence of said second ISVD comprises a sequence identity of more than 90% with SEQ ID NO: 3;

15 c) the amino acid sequence of said third ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 4; and

d) the amino acid sequence of said fourth ISVD comprises a sequence identity of more than 90% identity with SEQ ID NO: 6.

In one embodiment of the multispecific-multivalent polypeptide:

20 a) the first ISVD comprises the amino acid sequence of SEQ ID NO: 2;

b) the second ISVD comprises the amino acid sequence of SEQ ID NO: 3;

c) the third ISVD comprises the amino acid sequence of SEQ ID NO: 4; and

d) the fourth ISVD comprises the amino acid sequence of SEQ ID NO: 6.

25 The terms "first ISVD", "second ISVD", "third ISVD", etc., in this regard only indicate the relative position of the ISVDs to each other, wherein the numbering is started from the N-terminus of the polypeptide of the present technology. The "first ISVD" is thus closer to the

N-terminus than the “second ISVD”, whereas the “second ISVD” is closer to the N-terminus than the “third ISVD”. Accordingly, the ISVD arrangement is inverse when considered from the C-terminus. Since the numbering is not absolute and only indicates the relative position of the at least four ISVDs it is not excluded that other binding units/building blocks such as additional ISVDs binding to IL-13 or TSLP, or ISVDs binding to another target may be present in the polypeptide. Moreover, it does not exclude the possibility that other binding units/building blocks such as ISVDs can be placed in between. For instance, as described further below (see in particular, section 5.4 “(In vivo) half-life extension” below), the polypeptide can further comprise another ISVD binding to human serum albumin that can even be located between e.g. the “third ISVD” and “fourth ISVD”.

In another aspect the present technology provides a bispecific-bivalent polypeptide comprising an ISVD that specifically binds to IL-13 or TSLP as described in detail for the monospecific-monovalent polypeptides above (section 5.1; “Monospecific-monovalent polypeptides”) and an ISVD binding to human serum albumin as described in detail below (section 5.4; “(In vivo) half-life extension”).

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

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

The use of linkers to connect two or more (poly)peptides is well known in the art. Exemplary peptidic linkers are shown in Table A-5. One often used class of peptidic linker are known as the “Gly-Ser” or “GS” linkers. These are linkers that essentially consist of glycine (G) and serine (S) residues, and usually comprise one or more repeats of a peptide motif such as the GGGGS (SEQ ID NO: 73) motif (for example, comprising the formula (Gly-Gly-Gly-Gly-Ser)_n in which n may be 1, 2, 3, 4, 5, 6, 7 or more). Some often used examples of such GS linkers are 9GS linkers (GGGGSGGGGS, SEQ ID NO: 76) 15GS linkers (n=3) (SEQ ID NO: 78) and 35GS linkers (n=7) (SEQ ID NO: 83). Reference is for example made to Chen et al., Adv. Drug Deliv. Rev. 2013 Oct 15; 65(10): 1357–1369; and Klein et al., Protein Eng. Des. Sel. (2014) 27 (10):

325-330. In the polypeptide(s) of the present technology, the 9GS (SEQ ID NO: 76) and 35GS (SEQ ID NO: 83) linkers are used to link the components of the polypeptide to each other.

In one aspect of the multispecific-multivalent polypeptide of the present technology, the polypeptide comprising or consisting of at least four ISVDs, comprises the at least two ISVDs specifically binding to IL-13 and at least two ISVDs specifically binding to TSLP. In this aspect of the present technology, the at least two ISVDs binding to IL-13 are linked via a 35GS linker, whereas the at least two ISVDs specifically binding to TSLP are linked via a 9GS linker. In one embodiment, the at least two ISVDs specifically binding to TSLP are separated by an ISVD binding to albumin (9GS-Alb-9GS) (as described in section 5.4 “(In vivo) half-life extension” below). The inventors surprisingly found that such a configuration can increase the production yield of the polypeptide.

Accordingly, in one embodiment, the polypeptide comprises or consists of the following, in the order starting from the N-terminus of the polypeptide: a first ISVD specifically binding to IL-13, a second ISVD specifically binding to IL-13, a first ISVD specifically binding to TSLP, an optional binding unit providing the polypeptide with increased half-life as defined herein, and a second ISVD specifically binding to TSLP. In one embodiment, the binding unit providing the polypeptide with increased half-life is an ISVD.

In a further embodiment, the polypeptide comprises or consists of the following, in the order starting from the N-terminus of the polypeptide: an ISVD specifically binding to IL-13, a linker, a second ISVD specifically binding to IL-13, a linker, a first ISVD specifically binding to TSLP, a linker, an ISVD binding to human serum albumin, a linker, and a second ISVD specifically binding to TSLP. In one specific embodiment, the linker between the two ISVDs binding to IL-13 is a 35GS linker, whereas the other linkers are 9GS linkers.

Such configurations of the polypeptide can provide for increased production yield, good CMC characteristics, such as sufficient solubility and biophysical stability, strong potencies with regard to modulation of a type 2 immune response as well as low binding to pre-existing antibodies.

In one embodiment, the multispecific-multivalent polypeptide of the present technology exhibits reduced binding by pre-existing antibodies in human serum. To this end, in one

embodiment, the polypeptide comprises a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in at least one ISVD. In one embodiment, the polypeptide comprises a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in each ISVD. In another embodiment, the polypeptide comprises an extension of 1 to 5 (naturally occurring) amino acids, such as a single alanine (A) extension, at the C-terminus of the C-terminal ISVD. The C-terminus of an ISVD is normally VTVSS (SEQ ID NO: 138). In another embodiment, the polypeptide comprises a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD. In another embodiment, the ISVD comprises a lysine (K) or glutamine (Q) at position 112 (according to Kabat numbering) in at least one ISVD. In these embodiments, the C-terminus of the ISVD is VKVSS (SEQ ID NO: 139), VQVSS (SEQ ID NO: 140), VTVKS (SEQ ID NO:166), VTVQS (SEQ ID NO:167), VKVKS (SEQ ID NO:168), VKVQS (SEQ ID NO:169), VQVKS (SEQ ID NO:170), or VQVQS (SEQ ID NO:171) such that after addition of a single alanine the C-terminus of the polypeptide for example comprises the sequence VTVSSA (SEQ ID NO: 141), VKVSSA (SEQ ID NO: 142), VQVSSA (SEQ ID NO: 143), VTVKSA (SEQ ID NO:172), VTVQSA (SEQ ID NO:173), VKVKSA (SEQ ID NO:174), VKVQSA (SEQ ID NO:175), VQVKSA (SEQ ID NO:176), or VQVQSA (SEQ ID NO:177). In one embodiment, the C-terminus comprises VTVSSA (SEQ ID NO: 141). In another embodiment, the polypeptide comprises a valine (V) at amino acid position 11 and a leucine (L) at amino acid position 89 (according to Kabat numbering) in each ISVD, optionally a lysine (K) or glutamine (Q) at position 110 (according to Kabat numbering) in at least one ISVD and comprises an extension of 1 to 5 (naturally occurring) amino acids, such as a single alanine (A) extension, at the C-terminus of the C-terminal ISVD (such that the C-terminus of the polypeptide for example has the sequence VTVSSA (SEQ ID NO: 141), VKVSSA (SEQ ID NO: 142) or VQVSSA (SEQ ID NO: 143), such as VTVSSA (SEQ ID NO: 141)). See e.g. WO2012/175741 and WO2015/173325 for further information in this regard.

In one embodiment, the multispecific-multivalent polypeptide of the present technology comprises or consists of an amino acid sequence comprising a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 1, wherein the CDRs of the five ISVDs are as defined in items A to E (or A' to E' if using the Kabat definition) set forth

in sections “5.2 Immunoglobulin single variable domains” and “5.4 (In vivo) half-life extension” below, respectively, wherein in particular:

- the first ISVD specifically binding to IL-13 has a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17;
- the second ISVD specifically binding to IL-13 has a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18;
- the third ISVD specifically binding to TSLP has a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19;
- the fourth ISVD specifically binding to TSLP has a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21; and
- the ISVD binding to human serum albumin has a CDR1 that is the amino acid sequence of SEQ ID NO: 10, a CDR2 that is the amino acid sequence of SEQ ID NO: 15 and a CDR3 that is the amino acid sequence of SEQ ID NO: 20,

or alternatively if using the Kabat definition:

- the first ISVD specifically binding to IL-13 has a CDR1 that is the amino acid sequence of SEQ ID NO: 37, a CDR2 that is the amino acid sequence of SEQ ID NO: 42 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17;
- the second ISVD specifically binding to IL-13 has a CDR1 that is the amino acid sequence of SEQ ID NO: 38, a CDR2 that is the amino acid sequence of SEQ ID NO: 43 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18;
- the third ISVD specifically binding to TSLP has a CDR1 that is the amino acid sequence of SEQ ID NO: 39, a CDR2 that is the amino acid sequence of SEQ ID NO: 44 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19;
- the fourth ISVD specifically binding to TSLP has a CDR1 that is the amino acid sequence of SEQ ID NO: 41, a CDR2 that is the amino acid sequence of SEQ ID NO: 46 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21; and

- the ISVD binding to human serum albumin has a CDR1 that is the amino acid sequence of SEQ ID NO: 40, a CDR2 that is the amino acid sequence of SEQ ID NO: 45 and a CDR3 that is the amino acid sequence of SEQ ID NO: 20.

In another embodiment, the polypeptide comprises or consists of the amino acid sequence of SEQ ID NO: 1. In another embodiment, the polypeptide consists of the amino acid sequence of SEQ ID NO: 1.

In one embodiment, the polypeptide of the present technology has at least half the binding affinity, or at least the same binding affinity, to human IL-13 and to human TSLP as compared to a polypeptide consisting of the amino acid of SEQ ID NO: 1 wherein the binding affinity is measured using the same method, such as Surface Plasmon Resonance (SPR).

5.2 Immunoglobulin single variable domains

The term “immunoglobulin single variable domain” (ISVD), interchangeably used with “single variable domain”, defines immunoglobulin molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets ISVDs apart from “conventional” immunoglobulins (e.g. monoclonal antibodies) or their fragments (such as Fab, Fab', F(ab')₂, scFv, di-scFv), wherein two immunoglobulin domains, in particular two variable domains, interact to form an antigen binding site. Typically, in conventional immunoglobulins, a heavy chain variable domain (V_H) and a light chain variable domain (V_L) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both V_H and V_L will contribute to the antigen binding site, i.e. a total of 6 CDRs will be involved in antigen binding site formation.

In view of the above definition, the antigen-binding domain of a conventional 4-chain antibody (such as an IgG, IgM, IgA, IgD or IgE molecule; known in the art) or of a Fab fragment, a F(ab')₂ fragment, an Fv fragment such as a disulphide linked Fv or a scFv fragment, or a diabody (all known in the art) derived from such conventional 4-chain antibody, would normally not be regarded as an ISVD, as, in these cases, binding to the respective epitope of an antigen would normally not occur by one (single) immunoglobulin domain but by a pair of (associating) immunoglobulin domains such as light and heavy chain

variable domains, i.e., by a V_H - V_L pair of immunoglobulin domains, which jointly bind to an epitope of the respective antigen.

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

As such, the single variable domain may be a light chain variable domain sequence (e.g., a V_L -sequence) or a suitable fragment thereof; or a heavy chain variable domain sequence (e.g., a V_H -sequence or V_{HH} sequence) or a suitable fragment thereof; as long as it is capable of forming a single antigen binding unit (i.e., a functional antigen binding unit that essentially
10 consists of the single variable domain, such that the single antigen binding domain does not need to interact with another variable domain to form a functional antigen binding unit).

An ISVD can for example be a heavy chain ISVD, such as a V_H , V_{HH} , including a camelized V_H or humanized V_{HH} . In one embodiment, it is a V_{HH} , including a camelized V_H or humanized V_{HH} . Heavy chain ISVDs can be derived from a conventional four-chain antibody or from a heavy
15 chain antibody.

For example, the ISVD may be a single domain antibody (or an amino acid sequence that is suitable for use as a single domain antibody), a "dAb" or dAb (or an amino acid sequence that is suitable for use as a dAb) or a Nanobody® (as defined herein, and including but not limited to a V_{HH}); other single variable domains, or any suitable fragment of any one thereof.

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

" V_{HH} domains", also known as V_{HHS} , V_{HH} antibody fragments, and V_{HH} antibodies, have originally been described as the antigen binding immunoglobulin variable domain of "heavy
25 chain antibodies" (i.e., of "antibodies devoid of light chains"; Hamers-Casterman et al. Nature 363: 446-448, 1993). The term " V_{HH} domain" has been chosen in order to distinguish these variable domains from the heavy chain variable domains that are present in conventional 4-chain antibodies (which are referred to herein as " V_H domains") and from the light chain variable domains that are present in conventional 4-chain antibodies (which are

referred to herein as “V_L domains”). For a further description of V_{HH}’s, reference is made to the review article by Muyldermans (Reviews in Molecular Biotechnology 74: 277-302, 2001).

Typically, the generation of immunoglobulins involves the immunization of experimental animals, fusion of immunoglobulin producing cells to create hybridomas and screening for
5 the desired specificities. Alternatively, immunoglobulins can be generated by screening of naïve or synthetic libraries e.g. by phage display.

The generation of immunoglobulin sequences, such as Nanobodies®, has been described extensively in various publications, among which WO 94/04678, Hamers-Casterman *et al.* 1993 and Muyldermans *et al.* 2001 can be exemplified. In these methods, camelids are
10 immunized with the target antigen in order to induce an immune response against said target antigen. The repertoire of Nanobodies obtained from said immunization is further screened for Nanobodies that bind the target antigen.

In these instances, the generation of antibodies requires purified antigen for immunization and/or screening. Antigens can be purified from natural sources, or in the course of
15 recombinant production.

Immunization and/or screening for immunoglobulin sequences can be performed using peptide fragments of such antigens.

The present technology may use immunoglobulin sequences of different origin, comprising mouse, rat, rabbit, donkey, human and camelid immunoglobulin sequences. The present
20 technology also includes fully human, humanized or chimeric sequences. For example, the present technology comprises camelid immunoglobulin sequences and humanized camelid immunoglobulin sequences, or camelized domain antibodies, e.g. camelized dAb as described by Ward *et al.* (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996)). Moreover, the present technology also uses fused immunoglobulin sequences,
25 e.g. forming a multivalent and/or multispecific construct (for multivalent and multispecific polypeptides containing one or more V_{HH} domains and their preparation, reference is also made to Conrath *et al.*, J. Biol. Chem., Vol. 276, 10. 7346-7350, 2001, as well as to for example WO 96/34103 and WO 99/23221), and immunoglobulin sequences comprising tags

or other functional moieties, e.g. toxins, labels, radiochemicals, etc., which are derivable from the immunoglobulin sequences of the present technology.

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

A “camelized V_H ” comprises an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_H domain, but that has been “camelized”, i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring V_H domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_{HH} domain of a heavy chain antibody. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art (e.g. WO 2008/020079). Such “camelizing” substitutions are usually inserted at amino acid positions that form and/or are present at the V_H - V_L interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO 94/04678 and Davies and Riechmann (1994 and 1996), supra). In one embodiment, the V_H sequence that is used as a starting material or starting point for generating or designing the camelized V_H is a V_H sequence from a mammal, or the V_H sequence of a human being, such as a V_{H3} sequence. However, it should be noted that such camelized V_H can be obtained in any suitable manner known per se and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_H domain as a starting material.

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

As further described in paragraph q) on pages 58 and 59 of WO 08/020079, the amino acid residues of an ISVD can be numbered according to the general numbering for V_H domains
10 given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to V_{HH} domains from Camelids in the article of Riechmann and Muyldermans, 2000 (J. Immunol. Methods 240 (1-2): 185-195; see for example Figure 2 of this publication). It should be noted that - as is well known in the art for V_H domains and for V_{HH} domains - the total number of amino acid residues in each of
15 the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat numbering). This means that, generally, the numbering according to Kabat may or may not correspond to the actual
20 numbering of the amino acid residues in the actual sequence. The total number of amino acid residues in a V_H domain and a V_{HH} domain will usually be in the range of from 110 to 120, often between 112 and 115. It should however be noted that smaller and longer sequences may also be suitable for the purposes described herein.

In the present application, unless indicated otherwise, CDR sequences were determined
25 according to the AbM definition as described in Kontermann and Dübel (Eds. 2010, Antibody Engineering, vol 2, Springer Verlag Heidelberg Berlin, Martin, Chapter 3, pp. 33-51). According to this method, FR1 comprises the amino acid residues at positions 1-25, CDR1 comprises the amino acid residues at positions 26-35, FR2 comprises the amino acids at positions 36-49, CDR2 comprises the amino acid residues at positions 50-58, FR3 comprises

the amino acid residues at positions 59-94, CDR3 comprises the amino acid residues at positions 95-102, and FR4 comprises the amino acid residues at positions 103-113.

Determination of CDR regions may also be done according to different methods. In the CDR determination according to Kabat, FR1 of an ISVD comprises the amino acid residues at positions 1-30, CDR1 of an ISVD comprises the amino acid residues at positions 31-35, FR2 of an ISVD comprises the amino acids at positions 36-49, CDR2 of an ISVD comprises the amino acid residues at positions 50-65, FR3 of an ISVD comprises the amino acid residues at positions 66-94, CDR3 of an ISVD comprises the amino acid residues at positions 95-102, and FR4 of an ISVD comprises the amino acid residues at positions 103-113.

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

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

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

Again, as generally described herein for the immunoglobulin sequences, it is also possible to use suitable fragments (or combinations of fragments) of any of the foregoing, such as

fragments that contain one or more CDR sequences, suitably flanked by and/or linked via one or more framework sequences (for example, in the same order as these CDR's and framework sequences may occur in the full-sized immunoglobulin sequence from which the fragment has been derived).

