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(54) Title: T CELL RECRUITING POLYPEPTIDES CAPABLE OF BINDING CD123 AND TCR ALPHA/BETA

(57) Abstract: Polypeptides are provided that bind CD123 on a target cell and the constant domain of TCR on a T cell. The polypeptides can be used in methods for treatment of CD123 associated cancers or inflammatory conditions.

T CELL RECRUITING POLYPEPTIDES CAPABLE OF BINDING CD123 AND TCR ALPHA/BETA**FIELD OF THE INVENTION**

The present invention provides multispecific T cell recruiting polypeptides comprising one
5 immunoglobulin single variable domain that specifically binds the constant domain of the T cell
receptor (TCR) on a T cell and one or more immunoglobulin single variable domains that bind CD123
expressed on a target cell. The present invention also relates to the monovalent CD123 binding
polypeptides for use in these multispecific polypeptides. The invention also provides nucleic acids
encoding said polypeptides as well as vectors, hosts and methods for the production of the
10 polypeptides of the invention. The invention also relates to methods for treatment making use of the
polypeptides of the invention and kits providing the same.

BACKGROUND

CD123 (α subunit of the interleukin 3 receptor, IL-3R α) is a 75kDa glycoprotein, which becomes
15 43kDa upon digestion with N-glycosidase (Sato et al. 1993, Blood 82: 752-761). CD123 consists of
three extracellular domains, a transmembrane domain and a short intracellular region. The N-
terminal extracellular domain contributes significantly to the interaction of CD123 with IL-3, while the
intracellular region is necessary for signalling (Barry et al. 1997, Blood 89: 842-852). CD123
specifically binds IL-3 with low affinity. Heterodimerisation of CD123 with the common β (βc) subunit,
20 which on itself does not bind to IL-3, results in the formation of IL-3R, a high-affinity receptor for IL-3.
The βc subunit plays a significant role in signal transduction and as such triggers a range of biological
functions. (Hara et al, 1996 Stem cells 14: 605-618)

While the βc subunit is expressed on the surface of various cells, CD123 expression is more restricted
to IL-3 responsive cells, such as hematopoietic stem/progenitor cells, monocytes, megakaryocytes, B-
25 lymphocytes and plasmacytoid dendritic cells. Binding of IL-3 stimulates the proliferation and
differentiation of hematopoietic cells. During maturation of these cells, CD123 expression gradually
decreases and cannot be detected in mature lymphocytes and granulocytes.

CD123 is reported to be highly expressed on leukemia stem cells (LSC) and to be associated with the
initiation and development of many diseases, such as acute myeloid leukemia (AML), acute
30 lymphoblastic leukemia (ALL) and hairy cell leukemia (HCL). Reference is made to the review of Liu et
al. (2015 Life Sciences 122: 59-64) for more details on CD123 and related clinical applications in

leukemias. Given the difference in CD123 expression on normal hematopoietic stem cells and LSCs, CD123 is an interesting therapeutic target in hematological cancers.

AML is a clonal malignant disorder derived from a small population of LSC cells with overexpression of CD123. AML is characterised by proliferation of myeloid progenitor cells in the bone marrow and peripheral blood and results in the destruction of normal hematopoiesis. Although therapeutic regimens and supportive care for AML patients have improved over the years, no major changes occurred in the standard treatment options in the last three decades. Reference is made to Medinger et al. (2016 Leukemia Research Reports 6: 39-49) for an overview of novel approaches and therapeutic options in AML. Currently, only 35-40% of patients younger than 60 years cure from the disease. For elderly patients (>60 years), the overall prognosis remains adverse. Allogeneic hematopoietic stem cell transplantation currently provides the best chance for cure. Hence, there remains a need for novel therapeutics to cure AML.

A possible strategy for prevention of AML and treatment of relapse is the use of immunotherapy, which is a rapidly growing area of cancer research. Immunotherapy directs the body's immune surveillance system, and in particular T cells, to cancer cells.

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

Both α and β chains of the T cell receptor consist of a constant domain and a variable domain. Physiologically, the $\alpha\beta$ chains of the T cell receptor recognize the peptide loaded MHC complex and couple upon engagement to the CD3 chains. These CD3 chains subsequently transduce the engagement signal to the intracellular environment.

Considering the potential of naturally occurring cytotoxic T lymphocytes (CTLs) to mediate cell lysis, various strategies have been explored to recruit immune cells to mediate tumour cell killing. The elicitation of specific T cell responses however relies on the expression by cancer cells of MHC molecules and on the presence, generation, transport and display of specific peptide antigens. More recent developments have attempted an alternative approach by combining the advantages of

immunotherapy with antibody therapy by engaging all T cells of a patient in a polyclonal fashion via recombinant antibody based technologies: "bispecifics".

Bispecific antibodies have been engineered that have a tumour recognition part on one arm (target-binding arm) whereas the other arm of the molecule has specificity for a T cell antigen (effector-binding arm), mostly CD3. Through the simultaneous binding of the two arms to their respective antigens, T lymphocytes are directed towards and activated at the tumour cell where they can exert their cytolytic function.

The concept of using bispecific antibodies to activate T cells against tumour cells was described more than 20 years ago, but manufacturing problems and clinical failures sent the field into stagnation. Further progress was made when smaller format bispecifics, resulting from the reduction of antibodies to their variable fragments, were developed.

Although a first T cell engaging format, Blinatumomab (a BiTE molecule recognizing CD19 and CD3), was approved in December 2014 for second line treatment by the FDA, many hurdles had to be overcome. The first clinical trials of Blinatumomab were prematurely stopped due to neurologic adverse events, cytokine release syndrome and infections on the one hand and the absence of objective clinical responses or robust signs of biological activity on the other hand.

As a treatment option for AML, MacroGenics recently developed MGD006, a CD3 x CD123 bispecific DART (dual affinity retargeting molecules). As described in Hussaini et al. (2016 Blood 127: 122-131), MGD006 is able to recognize CD123 positive leukemia cells and to induce T cell activation resulting in killing of the CD123 overexpressing tumour cells in vitro and in vivo. However, the DART also upregulates the T cell activation marker CD25 on T cells upon incubation with the CD123 negative cell line K562^{GFP} (Figure 1D, Hussaini et al. 2016). Moreover, target independent killing was observed with two CD123 negative cell lines (Figure 2B, Hussaini et al. 2016). Therefore, with this DART, safety issues may arise from this target independent T cell activation.

In order to minimise the risk for adverse events and systemic side effects, such as cytokine storms, utmost care must be taken upon selection of both the tumour and the T cell antigen arms. The latter must bind to a constant domain of the TCR complex in a monovalent fashion and may not trigger T cell signaling in the absence of the targeted cancer cells. Only the specific binding of both arms to their targets (the tumour and the T cell antigen) may trigger the formation of the cytolytic synapses and subsequent killing of the tumour cells. The specificity of the tumour recognition arm for its antigen is a requisite to avoid off-target binding, which would inevitably result in target-independent T cell activation.

Efficacy aside, MGD006, as well as blinatumomab, are very small in size and lack an Fc domain. Therefore, continuous intravenous infusion will be required for MGD006, which will not contribute to patient compliance. MacroGenics now attempts to solve this problem by fusing an Fc domain onto its next generation DARTs (WO2015026892), which makes the molecule not only bigger, but also may result in manufacturing problems and importation of other Fc functions. The larger format with Fc is expected to have a better PK, but re-introduces the risk of off-target activity.

Hence, there remains a need for alternative bispecific CD123 x T cell antigen binding polypeptides with minimal target-independent T cell activation, wherein half-life can be tailored.

SUMMARY OF THE INVENTION

The invention solves this problem by providing multispecific polypeptides comprising one immunoglobulin single variable domain (ISV) that specifically binds to a constant domain of the T cell receptor (TCR) and one or more ISV that specifically bind CD123. In a particular aspect, the polypeptide redirects the T cells to the CD123 expressing cells and induces T cell mediated killing.

The combination of a T cell receptor binding ISV and CD123 binding ISV have been particularly selected to result in efficient T cell activation at (the site of) CD123 expressing cells, while target-independent T cell activation appears minimal.

Thus, in a first aspect the present invention provides a polypeptide that redirects T cells for killing of CD123 expressing cells, comprising one immunoglobulin single variable domain (ISV) that specifically binds T cell receptor (TCR) and one or more ISV that specifically bind CD123, wherein the ISV that specifically binds TCR (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 218-225; or

- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the one or more ISV that specifically bind CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or

- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the

same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the one or more ISV that specifically bind CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 2, 4, 5, 6, 8 and/or 10 of the CDR1 (position 27, 29, 30, 31, 33 and/or 35 according to Kabat numbering); provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 3, 5, 7, 8 and/or 9 of the CDR2 (position 50, 52, 54, 56, 57 and/or 58 according to Kabat numbering); provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 4, 5 and/or 8 of the CDR3 (position 95, 98, 99 and/or 101 according to Kabat numbering); provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the ISV that specifically binds CD123 is as further described herein.