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

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

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

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

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

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

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

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

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

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

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

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

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

5 Table A-0 below.

Table A-0: Hallmark Residues in Nanobodies

Position	Human V _H 3	Hallmark Residues
11	L, V; predominantly L	L, S, V, M, W, F, T, Q, E, A, R, G, K, Y, N, P, I; preferably L
37	V, I, F; usually V	F ⁽¹⁾ , Y, V, L, A, H, S, I, W, C, N, G, D, T, P, preferably F ⁽¹⁾ or Y
44 ⁽⁸⁾	G	E ⁽³⁾ , Q ⁽³⁾ , G ⁽²⁾ , D, A, K, R, L, P, S, V, H, T, N, W, M, I; preferably G ⁽²⁾ , E ⁽³⁾ or Q ⁽³⁾ ; most preferably G ⁽²⁾ or Q ⁽³⁾ .
45 ⁽⁸⁾	L	L ⁽²⁾ , R ⁽³⁾ , P, H, F, G, Q, S, E, T, Y, C, I, D, V; preferably L ⁽²⁾ or R ⁽³⁾
47 ⁽⁸⁾	W, Y	F ⁽¹⁾ , L ⁽¹⁾ or W ⁽²⁾ G, I, S, A, V, M, R, Y, E, P, T, C, H, K, Q, N, D; preferably W ⁽²⁾ , L ⁽¹⁾ or F ⁽¹⁾
83	R or K; usually R	R, K ⁽⁵⁾ , T, E ⁽⁵⁾ , Q, N, S, I, V, G, M, L, A, D, Y, H; preferably K or R; most preferably K
84	A, T, D; predominantly A	P ⁽⁵⁾ , S, H, L, A, V, I, T, F, D, R, Y, N, Q, G, E; preferably P
103	W	W ⁽⁴⁾ , R ⁽⁶⁾ , G, S, K, A, M, Y, L, F, T, N, V, Q, P ⁽⁶⁾ , E, C; preferably W
104	G	G, A, S, T, D, P, N, E, C, L; preferably G
108	L, M or T; predominantly L	Q, L ⁽⁷⁾ , R, P, E, K, S, T, M, A, H; preferably Q or L ⁽⁷⁾

Notes:

(1) In particular, but not exclusively, in combination with KERE or KQRE at positions 43-46.

(2) Usually as GLEW at positions 44-47.

(3) Usually as KERE or KQRE at positions 43-46, e.g. as KEREL, KEREV, KQREL, KQREV,

KEREG, KQREW or KQREG at positions 43-47. Alternatively, also sequences such as TERE (for example TEREL), TQRE (for example TQREL), KECE (for example KECEL or KECER), KQCE (for example KQCEL), RERE (for example REREG), RQRE (for example RQREL, RQREF or RQREW), QERE (for example QEREG), QQRE, (for example QQREW, QQREL or QQREF), KGRE (for example KGREG), KDRE (for example KDREV) are possible. Some other possible, but less preferred sequences include for example DECKL and NVCEL.

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

The present technology *inter alia* uses ISVDs that can bind to IL-13 or TSLP. In the context of the present technology, "binding to" a certain target molecule has the usual meaning in the art as understood in the context of antibodies and their respective antigens.

- 5 The multispecific-multivalent polypeptide of the present technology may comprise two or more ISVDs specifically binding to IL-13 and two or more ISVDs specifically binding to TSLP. For example, the polypeptide may comprise two ISVDs that specifically bind to IL-13 and two ISVDs that specifically bind to TSLP.

In some embodiments, at least one ISVD can functionally block its target molecule. For
 10 example, targeting moieties can block the interaction between IL-13 and IL-13 α 1 (Interleukin 13 receptor, α 1) and/or the interaction between IL-13/IL-13 α 1 complex and IL-4 α (alpha interleukin-4 receptor), or can block the interaction between TSLP and TSLPR (TSLP receptor) and/or TSLP/TSLPR complex and IL-7 α (Interleukin-7 receptor

subunit alpha). Accordingly, in one embodiment, the polypeptide of the present technology comprises at least two ISVDs that specifically bind to IL-13 and functionally block its interaction with IL-13R α 1 and/or the interaction between IL-13/IL-13R α 1 complex and IL-4R α , and two ISVDs that specifically bind to TSLP and functionally block its interaction with TSLPR and/or the interaction between TSLP/TSLPR complex and IL-7R α .

The ISVDs used in the present technology form part of a polypeptide of the present technology, which comprises or consists of at least four ISVDs, such that the polypeptide can specifically bind to IL-13 and TSLP.

Accordingly, the target molecules of the at least four ISVDs as used in the polypeptide of the present technology are IL-13 and TSLP. Examples are mammalian IL-13 and TSLP. Besides human IL-13 (Uniprot accession P35225) and human TSLP (Uniprot accession Q969D9), the versions from other species are also amenable to the present technology, for example IL-13 and TSLP from mice, rats, rabbits, cats, dogs, goats, sheep, horses, pigs, non-human primates, such as cynomolgus monkeys (also referred to herein as "*cyno*"), or camelids, such as llama or alpaca.

Specific examples of ISVDs specifically binding to IL-13 that can be used in the present technology are as described in the following items A and B:

A. An ISVD that specifically binds to human IL-13 and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 7 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 7;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 12 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 12; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 7, a CDR2 that is the amino acid sequence of SEQ ID NO: 12 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17.

B. An ISVD that specifically binds to human IL-13 and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 8 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 8;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 13 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 13; and
- 5 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 8, a CDR2 that is the amino acid sequence of SEQ ID NO: 13 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18.

10 Examples of such an ISVD that specifically binds to human IL-13 have one or more, or all, framework regions as indicated for construct 4B02 or 4B06, respectively, in Table A-2 (in addition to the CDRs as defined in the preceding items A and B, respectively). In one embodiment, it is an ISVD comprising or consisting of the full amino acid sequence of construct 4B02 or construct 4B06 (SEQ ID NOs: 2 and 3, respectively; see Table A-1 and A-2).

15 In another embodiment, the amino acid sequence of the ISVD(s) specifically binding to human IL-13 may have a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 2 or 3 respectively, wherein the CDRs are as defined in the preceding item A or B, respectively. In one embodiment, the ISVD specifically binding to IL-13 comprises or consists of the amino acid sequence of SEQ ID NO: 2 or 3.

20 When such an ISVD binding to IL-13 has 2 or 1 amino acid difference in at least one CDR relative to a corresponding reference CDR sequence (item A or B above), the ISVD has at least half the binding affinity, or at least the same binding affinity to human IL-13 as the construct 4B02 or 4B06 set forth in SEQ ID NO: 2 or 3, respectively, wherein the binding affinity is measured using the same method, such as SPR.

25 Specific examples of ISVDs specifically binding to TSLP that can be used in the present technology are as described in the following items C and D:

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

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 9 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 9;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 14 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 14; and
- 5 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 9, a CDR2 that is the amino acid sequence of SEQ ID NO: 14 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19.

- 10 D. An ISVD that specifically binds to human TSLP and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 11 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 11;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 16 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 16; and
 - 15 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 11, a CDR2 that is the amino acid sequence of SEQ ID NO: 16 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

- 20 Examples of such an ISVD that specifically binds to human TSLP have one or more, or all, framework regions as indicated for construct 501A02 and 529F10, respectively, in Table A-2 (in addition to the CDRs as defined in the preceding items C and D). In one embodiment, it is an ISVD comprising or consisting of the full amino acid sequence of construct 501A02 or 529F10 (SEQ ID NOs: 4 or 6, see Table A-1 and A-2).

- 25 In another embodiment, the amino acid sequence of an ISVD(s) specifically binding to human TSLP may have a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 4 or 6, respectively, wherein the CDRs are as defined in the

preceding item C or D. In one embodiment, the ISVD binding to human TSLP comprises or consists of the amino acid sequence of SEQ ID NOs: 4 or 6.

When such an ISVD binding to human TSLP has 2 or 1 amino acid difference in at least one CDR relative to a corresponding reference CDR sequence (item C or D above), the ISVD has at least half the binding affinity, or at least the same binding affinity to human TSLP as construct 501A02 or 529F10 set forth in SEQ ID NO: 4 and 6, respectively, wherein the binding affinity is measured using the same method, such as SPR.

In an embodiment, each of the ISVDs as defined under items A to D above is comprised in the polypeptide of the present technology.

Such a polypeptide of the present technology comprising each of the ISVDs as defined under items A to D above has at least half the binding affinity, or at least the same binding affinity, to human IL-13 and to human TSLP as a polypeptide consisting of the amino acid of SEQ ID NO: 1, wherein the binding affinity is measured using the same method, such as SPR.

The SEQ ID NOs referred to in the above items A to D and item E below (see section 5.4 “(In vivo) half-life extension”) are based on the CDR definition according to the AbM definition (see Table A-2). It is noted that the SEQ ID NOs defining the same CDRs according to the Kabat definition (see Table A-2.1) can likewise be used in the above items A to D and item E below (see section 5.4 “(In vivo) half-life extension”).

Accordingly, the specific examples of ISVDs specifically binding to IL-13 or TSLP that can be used in the present technology are as described above using the AbM definition can be also described using the Kabat definition as set forth in items A' to D' below:

A'. An ISVD that specifically binds to human IL-13 and comprises

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 37 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 37;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 42 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 42; and
- iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 17 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 17.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 37, a CDR2 that is the amino acid sequence of SEQ ID NO: 42 and a CDR3 that is the amino acid sequence of SEQ ID NO: 17.

B'. An ISVD that specifically binds to human IL-13 and comprises

- 5 i. a CDR1 that is the amino acid sequence of SEQ ID NO: 38 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 38;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 43 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 43; and
- 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 18 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 18.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 38, a CDR2 that is the amino acid sequence of SEQ ID NO: 43 and a CDR3 that is the amino acid sequence of SEQ ID NO: 18.

15 Examples of such an ISVD(s) that specifically binds to human IL-13 have one or more, or all, framework regions as indicated for construct 4B02 or 4B06, respectively, in Table A-2-1 (in addition to the CDRs as defined in the preceding items A' and B', respectively). In one embodiment it is an ISVD comprising or consisting of the full amino acid sequence of construct 4B02 or construct 4B06 (SEQ ID NOs: 2 and 3, respectively; see Table A-1 and A-2-1).

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

- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 39 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 39;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 44 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 44; and
- 25 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 19 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 19.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 39, a CDR2 that is the amino acid sequence of SEQ ID NO: 44 and a CDR3 that is the amino acid sequence of SEQ ID NO: 19.

D'. An ISVD that specifically binds to human TSLP and comprises

- 5 i. a CDR1 that is the amino acid sequence of SEQ ID NO: 41 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 41;
- ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 46 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 46; and
- 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 21 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 21.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 41, a CDR2 that is the amino acid sequence of SEQ ID NO: 46 and a CDR3 that is the amino acid sequence of SEQ ID NO: 21.

15 Examples of such an ISVD(s) that specifically binds to human TSLP have one or more, or all, framework regions as indicated for construct 501A02 and 529F10, respectively, in Table A-2-1 (in addition to the CDRs as defined in the preceding items C' and D'). In one embodiment, it is an ISVD comprising or consisting of the full amino acid sequence of construct 501A02 or 529F10 (SEQ ID NOs: 4 or 6, see Table A-1 and A-2-1).

20 The percentage of "sequence identity" between a first amino acid sequence and a second amino acid sequence may be calculated by dividing [*the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence*] by [*the total number of amino acid residues in the first amino acid sequence*] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (i.e. at a single position).

25

Usually, for the purpose of determining the percentage of "sequence identity" between two amino acid sequences in accordance with the calculation method outlined hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the

“first” amino acid sequence, and the other amino acid sequence will be taken as the “second” amino acid sequence.

An “amino acid difference” as used herein refers to a deletion, insertion or substitution of a single amino acid residue vis-à-vis a reference sequence. In one embodiment, an “amino acid difference” is a substitution.

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

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

5.3 Specificity

The terms “specificity”, “binding specifically” or “specific binding” refer to the number of different target molecules, such as antigens, from the same organism to which a particular binding unit, such as an ISVD, can bind with sufficiently high affinity (see below). “Specificity”, “binding specifically” or “specific binding” are used interchangeably herein with “selectivity”, “binding selectively” or “selective binding”. Binding units, such as ISVDs, specifically bind to their designated targets.

The specificity/selectivity of a binding unit can be determined based on affinity. The affinity denotes the strength or stability of a molecular interaction. The affinity is commonly given as by the KD, or dissociation constant, which has units of mol/liter (or M). The affinity can also

be expressed as an association constant, K_A , which equals $1/K_D$ and has units of $(\text{mol/liter})^{-1}$ (or M^{-1}).

The affinity is a measure for the binding strength between a moiety and a binding site on the target molecule: the lower the value of the K_D , the stronger the binding strength between a target molecule and a targeting moiety.

Typically, binding units used in the present technology (such as ISVDs) will bind to their targets with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, or 10^{-7} to 10^{-12} moles/liter or less, or 10^{-8} to 10^{-12} moles/liter (i.e. with an association constant (K_A) of 10^5 to 10^{12} liter/ moles or more, or 10^7 to 10^{12} liter/moles or more, or 10^8 to 10^{12} liter/moles).

Any K_D value greater than 10^{-4} mol/liter (or any K_A value lower than 10^4 liters/mol) is generally considered to indicate non-specific binding.

The K_D for biological interactions, such as the binding of immunoglobulin sequences to an antigen, which are considered specific are typically in the range of 10^{-5} moles/liter (10000 nM or $10\mu\text{M}$) to 10^{-12} moles/liter (0.001 nM or 1 pM) or less.

Accordingly, specific/selective binding may mean that - using the same measurement method, e.g. SPR - a binding unit (or polypeptide comprising the same) binds to IL13 and/or TSLP with a K_D value of 10^{-5} to 10^{-12} moles/liter or less and binds to related cytokines with a K_D value greater than 10^{-4} moles/liter. Examples of IL13 related targets are human IL4. Examples of related cytokines for TSLP are human IL7. Thus, in an embodiment of the present technology, at least two ISVDs comprised in the polypeptide binds to IL13 with a K_D value of 10^{-5} to 10^{-12} moles/liter or less and binds to IL4 of the same species with a K_D value greater than 10^{-4} moles/liter, and at least two ISVDs comprised in the polypeptide bind to TSLP with a K_D value of 10^{-5} to 10^{-12} moles/liter or less and binds to human IL7 of the same species with a K_D value greater than 10^{-4} moles/liter.

Thus, the polypeptide of the present technology has at least half the binding affinity, or at least the same binding affinity, to human IL13 and to human TSLP as compared to a polypeptide consisting of the amino acid of SEQ ID NO: 1, wherein the binding affinity is measured using the same method, such as SPR.

Specific binding to a certain target from a certain species does not exclude that the binding unit can also specifically bind to the analogous target from a different species. For example, specific binding to human IL13 does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to IL13 from cynomolgus monkeys. Likewise,
5 for example, specific binding to human TSLP does not exclude that the binding unit (or a polypeptide comprising the same) can also specifically bind to TSLP from cynomolgus monkeys ("cyno").

Specific binding of a binding unit to its designated target can be determined in any suitable manner known *per se*, including, for example, Scatchard analysis and/or competitive binding
10 assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known *per se* in the art; as well as the other techniques mentioned herein.

The dissociation constant may be the actual or apparent dissociation constant, as will be clear to the skilled person. Methods for determining the dissociation constant will be clear to
15 the skilled person, and for example include the techniques mentioned below. In this respect, it will also be clear that it may not be possible to measure dissociation constants of more than 10^{-4} moles/liter or 10^{-3} moles/liter (e.g. of 10^{-2} moles/liter). Optionally, as will also be clear to the skilled person, the (actual or apparent) dissociation constant may be calculated on the basis of the (actual or apparent) association constant (K_A), by means of the
20 relationship [$K_D = 1/K_A$].

The affinity of a molecular interaction between two molecules can be measured via different techniques known *per se*, such as the well-known surface plasmon resonance (SPR) biosensor technique (see for example Ober et al. 2001, Intern. Immunology 13: 1551-1559). The term "surface plasmon resonance", as used herein, refers to an optical phenomenon
25 that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, where one molecule is immobilized on the biosensor chip and the other molecule is passed over the immobilized molecule under flow conditions yielding k_{on} , k_{off} measurements and hence K_D (or K_A) values. This can for example be performed using the well-known BIAcore® system (BIAcore International AB, a GE
30 Healthcare company, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see

Jonsson et al. (1993, Ann. Biol. Clin. 51: 19-26), Jonsson et al. (1991 Biotechniques 11: 620-627), Johnsson et al. (1995, J. Mol. Recognit. 8: 125-131), and Johnson et al. (1991, Anal. Biochem. 198: 268-277).

Another well-known biosensor technique to determine affinities of biomolecular interactions is bio-layer interferometry (BLI) (see for example Abdiche et al. 2008, Anal. Biochem. 377: 209-217). The term "bio-layer Interferometry" or "BLI", as used herein, refers to a label-free optical technique that analyzes the interference pattern of light reflected from two surfaces: an internal reference layer (reference beam) and a layer of immobilized protein on the biosensor tip (signal beam). A change in the number of molecules bound to the tip of the biosensor causes a shift in the interference pattern, reported as a wavelength shift (nm), the magnitude of which is a direct measure of the number of molecules bound to the biosensor tip surface. Since the interactions can be measured in real-time, association and dissociation rates and affinities can be determined. BLI can for example be performed using the well-known Octet® Systems (ForteBio, a division of Pall Life Sciences, Menlo Park, USA).

Alternatively, affinities can be measured in Kinetic Exclusion Assay (KinExA) (see for example Drake et al. 2004, Anal. Biochem., 328: 35-43), using the KinExA® platform (Sapidyne Instruments Inc, Boise, USA). The term "KinExA", as used herein, refers to a solution-based method to measure true equilibrium binding affinity and kinetics of unmodified molecules. Equilibrated solutions of an antibody/antigen complex are passed over a column with beads precoated with antigen (or antibody), allowing the free antibody (or antigen) to bind to the coated molecule. Detection of the antibody (or antigen) thus captured is accomplished with a fluorescently labeled protein binding the antibody (or antigen).

The GYROLAB® immunoassay system provides a platform for automated bioanalysis and rapid sample turnaround (Fraley et al. 2013, Bioanalysis 5: 1765-74).

5.4 (In vivo) half-life extension

The polypeptide may further comprise one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers, in which said one or more

other groups, residues, moieties or binding units provide the polypeptide with increased (*in vivo*) half-life, compared to the corresponding polypeptide without said one or more other groups, residues, moieties or binding units. *In vivo* half-life extension means, for example, that the polypeptide has an increased half-life in a mammal, such as a human subject, after
5 administration. Half-life can be expressed for example as $t_{1/2\beta}$.

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

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

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

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

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

In one embodiment, the polypeptide comprises a serum albumin binding moiety selected from Alb-1, Alb-3, Alb-4, Alb-5, Alb-6, Alb-7, Alb-8, Alb-9, Alb-10 and Alb-23. In one embodiment, the serum albumin binding moiety is Alb-8 or Alb-23 or its variants, as shown in pages 7-9 of WO2012/175400 and the albumin binders described in WO 2012/175741,

WO2015/173325, WO2017/080850, WO2017/085172, WO2018/104444, WO2018/134235, WO2018/134234. Some serum albumin binders are also shown in Table A-4. In one embodiment, a further component of the polypeptide of the present technology is as described in item E:

- 5 E. An ISVD that binds to human serum albumin and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 10 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 10;
 - ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 15 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 15; and
 - 10 iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 20 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 20.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 10, a CDR2 that is the amino acid sequence of SEQ ID NO: 15 and a CDR3 that is the amino acid sequence of SEQ ID NO: 20.

- 15 Examples of such an ISVD that binds to human serum albumin have one or more, or all, framework regions as indicated for construct ALB23002 in Table A-2 (in addition to the CDRs as defined in the preceding item E). In one embodiment, it is an ISVD comprising or consisting of the full amino acid sequence of construct ALB23002 (SEQ ID NO: 5, see Table A-1 and A-2).

- 20 Item E can be also described using the Kabat definition as:

- E'. An ISVD that binds to human serum albumin and comprises
- i. a CDR1 that is the amino acid sequence of SEQ ID NO: 40 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 40;
 - 25 ii. a CDR2 that is the amino acid sequence of SEQ ID NO: 45 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 45; and
 - iii. a CDR3 that is the amino acid sequence of SEQ ID NO: 20 or an amino acid sequence with 2 or 1 amino acid difference(s) with SEQ ID NO: 20.

In one embodiment, the ISVD comprises a CDR1 that is the amino acid sequence of SEQ ID NO: 40, a CDR2 that is the amino acid sequence of SEQ ID NO: 45 and a CDR3 that is the amino acid sequence of SEQ ID NO: 20.

5 Examples of such an ISVD that binds to human serum albumin have one or more, or all, framework regions as indicated for construct ALB23002 in Table A-2-1 (in addition to the CDRs as defined in the preceding item E'). In one embodiment, it is an ISVD comprising or consisting of the full amino acid sequence of construct ALB23002 (SEQ ID NO: 5, see Table A-1 and A-2-1).

10 In a further embodiment, the amino acid sequence of an ISVD binding to human serum albumin may have a sequence identity of more than 90%, such as more than 95% or more than 99%, with SEQ ID NO: 5, wherein the CDRs are as defined in the preceding item E or E'. In one embodiment, the ISVD binding to human serum albumin has the amino acid sequence of SEQ ID NO: 5.

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

20 In one embodiment, when such an ISVD binding to human serum albumin has a C-terminal position it exhibits a C-terminal alanine (A) or glycine (G) extension. In one embodiment, such an ISVD is selected from SEQ ID NOs: 59, 60, 62, 64, 65, 66, 67, 68, 69 and 71 (see table A-4 below). In one embodiment, the ISVD binding to human serum albumin has another position than the C-terminal position (i.e. is not the C-terminal ISVD of the polypeptide of the present technology). In one embodiment, such an ISVD is selected from
25 SEQ ID NOs: 5, 57, 58, 61, and 63 (see table A-4 below).

5.5 Nucleic acid molecules

Also provided is a nucleic acid molecule encoding the polypeptide of the present technology.

A "nucleic acid molecule" (used interchangeably with "nucleic acid") is a chain of nucleotide monomers linked to each other via a phosphate backbone to form a nucleotide sequence. A

nucleic acid may be used to transform/transfect a host cell or host organism, e.g. for expression and/or production of a polypeptide. Suitable hosts or host cells for production purposes will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism. A host or host cell comprising a nucleic acid encoding the polypeptide of the present technology is also encompassed by the present technology.

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

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

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

5.6 Vectors

Also provided is a vector comprising the nucleic acid molecule encoding the polypeptide of the present technology. A vector as used herein is a vehicle suitable for carrying genetic

material into a cell. A vector includes naked nucleic acids, such as plasmids or mRNAs, or nucleic acids embedded into a bigger structure, such as liposomes or viral vectors.

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

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

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

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

25 **5.7 Compositions**

The present technology also provides a composition comprising at least one polypeptide of the present technology, at least one nucleic acid molecule encoding a polypeptide of the present technology or at least one vector comprising such a nucleic acid molecule. The composition may be a pharmaceutical composition. The composition may further comprise

at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally comprise one or more further pharmaceutically active polypeptides and/or compounds.

5.8 Host organisms

- 5 The present technology also pertains to host cells or host organisms comprising the polypeptide of the present technology, the nucleic acid encoding the polypeptide of the present technology, and/or the vector comprising the nucleic acid molecule encoding the polypeptide of the present technology.

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

5.9 Methods and uses of the polypeptide

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

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

Suitable host cells or host organisms for production purposes will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or eukaryotic organism. Specific examples include HEK293
25 cells, CHO cells, *Escherichia coli* or *Pichia pastoris*. In one embodiment, the host is *Pichia pastoris*.

The polypeptide of the present technology, a nucleic acid molecule or vector as described, or a composition comprising the polypeptide of the present technology, nucleic acid molecule or vector are useful as a medicament.

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

Also provided is the polypeptide of the present technology, a nucleic acid molecule or vector as described, or a composition comprising the polypeptide of the present technology, nucleic acid molecule or vector for use in the (prophylactic or therapeutic). treatment of an
10 inflammatory disease.

Further provided is a (prophylactic and/or therapeutic) method of treating an inflammatory disease, wherein said method comprises administering, to a subject in need thereof, a pharmaceutically active amount of the polypeptide of the present technology, a nucleic acid molecule or vector as described, or a composition comprising the polypeptide of the present
15 technology, nucleic acid molecule or vector.

Further provided is the use of the polypeptide of the present technology, a nucleic acid molecule or vector as described, or a composition comprising the polypeptide of the present technology, nucleic acid molecule or vector in the preparation of a pharmaceutical composition. In one embodiment, the prepared pharmaceutical composition is for treating
20 an inflammatory disease.

The inflammatory disease is a type 2 inflammatory disease such as atopic dermatitis and asthma.

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

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

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

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

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

Table A-1: Amino acid sequences of the different monovalent V_{HH} building blocks identified within the pentavalent polypeptide F027400161 (“ID” refers to the SEQ ID NO as used herein)

Name	ID	Amino acid sequence
4B02	2	DVQLVESGGGVVQPGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAA LSGDGYSTYTANSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAAKLQY VSGWSYDYPYWGQGTLTVSS
4B06	3	EVQLVESGGGVVQPGGSLRLSCAASGFTFN NYAMKWVRQAPGKGLEWVS SITTGGGSTDYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCANVPFG YYSEHFSGLSFDYRGQGTLTVSS
501A02	4	EVQLVESGGGVVQPGGSLRLSCAASGSGFGVNILYWYRQAAGIERELIASITS GGITNYVDSVKGRFTISRDN SENTMYLQMNSLRAEDTGLYYCASRNIFDGTT EWGQGTLTVSS
ALB23002	5	EVQLVESGGGVVQPGGSLRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSS ISGSGSDTLYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCTIGGSLSR SSQGTLTVSS
529F10	6	EVQLVESGGGVVQPGGSLRLSCAASGFTFADYDYDIGWFRQAPGKEREGVS CISNRDGSYYADSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAVEIHC DDYGVENFD FDPWGQGTLTVSS

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

ID	V _{HH}	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
2	4B02	22	DVQLVESGGGV VQPGGSLRLSC AAS	7	GRTFSY RMG	24	WFRQAPGK EREFVA	12	ALSGDG YSTY	29	TANSVKGRFTISRDNKNT VYQMNSLRPEDTALYYC AA	17	KLQYVSGWS YDYPY	34	WGQGT LVTVSS
3	4B06	23	EVQLVESGGGV VQPGGSLRLSC AAS	8	GFTFNYY AMK	25	WVRQAPGK GLEWVS	13	SITGG GSTD	30	YADSVKGRFTISRDNKNT LYQMNSLRPEDTALYYCA N	18	VPFGYYSEHF SGLSFDY	35	RGQGT VTVSS
4	501A02	23	EVQLVESGGGV VQPGGSLRLSC AAS	9	GSGFGV NILEY	26	WYRQAAGIE RELIA	14	SITSGGI TN	31	YVDSVKGRFTISRDNSENT MYLQMNSLRAEDTGLYYC AS	19	RNIFDGTTE	34	WGQGT LVTVSS
5	ALB23002	23	EVQLVESGGGV VQPGGSLRLSC AAS	10	GFTFRSF GMS	27	WVRQAPGK GPEWVS	15	SISGSGS DTL	32	YADSVKGRFTISRDNKNT LYQMNSLRPEDTALYYCT I	20	GGSLSR	36	SSQGT VTVSS
6	529F10	23	EVQLVESGGGV VQPGGSLRLSC AAS	11	GFTFADY DYDIG	28	WFRQAPGK EREGVS	16	CISNRD GSTY	33	YADSVKGRFTISRDNKNT VYQMNSLRPEDTALYYC AV	21	EIHCDYGV ENFDYDP	34	WGQGT LVTVSS

Table A-2.1: Sequences for CDRs according to Kabat definition and frameworks ("ID" refers to the given SEQ ID NO)

ID	V _{HH}	ID	FR1	ID	CDR1	ID	FR2	ID	CDR2	ID	FR3	ID	CDR3	ID	FR4
2	4B02	47	DVQLVESGGGV VQPGGSLRLSC AASGRTFS	37	SYRMG	24	WFRQAPGK EREFVA	42	ALSGDG YSTYTAN SVKG	52	RFTISRDN SKNTVYLQM NSLRPEDTALYYCAA	17	KLQYVSGWSY DYPY	34	WGQGTLL VTVSS
3	4B06	48	EVQLVESGGGV VQPGGSLRLSC AASGFTFN	38	NYAMK	25	WVRQAPGK GLEWVS	43	SITGGG STDYADS VKG	53	RFTISRDN SKNTVYLQM SLRPEDTALYYCAN	18	VPFGYSEHFS GLSFDY	35	RGQGTLL VTVSS
4	501A02	49	EVQLVESGGGV VQPGGSLRLSC AASGSGFG	39	VNILY	26	WYRQAAGIE RELIA	44	SITSGGIT NYVDSV KG	54	RFTISRDN SKNTVYLQM NSLRAEDTGLYYCAS	19	RNIFDGTTE	34	WGQGTLL VTVSS
5	ALB23002	50	EVQLVESGGGV VQPGGSLRLSC AASGFTFR	40	SFGMS	27	WVRQAPGK GPEWVS	45	SISGSGS DTLYADS VKG	55	RFTISRDN SKNTVYLQM SLRPEDTALYYCTI	20	GGSLSR	36	SSQGTLL TVSS
6	529F10	51	EVQLVESGGGV VQPGGSLRLSC AASGFTFA	41	DYDYDIG	28	WFRQAPGK EREGVS	46	CISNRDG STYYADS VKG	56	RFTISRDN SKNTVYLQM NSLRPEDTALYYCAV	21	EHCDYDGYVE NFDYDP	34	WGQGTLL VTVSS

Table A-3: Amino acid sequences of selected multivalent polypeptide ("ID" refers to the given SEQ ID NO)

[illegible]

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

Name	ID	Amino acid sequence
Alb8	57	EVQLVESGGGLVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LTVSS
Alb23	58	EVQLLESGGGLVQPGGSLRLSCLASGFTFRSFGMSWVRQAPGKGPEWVSSISG SGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQG TLTVSS
Alb129	59	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTATYYCTIGGSLRSSQGT LTVSSA
Alb132	60	EVQLVESGGGVVQPGGSLRLSCLASGFTFRSFGMSWVRQAPGKGPEWVSSIS GSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTATYYCTIGGSLRSSQ GTLTVSSA
Alb11	61	EVQLVESGGGLVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LTVSS
Alb11 (S110K)-A	62	EVQLVESGGGLVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LVKVSSA
Alb82	63	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LTVSS
Alb82-A	64	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LTVSSA
Alb82-AA	65	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT

		LTVSSAA
Alb82-AAA	66	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LTVSSAAA
Alb82-G	67	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LTVSSG
Alb82-GG	68	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LTVSSGG
Alb82-GGG	69	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISG SGSDTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LTVSSGGG
Alb23002	5	EVQLVESGGGVVQPGGSLRSLCAASGFTFRSFGMSWVRQAPGKGPEWVSSIS GSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQ GTLTVSS
Alb223	71	EVQLVESGGGVVQPGGSLRSLCAASGFTFRSFGMSWVRQAPGKGPEWVSSIS GSGSDTLYADSVKGRFTISRDNKNTLYLQMNSLRPEDTALYYCTIGGSLRSSQ GTLTVSSA

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

Name	ID	Amino acid sequence
3A linker	72	AAA
5GS linker	73	GGGGS
7GS linker	74	SGGSGGS
8GS linker	75	GGGGSGGS
9GS linker	76	GGGGSGGGS

10GS linker	77	GGGSGGGGS
15GS linker	78	GGGSGGGSGGGGS
18GS linker	79	GGGSGGGSGGGSGGS
20GS linker	80	GGGSGGGSGGGSGGGGS
25GS linker	81	GGGSGGGSGGGSGGGSGGGGS
30GS linker	82	GGGSGGGSGGGSGGGSGGGSGGGGS
35GS linker	83	GGGSGGGSGGGSGGGSGGGSGGGSGGGGS
40GS linker	84	GGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGGS
G1 hinge	85	EPKSCDKTHTCPPCP
9GS-G1 hinge	86	GGGSGGGSEPKSCDKTHTCPPCP
Llama upper long hinge region	87	EPKTPKPQAAA
G3 hinge	88	ELKTPLGDTTHTCPRCEPKSCDTPPPCPRCEPKSCDTPPPCPRCEPKSCDTPPPCPRCP

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

Name	ID	Amino acid sequence
F010700003 [F0107004B06-35GS-F0107004B02] ¹	148	EVQLVESGGGLVQPGGSLRLSCAASGFTFNYYAMKWVRQAPGKGLEWVSSIT TGGGSTDYADSVKGRFTISRDNKNTLYLQMNSLKPEDTAVYYCANVPFGYYSE HFSGLSFDYRGQGLTVTVSSGGGSGGGSGGGSGGGSGGGSGGGSGGGGS GGGGSEVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREF VAALSGDGYSTYTANSVNSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQ YVSGWSYDYPYWGQGLTVTVSS

Name	ID	Amino acid sequence
F010700014 [F0107004B02-35GS-F0107004B02] ¹	149	EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAALS GDGYSTYTANSVNSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQYVSG WSYDYPYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG GGGSEVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFV AALSGDGYSTYTANSVNSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQY VSGWSYDYPYWGQGTTLTVSS
F010700029 [F0107004B02-35GS-F0107004B06] ¹	150	EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAALS GDGYSTYTANSVNSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQYVSG WSYDYPYWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG GGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFNNYAMKWVRQAPGKGLEW VSSITTGGGSTDYADSVKGRFTISRDNKNTLYLQMNSLKPEDTAVYYCANVPF GYSEHFSGLSFDYRGQGTTLTVSS
F010700031 [F0107004B06-35GS-F0107004B06] ¹	151	EVQLVESGGGLVQPGGSLRLSCAASGFTFNNYAMKWVRQAPGKGLEWVSSIT TGGGSTDYADSVKGRFTISRDNKNTLYLQMNSLKPEDTAVYYCANVPFGYYSE HFSGLSFDYRGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG GGGSEVQLVESGGGLVQPGGSLRLSCAASGFTFNNYAMKWVRQAPGKGLE WVSSITTGGGSTDYADSVKGRFTISRDNKNTLYLQMNSLKPEDTAVYYCANV PFGYYSEHFSGLSFDYRGQGTTLTVSS
F010703842 [F0107529F10-35GS-F0107501A02] ¹	152	EVQLVESGGGLVQAGGSLRLSCAASGFTFADYDYDIGWFRQAPGKEREGVSCI SNRDGSTYYTDSVKGRFTISSDNKNTVSLQMNSLKPEDTAVYYCAVEIHCDY GVENFDLDPWGQGTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSG SGGGSEVQLVESGGGLVQAGESLRLSCAASGSGFGVNILYWYRQAAGIERELI ASITSGGITNYVDSVKGRFTISRDNKNTMYLQMNSLKAEDTGVIYCASRNIFD GTTEWGQGTTLTVSS