In one aspect, the CDR1 encompassed in the ISV that specifically binds TCR may be chosen from the group consisting of:

- a) SEQ ID NO: 181; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein

- at position 2 the D has been changed into A, S, E or G;
- at position 4 the H has been changed into Y;
- at position 5 the K has been changed into L;
- at position 6 the I has been changed into L;
- at position 8 the F has been changed into I or V; and/or
- at position 10 the G has been changed into S;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Apart from this or in addition, CDR2 encompassed in the ISV that specifically binds TCR may be chosen from the group consisting of:

- a) SEQ ID NO: 192; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein

- at position 1 the H has been changed into T or R;
- at position 3 the S has been changed into T or A;
- at position 5 the G has been changed into S or A;
- at position 7 the Q has been changed into D, E, T, A or V;
- at position 8 the T has been changed into A or V; and/or
- at position 9 the D has been changed into A, Q, N, V or S;

provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Apart from this or in addition, the CDR3 encompassed in the ISV that specifically binds TCR may be chosen from the group consisting of:

- a) SEQ ID NO: 218; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein

- at position 1 the F has been changed into Y, L or G;
- at position 4 the I has been changed into L;

- at position 5 the Y has been changed into W; and/or
- at position 8 the D has been changed into N or S;

provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 181; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein
 - at position 2 the D has been changed into A, S, E or G;
 - at position 4 the H has been changed into Y;
 - at position 5 the K has been changed into L;
 - at position 6 the I has been changed into L;
 - at position 8 the F has been changed into I or V; and/or
 - at position 10 the G has been changed into S;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 192; or
- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein
 - at position 1 the H has been changed into T or R;
 - at position 3 the S has been changed into T or A;
 - at position 5 the G has been changed into S or A;
 - at position 7 the Q has been changed into D, E, T, A or V;
 - at position 8 the T has been changed into A or V; and/or
 - at position 9 the D has been changed into A, Q, N, V or S;

provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

5 and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein

- 10 - at position 1 the F has been changed into Y, L or G;
- at position 4 the I has been changed into L;
- at position 5 the Y has been changed into W; and/or
- at position 8 the D has been changed into N or S;

provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

15 and wherein the ISV that specifically binds CD123 is as further described herein.

In a preferred aspect, the ISV that specifically binds TCR (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of SEQ ID NOs: 181-191, CDR2 is chosen from the group consisting of SEQ ID NOs: 192-217, and CDR3 is chosen from the group consisting of SEQ ID NOs: 218-225.

Accordingly, the present invention provides a polypeptide comprising an ISV that specifically binds TCR (essentially) consisting of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of SEQ ID NOs: 181-191, CDR2 is chosen from the group consisting of SEQ ID NOs: 192-217, and CDR3 is chosen from the group consisting of SEQ ID NOs: 218-225 and comprising an ISV that specifically binds CD123 as further described herein.

30 In a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 181, CDR2 is SEQ ID NO: 192, and CDR3 is SEQ ID NO: 218 and wherein the ISV that specifically binds CD123 is as further described herein.

Preferred ISVs for use in the polypeptide of the invention may be chosen from the group consisting of SEQ ID NOs: 42 and 78-180 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 42 and 78-180. Accordingly, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is chosen from the group consisting of SEQ ID NOs: 42 and 78-180 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 42 and 78-180, and wherein the ISV that specifically binds CD123 is as further described herein.

The ISV that specifically binds TCR may be present at any position in the polypeptide of the invention. Preferably, the ISV that specifically binds TCR is present at the N-terminus of the polypeptide of the invention. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is located at the N-terminus of the polypeptide.

The polypeptide of the invention further encompasses one or more ISVs. The ISVs for use in the polypeptide of the invention have been particularly selected for their high specificity towards CD123 present on CD123 expressing target cells.

In a further aspect, therefore, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein, and wherein the one or more ISV that specifically bind CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s)

difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the ISV comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the ISV comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

The present invention has identified ISVs that specifically bind CD123 with selected antigen binding sites or paratopes. In one aspect, the ISV that specifically binds CD123 binds to an epitope that is bound by the ISV 56A10 (i.e. an ISV that belongs to the same family as 56A10 or an ISV that is related to 56A10).

In one aspect, the CDR1 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

a) SEQ ID NO: 11; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- at position 3 the T has been changed into S or P;
- at position 6 the I has been changed into S;
- at position 7 the N has been changed into D; and/or
- at position 8 the D has been changed into V or A;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Apart from this or in addition, the CDR2 encompassed in the ISV that specifically binds TCR may be SEQ ID NO: 17.

Apart from this or in addition, the CDR3 encompassed in the ISV that specifically binds TCR may be chosen from the group consisting of:

- 5 a) SEQ ID NO: 21; or
- b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein
 - at position 3 the P has been changed into A;
 provided that the ISV comprising the CDR3 with 1 amino acid difference binds CD123 with
 10 the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein, and wherein the one or more ISV that specifically bind
 15 CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:
 - a) SEQ ID NO: 11; or
 - b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino
 20 acid sequence of SEQ ID NO: 11, wherein
 - at position 3 the T has been changed into S or P;
 - at position 6 the I has been changed into S;
 - at position 7 the N has been changed into D; and/or
 - at position 8 the D has been changed into V or A;
 provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s)
 25 difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;
 and
- 30 ii) CDR2 is SEQ ID NO: 17;
 and
- iii) CDR3 is chosen from the group consisting of:
 - c) SEQ ID NOs: 21; or

d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein

- at position 3 the P has been changed into A;

provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

In a preferred aspect, the ISV that specifically binds CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of SEQ ID NOs: 11-15, CDR2 is SEQ ID NO: 17, and CDR3 is chosen from the group consisting of SEQ ID NOs: 21-22.

Accordingly, the present invention provides a polypeptide comprising an ISV that specifically binds TCR as described herein, and comprising one or more ISV that specifically bind CD123 (essentially) consisting of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of SEQ ID NOs: 11-15, CDR2 is SEQ ID NO: 17, and CDR3 is chosen from the group consisting of SEQ ID NOs: 21-22.

In a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 11, CDR2 is SEQ ID NO: 17, and CDR3 is SEQ ID NO: 21.

Preferred ISVs for use in the polypeptide of the invention may be chosen from the group consisting of SEQ ID NOs: 1-6 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6. Accordingly, the present invention also provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 is chosen from the group consisting of SEQ ID NOs: 1-6 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6.

In another aspect, the ISV that specifically binds CD123 binds to an epitope that is bound by the Nanobody 55F03 (i.e. an ISV that belongs to the same family as 55F03 or an ISV that is related to 55F03).

In one aspect, the CDR1 encompassed in the ISV that specifically binds CD123 is SEQ ID NO: 16.

Apart from this or in addition, the CDR2 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

- a) SEQ ID NO: 18; or
- b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein
 - at position 3 the Y has been changed into W;
 - at position 6 the N has been changed into S; and/or
 - at position 10 the Q has been changed into E;

provided that the ISV comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Apart from this or in addition, the CDR3 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

- a) SEQ ID NO: 23; or
- b) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein
 - at position 4 the E has been changed into R; and/or
 - at position 5 the T has been changed into D or Y;

provided that the ISV comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein, and wherein the one or more ISV that specifically bind CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is SEQ ID NO: 16;

and

- ii) CDR2 is chosen from the group consisting of:

- a) SEQ ID NO: 18; or
- b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- at position 3 the Y has been changed into W;
- at position 6 the N has been changed into S; and/or
- at position 10 the Q has been changed into E;

provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

- c) SEQ ID NOs: 23; or
- d) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein
 - at position 4 the E has been changed into R; and/or
 - at position 5 the T has been changed into D or Y;

provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a preferred aspect, the ISV that specifically binds CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16, CDR2 is chosen from the group consisting of SEQ ID NOs: 18-20, and CDR3 is chosen from the group consisting of SEQ ID NOs: 23-25.

Accordingly, the present invention provides a polypeptide comprising an ISV that specifically binds TCR as described herein, and comprising one or more ISV that specifically bind CD123 (essentially) consisting of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16, CDR2 is chosen from the group consisting of SEQ ID NOs: 18-20, and CDR3 is chosen from the group consisting of SEQ ID NOs: 23-25.

In a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein, and wherein the one or more ISV that specifically bind CD123 (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16, CDR2 is SEQ ID NO: 18, and CDR3 is SEQ ID NO: 23.

Preferred ISVs for use in the polypeptide of the invention may be chosen from the group consisting SEQ ID NOs: 7-10 or from ISVs that have a sequence identity of more than 80%, more than 85%, more

than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR is as described herein, and wherein the one or more ISV that specifically bind CD123 is chosen from the group consisting of SEQ ID NOs: 7-10 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10.

The polypeptide of the invention may encompass one ISV that specifically binds CD123 or more than one ISV that specifically binds CD123, such as for example two, three or even more. In a further aspect, the present invention provides a polypeptide as described herein, comprising an ISV that specifically binds TCR as described herein, and comprising two or more ISVs that specifically bind CD123, preferably two.

The two or more, preferably two, ISVs encompassed in the polypeptide of the invention can be any ISV that specifically bind CD123 as described herein. The two or more, preferably two, ISVs encompassed in the polypeptide of the invention can be the same ISVs (i.e. with the same amino acid sequence) or they can be different ISVs (i.e. with a different amino acid sequence). In one aspect, the present invention provides a polypeptide as described in, wherein the two or more ISVs that specifically bind CD123 are biparatopic comprising a first ISV and a second ISV, wherein the first ISV binds to an epitope on CD123 that is different from the epitope on CD123 bound by the second ISV.

Preferably, the two or more, preferably two, ISVs that specifically bind CD123 are an ISV related to 56A10 and an ISV related to 55F03. Accordingly, in a one aspect, the present invention provides a polypeptide as described herein, wherein the first ISV is selected from the ISVs related to 56A10 and the second ISV is selected from the ISVs related to 55F03.

The two or more, preferably two, ISVs that specifically bind CD123 may be present at any position in the polypeptide of the invention. In one aspect, the present invention provides a polypeptide as described herein, wherein the second ISV is located N-terminally of the first ISV. In another aspect, the present invention provides a polypeptide as described herein, wherein the second ISV is located C-terminally of the first ISV.

The ISVs present in the polypeptide of the invention can be any ISV that is known in the art and as further described herein. In one aspect, the ISVs present in the polypeptide of the invention are selected from a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH, a camelized VH or a VHH which has been obtained by affinity maturation. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, wherein the ISV that specifically binds TCR and the one or more ISV that specifically bind CD123 (essentially) consist of a single domain

antibody, a dAb, a Nanobody, a VHH, a humanized VHH, a camelized VH or a VHH which has been obtained by affinity maturation.

Preferred polypeptides of the invention are chosen from the group consisting of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61.

More preferably, the polypeptide is chosen from the group consisting of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61.

As discussed above, the polypeptide of the invention redirects T cells for killing of CD123 expressing cells. In one aspect, the present invention provides a polypeptide as described herein, wherein said polypeptide induces T cell activation.

In a further aspect, the present invention provides a polypeptide as described herein, wherein said T cell activation is independent from MHC recognition.

In a further aspect, the present invention provides a polypeptide as described herein, wherein said T cell activation depends on presenting said polypeptide bound to CD123 on a target cell to a T cell.

In a further aspect, the present invention provides a polypeptide as described herein, wherein said T cell activation causes one or more cellular response by said T cell, wherein said cellular response is selected from the group consisting of proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, expression of activation markers and redirected target cell lysis.

In a specific aspect, the T cell activation induced by the polypeptide of the invention causes killing of CD123 expressing cells with an average EC50 value of between 1 nM and 1 pM, such as at an average EC50 value of 500 pM or less, such as less than 400, 300, 200 or 100 pM or even less, such as less than 90, 80, 70, 60, 50, 40 or 30 pM or even less, said EC50 value preferably determined in a flow cytometry based assay with TOPRO3 read-out using MOLM-13 cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

In another specific aspect, the T cell activation induced by the polypeptide of the invention causes lysis of CD123 expressing cells with an average lysis percentage of more than about 10%, such as 15%, 16%, 17%, 18%, 19% or 20% or even more, such as more than 25%, or even more than 30%, said lysis percentage preferably determined in a flow cytometry based assay with TOPRO3 read-out using MOLM-13 cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

In another specific aspect, the present invention provides a polypeptide as described herein, wherein said T cell activation induced by the polypeptide of the invention causes IFN- γ secretion with an average EC50 value of between 100 nM and 10 pM, such as at an average EC50 value of 50 nM or less, such as less than 40, 30, 20, 10 or 9 nM or even less, such as less than 8, 7, 6, 5, 4, 3, 2 or 1 nM or even less, such as less than 500pM or even less, such as less than 400, 300, 200 or 100 pM or even less, said EC50 value preferably determined in an ELISA based assay.

In a further aspect, the present invention provides a polypeptide as described herein, wherein said T cell activation causes proliferation of said T cells.

As discussed above, the polypeptides of the present invention are selected such that target-independent T cell activation should be minimal. In a further aspect, therefore, the present invention provides a polypeptide as described herein, wherein the T cell activation in the absence of CD123 positive cells is minimal.

More specifically, T cell activation induced lysis of CD123 negative cells by the polypeptides of the present invention is no more than about 10%, such as 9% or less, such as 8, 7, or 6 % or even less, said lysis preferably determined as average lysis percentage in a flow cytometry based assay with TOPRO3 read-out using U-937 cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

The present invention also relates to the building blocks, i.e. the ISVs that make up the polypeptides of the invention. Accordingly, the present invention also provides a polypeptide that is an ISV that specifically binds CD123 and that comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or

- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

More preferably, the polypeptide that is an ISV that specifically binds CD123 comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or

- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention also provides a polypeptide as described above, that comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the polypeptide comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

The present invention has identified ISVs that specifically bind CD123 with selected antigen binding sites or paratopes. In one aspect, the ISV that specifically binds CD123 binds to an epitope that is bound by the ISV 56A10 (i.e. an ISV that belongs to the same family as 56A10 or an ISV that is related to 56A10).

Accordingly, in one aspect, the CDR1 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

a) SEQ ID NO: 11; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- at position 3 the T has been changed into S or P;
- at position 6 the I has been changed into S;
- at position 7 the N has been changed into D; and/or
- at position 8 the D has been changed into V or A;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Apart from this or in addition, the CDR2 encompassed in the ISV that specifically binds CD123 is SEQ ID NO: 17.

Apart from this or in addition, the CDR3 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

a) SEQ ID NO: 21; or

b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein

- at position 3 the P has been changed into A;
provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention also provides a polypeptide as described above, that comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 11; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein
 - at position 3 the T has been changed into S or P;
 - at position 6 the I has been changed into S;
 - at position 7 the N has been changed into D; and/or
 - at position 8 the D has been changed into V or A;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is SEQ ID NO: 17;

and

iii) CDR3 is chosen from the group consisting of:

- c) SEQ ID NOs: 21; or
- d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein
 - at position 3 the P has been changed into A;

provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention provides a polypeptide as described herein, in which CDR1 is chosen from the group consisting of SEQ ID NOs: 11-15, CDR2 is SEQ ID NO: 17, and CDR3 is chosen

from the group consisting of SEQ ID NOs: 21-22. Preferably, CDR1 is SEQ ID NO: 11, CDR2 is SEQ ID NO: 17, and CDR3 is SEQ ID NO: 21.

Preferred ISVs of the invention related to 56A10 may be chosen from the group consisting of SEQ ID NOs: 1-6 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, wherein the polypeptide is chosen from the group consisting of SEQ ID NOs: 1-6 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6. Preferably, the polypeptide is chosen from the group consisting of SEQ ID NOs: 1-6.

In one aspect, the polypeptide of the invention binds to human CD123 expressed on MOLM-13 cells with an average EC50 value between 10 nM and 100 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, 3, 2, or 1 nM or even less, preferably as measured by flow cytometry.

In another aspect, the polypeptide of the invention binds to human CD123 with an average KD value of between 10 nM and 100 pM, such as at an average KD value of 5 nM or less, such as less than 4, 3 or 2 nM or even less, said KD value preferably determined by surface plasmon resonance.

In yet another aspect, the ISV that specifically binds CD123 binds to an epitope that is bound by the ISV 55F03 (i.e. an ISV that belongs to the same family as 55F03 or an ISV that is related to 55F03).

Accordingly, in one aspect, the CDR1 encompassed in the ISV that specifically binds CD123 is SEQ ID NO: 16.

Apart from this or in addition, the CDR2 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

- a) SEQ ID NO: 18; or
- b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- at position 3 the Y has been changed into W;
- at position 6 the N has been changed into S; and/or
- at position 10 the Q has been changed into E;

provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Apart from this or in addition, the CDR3 encompassed in the ISV that specifically binds CD123 may be chosen from the group consisting of:

- a) SEQ ID NO: 23; or
- b) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein
 - at position 4 the E has been changed into R; and/or
 - at position 5 the T has been changed into D or Y;
 provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention also provides a polypeptide as described above, that comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is SEQ ID NO: 16;
and
- ii) CDR2 is chosen from the group consisting of:
 - a) SEQ ID NO: 18; or
 - b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein
 - at position 3 the Y has been changed into W;
 - at position 6 the N has been changed into S; and/or
 - at position 10 the Q has been changed into E;
 provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;
 and
- iii) CDR3 is chosen from the group consisting of:
 - c) SEQ ID NOs: 23; or
 - d) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein
 - at position 4 the E has been changed into R; and/or
 - at position 5 the T has been changed into D or Y;

provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with the same, about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

5 In a further aspect, the present invention provides a polypeptide as described herein, in which CDR1 is SEQ ID NO: 16, CDR2 is chosen from the group consisting of SEQ ID NOs: 18-20, and CDR3 is chosen from the group consisting of SEQ ID NOs: 23-25. Preferably, CDR1 is SEQ ID NO: 16, CDR2 is SEQ ID NO: 18, and CDR3 is SEQ ID NO: 23.