Name	ID	Amino acid sequence
F027400016 [F0107501A02 (E1D,L11V,A14P, E16G,A41P,I43K ,E44Q,A74S,E75 K,M78L,K83R,A8 4P,G88A,V89L)* -35GS-529F10)] ¹	153	DVQLVESGGGVVQPGGSLRLSCAASGSGFGVNILYWYRQAPGKQRELIASITSG GITNYVDSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCASRNIFDGTTEWG QGTLVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSEVQLVE SGGGVVQPGGSLRLSCAASGFTFADYDYDIGWFRQAPGKEREGVSCISNRDGS TYYADSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAVEIH CDDYGVENFD FDPWGGQGLTVTVSS
F010700003-SO [4B06-35GS- 4B02(D1E)*] ¹	154	EVQLVESGGGVVQPGGSLRLSCAASGFTFN NYAMKWVRQAPGKGLEWVSSIT TGGGSTDYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCANVPFGYYSE HFSGLSFDYRGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS GGGGSEVQLVESGGGVVQPGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREF VAALSGDGYSTYTANSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAAKLQ YVSGWSYDYPYWGQGLTVTVSS
F010700014 -SO [4B02-35GS- 4B02(D1E)*] ¹	155	DVQLVESGGGVVQPGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAALS GDGYSTYTANSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAAKLQYVSG WSYDYPYWGQGLTVTVSSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGGS GGGSEVQLVESGGGVVQPGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFV AALSGDGYSTYTANSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAAKLQY VSGWSYDYPYWGQGLTVTVSS

Name	ID	Amino acid sequence
F010700029-SO [4B02-35GS-4B06] ¹	156	DVQLVESGGGVVQPGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAALS GDGYSTYTANSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAAKLQYVSG WSYDYPYWGQGTLVTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSG GGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFN NYAMKWVRQAPGKGLE WVSSITTGGGSTDYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCANVP FGYYSEHFGSLFDYRGQGTLVTVSS
F010700031-SO [4B06-35GS-4B06] ¹	157	EVQLVESGGGVVQPGGSLRLSCAASGFTFN NYAMKWVRQAPGKGLEWVSSIT TGGGSTDYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCANVPFGYYSE HFGSLFDYRGQGTLVTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSG GGGGSEVQLVESGGGVVQPGGSLRLSCAASGFTFN NYAMKWVRQAPGKGLE WVSSITTGGGSTDYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTALYYCANVP FGYYSEHFGSLFDYRGQGTLVTVSS
F010703842-SO [529F10-35GS-501A02] ¹	158	EVQLVESGGGVVQPGGSLRLSCAASGFTFADYDYDIGWFRQAPGKEREGVSCI SNRDGSTYYADSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAVEIHCD DY GVENFD FDPWGQGTLVTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSG SGGGSEVQLVESGGGVVQPGGSLRLSCAASGSGFGVNILYWYRQAAGIEREL IASITSGGITNYVDSVKGRFTISRDN SENTMYLQMNSLRAEDTGLYYCASRNIFD GTTEWGQGTLVTVSS
F027400016-SO [501A02-35GS-529F10] ¹	159	EVQLVESGGGVVQPGGSLRLSCAASGSGFGVNILYWYRQAAGIERELIASITSG GITNYVDSVKGRFTISRDN SENTMYLQMNSLRAEDTGLYYCASRNIFDGTTEW GQGTLVTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQLV ESGGGVVQPGGSLRLSCAASGFTFADYDYDIGWFRQAPGKEREGVSCISNRDG STYYADSVKGRFTISRDN SKNTVYLQMNSLRPEDTALYYCAVEIHCD DYGVENF DFDPWGQGTLVTVSS

¹ [] indicates the monovalent building blocks and their linkage which were used for the bivalent construct.

*() indicates the amino acid substitutions introduced into the (parental) monovalent building block (e.g.: 4B02(D1E) means that the monovalent building block 4B02 (SEQ ID NO:2) contains a D1E substitution).

6 Examples

5 6.1 Generation of monovalent ISVDs specifically binding to IL-13 and TSLP, respectively

6.1.1 Example 1: Immunizations

Three llamas were immunized with recombinant human IL-13 (Peprotech, cat nr 200-13, *E. coli*-derived) according to standard protocols, with the aim to induce a heavy-chain
10 antibody dependent humoral immune response. In addition, these llamas were boosted with human/cyno IL-13 from another source (Sino Biological, cat nrs 10369-HNAC and 11057-CNAH, mammalian cell derived).

Another three llamas were immunized with recombinant hTSLP-Fc. In addition, these llamas were boosted with cyno TSLP-Fc. Two additional llamas were immunized with
15 hTSLP alternated with cyno TSLP-Fc.

Immune blood (PBL) samples were taken at regular intervals, and total RNA was prepared from the isolated B-cells. The humoral immune response was monitored during the immunization process by comparing the antigen specific serum titers of a sample collected prior to initiation of immunization and a serum sample typically collected after
20 multiple antigen administrations. Briefly, 96-well Maxisorp plates were coated with human IL-13 (Sino Biological, cat nr 10369-HNAC) or human TSLP. Recombinant human TSLP is commercially available, such as from R&D Systems (cat nr 1398-TS). After blocking and adding diluted serum samples, the presence of anti-IL-13 and anti-TSLP ISVDs was demonstrated by using HRP (horseradish peroxidase) conjugated goat anti-
25 llama immunoglobulin (Bethyl Laboratories Inc.) and a subsequent enzymatic reaction in the presence of the substrate TMB (3,3',5,5'-tetramethylbenzidine).

6.1.2 Example 2: Library construction and phage display selections

Peripheral blood mononuclear cells were prepared from the blood samples using Ficoll-Hypaque according to the manufacturer's instructions. Total RNA extracted from these cells and from lymph nodes was used as starting material for RT-PCR to amplify ISVD
5 encoding gene fragments. These fragments were cloned into phagemid vector pAX212. Phage was prepared according to standard protocols (Antibody Phage Display: Methods and Protocols (First Edition, 2002, O'Brian and Aitken eds., Humana Press, Totowa, NJ) and stored after filter sterilization at 4°C until further use. Five phage libraries were constructed for IL13, with library sizes between 6.1×10^8 and 1.3×10^9 , and a percentage of
10 insert ranging from 87 to 96%. Five phage libraries were constructed for TSLP, with library sizes between 4.2×10^8 and 9.8×10^8 , and a percentage of insert ranging from 91 to 100%.

To identify ISVDs recognizing human and cyno IL-13, the phage libraries were incubated with 50 nM soluble biotinylated hIL13-Fc in presence of IgG from human serum (Sigma, I4506). Complexes of hIL-13-Fc and phage were captured from solution on streptavidin
15 coated magnetic beads. After extensive washing with PBS/ 0.05% Tween20, bound phage were eluted by addition of trypsin (1 mg/ml). Outputs of these round 1 selections were incubated with 0.05, 0.5 or 5 nM soluble biotinylated hIL-13-Fc or cyno IL-13-Fc. Not enriched outputs from the round 2 selections were further incubated with a 0.005, 0.05, 0.5 or 5 nM soluble biotinylated human or cyno IL-13-Fc in round 3. Individual clones from
20 enriched round 2 and round 3 selections were picked.

To identify ISVDs recognizing human and cyno TSLP, the phage libraries were incubated with 50 nM soluble biotinylated hTSLP-Fc in presence of IgG from human serum (Sigma, I4506) or with 500 nM biotinylated hTSLP or with 500 nM biotinylated cyno TSLP. The human and cyno TSLP sequences are known (Uniprot accession Uniprot accession
25 Q969D9 and NCBI RefSeq XP_005557555.1, respectively). Recombinant protein was used to perform the assay. Complexes of TSLP and phage were captured from solution on streptavidin coated magnetic beads. After extensive washing with PBS/ 0.05% Tween20, bound phage were eluted by addition of trypsin (1 mg/ml). Outputs of these round 1 selections were incubated with 5 nM soluble biotinylated hILTSLP-Fc or cyno TLSP-Fc or

0.5 nM biotinylated hTSLP. Outputs from the round 2 selections were incubated with 0.05, 0.5 or 5 nM soluble biotinylated human TSLP, TSLP-Fc or cyno TSLP-Fc. From each round individual clones from the enriched outputs were picked.

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

As a control, selected periplasmic extracts were screened in an ELISA for binding to human and cyno IL13-Fc, respectively TSLP-Fc. The assessment was performed in a
10 Spectraplate 384-HB (PerkinElmer) in a 25µl format. The antigens were coated overnight at 1µg/ml in PBS at 4°C. Wells were blocked with a casein solution (1%). After addition of a 5-fold dilution of peri plasmic extracts, ISVD binding was detected using mouse anti-Flag-HRP (Sigma) and a subsequent enzymatic reaction in the presence of substrate esTMB (3,3',5,5'-tetramethylbenzidine).

15 6.1.3 Example 3: Screening for blocking ISVDs in periplasmic extracts by AlphaScreen assays using human IL13 and human TSLP

In order to determine the blocking capacity of the ISVDs, crude periplasmic extracts were screened in different protein-based competition assays using the AlphaScreen technology (PerkinElmer, Waltham, MA USA). Fluorescence was measured using the EnVision
20 Multilabel Plate Reader (PerkinElmer) using an excitation wavelength of 680 nm and an emission wavelength of 520 nm.

In the hIL-13:hIL-13Rα1 binary complex AlphaScreen, it was investigated if ISVDs could block the interaction between hIL-13 and the hIL-13Rα1 extracellular domain. To this end, dilutions of the periplasmic extracts were pre-incubated with biotinylated hIL-13
25 (Peprotech cat nr 200-13). To this mixture, hIL-13Rα1-hFc (R&D systems; cat nr 146-IR) and anti hFc-coupled Acceptor beads were added and further incubated for 1 hour at room temperature, followed by addition of streptavidin-coupled Donor beads and an additional 1-hour incubation. When the binary complex is formed, Acceptor and Donor

beads are brought into proximity and upon laser excitation a detectable signal is generated. Decrease in the AlphaScreen signal indicates that the binding of biotinylated hIL-13 to hIL-13R α 1 is blocked by the ISVD present in the periplasmic extract. In a similar set-up it was investigated if ISVDs could block the interaction between hTSLP (eBioscience, cat nr 10-8499) and the hTSLPR extracellular domain (R&D systems cat nr 981-TR).

In the hIL-13:hIL-13R α 1:hIL4R α ternary complex AlphaScreen, it was screened if ISVDs could block the recruitment of hIL4R α to the hIL13:hIL13R α 1 binary complex. hIL-13 binds to hIL-13R α 1 and this binary complex recruits hIL4R α , resulting in formation of the ternary complex hIL-13:hIL-13R α 1:hIL4R α . Dilutions of the periplasmic extracts were pre-incubated with hIL-13 (peprotech cat nr 200-13) and biotinylated hIL4R α (R&D Systems; cat nr 230-4R/CF). To this mixture, hIL-13R α 1-hFc (R&D systems; cat nr 146-IR) and anti hFc-coupled Acceptor beads were added and further incubated for 1 hour at room temperature, followed by addition of streptavidin-coupled Donor beads and an additional 1-hour incubation. When the ternary complex is formed, Acceptor and Donor beads are brought into proximity and upon laser excitation a detectable signal is generated. Decrease in the AlphaScreen signal indicates that the formation of the ternary complex is blocked by the ISVD present in the periplasmic extract. Similarly, it was investigated if ISVDs could block the formation of the hTSLP:hTSLPR:hIL7R α complex (hIL7R α source = Sino Biological cat nr 1095-H08H).

Based on the Alphascreen analysis (Table 3 and Table 4), a number of blocking ISVDs were selected and sequenced (Table 1 and Table 2).

Table 1: Amino acid sequences of functional anti-IL-13 ISVDs.

ISVD ID	SEQUENCE
F0107004B02 (SEQ ID NO: 144)	EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAALSGDG YSTYTANSVNSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQYVSGWSYDYPY WGQGTLTVSS
F0107004B06 (SEQ ID NO: 145)	EVQLVESGGGLVQPGGSLRLSCAASGFTFNYYAMKWVRQAPGKGLEWVSSITTGG GSTDYADSVKGRFTISRDNKNTLYLQMNSLKPEDTAVYYCANVPFGYYSEHFSGLS FDYRGQGTTLTVSS

Table 2: Amino acid sequences of functional anti-TSLP ISVDs.

ISVD ID	SEQUENCE
F0107501A02 (SEQ ID NO: 146)	EVQLVESGGGLVQAGESLRLSAASGSGFGVNILYWYRQAAGIERELIASITSGGITN YVDSVKGRFTISRDN AENTMYLQMNSLKAEDTGVIYCASRNIFDGTTEWGQGLTV TVSS
F0107529F10 (SEQ ID NO: 147)	EVQLVESGGGLVQAGGSLRLS AASGFTFADYDYDIGWFRQAPGKERE GVCISNR DGSTYYTDSVKGRFTISSDN AKNTVSLQMNSLKPEDTAVYYCAVEIHCDDYGVENFD FDPWGQGLTVTVSS

6.1.4 Example 4: Surface Plasmon Resonance analysis of periplasmic extracts on IL13 and TSLP

Off-rates of the periplasmic extracts containing anti-IL13 or anti-TSLP ISVDs were measured by Surface Plasmon Resonance (SPR) using a Proteon XPR36 instrument (Bio-Rad Laboratories, Inc.). Phosphate buffered saline (PBS), pH7.4 supplemented with 0.005% Tween20 was used as running buffer and the experiments were performed at 25 °C.

hIL13-Fc, cyno IL13-Fc, hTSLP-Fc and cyno TSLP-Fc were immobilized on ProteOn GLC Sensor Chips by amine coupling using EDC and NHS at flow rate 30µl/min for activation. IL13 proteins were injected at 10µg/ml in ProteOn Acetate buffer at pH5.0. TSLP proteins were injected at 5µg/mL in ProteOn Acetate Buffer at pH5.5. After immobilization, surfaces were deactivated with ethanolamine.

Periplasmic extracts of the ISVD candidates were diluted 10 times in PBS-Tween20 (0.1%) and injected for 2 minutes at 45µl/min and allowed to dissociate for 900 seconds. Between different samples, the surfaces were regenerated with a 2 minute injection of Phosphoric Acid (0.425%) at 45µl/min, in case of IL13, or with a 1 minute injection of Glycine pH3.0 (5mM)/SDS(0.25%) at 45µL/min, in case of TSLP. From the sensorgrams obtained for the different periplasmic extracts off-rates were calculated.

Off-rate analysis on hIL-13-Fc and cyno IL-13-Fc is shown in Table 3.

Table 3: Summary of the screening results of anti-IL-13 ISVDs F0107004B02 and F0107004B06.

ISVD ID	IL-13-IL-13R α 1 AlphaScreen (% inhibition)	IL13-IL-13R α 1-IL4R α AlphaScreen (% inhibition)	k _{off} hIL-13-Fc (1/s)	k _{off} cyno IL-13-Fc (1/s)
F0107004B02	37	61	1.7E-03	1.1E-03
F0107004B06	21	66	6.3E-03	8.9E-03

- 5 Off-rate analysis on hTSLP-Fc and cyno TSLP-Fc is shown in Table 4.

Table 4: Summary of the screening results of anti-TSLP ISVDs F0107501A02 and F0107529F10.

ISVD ID	TSLP-TSLPR AlphaScreen (% inhibition)	TSLP-TSLPR-IL7R α AlphaScreen (% inhibition)	k _{off} hTSLP-Fc (1/s)	k _{off} cyno TSLP-Fc (1/s)
F0107501A02	92	97	3.6E-05	6.6E-04
F0107529F10	99	99	<5E-05	1.8E-03

6.1.5 Example 5: Expression and purification of anti-IL-13 and anti-TSLP ISVDs

- 10 Anti-IL-13 ISVDs and anti-TSLP ISVDs were selected for expression and purification, based on their blocking capacity in AlphaScreen assays and off-rate values. Sequences are shown in Tables 1 and 2.

- ISVDs were expressed in *E. coli* TG1 cells as c-myc, His6-tagged proteins. Expression was induced by addition of 1 mM IPTG and allowed to continue for 3h at 37°C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets and resuspension in dPBS. These extracts were used as starting material for immobilized metal affinity chromatography (IMAC) using Nickel Sepharose TM 6 FF columns (Atoll). ISVDs were eluted from the column with 250 mM imidazole and subsequently desalted towards dPBS. For the cell-based assays described below, endotoxins were removed by gel filtration in the presence of 50 mM Octyl β -D-glucopyranoside (OGP, Sigma). Endotoxin levels were determined using a standard LAL-assay.
- 15
- 20

6.1.6 Example 6: Blocking capacity of purified anti-IL13 and anti-TSLP ISVDs in AlphaScreen assays

The binary and ternary complex AlphaScreen assays, as described in example 3, were used to determine the IC₅₀ values of the anti-IL-13 and anti-TSLP ISVDs, purified as described in Example 5. Instead of dilutions of the periplasmic extracts, a dilution series of each purified ISVD starting from 500 nM down to 1.8 pM was pre-incubated with IL-13 or TSLP.

The tested anti-IL-13 ISVDs inhibited the formation of the ternary complex and partially blocked the binary complex with IC₅₀ values as shown in Table 5.

Table 5: IC₅₀ values for the blocking anti-IL13 ISVDs as determined in the binary and ternary complex AlphaScreen assays.

ISVD ID	IL-13-IL-13R α 1 AlphaScreen		IL-13-IL-13R α 1-IL4R α AlphaScreen	
	IC ₅₀ (M)	% Inhibition	IC ₅₀ (M)	% Inhibition
F0107004B02	4.0E-09	82	1.7E-09	100
F0107004B06	6.1E-08	55	6.7E-09	100

The tested anti-TSLP ISVDs fully inhibited the formation of the ternary complex, and also inhibited the formation of the binary complex, with IC₅₀ values as shown in Table 6.

Table 6: IC₅₀ values for the blocking anti-TSLP ISVDs as determined in the binary and ternary complex AlphaScreen assays.