Preferred ISVs of the invention related to 56A10 may be chosen from the group consisting of SEQ ID
10 NOs: 7-10 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, wherein the polypeptide is chosen from the group consisting of SEQ ID NOs: 7-10 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even
15 more than 99% with one of SEQ ID NOs: 7-10. Preferably, the polypeptide is chosen from the group consisting of SEQ ID NOs: 7-10.

In one aspect, the polypeptide of the invention binds to human CD123 expressed on MOLM-13 cells with an average EC50 value between 10 μ M and 100 nM, such as at an average EC50 value of 5 μ M or less, such as less than 4, 3, 2, or 1 μ M or even less, preferably as measured by flow cytometry.

20 In another aspect, the polypeptide of the invention binds to human CD123 with an average KD value of between 1 μ M and 10 nM, such as at an average KD value of 500 nM or less, such as less than 400, 300 or 200 nM or even less, said KD value preferably determined by surface plasmon resonance.

In a further aspect, the present invention provides a polypeptide that cross-blocks the binding to CD123 of at least one of the polypeptides as described herein or that cross-blocks the binding to
25 CD123 of one of the polypeptides with SEQ ID NOs: 1-10.

In a further aspect, the present invention provides a polypeptide that is cross-blocked from binding to CD123 by at least one of the polypeptides as described herein or that is cross-blocked from binding to CD123 by one of the polypeptides with SEQ ID NOs: 1-10.

The polypeptide that specifically binds CD123 as described herein, preferably (essentially) consists of
30 a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH, a camelized VH or a VHH which has been obtained by affinity maturation.

The polypeptide of the invention that specifically binds CD123 may contain one or more ISVs that specifically bind CD123. Accordingly, in a further aspect, the present invention provides a polypeptide

comprising two or more ISVs, preferably two, that specifically bind CD123. In a preferred aspect, the two or more ISVs, preferably two ISVs, that specifically bind CD123, are chosen from the group of ISVs related to 56A10 or from the group of ISVs related to 55F03.

In a further aspect, the present invention provides a polypeptide that specifically binds CD123, comprising two ISVs that specifically bind CD123, wherein the ISVs are chosen from the group of ISVs related to 56A10 or from group of ISVs related to 55F03.

The polypeptide of the invention comprising two or more ISVs, preferably two ISVs, that specifically bind CD123 is preferably biparatopic comprising a first ISV and a second ISV, wherein the first ISV binds to an epitope on CD123 that is different from the epitope on CD123 bound by the second ISV.

In a preferred aspect, the first ISV is selected from the group of ISVs related to 56A10 and the second ISV is selected from the group of ISVs related to 55F03.

The ISVs may be present at any position in the biparatopic polypeptide of the invention that binds CD123. In one aspect, the second ISV is located N-terminally of the first ISV. In another aspect, the second ISV is located C-terminally of the first ISV.

The ISVs present in the polypeptide of the invention may be directly linked to each other, or they can be linked via one or more linkers, preferably peptide linkers. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, wherein the ISVs are directly linked to each other or linked to each other via a linker. Preferred linkers for use in the polypeptides of the invention are shown in Table B-3 (SEQ ID NOs: 325 to 336). As such, in a further aspect, the present invention provides a polypeptide as described herein, in which the linker is selected from the group consisting of SEQ ID NOs: 325 to 336.

The present invention further encompasses constructs (also referred to herein as “construct(s) of the invention”) that comprise a polypeptide as described herein, and further comprise one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers.

In a further aspect, the said one or more other groups, residues, moieties or binding units may provide the construct with increased half-life, compared to the corresponding polypeptide without the one or more other groups, residues, moieties or binding units. Said one or more other groups, residues, moieties or binding units that provide the polypeptide with increased half-life can any molecule that provides for a retention of the polypeptide in the serum. In one aspect, the 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.

Accordingly, in one aspect, the present invention provides a construct as described herein, in which said one or more other groups, residues, moieties or binding units that provide the construct with increased half-life is chosen from the group consisting of serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

5 In another aspect, the present invention provides a construct as described herein, in which said one or more other binding units that provide the construct 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). Preferably, said one or more other binding units that provide the polypeptide with increased half-life is an ISV that binds serum albumin. In a further aspect, said
10 ISV that binds serum albumin may (essentially) consist of a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH or a camelized VH.

A preferred ISV for use in the constructs as described herein, is an ISV that binds serum albumin and that (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementary determining regions (CDR1 to CDR3, respectively), in which CDR1 is GFTFSSFGMS (SEQ ID NO: 363) or
15 GFTFRSFGMS (SEQ ID NO: 364), CDR2 is SISGSGSDDL (SEQ ID NO: 365) and CDR3 is GGSLSR (SEQ ID NO: 366). Preferred ISVs that binds serum albumin are selected from the group consisting of SEQ ID NOs: 43 and 351 to 362.

As for the polypeptides of the invention, the other groups, residues, moieties or binding units, such as ISVs may be directly linked to each other or linked to each other via a linker. In a further aspect,
20 the present invention provides a construct as described herein, in which the linker is selected from the group consisting of SEQ ID NOs: 325 to 336.

Preferred constructs of the invention may be chosen from the group consisting of SEQ ID NOs: 63-67 or constructs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 63-67, preferably, SEQ ID NOs: 63-67.

25 The constructs of the invention may be sequence optimized, e.g. to make the construct more human-like, to improve the expression of the constructs, to increased the stability of the constructs upon storage and/or to make the constrcuts less prone to binding by antibodies pre-existing in the serum. In one aspect, the present invention provides a construct as described herein, further comprising a C-terminal extension (X)_n, in which n is 1 to 5, such as 1, 2, 3, 4 or 5, and in which X is a naturally
30 occurring amino acid, preferably no cysteine. Preferred constructs are chosen from the group consisting of SEQ ID NOs: 338-342.

The present invention also provides nucleic acids encoding the polypeptides and constructs (that are such that they can be obtained by expression of a nucleic acid encoding the same) as defined herein. In one aspect, the nucleic acid as described herein, is in the form of a genetic construct.

The present invention also provides an expression vector comprising the nucleic acid as defined
5 herein.

The present invention also provides a host or host cell comprising the nucleic acid as defined herein, or the expression vector as defined herein.

In a further aspect, the present invention provides a method for the production of the polypeptide or construct (that is such that it can be obtained by expression of a nucleic acid encoding the same) as
10 defined herein, said method at least comprising the steps of:

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

In a further aspect, the present invention provides a composition comprising at least one polypeptide
15 or construct as defined herein or a nucleic acid as defined herein. In one aspect, the composition is a pharmaceutical composition. In one aspect, the composition 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.

The present invention also provides a polypeptide as described herein, a construct as described
20 herein, or a composition as described herein, for use as a medicament. In a further aspect, the present invention provides the use of a polypeptide as described herein, or a composition as described herein, for the manufacture of a medicament. In a further aspect, the present invention provides a polypeptide as described herein, a construct as described herein, or a composition as described herein, for use in the prevention, treatment and/or amelioration of a CD123 associated
25 disease or condition. The present invention also provides a method for the prevention, treatment or and/or amelioration of a CD123 associated disease or condition, comprising the step of administering to a subject in need thereof, a pharmaceutically active amount of the polypeptide as described herein, a construct as described herein, or a composition as described herein. The present invention also provides the use of a polypeptide as described herein, a construct as described herein, or a
30 composition as described herein, for the manufacture of a medicament for the prevention, treatment and/or amelioration of a CD123 associated disease or condition. Without being limiting, the CD123 associated disease or condition may be a proliferative disease or an inflammatory condition. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, a

construct as described herein, or a composition as described herein, for use in the prevention, treatment and/or amelioration of a proliferative disease or an inflammatory condition. The present invention also provides a method for the prevention, treatment and/or amelioration of a proliferative disease or an inflammatory condition, comprising the step of administering to a subject
5 in need thereof, a pharmaceutically active amount of the polypeptide as described herein, a construct as described herein, or a composition as described herein. The present invention also provides the use of a polypeptide as described herein, a construct as described herein, or a composition as described herein, for the manufacture of a medicament for the prevention, treatment and/or amelioration of a proliferative disease or an inflammatory condition.

10 Without being limiting, the proliferative disease may be cancer. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, a construct as described herein, or a composition as described herein, for use in the prevention, treatment and/or amelioration of cancer. The present invention also provides a method for the prevention, treatment and/or amelioration of cancer, comprising the step of administering to a subject in need thereof, a pharmaceutically active
15 amount of the polypeptide as described herein, a construct as described herein, or a composition as described herein. The present invention also provides the use of a polypeptide as described herein, a construct as described herein, or a composition as described herein, for the manufacture of a medicament for the prevention, treatment and/or amelioration of cancer.

The cancer to be treated by the method of the invention can be any cancer known to be treated by
20 CD123 targeted cell killing. Cancer known to involve CD123 expression on aberrantly proliferating cells include (without being limiting) lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and
25 multiple myeloma. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, a construct as described herein, a composition as described herein, for use in the prevention, treatment and/or amelioration of a cancer selected from lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia
30 and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma. The present invention also provides a method for the prevention, treatment and/or amelioration of cancer selected from lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia

and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma, comprising the step of administering to a subject in need thereof, a pharmaceutically active amount of the polypeptide as described herein, a construct as described herein, or a composition as described herein. The present invention also provides the

5 use of a polypeptide as described herein, a construct as described herein, or a composition as described herein, for the manufacture of a medicament for the prevention, treatment and/or amelioration of a cancer chosen from the group consisting of lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia

10 and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma.