ISVD ID	TSLP-TSLPR AlphaScreen		TSLP-TSLPR-IL7R α AlphaScreen	
	IC ₅₀ (M)	% Inhibition	IC ₅₀ (M)	% Inhibition
F0107501A02	3.00E-10	97	3.50E-10	100
F0107529F10	1.30E-11	100	1.80E-10	100

6.1.7 Example 7: Blocking capacity of purified anti-IL-13 and anti-TSLP ISVDs in cell - based assays

The inhibitory potency of the anti-IL-13 ISVDs was determined in a cell-based assay monitoring IL-13 mediated proliferation of TF-1 cells. To this end, TF-1 cells were cultured in RPMI 1640 medium with the addition of 1/5 HEPES, 1/500 Na-Pyruvate, 1/500 Glutamax and 2ng/mL recombinant human GM-CSF. TF-1 cells were seeded at 40.000 cells per well in growth medium w/o GM-CSF. A dilution series of the purified anti-IL-13 ISVDs or reference compounds was added. After 15 min incubation at 37°C, 200 pM of IL-13 (Peprotech cat nr 200-13) was added. After 96 hours, proliferation of the TF-1 cells was determined with Cell Titer 96 Aqueous One Solution (Promega #G3580) on an EnVision Multilabel Reader (Perkin Elmer).

The ISVDs shown in Table 7 inhibit IL-13-induced TF1 proliferation.

Table 7: Overview of IC50 values of anti-IL-13 monovalent ISVDs in IL-13-induced TF-1 proliferation assay.

compound	IC50 (M)
F0107004B02	2.7E-08
F0107004B06	1.0E-05
anti-hIL-13 reference mAb2	4.1E-10
anti-hIL-13 reference mAb3	9.2E-11
anti-hIL-13 reference mAb1	2.2E-11

The blocking potency of the anti-TSLP ISVDs was determined in a cell-based assay monitoring TSLP mediated proliferation of BaF3 cells transfected with plasmids encoding hTSLPR and hIL7Ra. Cells were seeded at a density of 20.000 cells/well in RPMI 1640 growth medium in cell culture treated white 96 well plates. A dilution series of anti-TSLP ISVDs were added, followed by addition of 50 pM hTLSP-Fc or 50 pM cyno TSLP-Fc for stimulation of the cells. The human and cyno TSLP sequences are known (Uniprot accession Uniprot accession Q969D9 and NCBI RefSeq XP_005557555.1, respectively). Recombinant protein was used to perform the assay.

After incubation for 72 hours, cell density and viability was monitored using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7571/G7572/G7573) and read-out on an EnVision Multilabel Reader (Perkin Elmer). Results are shown in Table 8.

Table 8: Potency and efficacy of anti-TSLP monovalent ISVDs in TSLP-induced BaF3 cell proliferation assay. ND = not determined

ISVD ID	IC50 (M) hTSLP-Fc	% inhibition hTSLP-Fc	IC50 (M) cyno TSLP-Fc	% inhibition cyno TSLP-Fc
F0107501A02	>1.0E-06	46	>1.0E-06	10
F0107529F10	ND	ND	ND	ND

6.1.8 Example 8: Binding affinity of purified anti-IL-13 and anti-TSLP ISVDs to human and cyno IL-13 and TSLP

Full binding kinetic study by SPR was performed on a BIAcore T100 instrument (GE Healthcare).

For IL-13, around 2000 RU of hIL13-Fc or 4000 RU cyno IL13-Fc was immobilized directly on a CM5 sensor chip. The ISVDs were then injected at different concentrations (between 3μM and 12nM) for 120s and allowed to dissociate for 900s. Regeneration of the hIL-13-Fc and cyno IL-13-Fc surfaces were performed using a 47s injection of 0.85% H₃PO₄:MilliQ (1:1).

For TSLP, around 8000 RU of anti-hulgG antibody (GE Healthcare) was immobilized directly on a CM5 sensor chip. hTSLP-Fc (at 1μg/mL) or cyno TSLP-Fc (at 0.75μg/mL) were floated and captured for 120s over the chip. The ISVDs were then injected at different concentrations (between 0.4nM and 3000nM) for 120s and allowed to dissociate for 900s.

Evaluation of the binding curves was done using BIAcore T100 Evaluation software V2.0.3. Kinetic analysis was performed by fitting a 1:1 interaction model (Langmuir binding) (Rmax = global; RI = constant = 0, offset = 0). Interactions which could not meet the

acceptance criteria for the 1:1 interaction model, were fitted using the heterogeneous ligand fit model ($R^2 = \text{constant} = 0$, offset = 0).

Kinetic data are shown in Table 9 for IL-13 ISVDs and in Table 10 for TSLP ISVDs.

Table 9: KD determination of monovalent anti-IL13 ISVDs via SPR.

Sample	Surface	% major interaction present	k_a (1/Ms)	k_d (1/s)	K_D (M)	Model used for fitting of binding curves
F0107004B02	hIL13-Fc	80	2.2E+04	2.4E-02	1.1E-06	heterogeneous ligand fit
	cyno IL13-Fc	81	1.8E+04	3.4E-02	1.9E-06	heterogeneous ligand fit

5

Table 10: KD determination of monovalent anti-TSLP ISVDs via SPR.

ID	hTSLP-Fc			cyno TSLP-Fc		
	k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)
F0107501A02	1.1E+05	5.7E-05	5.1E-10	7.3E+04	1.8E-03	2.5E-08
F0107529F10	1.2E+07	4.2E-04	3.5E-11	8.7E+05	1.5E-03	1.7E-09

6.2 Generation of monospecific multivalent polypeptides binding to IL-13 and TSLP, respectively

10 6.2.1 Example 10: Generation and *in vitro* characterization of wild-type anti-IL-13 bivalent or biparatopic ISVD constructs

The selected anti-IL-13 ISVDs (F0107004B02 and F0107004B06) were formatted into biparatopic and bivalent ISVD constructs. The building blocks in the constructs are genetically linked by a flexible 35GS (GlySer) linker. ISVDs were expressed as FLAG3-HIS6-tagged protein in *Pichia pastoris* (amino acid sequences are shown in Table 14). Induction of ISVD construct expression occurred by stepwise addition of methanol. Clarified medium with secreted ISVD construct was used as starting material for immobilized metal affinity chromatography (IMAC) followed by desalting resulting in 90% purity as assessed by SDS-PAGE.

15

The biparatopic and bivalent IL-13 constructs were characterised in the binary and ternary complex blocking AlphaScreen assays (as described in example 6) as well as in the TF-1 proliferation assay (as described in example 7). An overview of the generated constructs and their blocking potencies in the binary and ternary AlphaScreen assay and in the TF-1 proliferation assay is shown in Table 11. The biparatopic construct displays excellent potencies against hIL-13 and cyIL-13, similar to the potency of anti-hIL-13 reference mAb1. Also bivalent constructs improve in potency, but not to the same extent as the biparatopic construct F010700029 in the TF1 proliferation assay. In addition, the bivalent constructs do not reach full inhibition in the IL13-IL13R α 1 Alphascreen.

Table 11: Overview of potency of different anti-IL-13 constructs in the Alphascreen assays and of potency and efficacy in the TF-1 proliferation assay. ND = not determined

ISVD construct ID	ISVD construct description	IL13-IL13R α 1 AlphaScreen		IL13-IL13R α 1-IL4R α AlphaScreen		TF1 proliferation assay IC50 (M)	
		IC50 (M)	% inhibition	IC50 (M)	% inhibition	200pM hIL13	200pM cyno IL13
F010700003	F0107004B06-35GS-F0107004B02	1.3E-10	99	3.40E-09	100	1.20E-08	ND
F010700014	F0107004B02-35GS-F0107004B02	2.7E-10	84	4.80E-09	98	9.40E-08	ND
F010700029	F0107004B02-35GS-F0107004B06	3.1E-10	99	3.00E-09	100	2.00E-11	2.50E-10
F010700031	F0107004B06-35GS-F0107004B06	No fit	partial	6.60E-10	99	1.00E-07	ND
anti-hIL-13 reference mAb1		ND	ND	ND	ND	1.50E-11	1.30E-10

The most potent anti-IL-13 biparatopic ISVD construct was tested in the IL-13 induced A549 eotaxin release assay. To this end A549 suspension cells were cultured in Ham's F12K medium supplemented with 10% FCS. Cells were seeded into a 96 well plate at 200.000 cells/well. The next day 200pM hIL-13 (Sino Biological cat nr 10369-HNAC) or cyno IL-13 (Sino Biological cat nr 11057-CNAH) were added, followed by a dilution series of the ISVD constructs. After 24h, Eotaxin-3 was determined in the supernatans using the MSD ELISA (Human Eotaxin-3 Tissue Culture Kit (Meso Scale, K151ABB-1)). Results are shown in Table 12.

Table 12: Potency of F010700029 in the IL-13-mediated Aa549 eotaxin release assay. Inhibition was 100%.

ISVD construct ID	ISVD construct description	IC50 (M) hIL-13	IC50 (M) cyno IL-13
F010700029	F0107004B02-35GS-F0107004B06	2.10E-10	1.50E-10
anti-hIL-13 reference mAb1		8.00E-11	7.00E-11

Competition ELISA was used to test if anti-IL-13 monovalent and biparatopic ISVD constructs inhibit binding of hIL-13 to IL13R α 2. IL13R α 2 (SinoBiological cat nr 10350-H08H) was coated on a 384 well Spectraplate (Perkin Elmer). hIL13-Fc (SinoBiological, cat nr 10369-H01H) was mixed with serial dilutions of ISVD constructs or positive control compound IL-13R α 2 and incubated for 1 hour. After washing and blocking, the coated receptor was incubated with the IL13-Fc ISVD/control mix and incubated for 1 hour. After washing, the presence of bound IL-13-Fc was detected using anti-human IgG-peroxidase antibody and a subsequent enzymatic reaction in the presence of substrate esTMB. In the case the ISVD inhibits the IL-13-IL-13R α 2 interaction, detection of IL-13-Fc disappears in a dose dependent manner. Results are shown in Table 13.

Table 13: capacity of anti-IL-13 monovalent and biparatopic ISVD constructs to inhibit IL-13-IL-13R α 2 complex formation in a competition ELISA

Compound	construct description	% inhibition of IL13-IL13R α 2 complex formation	IC50 (M)
F010704B02	-	37	4.9E-08
F010704B06	-	49	No fit
F027100271	F0107004B02-9GS-ALB23002-9GS-F0107004B06	53	No fit
IL-13R α 2	-	100	7.9E-09

Table 14: Amino acids sequences of wild-type anti-IL13 biparatopic and bivalent ISVD constructs.

ISVD construct ID	ISVD construct description	Sequence
F010700003 (SEQ ID NO: 148)	F0107004B06-35GS- F0107004B02	EVQLVESGGGLVQPGGSLRLSCAASGFTFNYYAMKWVRQAPGKG LEWVSSITGGGSTDYADSVKGRFTISRDNRKNTLYLQMNSLKPED TAVYYCANVPFGYYSEHFSGLSFDYRGQGTTLTVSSGGGGSGGGG SGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQAGG SLRLSCAASGRTFSSYRMGWFRQAPGKEREFVAALSGDGYSTYTA NSVNSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQYVSGWS YDYPYWGQGTTLTVSS
F010700014 (SEQ ID NO: 149)	F0107004B02-35GS- F0107004B02	EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKE REFVAALSGDGYSTYTANSVNSRFTISRDNKNTVYLQMNSLKPED TAIYYCAAKLQYVSGWSYDYPYWGQGTTLTVSSGGGGSGGGSG GGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQAGGSLR LSCAASGRTFSSYRMGWFRQAPGKEREFVAALSGDGYSTYTANSV NSRFTISRDNKNTVYLQMNSLKPEDTAIYYCAAKLQYVSGWSYDY PYWGQGTTLTVSS
F010700029 (SEQ ID NO: 150)	F0107004B02-35GS- F0107004B06	EVQLVESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKE REFVAALSGDGYSTYTANSVNSRFTISRDNKNTVYLQMNSLKPED TAIYYCAAKLQYVSGWSYDYPYWGQGTTLTVSSGGGGSGGGSG GGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGGSLR LSCAASGFTFNYYAMKWVRQAPGKGLEWVSSITGGGSTDYADS VKGRFTISRDNRKNTLYLQMNSLKPEDTAVYYCANVPFGYYSEHFS GLSFDYRGQGTTLTVSS
F010700031 (SEQ ID NO: 151)	F0107004B06-35GS- F0107004B06	EVQLVESGGGLVQPGGSLRLSCAASGFTFNYYAMKWVRQAPGKG LEWVSSITGGGSTDYADSVKGRFTISRDNRKNTLYLQMNSLKPED TAVYYCANVPFGYYSEHFSGLSFDYRGQGTTLTVSSGGGGSGGGG SGGGSGGGSGGGSGGGSGGGSGGGSEVQLVESGGGLVQPGG SLRLSCAASGFTFNYYAMKWVRQAPGKGLEWVSSITGGGSTDY ADSVKGRFTISRDNRKNTLYLQMNSLKPEDTAVYYCANVPFGYYSE HFSGLSFDYRGQGTTLTVSS

6.2.2 Example 11: Generation and characterization of wild-type biparatopic anti-TSLP ISVD constructs

The selected TSLPR blockers (F0107501A02 and F0107529F10) were combined into
5 biparatopic ISVD constructs. The constructs were expressed as FLAG3-HIS6-tagged
protein in *Pichia pastoris* (amino acid sequences are shown in Table 16). Induction of ISVD
construct expression occurred by stepwise addition of methanol. Clarified medium with
secreted ISVD construct was used as starting material for immobilized metal affinity
chromatography (IMAC) followed by desalting resulting in 90% purity as assessed by SDS-
10 PAGE.

The biparatopic constructs were titrated as purified proteins against 50 or 5 pM hTSLP
and 50 or 5 pM cyno TSLP in the BaF3 proliferation assay (as described in example 7) and
compared to different anti-TSLP reference compounds (anti-hTSLP reference mAb2 and
anti-hTSLP reference mAb1). The data is summarized in Table 15 (hTSLP and cyno TSLP).
15 The biparatopic constructs clearly outperform the reference mAbs. The biparatopic
construct with F0107501A02 at the N-terminus shows improved reactivity towards cyno
TSLP compared to the construct with F0107501A02 at the C-terminus.

Table 15: Overview of IC50 values (M) of different biparatopic anti-TSLP formats in the human and cyno TSLP, respectively, mediated BaF3 proliferation assay.

ISVD construct ID	ISVD construct description	IC50 (M) hTSP	IC50 (M) cyno TSLP	Conc TSLP used for stimulation (pM)
F010703842	F0107529F10-35GS-F0107501A02	4.8E-11	3.9E-09	50
F027400016	F0107501A02 (E1D, L11V, A14P, E16G, A41P, I43K, E44Q, A74S, E75K, M78L, K83R, A84P, G88A, V89L)*-35GS-529F10	4.6E-12	6.0E-11	5
anti-hTSLP reference mAb2		1.6E-10	>1.0E-07	50
anti-hTSLP reference mAb1		5.5E-10	3.8E-09	5

*() indicates the amino acid substitutions introduced into the (parental) monovalent building block.

Table 16: Amino acids sequences of biparatopic anti-TSLP ISVD constructs F010703842 and F027400016.

ISVD construct ID	ISVD construct description	Sequence
F010703842 (SEQ ID NO: 152)	F0107529F10-35GS-F0107501A02	EVQLVESGGGLVQAGGSLRLSCAASGFTFADYDYDIGWF RQAPGKEREGVSCISNRDGYSTYYTDSVKGRFTISSDNAKN TVSLQMNSLKPEDTAVYYCAVEIHCDYGVENFD FDPWG QGTLTVTVSSGGGGSGGGSGGGSGGGSGGGSGGGSGGG GSGGGGSEVQLVESGGGLVQAGSLRLSCAASGSGFGVN ILYWYRQAAGIERELIASITSGGITNYVDSVKGRFTISRDN ENTMYLQMNSLKAEDTGYYCASRNIFDGTTEWGGQGT LTVTVSS
F027400016 (SEQ ID NO: 153)	F0107501A02 (E1D, L11V, A14P, E16G, A41P, I43K, E44Q, A74S, E75K, M78L, K83R, A84P, G88A, V89L)*-35GS-529F10	DVQLVESGGGVVQPGGSLRLSCAASGSGFGVNILYWYRQ APGKQRELIASITSGGITNYVDSVKGRFTISRDN SKNTLYLQ MNSLRPEDTALYYCASRNIFDGTTEWGGQGT LTVTVSSGGG GSGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGSEVQL VESGGGVVQPGGSLRLSCAASGFTFADYDYDIGWFRQAP GKEREGVSCISNRDGYSTYYADSVKGRFTISRDN SKNTVYLQ MNSLRPEDTALYYCAVEIHCDYGVENFD FDPWGQGT LTVTVSS

*() indicates the amino acid substitutions introduced into the (parental) monovalent building block.

6.3 Sequence optimization of the anti-IL-13 and anti-TSLP monovalent ISVDs

6.3.1 Example 12: Sequence optimization of anti-IL-13 and anti-TSLP monovalent ISVDs

Anti-IL-13 ISVDs F0107004B02 and F0107004B06 and anti-TSLP ISVDs F0107501A02 and F0107529F10 were further sequence optimized.

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

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

- 1) Substitutions in parental wild type ISVD sequences to yield ISVD sequences that are more identical to the human VH3-JH germline consensus sequences, a process called humanization. To this end, specific amino acids, with the exception of the so-called hallmark residues, in the FRs that differ between the ISVD and the human VH3-JH germline consensus are altered to the human counterpart in such a way that the protein structure, activity and stability are kept intact.
- 2) Substitutions towards the llama germline to increase the stability of the ISVD, which is defined as **camelisation**. To this end, the parental wild type ISVD amino acid sequence is aligned to the llama IGHV germline amino acid sequence of the ISVD (identified as the top hit from a BlastP analysis of the ISVD against the llama IGHV germlines).
- 3) Substitutions that improve long-term stability or properties under storage, substitutions that increase expression levels in a desired host cell or host organism, and/or substitutions that remove or reduce (undesired) **post-translational modification(s)** (such as glycosylation or phosphorylation), again depending on the desired host cell or host organism. *To avoid N-terminal pyroglutamate formation, standardly an E1D mutation is introduced in the N-terminal building block of a multivalent Nb, without impact on potency or stability. During sequence optimization of the building blocks, the E1D mutation is therefore not consistently introduced.*
- 4) Mutations on position 11 towards Val and on position 89 towards Leu to minimize the binding of any naturally occurring **pre-existing antibody** activity.