The inflammatory condition to be treated by the method of the invention can be any inflammatory condition known to be treated by CD123 targeted cell killing. Inflammatory conditions known to involve CD123 expression on cells include (without being limiting) Autoimmune Lupus (SLE), allergy,

15 asthma and rheumatoid arthritis. Accordingly, in a further aspect, the present invention provides a polypeptide as described herein, a construct as described herein, a composition as described herein, for use in the prevention, treatment and/or amelioration of an inflammatory condition chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis. The present invention also provides a method for the prevention, treatment and/or amelioration of an

20 inflammatory condition chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis, comprising the step of administering to a subject in need thereof, a pharmaceutically active amount of the polypeptide as described herein, a construct as described herein, or a composition as described herein. The present invention also provides the use of a

25 polypeptide as described herein, a construct as described herein, or a composition as described herein, for the manufacture of a medicament for the prevention, treatment and/or amelioration of an inflammatory condition chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis.

The polypeptides, constructs and compositions of the present invention can also be used in combination with another therapeutic drug. Accordingly, in a further aspect, the present invention

30 provides a polypeptide as described herein, a construct as described herein, a composition as described herein, for use in a combination treatment.

The present invention also provides a method as described herein, wherein the treatment is a combination treatment.

In a further aspect, the present invention provides the use of a polypeptide as described herein, a construct as described herein, or a composition as described herein, for the manufacture of a medicament for the prevention, treatment and/or amelioration as described herein, wherein the treatment is a combination treatment.

- 5 In a further aspect, the present invention provides a kit comprising a polypeptide as described herein, a construct as described herein, a nucleic acid as described herein, an expression vector as described herein or a host or host cell as described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

10 **Figure 1:** Assessment of the expression of human TCR/CD3 and human CD3 on transfected CHO, HEK293 and Llana cell lines using 100nM anti-human TCR α/β antibody (clone BW242/412) (black) and 100nM anti-human CD3 antibody (clone OKT-3) (grey). The MCF value (mean channel fluorescence) was plotted for each cell line. The X-axis depicts the cell type and the transfected genes; CD3 indicates transfection with the CD3 complex (epsilon, delta, gamma and zeta chains),
15 huTCR indicates transfection with the TCR α/β chains, wherein the variable domain used is between brackets.

Figure 2: Quality assessment of soluble recombinant cynomolgus TCR α/β proteins using anti-non-human primate/Rat TCR α/β antibody clone R73; anti-human TCR α/β antibodies (solid circles) and an irrelevant anti-egg lysozyme Nanobody (cAbllys) (open circles). The OD value was plotted against the
20 concentration of the Nanobody.

Figure 3: Dose dependent binding of monovalent anti-TCR Nanobody T0170055A02 to human TCR/CD3 expressed on CHO-K1 cells (Figure 3A) and to primary human T cells (Figure 3B). Dose dependent binding of monovalent anti-TCR Nanobody T0170056G05 to human TCR/CD3 expressed on CHO-K1 cells (Figure 3C) and to primary human T cells (Figure 3D). The MCF value (mean channel
25 fluorescence) was plotted against the concentration of the Nanobody.

Figure 4: Dose dependent binding of monovalent anti-TCR Nanobodies T0170055A02 (Figure 4A) and T0170056G05 (Figure 4B) to HEK293H human TCR(2IAN)/CD3 (closed circle), HEK293H human CD3 (cross) and to HEK293H reference cell line (open circles). The MCF value (mean channel fluorescence) was plotted against the concentration of the Nanobody.

30 **Figure 5:** Dose dependent binding of monovalent anti-TCR Nanobodies T0170055A02 (Figure 4A, closed circles) and T0170056G05 (Figure 4B, closed circles) and an irrelevant Nanobody (Figure 4A

and Figure 4B, open circles) to soluble recombinant human TCR α/β (2XN9)-zipper protein. The OD at 450nm was plotted against the concentration of the Nanobody.

Figure 6: Kinetic analysis of T01700055A02 (Figure 6A) and T01700056G05 (Figure 6B) on soluble recombinant human TCR α/β (2XN9)-zipper protein interaction via BioLayer Interferometry on an Octet RED384 instrument. Applied analyte concentrations were: 1000, 333, 111, 37, 12.3, 4.1 and 1.4 nM. Langmuir fits to the kinetic data are indicated with the black lines, whereas sensorgrams are presented by the grey lines.

Figure 7: Dose dependent binding of monovalent anti-TCR Nanobodies T0170055A02 (Figure 7A, closed circles) and T0170056G05 (Figure 7B, closed circles) and an irrelevant Nanobody (Figure 7A and Figure 7B, open circles) to soluble recombinant cynomolgus TCR α/β -zipper protein. The OD at 450nm was plotted against the concentration of the Nanobody.

Figure 8: Kinetic analysis of T0170055A02 (Figure 8A) and T0170056G05 (Figure 8B) on soluble recombinant cynomolgus TCR α/β -zipper protein interaction via BioLayer Interferometry on an Octet RED384 instrument. Applied analyte concentrations were: 1000, 333, 111, 37, 12.3, 4.1 and 1.4 nM. Langmuir fits to the kinetic data are indicated with the black lines, whereas sensorgrams are presented by the grey lines.

Figure 9: T cell activation data of bead coupled monovalent anti-TCR Nanobodies (Figure 9A). T cell activation data of monovalent anti-TCR Nanobodies presented in solution (Figure 9B). Activation was measured by monitoring the CD69 upregulation on primary human T cells. The MCF value (mean channel fluorescence) was plotted for each Nanobody.

Figure 10: Assessment the expression of human CD123 expression on HEK293 Flp-In, HEK293 Flp-In cyno CD123, CHO Flp-In and CHO Flp-In human CD123 using the anti-CD123 antibody (BD Biosciences, Cat. no. 554527) (black) and the isotype control (eBioscience, Cat. no. 16-4724-85) followed by PE-labelled goat anti-mouse (Jackson Immunoresearch lab. Inc., Cat. no. 115-116-071) (grey) in flow cytometry. The MFI value (median channel fluorescence intensity) was plotted for each cell line.

Figure 11: Assessment of human CD123 expression on U-937, MOLM-13, KG1a and NCI-H929 cells using the APC-labelled anti-CD123 antibody (BD Biosciences, Cat. no. 560087) (black) and the APC-labelled isotype control (Biolegend, Cat. no. 400220) (grey) in flow cytometry. The MFI value (median channel fluorescence intensity) was plotted for each cell line.

Figure 12: Dose-dependent binding of the monovalent anti-CD123 Nanobody A0110056A10 to MOLM-13 cells (Figure 12A) and KG1a cells (Figure 12C). Dose-dependent binding of the monovalent

anti-CD123 Nanobody A0110055F03 to MOLM-13 cells (Figure 12B) and KG1a cells (Figure 12D). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 13: Dose-dependent binding of Alexa647-labelled A0110056A10 to Flp-In parental CHO cells (open symbol) and human CD123 transfected CHO cells (closed symbol) (Figure 13A). Dose-dependent binding of Alexa647-labelled A0110056A10 to Flp-In parental HEK cells (open symbol) and cynomolgus CD123 transfected HEK cells (closed symbol) (Figure 13B). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 14: Dose-dependent binding of A0110055F03 to Flp-In parental CHO cells (open symbol) and human CD123 transfected CHO cells (closed symbol) (Figure 14A). Dose-dependent binding of A0110055F03 to Flp-In parental HEK cells (open symbol) and cynomolgus CD123 transfected HEK cells (closed symbol) (Figure 14B). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 15: Dose-dependent binding of the monovalent anti-CD123 Nanobody A0110056A10-Alexa 647 to MOLM-13 cells (Figure 15A) and on human CD123 transfected CHO Flp-In cells (Figure 15B). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 16: Dose-dependent competition of the monovalent Nanobodies A0110056A10 (squares) and A0110055F03 (circles) with Alexa 647-labelled A0110056A10 for binding to human CD123 on MOLM-13 cells (Figure 16A) and on human CD123 transfected CHO Flp-In cells (Figure 16B). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 17: Dose-dependent binding of the APC-labelled mouse anti-human CD123 (clone 7G3) antibody to human CD123 on MOLM-13 cells (Figure 17A) and on human CD123 transfected CHO Flp-In cells (Figure 17B). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 18: Dose-dependent competition of the monovalent Nanobodies A0110056A10 (squares) and A0110055F03 (circles) with APC-labelled mouse anti-human CD123 (clone 7G3) antibody for binding to CD123 expressed on MOLM-13 cells (Figure 18A) or to CHO Flp-In cells transfected with huCD123 (Figure 18B). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 19: Dose-dependent binding of the mouse anti-human CD123 (clone 7G3) antibody to in house biotinylated CD123 recombinant protein (R&D Systems, Cat. no. 301-R3/CF). The OD at 450nm was plotted against the concentration.