F0107004B02 and F0107004B06

Sequence optimisation of anti-IL-13 ISVD F0107004B02 resulted in a final sequence optimised variant F027100019, which comprises 8 amino acid substitutions (i.e. E1D, L11V, A14P, N64K, S65G, A74S, K83R, I89L) compared to the parental ISVD F0107004B02. Sequence optimisation of anti-IL-13 ISVD F0107004B06 resulted in a final sequence

optimised variant F027100183, which comprises 4 amino acid substitutions (i.e. L11V, R74S, K83R, V89L) compared to the parental ISVD F107004B06.

The sequence optimised variants were assembled from oligonucleotides using a PCR overlap extension method. The variants were expressed in *E.coli* and purified by IMAC and desalting. F027100019 and F027100183 were evaluated for their hIL-13 binding capacity by surface plasmon resonance, using hIL-13 from Peprtech (cat nr 200-13). Additionally, F027100019 was tested for its neutralizing activity in the eotaxin release assay. Monomeric behavior of both variants was monitored by Size Exclusion-HPLC (SE-HPLC). Thermal stability of the variants was tested in a thermal shift assay (TSA) using the Lightcycler (Roche). In this assay, the parental ISVDs and their variants are incubated at different pH's in the presence of sypro orange and a temperature gradient is applied. When the ISVDs start denaturing, sypro orange binds and the measured fluorescence increases suddenly, as such a melting temperature can be determined for a certain pH. Results are summarized in Table 17 and Table 18.

15

Table 17: results of the analysis of the sequence optimization variant F027100019 of anti-IL-13 ISVD F0107004B02. nd = not determined, * measured on hIL-13-Fc

ISVD ID	Mutation(s)	IC50 (nM) Eotaxin release assay	Kon hIL-13 (1/Ms)	Koff hIL-13 (1/s)	KD hIL-13 (M)	Tm (°C) at pH 7 TSA	SE-HPLC % main peak
F0107004B02	WT	nd	2.20E+04*	2.40E-02*	1.10E-06*	67	94
F027100019	E1D, L11V, A14P, N64K, S65G, A74S, K83R, I89L	47	3.50E+04	1.20E-03	3.40E-08	70	100

F027100019 exhibited good potency in the Eotaxin release assay and its affinity to hIL-13 was determined to be 34nM in SPR. The Tm of F027100019 is 3°C higher than for the parental ISVD F0107004B2. The % framework identity in the framework regions for F027100019 is 85% based on the AbM definition (see Antibody Engineering, Vol2 by Kontermann & Dübel (Eds), Springer Verlag Heidelberg Berlin, 2010) and 86% based on the Kabat definition.

20

Table 18: results of the analysis of the sequence optimization variant of anti-IL13 ISVD F0107004B06. Nd = not determined

ISVD ID	Mutation(s)	Kon hIL-13 (1/Ms)	Koff hIL-13 (1/s)	KD hIL-13 (M)	Tm (°C) at pH 7 TSA	SE-HPLC % main peak
F0107004B06	WT	2.50E+05	3.30E-03	2.60E-08	nd	nd
F027100183	L11V, R74S, K83R, V89L	2.80E+05	7.50E-03	2.70E-08	61	100

Affinity of F027100183 is similar compared to the WT sequence and the variant has a Tm of 61°C. The variant elutes as a 100% monomeric peak on SE-HPLC. The % framework identity in the framework regions for F027100183 is 94.4% based on the AbM definition and 93.1% based on the Kabat definition.

F0107529F10

Sequence optimisation of anti-TSLP ISVD F0107529F10 resulted in a final sequence optimised variant F027400021, which comprises 8 amino acid substitutions (i.e., L11V, A14P, T60A, S71R, A74S, S79Y, K83R, V89L) compared to the parental ISVD F0107529F10. Sequence optimisation of anti-TSLP ISVD F0107501A02 resulted in a final sequence optimised variant F027400160, which comprises 6 amino acid substitutions (i.e., L11V, A14P, E16G, A74S, K83R, V89L) compared to the parental ISVD F0107501A02.

The sequence optimised variants were assembled from oligonucleotides using a PCR overlap extension method. The constructs were expressed in *E.coli* and purified by IMAC and desalting. The variants were evaluated for their binding capacity to human and cyno TSLP by surface plasmon resonance. Monomeric behaviour of all variants was monitored by Size Exclusion-HPLC (SE-HPLC) and the thermal stability in a thermal shift assay (TSA). Additionally, the variants of F0107501A02 were tested for their blocking activity on hTSLP in the ternary complex Alphascreen. Results are summarized in Table 19 and Table 20.

Table 19: Results of the analysis of the sequence optimization variants of anti-TSLP ISVD F0107529F10, nd = not determined

ISVD ID	Mutations	koff hTSLP (s ⁻¹)	koff cyno TSLP (s ⁻¹)	K _D hTSLP- hFc (M)	K _D cyno TSLP-hFc (M)	T _m (°C) at pH 7 TSA	SE-HPLC % main peak
F0107529F10	WT	6.60E-05	1.80E-03	3.50E-11	1.70E-09	64.0	nd
F010704099	A14P, T60A, S71R, A74S, S79Y, K83R	7.70E-05	1.80E-03	3.80E-11	3.30E-09	75.4	100
F027400021	L11V, A14P, T60A, S71R, A74S, S79Y, K83R, V89L	nd	nd	nd	nd	74.0	100

Intermediate variant F010704099 with mutations A14P, T60A, S71R, A74S, S79Y, K83R showed a similar affinity on hTSLP and a 2-fold lower affinity on cyno TSLP compared to the parental ISVD F0107529F10. The T_m showed an overall increase of 11.4°C compared to the parental ISVD. Two additional mutations, i.e. L11V and V89L, were introduced into variant F010704099 to minimize pre-existing antibody binding, resulting in the final variant F027400021. The % framework identity in the framework regions for F027400021 is 89.9% based on the AbM definition and 88.5% based on the Kabat definition.

Table 20: Results of the analysis of the sequence optimization (SO) variants of anti-TSLP ISVD F0107501A02.

ISVD ID	Mutation(s)	Koff hTSLP (s ⁻¹)	koff cyno TSLP (s ⁻¹)	Ternary complex AlphaScreen IC50 (M)	T _m (°C) at pH 7 TSA	SE-HPLC % main peak
F0107501A02	WT	5.4E-05	1.5E-03	2.8E-10	68.5	100
F010704076	A14P, E16G, A74S, K83R	6.2E-05	1.6E-03	2.7E-10	70.8	99.3
F027400160	L11V, A14P, E16G, A74S, K83R, V89L	1.2E-04	4.3e-03	nd	70.0	100

Intermediate variant F010704076 with mutations A14P, E16G, A74S, K83R showed a similar off-rate on hTSLP and on cyno TSLP compared to the parental ISVD F0107501A02 and a similar potency in ternary complex Alphascreen. The T_m showed an overall increase of 12.3°C compared to the parental ISVD. Two additional mutations i.e., L11V and V89L,

were introduced into variant F010704076 to minimize pre-existing antibody binding, resulting in the final variant F027400160.

The % framework identity in the framework regions for F027400160 is 83.1% based on the AbM definition and 80.5% based on the Kabat definition.

5 **Table 21: Amino acid sequences of SO version of ISVD F0107004B02, F0107004B06, F0107501A02 and F0107529F10.**

ISVD ID	ISVD description	Sequence
F010704076 (SEQ ID NO: 160)	F0107501A02 (A14P, E16G, A74S, K83R)*	EVQLVESGGGLVQPGGSLRLSCAASGSGFGVNILYWYRQA AGIERELIASITSGGITNYVDSVKGRFTISRDNSENTMYLQM NSLRAEDTGYYCASRNIFDGTTEWGQGTTLTVSS
F010704099 (SEQ ID NO: 161)	F0107529F10 (A14P, T60A, S71R, A74S, S79Y, K83R)*	EVQLVESGGGLVQPGGSLRLSCAASGFTFADYDYDIGWFR QAPGKEREGVSCISNRDGSYYADSVKGRFTISRDNKNTV YLQMNSLRPEDTAVYYCAVEIHCDYGVENFDYDPWGQG TLTVSS
F027100019 (SEQ ID NO: 162)	F0107004B02 (E1D, L11V, A14P, N64K, S65G, A74S, K83R, I89L)*	DVQLVESGGGVVQPGGSLRLSCAASGRTFSSYRMGWFRQ APGKEREFVAALSGDGYSTYANSVKGRFTISRDNKNTVYL QMNSLRPEDTALYYCAAKLQYVSGWSYDYPYWGQGTTLV VSSAAA
F027100183 (SEQ ID NO: 163)	F0107004B06 (L11V, R74S, K83R, V89L)*	EVQLVESGGGVVQPGGSLRLSCAASGFTFNYYAMKWVRQ APGKGLEWVSSITGGGSTDYADSVKGRFTISRDNKNTLYL QMNSLRPEDTALYYCANVPFGYYSEHFSGLSFDYRGQGT TVSS
F027400021 (SEQ ID NO: 164)	F0107529F10 (L11V, A14P, T60A, S71R, A74S, S79Y, K83R, V89L)*	EVQLVESGGGVVQPGGSLRLSCAASGFTFADYDYDIGWFR QAPGKEREGVSCISNRDGSYYADSVKGRFTISRDNKNTV YLQMNSLRPEDTALYYCAVEIHCDYGVENFDYDPWGQGT LTVSS
F027400160 (SEQ ID NO: 165)	F0107501A02 (L11V, A14P, E16G, A74S, K83R, V89L)*	EVQLVESGGGVVQPGGSLRLSCAASGSGFGVNILYWYRQA AGIERELIASITSGGITNYVDSVKGRFTISRDNSENTMYLQM NSLRAEDTGLYYCASRNIFDGTTEWGQGTTLTVSS

*() indicates the amino acid substitutions introduced into the (parental) monovalent building block.

6.4 Multispecific ISVD construct F027400161

- 10 The above identified optimized ISVDs F027100019 (optimized variant of F0107004B02), F027100183 (optimized variant of F0107004B06), F027400021 (optimized variant of F0107529F10), and F027400160 (optimized version of F0107501A02) were used for the generation of multispecific ISVD construct F027400161. The optimized monovalent

building blocks used in F027400161 are designated in the following in an abbreviated form according to their ISVD origin as 04B02, 04B06, 529F10, and 501A02, respectively.

6.4.1 Example 15: Multispecific ISVD construct generation

- Identification of ISVD-containing polypeptide F027400161 (SEQ ID NO: 1) binding to IL-13 and TSLP resulted from a data-driven bispecific engineering and formatting campaign in which several anti-TLSP building blocks, several anti-IL13 building blocks and the anti-HSA building block ALB23002 were included. Different positions/orientations of the building blocks and different linker lengths (9GS vs 35GS) were applied and proofed to be critical for different parameters (potency, cross-reactivity, expression yield, etc..).
- A large panel of different ISVD constructs was transformed in *Pichia Pastoris* for small scale productions. Induction of ISVD expression occurred by stepwise addition of methanol. Clarified medium with secreted ISVD was used as starting material for purification via Protein A affinity chromatography followed by desalting. The purified samples were used for functional characterisation and expression evaluation.
- Some constructs showed impaired potencies depending on linker length and relative position of ISVD building blocks. For example: potency for cyno TSLP of the anti-TSLP biparatopic combinations 501A02-529F10 is strongly impaired when linked with a short 9GS linker. Some constructs showed low expression levels depending on the combination and order of the building blocks. For the bispecifics comprising 4B02-4B06 expression was best in combination with 501A02-529F10.

Table 22: Selection of different multispecific ISVD formats evaluated. BB = building block, ALB = ALB23002

ISVD ID	BB1	linker 1	BB2	linker 2	BB3	linker 3	BB4	linker 4	BB5
F027400161	4B02	35GS	4B06	9GS	501A02	9GS	ALB	9GS	529F10
F027400162	4B02	9GS	501A02	9GS	4B06	9GS	529F10	9GS	ALB
F027400163	501A02	9GS	ALB	9GS	529F10	9GS	4B02	35GS	4B06
F027400183	501A02	35GS	529F10	9GS	4B02	9GS	ALB	9GS	4B06

F027400189	4B02	9GS	ALB	9GS	4B06	9GS	501A02	35GS	529F10
F027400283	501A02	9GS	4B02	9GS	529F10	9GS	4B06	35GS	ALB
F027400284	501A02	35GS	4B02	35GS	529F10	35GS	4B06	35GS	ALB
F027400296	4B02	9GS	501A02 (N73Q)*	9GS	4B06	9GS	529F10	9GS	ALB
F027400298	501A02 (N73Q)*	9GS	4B02	9GS	529F10	9GS	4B06	9GS	ALB

*() indicates the amino acid substitutions introduced into the (parental) monovalent building block.

Table 23 illustrates that different yields ranging from low to high titer were obtained for six constructs comprising the same building blocks but ordered in different ways and connected with different linker lengths. Highest expression titers are obtained for constructs comprising the IL-13 ISVDs 4B02 and 4B06 linked via a 35GS linker (F027400161 and F027400163). In addition, the solubility of F027400161 and F027400163 was much higher than their respective counterparts F027400296 and 298, of which building blocks are linked with four 9GS linkers and ALB is positioned at the C-terminus.

Subsequently, the large bispecific panel was trimmed down to a panel of 2 bispecific constructs, consisting of ISVD constructs F027400161 and F027400163 proven to be potent on both targets (human and cyno) and having the potential of high expression levels, based on preliminary yield estimates.

Larger scale 2L and 5L productions in *Pichia Pastoris* were done for expression yield determination and assessment of biophysical properties and pre-existing antibody reactivity.

Table 24 and example 22 demonstrate that pre-existing antibody reactivity is driven by the orientation of the building blocks and the linker lengths.

Table 23: Expression levels and solubility of 6 ISVD constructs with the building blocks of 4B02, 4B06, 501A02 and 529F10 in different orientations and/or with different linker lengths.

ISVD construct ID	BB1	linker 1	BB2	linker 2	BB3	linker 3	BB4	linker 4	BB5	Yield 5 ml expression (µg/ml)	Yield 5L fermentation (g/L)	solubility (mg/ml)
F027400161	4B02	35GS	4B06	9GS	501A02	9GS	ALB	9GS	529F10	124	4.7	145
F027400163	501A02	9GS	ALB	9GS	529F10	9GS	4B02	35GS	4B06	114	4.4	>150
F027400189	4B02	9GS	ALB	9GS	4B06	9GS	501A02	35GS	529F10	77		
F027400183	501A02	35GS	529F10	9GS	4B02	9GS	ALB	9GS	4B06	55		
F027400296	4B02	9GS	501A02 (N73Q)*	9GS	4B06	9GS	529F10	9GS	ALB	80	2.4	Very low
F027400298	501A02 (N73Q)*	9GS	4B02	9GS	529F10	9GS	4B06	9GS	ALB	80	2.4	25

*() indicates the amino acid substitutions introduced into the (parental) monovalent building block.

5 Table 24: Binding of pre-existing antibodies present in 96 human serum samples to F027400161, F027400163 and F027400164 compared to control ISVD construct F027301186.

Construct ID	BB1	linker 1	BB2	linker 2	BB3	linker 3	BB4	linker 4	BB5	25% percentile	Median RU levels	75% percentile
F027301186	1E07	35GS	1E07	35GS	1C02	9GS	ALB	9GS	1C02	61	135	622
F027400161	4B02	35GS	4B06	9GS	501A02	9GS	ALB	9GS	529F10-A	8	22	34
F027400163	501A02	9GS	ALB	9GS	529F10	9GS	4B02	35GS	4B06-A	19	34	63
F027400164	501A02	9GS	4B02	9GS	529F10	9GS	4B06	9GS	ALB-A	4	13	24

Finally, ISVD construct F027400161 was selected based on potency, reduced binding to pre-existing antibodies and superior expression levels and CMC characteristics

6.4.2 Example 16: Multispecific ISVD construct binding affinity to TSLP, IL13 and serum albumin

5 The affinity, expressed as the equilibrium dissociation constant (K_D), of F027400161 towards human and cyno TSLP (Human and cyno TSLP sequences are known (Uniprot accession Uniprot accession Q969D9 and NCBI RefSeq XP_005557555.1, respectively). Recombinant protein was used to perform the assay, human IL-13 (Sino Biological cat nr 10369-HNAC), cyno IL-13 (Sino Biological cat nr 11057-CNAH) and rhesus IL-13 (R&D
10 systems cat nr 2674-RM) and human (Sigma Aldrich, cat nr A8763), cyno and mouse (Albumin Bioscience cat nr N1204H1CM) serum albumin was quantified by means of in-solution affinity measurements on a Gyrolab xP Workstation (Gyros).

Under K_D -controlled measurements a serial dilution of TSLP or IL-13 (ranging from 1 μ M – 0.25 fM) or serum albumin (ranging from 100 μ M – 320 pM) and a fixed amount of
15 F027400161 (5 pM or 10 pM in case of TSLP and IL13 and 20 nM in case of serum albumin) were mixed to allow interaction and incubated for either 48 or 72 hours (in case of TSLP and IL13) or 2 hours (in case of serum albumin) to reach equilibrium.