Figure 20: Dose-dependent competition of the monovalent anti-CD123 Nanobodies A0110056A10 (squares) and A0110055F03 (closed circles) with mouse monoclonal anti-CD123 antibody (clone 7G3) (BD Biosciences, Cat no. 554527) for binding to the CD123 protein in ELISA (Figure 20A). The irrelevant anti-egg lysozyme Nanobody cAbLys (open circles) and the mouse monoclonal anti-CD123 antibody (clone 7G3) in solution (stars) were taken along as negative and positive control, respectively (Figure 20B). The OD at 450nm was plotted against the concentration.

Figure 21: Dose-dependent binding of the monovalent anti-CD123 Nanobody A0110056A10-Alexa 647 to MOLM-13 cells (Figure 21A), to human CD123 transfected CHO Flp-In cells (Figure 21B) and to cyno CD123 transfected HEK Flp-In cells (Figure 21C). The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 22: Dose-dependent competition of the multivalent CD123/TCR binding polypeptides with Alexa647-A0110056A10 for binding to CD123 expressed on MOLM-13 cells (Figure 22A) and on huCD123 transfected on CHO Flp-In cells (Figure 22C) or cyCD123 transfected on HEK Flp-In cells (Figure 22B). The irrelevant multivalent polypeptide T017000129 was taken along as negative control. The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 23: Dose-dependent competition of the multivalent CD123/TCR binding polypeptides with biotinylated-T0170056G05 for binding to human TCR/CD3 expressed on CHO-K1 cells. The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 24: Dose-dependent competition of the multivalent CD123/TCR binding polypeptides with T017000099 for binding to CD3/TCR expressed on HSC-F. The monovalent His tagged T017000125 was taken along as positive control. The MFI value (median channel fluorescence intensity) was plotted against the concentration.

Figure 25: Dose-dependent redirected human effector T cell killing of human CD123 expressing MOLM-13 cells in a flow cytometry based assay by multivalent CD123/TCR binding polypeptides using an effector to target ratio of 10:1. A0110056A10, T017000132 and T017000129 were taken along as negative control. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figure 26: Dose-dependent redirected human effector T cell killing of human CD123 expressing KG1a cells in a flow cytometry based assay by multivalent CD123/TCR binding polypeptides using an effector to target ratio of 10:1. A0110056A10, T017000129 and T017000132 were taken along as negative controls. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figure 27 Dose-dependent redirected cynomolgus effector T cell killing of human CD123 positive MOLM-13 cells in a flow cytometry based assay by multivalent CD123/TCR binding polypeptides using an effector to target ratio of 10:1. A0110056A10 was taken along as negative controls. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

5 **Figure 28:** Dose-dependent redirected cynomolgus effector T cell killing of human CD123 positive KG1a cells in a flow cytometry based assay by multivalent CD123/TCR binding polypeptides using an effector to target ratio of 8. Several irrelevant constructs were taken along as negative controls. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

10 **Figure 29:** Dose-dependent T cell activation (CD25 upregulation) by the multivalent CD123/TCR binding polypeptides on CD4/CD8+ gated T cell during the redirected cynomolgus effector T cell killing of human CD123 positive MOLM-13 cells after an incubation time of 72h. The MFI (Mean fluorescence intensity) within CD4/CD8+ gated T cell was plotted against the concentration of the constructs.

15 **Figure 30:** Dose-dependent redirected human effector T cell killing of human CD123 transfected CHO Flp-In cells in an xCELLigence based assay by T017000139 (filled diamonds) using an effector to target ratio of 15:1. The monovalent Nanobodies A0110056A10, T0170056G05 and the irrelevant construct T017000129 were taken along as negative control. The Cell Index (CI) after an incubation time of 50h was plotted against the concentration of the multispecific polypeptide.

20 **Figure 31:** Monovalent building blocks and multispecific polypeptides in the redirected human effector T cell killing assay using the CD123 negative CHO Flp-In reference cell line in an xCELLigence based assay using an effector to target ratio of 15:1. The CI after an incubation time of 50h was plotted against the concentration of the multispecific polypeptide.

25 **Figure 32:** Monovalent building blocks and multispecific CD123/TCR binding polypeptides on the growth of CD123 transfected cell line (Figure 32A) and reference cell line (Figure 32B) in the absence of T cells. The CI after an incubation time of 50h was plotted against the concentration of the multispecific polypeptide.

30 **Figure 33:** Dose-dependent redirected cynomolgus effector T cell killing of cynomolgus CD123 transfected HEK Flp-In cells in an xCELLigence based assay by T017000139 (filled diamonds) using an effector to target ratio of 15:1. The monovalent Nanobody, T0170056G05 and the irrelevant construct T017000129 were taken along as negative control. The CI after an incubation time of 80h was plotted against the concentration of the multispecific polypeptide.

Figure 34: Monovalent building block and multispecific polypeptides in the redirected cynomolgus T cell killing assay using the CD123 negative HEK Flp-In reference cell line. The CI after an incubation time of 80h was plotted against the concentration of the multispecific polypeptide.

Figure 35: Monovalent building block and multispecific CD123/TCR binding polypeptides on the growth of a CD123 transfected cell line (Figure 35A) and reference cell line (Figure 35B) in the absence of T cells. The CI after an incubation time of 80h was plotted against the concentration of the multispecific polypeptide.

Figure 36: Dose-dependent cytokine production by human effector T cells (Figure 36A) or cyno effector T cells (Figure 36B) during multispecific CD123/TCR binding polypeptides dependent redirected T cell killing of human CD123 expressing CHO Flp-In target cells using an effector to target ratio of 10:1. INF- γ production was measured after 72h. The OD value was plotted against the concentration.

Figure 37: Dose-dependent cytokine production by effector T cells during multispecific CD123/TCR binding polypeptides dependent redirected T cell killing of human CD123 expressing CHO Flp-In target cells using an effector to target ratio of 10:1. IL-6 production was measured after 72h. The pg/ml value was plotted against the concentration.

Figure 38: Redirected autologous T cell mediated depletion of CD123+ pDCs and basophils by multivalent CD123/TCR binding polypeptides in healthy human (Figure 38A) and cynomolgus (Figure 38B) PBMC samples after an incubation time of 5h. The percentage of Lin-/CD123+ cells (pDCs and basophils) was plotted against the concentration of the constructs.

Figure 39: Redirected autologous T cell monocyte depletion by multivalent CD123/TCR binding polypeptides in healthy human PBMC samples after an incubation time of 5h (Figure 39A) and 24h (Figure 39B). The percentage of monocytes (CD14+ cells) was plotted against the concentration of the constructs.

Figure 40: Dose-dependent CD69 upregulation, human T cell activation by the multivalent CD123/TCR binding polypeptides on CD3+ gated T cell during redirected T cell killing of autologous CD123 positive cells after an incubation time of 24h. The MFI (Mean fluorescence intensity) within CD3+ gated T cell was plotted against the concentration of the constructs.

Figure 41: Dose-dependent characterization of monovalent Nanobodies and the irrelevant multivalent polypeptide T017000129 for redirected human (Figure 41A) or cynomolgus (Figure 41B) effector T cell killing of human CD123 KG1a cells in a flow cytometry based assay using an effector to target ratio of 10:1. T017000139 (filled diamonds) was taken along as positive control. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figure 42: Dose-dependent characterization of monovalent Nanobodies and the irrelevant multivalent polypeptide T017000129 for redirected human (Figure 42A) or cynomolgus (Figure 42B and Figure 42C) effector T cell killing of human CD123 MOLM-13 cells in a flow cytometry based assay using an effector to target ratio of 10:1. T017000139 (filled diamonds) was taken along as positive control. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figures 43: Dose-dependent cytokine production by human effector T cells during multispecific CD123/TCR binding polypeptides dependent redirected T cell killing of MOLM-13 (Figure 43A and Figure 43C) and KG1a (Figure 43B) target cells using an effector to target ratio of 10:1. Human IL-6 (Figure 43C) and IFN- γ (Figure 43A and Figure 43B) production was measured after 72h. The concentration of cytokine was plotted against the concentration.

Figure 44A and 44B: Dose-dependent characterization of target independent redirected human effector T cell killing by multispecific CD123/TCR binding polypeptides in a flow cytometry based assay using CD123 negative NCI-H929 cell line. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figure 45: Dose-dependent characterization of target independent redirected human (Figure 45A) or cynomolgus (Figure 45B) effector T cell killing by multispecific CD123/TCR binding polypeptides in a flow cytometry based assay using CD123 negative U937 cell line. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figure 46: Dose-dependent T cell activation readout by the multivalent CD123/TCR binding polypeptides on CD4/CD8+ gated T cell during the cynomolgus effector T cell killing of CD123 negative U-937 cells (Figure 46A) and during human effector T cell killing of CD123 negative NCI-H929 cells (Figure 46B) after an incubation time of 72h. The MFI (Mean fluorescence intensity) within CD4/CD8+ gated T cell was plotted against the concentration of the constructs.