Under receptor-controlled measurements a serial dilution of TSLP or IL-13 (ranging from 1 μ M – 0.25 fM) or serum albumin (ranging from 100 μ M – 320 pM) and a fixed amount
20 of F027400161 (250 pM in case of TSLP, 10 nM in case of IL-13 and 1 μ M in case of serum albumin) were mixed to allow interaction and incubated for either 48 or 72 hours (in case of TSLP and IL-13) or 2 hours (in case of serum albumin) to reach equilibrium.

Biotinylated human TSLP/IL-13/serum albumin was captured in the microstructures of a Gyrolab Bioaffy 1000 CD, which contains columns of beads and is used as a molecular
25 probe to capture free F027400161 from the equilibrated solution. The mixture of TSLP/IL-13/serum albumin and F027400161 (containing free TSLP/IL-13/serum albumin, free F027400161 and TSLP/IL-13/serum albumin - F027400161 complexes) was allowed to flow through the beads, and a small percentage of free F027400161 was captured, which

is proportional to the free ISVD concentration. A fluorescently labeled anti-V_{HH} antibody, ABH0086-Alexa647, was then injected to label any captured F027400161 and after rinsing away excess of fluorescent probe, the change in fluorescence was determined. Fitting of the dilution series was done using Gyrolab Analysis software, where K_D- and receptor-controlled curves were analyzed to determine the K_D value.

The results (Table 25) demonstrate that the multispecific ISVD construct binds human/cyno TSLP and human/cyno/rhesus IL13 with high affinity.

Table 25: Binding affinities of F027400161 to human, rhesus and cyno TSLP and IL13 and human, cyno and mouse serum albumin (sequences of cyno and rhesus TSLP and cyno and rhesus albumin are identical).

Antigen	human		cynomolgus monkey		rhesus monkey		Incubation time (h)
	K _D (pM)	95% CI (pM)	K _D (pM)	95% CI (pM)	K _D (pM)	95% CI (pM)	
TSLP	1.8	1.2 - 2.3	55.9	51 - 61			48
	2.3	1.4 - 3.3	44.0	26 - 62			48
	1.0	0.65 - 1.4	59.0	35 - 83			72
IL13	4.2	3.6 - 4.8	3.8	2.8 - 4.9	6.4	5.3 - 7.4	48
	6.3	5.3 - 7.3	1.5	0.7 - 2.3	1.9	0.6 - 3.1	48
	4.7	3.8 - 5.7	3.5	2.7 - 4.2	1.5	0.5 - 2.4	72
Antigen	human		cynomolgus monkey		mouse		Incubation time (h)
	K _D (nM)	95% CI (nM)	K _D (nM)	95% CI (nM)	K _D (nM)	95% CI (nM)	
SA	119	99.4 - 139	198	185 - 211	1800	1700 - 1900	2
	110	56 - 162	133	70 - 196			2
	145	69 - 222	145	78 - 213			2

6.4.3 Example 17: Multispecific ISVD construct binds selectively to TSLP and IL13

Absence of F027400161 binding to IL-4 and IL-7 as IL13 and TSLP related cytokines was assessed via SPR (Proteon XPR36), respectively.

Cytokines were immobilized on a proteon GLC sensor chip at 25 µg/mL for 600s using
5 amine coupling, with 80 seconds injection of EDC/NHS for activation and a 150 seconds
injection of 1 M Ethanolamine HCl for deactivation (ProteOn Amine Coupling Kit. cat. 176-
2410). Flow rate during activation and deactivation was set to 30 µl/min and during ligand
injection to 25 µl/min. The pH of the 10 mM Acetate immobilization buffer was 6.0 for
IL13 and IL4 (Peprotech cat nr 200-07) and 5.5 for TSLP and IL7 (R&D systems cat nr 204-
10 IL/CF).

Next, 1 µM of F027400161 was injected for 2 minutes and allowed to dissociate for 600s
at a flow rate of 45 µL/min. As running buffer PBS (pH7.4) + 0.005% Tween 20 was used.
As positive controls, 100 nM α-hIL4 Ab and 100 nM α-IL7 Ab were injected. Interaction
between F027400161 and the positive controls with the immobilized targets was
15 measured by detection of increases in refractory index which occurs as a result of mass
changes on the chip upon binding.

All positive controls did bind to their respective target. No binding was detected of
F027400161 to human IL4 and IL7.

In addition, it was investigated if F027400161 could bind to the short form of TSLP. To this
20 end, TSLP and the short form of TSLP (as described in Fornasa, 2015) were immobilized on
a proteon GLC sensor chip at 10 µg/mL, respectively 5 µg/ml for 150s using amine
coupling as described above. The pH of the 10 mM Acetate immobilization buffer was 5.5
for TSLP and 4.0 for the short form of TSLP.

Next, 500 nM of F027400161 was injected for 2 minutes and allowed to dissociate for
25 600s at a flow rate of 45 µL/min. As running buffer PBS (pH7.4) + 0.005% Tween 20 was
used. As reference compound, 500 nM anti-hTSLP reference mAb1 was injected and as
positive control 500 nM α-hTSLP pAb (Abcam ab47943).

Whereas the positive control did bind to both the long (normal) and short form of TSLP, no binding was detected of F027400161 and anti-hTSLP reference mAb1 to the short form of TSLP.

6.4.4 Example 18: Simultaneous binding of multispecific ISVD construct to IL13, TSLP and HSA

A Biacore T200 instalment was used to determine whether F027400161 can bind simultaneously to hTSLP and hIL13. To this end hTSLP (recombinant human TSLP is commercially available, such as from R&D Systems (cat nr 1398-TS) was immobilized on a CM5 Sensor chip via amine coupling. 100 nM of F027400161 was injected for 2 min at 10 µl/min over the TSLP surface in order to capture the ISVD construct via the TSLP building blocks 501A02-529F10. Subsequently either 100 nM of hIL13 (PeproTech, cat nr 200-13), HSA or hOX40L or 1000 nM of HSA were injected or mixtures of 100 nM IL13 + 100 nM HSA , 100 nM IL13 + 1000 nM HSA, 100 nM OX40L + 100 nM HSA , 100 nM OX40L + 1000 nM HSA or 100nM IL13 + 100nM OX40L, at a flow rate of 10 µl/min for 2 min followed by a subsequent 300 seconds dissociation step. The TSLP surfaces are regenerated with a 1 minute injection of 0.5% SDS + 10 mM glycine pH3 at 45 µl/min. The sensorgram (Figure 1) demonstrates that F027400161 can bind human IL13, human TSLP and HSA simultaneously as shown by the increase in response units after capture on TSLP: ~130 RU increase from IL13 only, ~60 RU increase from 100 nM HSA and ~350 RU from 1000 nM HSA only, ~180 RU increase for the IL13 and 100 nM HSA mixture, and ~500 RU for the IL13 and 1000 nM HSA mixture. Higher concentrations of HSA were needed to see decent RU increase levels, due to the lower affinity of F027400161 for HSA (see example 16).

Example 19: Multispecific ISVD construct inhibition of IL13 induced eotaxin release *in vitro*

Functional activity of soluble IL13 from the different species of interest (human, rhesus and cynomolgus monkey) and inhibition thereof by F027400161 was studied using a cell based assay investigating eotaxin release by A549 human lung carcinoma cells.

To this end, A549 suspension cells were cultured in Ham's F12K medium, supplemented with 10% FCS, and seeded into a 96 well plate at 400.000 cells/well. After 24 hours incubation, a dilution series of F027100161 or reference compounds (anti-hIL-13 reference mAb1 and anti-hIL-13 reference mAb2) were added. After 20 min incubation, human IL13 (Sino Biological cat nr 10369-HNAC), cyno IL13 (Sino Biological cat nr 11057-CNAH) or rhesus IL13 (R&D Systems, cat nr 2674-RM-025) is added to a final concentration of 160 pM. After further incubation for 24 hours in the presence of 30 μ M HSA, heparin is added at a final concentration of 50 μ g/ml, to enhance the eotaxin expression. After an additional 4 hours of incubation, eotaxin-3, secreted in the cell supernatant was quantified by use of the Human CCL26/Eotaxin-3 DuoSet ELISA (R&D systems, DY346).

F027400161 inhibited human, cyno and rhesus IL13-induced eotaxin-3 release in a concentration-dependent manner with an IC₅₀ of 194 pM (for human IL13), 1040 pM (for cyno IL13) and 713 pM (for rhesus IL13), comparable to the reference compound anti-hIL-13 reference mAb1, and better than the reference compound anti-hIL-13 reference mAb2 (Table 26, Figure 2).

Table 26: IC₅₀ values of F027400161 mediated neutralization of human, cyno and rhesus IL13 in the eotaxin release assay versus the reference compounds anti-hIL-13 reference mAb1 and anti-hIL-13 reference mAb2. nd = not determined

	F027400161			anti-hIL-13 reference mAb1			anti-hIL-13 reference mAb2		
antigen	Human IL13	Cyno IL13	Rhesus IL13	Human IL13	Cyno IL13	Rhesus IL13	Human IL13	Cyno IL13	Rhesus IL13
eotaxin release assay (IC ₅₀ , pM)	194	1040	713	45	160	99	668	nd	3560

6.4.5 Example 20: Multispecific ISVD construct inhibition of IL13 induced STAT-6 activation in HEK-Blue IL4/IL13 cells

HEK-Blue™ IL-4/IL-13 cells were generated by stable transfection of HEK293 cells with the human STAT6 gene and a STAT6-inducible SEAP reporter gene. Upon IL-4 and IL-13

stimulation, the cells produce STAT6-induced SEAP secreted in the supernatant quantified by QUANTI-Blue™.

HEK-Blue™ cells were cultured DMEM, supplemented with 10% FBS and seeded into a 96 well plate at 50.000 cells/well. A dilution series of F027100161 or reference compounds (anti-hIL-13 reference mAb1 and anti-hIL-13 reference mAb2) was pre-incubated with 10 pM hIL13 (Sino Biological cat nr 10369-HNAC) or cyno IL-13 (Sino Biological cat nr 11057-CNAH) for 1 hour at room temperature and added to the cells. After incubation for 22 to 24 hours in the presence of 30 µM HSA, 40 µl of the cell supernatant was mixed with 160 µl QUANTI-Blue™. Secreted SEAP was quantified by measuring absorption at 620 nm on a Clariostar instrument.

F027400161 inhibited human and cyno IL-13 induced SEAP secretion in a concentration-dependent manner with an IC50 of 32.8 pM (for human IL-13) and 53.4 pM (for cyno IL-13), better than the reference compound anti-hIL-13 reference mAb2 (Table 27, Figure 3).

Table 27: IC50 values of F027400161 mediated neutralization of human and cyno IL-13 in the SEAP reporter assay versus the reference compounds anti-hIL-13 reference mAb1 and anti-hIL-13 reference mAb2.

Construct ID	IC50 hIL13 (M)	IC50 cyno IL13 (M)
F027400161	3.28E-11	5.34E-11
anti-hIL-13 reference mAb2	1.10E-10	4.26E-10
anti-hIL-13 reference mAb1	3.45E-12	5.85E-12

6.4.6 Example 21: Multispecific ISVD construct inhibition of TSLP- induced Ba/F3 cell proliferation *in vitro*

Functional activity of soluble TSLP from the different species of interest (human, rhesus and cynomolgus monkey) and inhibition thereof by F027400161 was studied using a cell-based assay investigating proliferation of BaF3 cells transfected with plasmids encoding hTSLPR and hIL7Ra.

- Cells were seeded at a density of 15000 cells/well in RPMI 1640 growth medium in cell culture treated white 96 well plates. A dilution series of F027100161 or reference compounds (anti-hTSLP reference mAb1) were added, followed by addition of 5 pM human or cyno TSLP for stimulation of the cells. The human and cyno TSLP sequences are known (Uniprot accession Q969D9 and NCBI RefSeq XP_005557555.1, respectively). Recombinant protein was used to perform the assay. After incubation for 48 hours in the presence of 30 μ M HSA, cell density and viability were monitored using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, G7571/G7572/G7573) and read-out on an EnVision Multilabel Reader (Perkin Elmer).
- F027400161 inhibited human and cyno TSLP dependent proliferation of Ba/F3 cells in a concentration-dependent manner with an IC₅₀ of 7.8 pM (for human TSP) and 24 pM (for cyno TSLP), performing hence much better than the reference compound anti-hTSLP reference mAb1 (Table 28, Figure 4).

Table 28: IC₅₀ values of F027400161 mediated neutralization of human and cyno TSLP induced BaF3 proliferation versus the reference compound anti-hTSLP reference mAb1.

	F027400161		anti-hTSLP reference mAb1	
antigen	Human TSLP	Cyno TSLP	Human TSLP	Cyno TSLP
BaF3 proliferation assay (IC₅₀, pM)	7.8	24	356	3180

6.4.7 Example 22: Multispecific ISVD construct binding to pre-existing antibodies

- The pre-existing antibody reactivity of ISVD construct F027400161 was assessed in normal human serum (n=96) using the ProteOn XPR36 (Bio-Rad Laboratories, Inc.). PBS/Tween (phosphate buffered saline, pH7.4, 0.005% Tween20) was used as running buffer and the experiments were performed at 25°C.

ISVDs are captured on the chip via binding of the ALB23002 building block to HSA, which is immobilized on the chip. To immobilize HSA, the ligand lanes of a ProteOn GLC Sensor Chip are activated with EDC/NHS (flow rate 30 μ l/min) and HSA is injected at 100 μ l/ml in

ProteOn Acetate buffer pH4.5 to render immobilization levels of approximately 2900 RU. After immobilization, surfaces are deactivated with ethanolamine HCl (flow rate 30µl/min).

Subsequently, ISVD constructs are injected for 2 min at 45µl/min over the HSA surface to render an ISVD capture level of approximately 800 RU. The samples containing pre-existing antibodies are centrifuged for 2 minutes at 14,000rpm and supernatant is diluted 1:10 in PBS-Tween20 (0.005%) before being injected for 2 minutes at 45µl/min followed by a subsequent 400 seconds dissociation step. After each cycle (i.e. before a new ISVD capture and blood sample injection step) the HSA surfaces are regenerated with a 2 minute injection of HCl (100 mM) at 45µl/min. Sensorgrams showing pre-existing antibody binding are obtained after double referencing by subtracting 1) ISVD-HSA dissociation and 2) non-specific binding to reference ligand lane. Binding levels of pre-existing antibodies are determined by setting report points at 125 seconds (5 seconds after end of association). Percentage reduction in pre-existing antibody binding is calculated relative to the binding levels at 125 seconds of a reference ISVD.

The pentavalent ISVD construct F027400161, optimized for reduced pre-existing antibody binding by introduction of mutations L11V and V89L in each building block and a C-terminal alanine, shows substantially less binding to pre-existing antibodies compared to a control non-optimized pentavalent ISVD construct F027301186, (Table 24 and Figure 5).

Pre-existing antibody binding depends on the orientation of the building blocks and the linker lengths present in the multispecific constructs. Table 24 and Figure 5 demonstrate that construct F027400161 shows lower pre-existing antibody reactivity than construct F027400163, due to its specific orientation, but that F027400164 shows lower reactivity than F027400161, due to short linkers all over.

6.4.8 Example 23: F027400161 blocks TSLP-induced CCL17 in human dendritic cells *in vitro*

The type 2 inflammation cascade is initiated and propagated by a concerted action of epithelial cells, dendritic cells, type 2 helper T cells (Th2 cells), mast cells and innate lymphoid cells in a context-dependent manner. The cytokine thymic stromal lymphopoietin (TSLP) has been implicated in the initiation and progression of allergic inflammation through its ability to activate dendritic cells (DCs). Upon activation by TSLP, human DCs produce CCL17, a Th2-associated chemokine, and drive Th2 cell differentiation from naïve CD4⁺ T cells. F027400161 targets both TSLP and IL-13, can block the interaction between TSLP and DCs, and thereby, reduce CCL-17 production and is projected to confer efficacy in type 2 inflammatory diseases and beyond.

The ability of F027400161 to inhibit TSLP-induced CCL17 production was assessed in human DCs isolated from 8 individual healthy donors in comparison to the reference monospecific antibody, anti-hTSLP reference mAb1. Human DCs (CD3⁻CD14⁻CD11c⁺HLA-DR^{high}) were isolated and enriched from healthy human PBMCs in buffy coat (human leucocyte pack) samples. A total of 0.5-0.8x10⁶ DCs/well were incubated with eight 3-fold serially diluted concentrations of F027400161 (400 ng/mL or 5.714 nM top concentration) or eight 4-fold serially diluted concentrations of anti-hTSLP reference mAb1 (4000 ng/mL or 27 nM top concentration) prior to stimulation with 4 ng/mL of recombinant human TSLP for 36 hours in 37°C cell culture incubator. Recombinant human TSLP is commercially available, such as from R&D Systems (Catalog number 1398-TS). CCL17 production in freshly collected cell culture supernatant was measured by ELISA and IC₅₀ values of the ISVD construct and benchmark antibody were calculated in Graphpad Prism.

The collective results of the dose inhibition responses of F027400161 and anti-hTSLP reference mAb1 in human DCs, are shown in Figure 6. The reference monospecific antibody anti-hTSLP reference mAb1 inhibited TSLP induced CCL17 production at a mean IC₅₀ concentration of 793.4 pM, while F027400161 inhibited TSLP induced CCL17 production with a mean IC₅₀ of 53.26 pM.

In conclusion, these results demonstrate that the ISVD construct F027400161 is more potent in inhibiting TSLP-induced CCL17 response in human DCs, compared to anti-hTSLP reference mAb1.

6.4.9 Example 24: F027400161 blocks 0.5 ng/mL [IL-13+TSLP] induced synergistic CCL17 in human PBMCs *in vitro*

Type 2 cytokines such as thymic stromal lymphopoietin (TSLP) and Interleukin-13 (IL-13) exert unique, additive and synergistic response to drive asthma and atopic dermatitis (AD) pathophysiology. The roles of TSLP as an epithelial cell-derived initiator of the type 2 immune cascade and of IL-13 as a downstream effector cytokine have been extensively validated. F027400161 targets both TSLP and IL-13, thereby projected to confer efficacy in type 2 mediated inflammatory diseases and beyond.