Figure 47: Impact of multispecific CD123/TCR binding polypeptides on cytokine production using human effector T cells and NCI-H929 target cells using an effector to target ratio of 10:1. Human IL-6 (Figure 47B) and IFN- γ (Figure 47A) production was measured. The OD value of amount of cytokine was plotted against the concentration.

Figure 48A and 48B: Dose-dependent T cell proliferation of human effector T cells by multispecific polypeptides in a redirected MOLM-13 target cell killing setting using an effector to target ratio of 10:1. The CPM (count per minute) was plotted against the concentration.

Figure 49: Dose-dependent T cell proliferation of human effector T cells by multispecific polypeptides in absence of target cells. The CPM (count per minute) was plotted against the concentration.

Figure 50: Lytic potential of non-activated and pre-activated T cells in the presence of T017000114 and MOLM-13 cells at different E:T ratios. The % cell death was plotted against the concentration of the construct.

Figure 51: Lytic potential of non-activated and pre-activated T cells in the presence of T017000139 and KG1a cells at different E:T ratios. The % cell death was plotted against the concentration of the construct.

Figure 52: Dose-dependent redirected human (Figure 52A and 52B) and cynomolgus (Figure 52C and 52D) T cell killing of MOLM-13 cells in the absence or presence of serum albumin in a flow cytometry based assay by multivalent CD123/TCR binding polypeptides, using an effector to target ratio of 10:1. The irrelevant multivalent polypeptide T017000129 and the monovalent building blocks A0110056A10 and T0170056G05 were taken along as negative control. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the polypeptide.

Figure 53: Dose-dependent redirected human (Figure 53A, 53B and 53C) and cynomolgus (Figure 53D, 53E and 53F) T cell killing of KG1a cells in the absence or presence of serum albumin in a flow cytometry based assay by multivalent CD123/TCR binding polypeptides using an effector to target ratio of 10:1. The irrelevant multivalent polypeptides A022600009 (in the presence or absence of SA) and T017000129, and the monovalent building blocks A0110056A10 and T0170056G05 were taken along as negative control. The % cell death (% of TOPRO positive cells) was plotted against the concentration of the construct.

Figure 54: Dose-dependent cytokine production by human T cells during redirected T cell killing of MOLM-13 by the HLE multispecific CD123/TCR binding polypeptides using an effector to target ratio of 10:1. Human IL-6 (Figure 54B) and IFN- γ (Figure 54A) production was measured. The amount of cytokine is plotted against the concentration.

Figure 55: Dose-dependent T cell proliferation of human effector T cells by HLE multispecific polypeptides in a redirected MOLM-13 target cell killing setting using an effector to target ratio of 10:1. The CPM (count per minute) was plotted against the concentration.

Figure 56: Redirected autologous T cell redirected CD123+ pDC and basophil depletion by multivalent HLE CD123/TCR binding polypeptides in healthy human PBMC samples after an incubation time of 5h. The percentage of Lin-/CD123+ cells (pDCs and basophils) was plotted against the concentration of the constructs.

Figure 57: Redirected autologous T cell redirected CD123+ pDC and basophil depletion by multivalent HLE CD123/TCR binding polypeptides in healthy cynomolgus PBMC samples in an in vitro setting after an incubation time of 5h. The percentage of Lin-/CD123+ cells (pDCs and basophils) was plotted against the concentration of the constructs.

5 **Figure 58:** Redirected autologous T cell redirected monocyte depletion by multivalent CD123/TCR binding polypeptides in healthy human PBMC samples after an incubation time of 24h. The percentage of monocytes (CD14+ cells) was plotted against the concentration of the constructs.

Figure 59: T cell counts in peripheral blood of treated cynomolgus monkey over time. The absolute number of CD4⁺CD3⁺ T cells (Figure 59A) and CD8⁺CD3⁺ T cells (Figure 59B) per μ L blood is expressed as average \pm SEM over time for the different treatment groups: positive control (open circles, n = 2), irrelevant/TCR polypeptide (cross, n = 4), CD123/TCR polypeptide (black triangle, n = 4). Grey bars reflect continuous infusion treatment periods.

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Figure 60: CD123⁺CD14⁻ cell counts in peripheral blood of treated cynomolgus monkey over time. The absolute number of CD123⁺CD14⁻ cells per μ L blood is expressed as average \pm SEM for the different treatment groups: positive control (open circles, n = 2), irrelevant/TCR polypeptide (cross, n = 4), CD123/TCR polypeptide (black triangle, n = 4). Grey bars reflect continuous infusion treatment periods.

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Figure 61: PD-1 expression on CD4⁺CD3⁺ and CD8⁺CD3⁺ T cells over time. The frequency of CD4⁺CD3⁺ T cells (Figure 61A) and CD8⁺CD3⁺ T cells (Figure 61B) in blood is expressed as average \pm SEM for the different treatment groups: positive control (open circles, n = 2), irrelevant/TCR polypeptide (cross, n = 4), CD123/TCR polypeptide (black triangle, n = 4). Grey bars reflect continuous infusion treatment periods.

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Figure 62: Serum interleukin-6 in treated cynomolgus monkey over time. The concentration of IL-6 in serum is expressed as average \pm SEM (pg/mL) for the different treatment groups: positive control (open circles, n = 2), irrelevant/TCR polypeptide (cross, n = 4), CD123/TCR polypeptide (black triangle, n = 4). Grey bars reflect continuous infusion treatment periods.

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DETAILED DESCRIPTION

Definitions

30 Unless indicated or defined otherwise, all terms used have their usual meaning in the art, which will be clear to the skilled person. Reference is for example made to the standard handbooks, such as Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual (2nd Ed.) Vols. 1-3, Cold Spring Harbor

Laboratory Press), F. Ausubel et al. (1987, Current protocols in molecular biology, Green Publishing and Wiley Interscience, New York), Lewin (1985, Genes II, John Wiley & Sons, New York, N.Y.), Old et al. (1981, Principles of Gene Manipulation: An Introduction to Genetic Engineering (2nd Ed.) University of California Press, Berkeley, CA), Roitt et al. (2001, Immunology (6th Ed.) Mosby/Elsevier, Edinburgh), Roitt et al. (2001, Roitt's Essential Immunology (10th Ed.) Blackwell Publishing, UK), and Janeway et al. (2005, Immunobiology (6th Ed.) Garland Science Publishing/Churchill Livingstone, New York), as well as to the general background art cited herein.

Unless indicated otherwise, all methods, steps, techniques and manipulations that are not specifically described in detail can be performed and have been performed in a manner known per se, as will be clear to the skilled person. Reference is for example again made to the standard handbooks and the general background art mentioned herein and to the further references cited therein; as well as to for example the following reviews; Presta (2006, Adv. Drug Deliv. Rev. 58 (5-6): 640-56), Levin and Weiss (2006, Mol. Biosyst. 2(1): 49-57), Irving et al. (2001, J. Immunol. Methods 248(1-2): 31-45), Schmitz et al. (2000, Placenta 21 Suppl. A: S106-12), Gonzales et al. (2005, Tumour Biol. 26(1): 31-43), which describe techniques for protein engineering, such as affinity maturation and other techniques for improving the specificity and other desired properties of proteins such as immunoglobulins.

The term "sequence" as used herein (for example in terms like "immunoglobulin sequence", "antibody sequence", "variable domain sequence", "V_{HH} sequence" or "protein sequence"), should generally be understood to include both the relevant amino acid sequence as well as nucleic acids or nucleotide sequences encoding the same, unless the context requires a more limited interpretation.

Amino acid sequences are interpreted to mean a single amino acid or an unbranched sequence of two or more amino acids, depending of the context. Nucleotide sequences are interpreted to mean an unbranched sequence of 3 or more nucleotides.

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

The terms "protein", "peptide", "protein/peptide", and "polypeptide" are used interchangeably throughout the disclosure and each has the same meaning for purposes of this disclosure. Each term

refers to an organic compound made of a linear chain of two or more amino acids. The compound may have ten or more amino acids; twenty-five or more amino acids; fifty or more amino acids; one hundred or more amino acids, two hundred or more amino acids, and even three hundred or more amino acids. The skilled artisan will appreciate that polypeptides generally comprise fewer amino acids than proteins, although there is no art-recognized cut-off point of the number of amino acids that distinguish a polypeptides and a protein; that polypeptides may be made by chemical synthesis or recombinant methods; and that proteins are generally made *in vitro* or *in vivo* by recombinant methods as known in the art.

Amino acid residues will be indicated according to the standard three-letter or one-letter amino acid code. Reference is made to Table A-2 on page 48 of WO 08/020079.