The ability of F027400161 to inhibit 0.5 ng/mL IL-13 and TSLP-induced synergistic production of CCL17 (TARC) was evaluated in human PBMCs from 8 individual healthy donors. The study was designed to evaluate non-inferiority of the ISVD construct versus monospecific biologics, anti-hTSLP reference mAb1 and anti-hIL-13 reference mAb1. One million cells per well of human PBMCs were stimulated with 0.5 ng/mL of recombinant human TSLP (recombinant human TSLP is commercially available, such as from R&D Systems (cat nr 1398-TS) plus IL-13 (R&D Systems, cat nr 213-ILB-005/CF) and incubated with ten 3-fold serially diluted concentrations of the ISVD construct (10 nM top concentration), anti-hIL-13 reference mAb1 (10 nM top concentration) and anti-hTSLP reference mAb1 (100 nM top concentration) in a 96-well plate for 20 hours in 37°C cell-culture incubator. The assays were performed with technical triplicates within each donor for F027400161. The concentrations of the cytokines used were within 2-fold of the standard error of reported literature values from sera, BAL fluid, sputum and skin of normal humans, asthmatics and atopic dermatitis patients (Berraïes A et al, Immunol Letter 178: 85-91, 2016; Bellini A et al, Mucosal Immunology 5(2):140-9, 2012; Davoodi P et al, Cytokine 60(2):431-7, 2012; Szegedi K et al, J Eur Acad Dermatol Venereol 29(11):2136-44, 2015). CCL17 production in freshly collected cell culture supernatant was measured by Meso Scale Diagnostics (MSD) V-PLEX Human TARC Kit.

The collective results of the dose inhibition responses of F027400161 and benchmark antibodies, anti-hIL-13 reference mAb1 and anti-hTSLP reference mAb1, are shown in Figure 7. F027400161 demonstrated 100% inhibition of 0.5 ng/mL IL-13+TSLP-induced synergistic CCL17 production with a mean IC_{50} of 0.0061 nM. The reference antibody anti-hIL-13 mAb1, despite having a lower mean IC_{50} of 0.0028 nM, was unable to fully block the synergistic CCL17 response at equimolar doses of F027400161 and plateaued at approximately 80% inhibition. On the other hand, anti-hTSLP reference mAb1 was only able to block approximately 50% of the CCL17 production with a mean IC_{50} of 2.932 nM.

In conclusion, these results demonstrate that F027400161 is more potent than anti-hTSLP reference mAb1 and is superior compared to anti-hIL-13 reference mAb1 for blocking pathophysiological relevant concentrations of IL-13 and TSLP-induced synergistic response in human PBMCs, highlighting its therapeutic potential for the treatment of type 2 inflammatory diseases such as asthma and atopic dermatitis.

6.4.10 Example 25: F027400161 blocks 5 ng/mL [IL-13+TSLP] induced synergistic CCL17 in human PBMCs *in vitro*

The ability of F027400161 to inhibit 5 ng/mL IL-13+TSLP-induced CCL17 production was evaluated in human PBMCs from 8 healthy individual donors. The study was designed to evaluate non-inferiority of the ISVD construct versus monospecific biologics, anti-hTSLP reference mAb1 and anti-IL-13 reference mAb1. The concentrations of the cytokines used were ~10 fold over the upper end of the pathophysiological ranges of TSLP and IL-13 that have been reported in the literature in normal humans, asthmatics and atopic dermatitis patients (Berraies A et al, Immunol Letter 178: 85-91, 2016; Bellini A et al, Mucosal Immunology 5(2):140-9, 2012; Davoodi P et al, Cytokine 60(2):431-7, 2012; Szegedi K et al, J Eur Acad Dermatol Venereol 29(11):2136-44, 2015) as hypothetical concentrations during transient inflammatory state. One million cells per well of human PBMCs were stimulated with 5 ng/mL of recombinant human TSLP plus IL-13 (R&D Systems, cat nr 213-ILB-005/CF) and incubated with ten 3-fold serially diluted concentrations of F-27400161 (10 nM top concentrations), anti-hIL-13 reference mAb1 (10 nM top concentration) and anti-hTSLP reference mAb1 (100 nM top dose) in a 96-well plate for 20 hours in 37°C cell-

culture incubator. The assays were performed with technical triplicates within each donor for F027400161. CCL17 production in freshly collected cell culture supernatant was measured by Meso Scale Diagnostics (MSD) V-PLEX Human TARC Kit.

The collective results of the dose inhibition responses of F027400161 and benchmark antibodies, anti-hIL-13 reference mAb1 and anti-hTSLP reference mAb1, are shown in Figure 8. F027400161 demonstrated 100% inhibition of 5 ng/mL IL-13+TSLP-induced synergistic CCL17 production with a mean IC_{50} of 0.0387 nM. While PBMCs treated with equimolar doses of the comparator antibody anti-hIL-13 reference mAb1 showed a lower mean IC_{50} of 0.01339 nM, the inhibition response never reached 100% and plateaued at approximately 90% inhibition. On the other hand, anti-hTSLP reference mAb1 only partially blocked approximately 40% of the CCL17 production with a mean IC_{50} of 19 nM.

In conclusion, these results demonstrate that F027400161 is superior to both anti-hTSLP reference mAb1 and anti-hIL-13 reference mAb1 in blocking IL-13 and TSLP-induced synergistic CCL17 response in human PBMCs at [TSLP+IL-13] concentrations 10-fold over the pathophysiological ranges of the cytokines, highlighting its therapeutic potential for the treatment of asthma and atopic dermatitis during acute or inflammatory phases.

6.4.11 Experiment 27: F027400161 blocks allergen Der P-induced production of IL-5, CCL17, and CCL26 in a triculture assay system

TSLP drives type 2 immune response by inducing CCL17, IL-5, and IL-13 production. Subsequently, IL-13 triggers CCL26 production by local epithelial cells, leading to the ramification of type 2 immune response mediated inflammatory diseases and beyond.

The ability of F027400161 to inhibit TSLP-induced IL-5 and CCL17, and IL-13-induced CCL26 production was evaluated in a triculture assay system using MRC5 fibroblasts and A549 epithelial cells coculturing with Der P-stimulated human PBMCs from 6 individual normal donors for 6 days. The study was designed to evaluate non-inferiority of the ISVD versus monospecific biologics, anti-hTSLP reference mAb1 and anti-hIL-13 reference mAb1. MRC5 fibroblasts produced ~100pg/mL endogenous TSLP constitutively, and A549

epithelial cells produced CCL26 in response to IL-13 produced by PBMCs with Der P and endogenous TSLP stimulation. One day prior to coculture with human PBMCs, seventy-five thousand MRC5 fibroblasts and A549 epithelial cells per well were plated. One million cells per well of human PBMCs were added into plated MRC5 fibroblasts and A549 epithelial cells, stimulated with 3µg/mL of Der P, and incubated with 11.1 nM of ISVD, anti-hIL-13 reference mAb1, or anti-hTSLP reference mAb1 in a 24-well plate for 6 days in a 37°C cell-culture incubator. The assays were performed with technical triplicates within each donor for F027400161. The production of IL-5, CCL17, and CCL26 in freshly collected cell culture supernatant was measured by Human Magnetic Luminex Assays from RnD System.

The collective results of the inhibition responses of F027400161 and benchmark antibodies, anti-hIL-13 reference mAb1 and anti-hTSLP reference mAb1, are shown in Figure 9. F027400161 demonstrated 60% inhibition of CCL17, 50% inhibition of IL-5, and 95% inhibition of CCL26 production. The reference antibody anti-hTSLP reference mAb1 displayed 50% inhibition of CCL17, 50% inhibition of IL-5, and approximately 55% inhibition of CCL26 production. While anti-hIL-13 reference mAb1 demonstrate comparable 95% inhibition of CCL26 production, this reference antibody was only able to block approximately 35% of the CCL17 production and less than 10% of IL-5 production (Figure 9). Lack of complete inhibition of IL-5 and CCL17 by these tested molecules may suggest that Der P stimulation triggers PBMCs to elicit pathways other than TSLP and IL-13 to drive the production of IL-5 and CCL17.

In conclusion, these results demonstrate that the anti-TSLP/IL-13 ISVD F027400161 is superior than anti-hTSLP reference mAb1 and anti-hIL-13 reference mAb1 by its ability to block three cytokines and chemokines (CCL17, IL-5 and CCL26) in a complex assay system comprising of human PBMCs cocultured with tissue structural cells, highlighting its therapeutic potential for the treatment of type 2 inflammatory diseases such as asthma and atopic dermatitis, as well as a broad range of immunological disease indications.

6.4.12 Example 26: NSG-SGM3 Mouse model to evaluate F027400161 mediated target occupancy and pharmacodynamics *in vivo*

F027400161 targets both human TSLP and IL-13 and does not cross react with the murine orthologs. Hence, to evaluate the biological activities of F027400161, a xenografted, humanized model system was used. Female NSG-SGM3 (NOD/SCID-IL2R γ -/-, NOD.Cg-PrkdcscidIl2rytm1Wjl/SzJ) were obtained from Jackson labs, Bar Harbor, ME, USA. These mice express human hematopoietic cytokines: stem cell factor (SCF), granulocyte/macrophage stimulating factor (GM-CSF), and interleukin-3 (IL-3), all driven by a human cytomegalovirus promoter/enhancer sequence. The triple transgenic mouse produces above cytokines constitutively, providing cell proliferation and survival signals, supporting the stable engraftment of CD33+ myeloid lineages, and several types of lymphoid cells. Briefly, the protocol followed for engraftment is as follows:

On day 0 of the study, mice were irradiated with 150 centigray at a rate of 120 rads/minute for 1 minute and 15 seconds. Mice were engrafted with 1×10^5 cord blood CD34+ stem/progenitor cells by the intravenous (IV) route in 200 μ l of Dulbecco's phosphate buffered saline (DPBS) approximately 6 hours post-engraftment. One group of mice were irradiated in the same manner, but not engrafted. These mice are considered irradiated naïve mice. On day 88 post engraftment, mice received a hydrodynamic (HDD) *i.v.* injection of either saline (engrafted control mice) or 50 μ g of IL-4 minicircle DNA in combination with 50 μ g of TSLP minicircle DNA. On day 91, a submandibular bleed was performed, and 100 to 150 μ l of blood was collected from each mouse and placed into lithium heparin tubes. An engraftment check was performed by flow cytometry and the plasma levels of IL-4 and TSLP were evaluated. Information from the engraftment check and/or the cytokine determination was used to select (assign or eliminate) mice from the study. Mice from the engrafted control group with less than 25% human CD45+ cells were removed from the study. Similarly, engrafted mice that received minicircle DNA and showed plasma TSLP levels 1 standard deviation below the mean were also removed from the study. Mice that received minicircle DNA by HDD *i.v.* injection received subcutaneous

doses of either vehicle (20 mM phosphate, 125 mM L-arginine HCL, and 0.01% tween 20 pH 7.0) or F027400161 (0.01, 0.05, 0.1, or 10 mg/kg) on days 95, 97, 99, and 101. On day 103, mice were anesthetized by isoflurane anesthesia. While under isoflurane anesthesia, blood was collected by retro-orbital bleeds. Following blood collection and while still
5 under isoflurane anesthesia, the mice were terminated by cervical dislocation. A portion of the lung was harvested and placed in RNA later for gene expression evaluation. Plasma levels of human TSLP from the day 103 plasma samples were determined by MSD kit evaluation (Cat# K15067-L-2, Meso Scale Diagnostics, Rockville, MD, USA). Plasma levels of human IL-13 from the day 103 plasma samples were determined by ELISA (Cat# 88-
10 7439--88Human IL-13 ELISA kit, Invitrogen/Thermo-Fischer, Waltham, MA, USA). Internal assay validation experiments demonstrated that both the human TSLP and IL-13 detection kits were not able to detect F027400161 bound hTSLP and hIL-13.

The collective results of these experiments as shown in Figure 10 and Figure 11 demonstrate that F027400161 was able to significantly inhibit detectable levels of human
15 TSLP and IL-13 in the plasma of humanized NSG-SGM3 mice, demonstrating target occupancy for both human TSLP and human IL-13.

In the NSG-SGM3 mouse model, hydrodynamic delivery of TSLP and IL-4 cDNAs turn on the expression of human IL-13 (Figure 11). Pharmacodynamic effects of F027400161 were studied using samples derived from the NSG-SGM3 study by taking advantage of the
20 ability of human IL-13 to signal through the mouse IL-13 receptor (Hershey GK. 2003, J Allergy Clin Immunol.;111(4):677-90). In these studies, the impact of F027400161 treatment on human IL-13 regulated mouse gene transcripts were studied. Mouse lung tissues from the above study were used in this analysis.

The lungs from treated and control NSG-SGM3 mice were harvested and processed to
25 make RNA as detailed in the attached protocol. The RNAs were processed for quantification by TaqMan and the data analyzed as described in the protocols. In brief, lungs harvested from the mice were stored in RNALater, processed and purified according to standard protocols to generate high quality RNA. Purified lung RNA was then reverse

transcribed to cDNA using Quanta Q-Script 5X master mix according to manufacturer's protocol. Obtained lung cDNA was used to quantify the transcript expression levels of the human IL-13 responsive mouse target genes (mouse Retnla and mouse Clca1) and an endogenous control (Rpl37a) using a TaqMan assay according to manufacturer's protocols. Data analysis was performed in Quantstudio 6&7 flex software. For each probe, C_T values and delta C_T values (against Rpl37a) were exported into excel and relative expression values for each gene were calculated using the following formula:

$$\text{Normalized relative expression} = (\text{Power}(2, -(\text{delta } C_T))) * 1000.$$

The two human IL-13 regulated mouse genes evaluated were Retnla (Resistin like alpha), that plays a role in pulmonary vascular remodeling and Clca1 (chloride channel accessory 1) (Lewis CC, 2009, J Allergy Clin Immunol.;123(4):795-804).

The collective results of these experiments as shown in Figures 12 and 13 demonstrate that F027400161 was able to significantly inhibit mouse Retnla and mouse Clca1 transcript expression, demonstrating the *in vivo* pharmacodynamics effect of F027400161 on human IL-13 driven mouse transcript responses.

Methods:

Sample homogenate Preparation:

Lungs were harvested from the mice and stored in RNA later. Lung lobes were then dried and transferred to fastprep lysing Matrix A tubes (for homogenizing lungs) containing 1 mL RLT+2-ME. Samples were homogenized in MP-Bio homogenizer using program 1 (Two 40 second cycles separated by 5 minutes to avoid sample heatup). Samples were then spun at 10,000g for 3 minutes. Collected 350ul of lysate [in RLT + 2ME (1% v/v)]. Pipette using a multi-channel multiple times (~20x) to lyse the cells.

RNA-Preparation:

For RNA purification, 350ul homogenate was used. 1X volume (350ul) of 70% Ethanol was added and the homogenate mixed thoroughly and transferred to a 96 well RNeasy spin plate placed in elution plate and RNA was prepared using the Qiagen RNA mini tissue RNA

extraction protocol with following modifications. The 96 well plate was covered with sealable aluminum foil and centrifuged for 2 minutes at 4000 x g. To wash the column, 400 µl Buffer RWT was added to the RNeasy spin columns and the spin-column plate was centrifuged for 2 minutes at RT at 4000 x g. DNase I digestion was carried out by adding 80 µl of 1X DNase I mix and incubating at RT for 15 minutes. The DNase I was washed off by adding 400µl buffer RWT and spinning the plate at 4000 x g for 2 minutes. This was followed by washing the spin-plate with 500ul each of buffer RPE and 80% ethanol. This was followed by drying of column membranes by spinning at 4000 x g for 4 minutes. RNA was then eluted in 40µl Tris HCl (10mM; pH-8.0). RNA was quantified using Nanodrop and 500ng RNA was used for cDNA preparation.

First Strand Synthesis:

Quanta Q-Script 5X master mix was used and cDNA was synthesized using manufacturer's protocol. Final concentration of cDNA was 25 ng/ul. cDNA was stored at -20 Celcius till the TaqMan assay was performed.

TaqMan assay:

TaqMan multiplex master mix was prepared in a 1.5 ml microcentrifuge tube by adding the components in the following order. Three separate master mixes made for each probe-set along with the internal Rpl37a control. Each multiplex qPCR reaction was conducted in a 10 µl reaction volume.

Component	Single Rxn (ul)	For 40 Rxns (ul)
50X RPL37A Primer/Probe	0.3	10
20X Target Gene Primer/Probe	0.6	24
Water	2.8	4
TaqMan Fast Advanced Mastermix; 2X	5.0	200
Total	8.7	238

A total of 8.8 ul of the master mix was added for each sample into the appropriate wells of a 384-well optical plate. 30 ng (1.2 ul) cDNA samples were added to each well. TaqMan assay was set-up on QuantStudio 7K. The conditions in the thermo cycler were: pre-denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 2 s, and annealing

and extension at 60 °C for 5 s. Fluorescent measurements were carried out during the extension step.

Data analysis:

Data analysis was performed in Quantstudio 6&7 flex software.

- 5 For each probe, CT values and delta CT values (against Rpl37a) were exported into excel and relative expression values for each gene were calculated using the following formula:

$$\text{Normalized relative expression} = (\text{power}(2, -(\text{delta CT}))) * 1000$$

References:

- 10 Liu, Y.J. 2006, J Exp Med.;203(2):269-73. Thymic stromal lymphopoietin: master switch for allergic inflammation.
- Hershey GK. 2003, J Allergy Clin Immunol.;111(4):677-90. IL-13 receptors and signaling pathways: an evolving web.
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- 15 patterns driven by IL-4 and IL-13 in the mouse lung.

7 Industrial applicability

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