Table B-1: Common amino acids

1-Letter Code	3-Letter Code	Name
A	Ala	Alanine
B	Asx	Aspartic acid or Asparagine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
J	Xle	Isoleucine or Leucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
O	Pyl	Pyrrolysine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
U	Scy	Selenocysteine
V	Val	Valine
W	Trp	Tryptophan
X	Xxx	Uncommon or Unspecified
Y	Tyr	Tyrosine
Z	Glx	Glutamic acid or Glutamine

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

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

For instance, when a nucleotide sequence, amino acid sequence or polypeptide is said to “comprise” another nucleotide sequence, amino acid sequence or polypeptide, respectively, or to “essentially consist of” another nucleotide sequence, amino acid sequence or polypeptide, this may mean that the latter nucleotide sequence, amino acid sequence or polypeptide has been incorporated into the first mentioned nucleotide sequence, amino acid sequence or polypeptide, respectively, but more usually this generally means that the first mentioned nucleotide sequence, amino acid sequence or polypeptide comprises within its sequence a stretch of nucleotides or amino acid residues, respectively, that has the same nucleotide sequence or amino acid sequence, respectively, as the latter sequence, irrespective of how the first mentioned sequence has actually been generated or obtained (which may for example be by any suitable method described herein). By means of a non-limiting example, when a polypeptide of the invention is said to comprise an immunoglobulin single variable domain, this may mean that said immunoglobulin single variable domain sequence has been incorporated into the sequence of the polypeptide of the invention, but more usually this generally means that the polypeptide of the invention contains within its sequence the sequence of the immunoglobulin single variable domains irrespective of how said polypeptide of the invention has been generated or obtained. Also, when a nucleic acid or nucleotide sequence is said to comprise another nucleotide sequence, the first mentioned nucleic acid or nucleotide sequence is preferably such that, when it is expressed into an expression product (e.g. a polypeptide), the amino acid sequence encoded by the latter nucleotide sequence forms part of said expression product (in other words, that the latter nucleotide sequence is in the same reading frame as the first mentioned, larger nucleic acid or nucleotide sequence).

By “essentially consist of” is meant that the immunoglobulin single variable domain used in the method of the invention either is exactly the same as the polypeptide of the invention or corresponds to the polypeptide of the invention which has a limited number of amino acid residues, such as 1-20 amino acid residues, for example 1-10 amino acid residues and preferably 1-6 amino acid residues, such as 1, 2, 3, 4, 5 or 6 amino acid residues, added at the amino terminal end, at the carboxy terminal end, or at both the amino terminal end and the carboxy terminal end of the immunoglobulin single variable domain.

By “consist of” is meant that the immunoglobulin single variable domain used in the method of the invention is exactly the same as the polypeptide of the invention.

For the purposes of comparing two or more nucleotide sequences, the percentage of “sequence identity” between a first nucleotide sequence and a second nucleotide sequence may be calculated by dividing [the number of nucleotides in the first nucleotide sequence that are identical to the nucleotides at the corresponding positions in the second nucleotide sequence] by [the total number of nucleotides in the first nucleotide sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of a nucleotide in the second nucleotide sequence - compared to the first nucleotide sequence - is considered as a difference at a single nucleotide (position). Alternatively, the degree of sequence identity between two or more nucleotide sequences may be calculated using a known computer algorithm for sequence alignment such as NCBI Blast v2.0, using standard settings. Some other techniques, computer algorithms and settings for determining the degree of sequence identity are for example described in WO 04/037999, EP 0967284, EP 1085089, WO 00/55318, WO 00/78972, WO 98/49185 and GB 2357768. Usually, for the purpose of determining the percentage of “sequence identity” between two nucleotide sequences in accordance with the calculation method outlined hereinabove, the nucleotide sequence with the greatest number of nucleotides will be taken as the “first” nucleotide sequence, and the other nucleotide sequence will be taken as the “second” nucleotide sequence.

For the purposes of comparing two or more amino acid sequences, the percentage of “sequence identity” between a first amino acid sequence and a second amino acid sequence (also referred to herein as “amino acid identity”) may be calculated by dividing [the number of amino acid residues in the first amino acid sequence that are identical to the amino acid residues at the corresponding positions in the second amino acid sequence] by [the total number of amino acid residues in the first amino acid sequence] and multiplying by [100%], in which each deletion, insertion, substitution or addition of an amino acid residue in the second amino acid sequence - compared to the first amino acid sequence - is considered as a difference at a single amino acid residue (position), *i.e.*, as an “amino acid difference” as defined herein. Alternatively, the degree of sequence identity between

two amino acid sequences may be calculated using a known computer algorithm, such as those mentioned above for determining the degree of sequence identity for nucleotide sequences, again using standard settings. Usually, for the purpose of determining the percentage of “sequence identity” between two amino acid sequences in accordance with the calculation method outlined
5 hereinabove, the amino acid sequence with the greatest number of amino acid residues will be taken as the “first” amino acid sequence, and the other amino acid sequence will be taken as the “second” amino acid sequence.

Also, in determining the degree of sequence identity between two amino acid sequences, the skilled person may take into account so-called “conservative” amino acid substitutions, which can generally
10 be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO 04/037999, GB 335768, WO 98/49185, WO 00/46383 and WO 01/09300; and (preferred) types and/or combinations of such substitutions
15 may be selected on the basis of the pertinent teachings from WO 04/037999 as well as WO 98/49185 and from the further references cited therein.

Such conservative substitutions preferably are substitutions in which one amino acid within the following groups (a) – (e) is substituted by another amino acid residue within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively
20 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. Particularly preferred conservative substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or
25 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.

Any amino acid substitutions applied to the polypeptides described herein may also be based on the analysis of the frequencies of amino acid variations between homologous proteins of different species developed by Schulz et al. (1978, Principles of Protein Structure, Springer-Verlag), on the
30 analyses of structure forming potentials developed by Chou and Fasman (1974, Biochemistry 13: 211; 1978, Adv. Enzymol., 47: 45-149), and on the analysis of hydrophobicity patterns in proteins developed by Eisenberg et al. (1984, Proc. Natl. Acad. Sci. USA 81: 140-144), Kyte and Doolittle (1981, J. Molec. Biol. 157: 105-132), and Goldman et al. (1986, Ann. Rev. Biophys. Chem. 15: 321-353), all incorporated herein in their entirety by reference. Information on the primary, secondary and

tertiary structure of Nanobodies is given in the description herein and in the general background art cited above. Also, for this purpose, the crystal structure of a V_{HH} domain from a llama is for example given by Desmyter et al. (1996, Nature Structural Biology, 3: 803), Spinelli et al. (1996, Natural Structural Biology, 3: 752-757) and Decanniere et al. (1999, Structure, 7 (4): 361). Further information
5 about some of the amino acid residues that in conventional V_H domains form the V_H/V_L interface and potential camelizing substitutions on these positions can be found in the prior art cited above.

Amino acid sequences and nucleic acid sequences are said to be “exactly the same” if they have 100% sequence identity (as defined herein) over their entire length.

When comparing two amino acid sequences, the term “amino acid difference” refers to an insertion,
10 deletion or substitution of a single amino acid residue on a position of the first sequence, compared to the second sequence; it being understood that two amino acid sequences can contain one, two or more such amino acid differences. More particularly, in the amino acid sequences and/or polypeptides of the present invention, the term “amino acid difference” refers to an insertion, deletion or substitution of a single amino acid residue on a position of the CDR sequence specified in
15 b), d) or f), compared to the CDR sequence of respectively a), c) or e); it being understood that the CDR sequence of b), d) and f) can contain one, two or maximal three such amino acid differences compared to the CDR sequence of respectively a), c) or e).

The “amino acid difference” can be any one, two, three or maximal four substitutions, deletions or insertions, or any combination thereof, that either improve the properties of the polypeptide of the
20 invention or that at least do not detract too much from the desired properties or from the balance or combination of desired properties of the polypeptide of the invention. In this respect, the resulting polypeptide of the invention should at least bind CD123 or T cell receptor with the same, about the same, or a higher affinity compared to the polypeptide comprising the one or more CDR sequences without the one, two, three or maximal four substitutions, deletions or insertions, said affinity as
25 measured by surface plasmon resonance.

In this respect, the amino acid sequence according to b), d) and/or f) may be an amino acid sequence that is derived from an amino acid sequence according to a), c) and/or e) respectively by means of affinity maturation using one or more techniques of affinity maturation known per se.

For example, and depending on the host organism used to express the polypeptide of the invention,
30 such deletions and/or substitutions may be designed in such a way that one or more sites for post-translational modification (such as one or more glycosylation sites) are removed, as will be within the ability of the person skilled in the art.

The "affinity" denotes the strength or stability of a molecular interaction. The affinity is commonly given as by the K_D , or dissociation constant, which 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}). In the present specification, the stability of the interaction between two molecules will mainly be expressed in terms of the K_D value of their interaction; it being clear to the skilled person that in view of the relation $K_A = 1/K_D$, specifying the strength of molecular interaction by its K_D value can also be used to calculate the corresponding K_A value. The K_D -value characterizes the strength of a molecular interaction also in a thermodynamic sense as it is related to the change of free energy (DG) of binding by the well-known relation $DG = RT \cdot \ln(K_D)$ (equivalently $DG = -RT \cdot \ln(K_A)$), where R equals the gas constant, T equals the absolute temperature and \ln denotes the natural logarithm.

The K_D for biological interactions which are considered meaningful (e.g. specific) are typically in the range of 10^{-12} M (0.001 nM) to 10^{-5} M (10000 nM). The stronger an interaction is, the lower is its K_D .

The K_D can also be expressed as the ratio of the dissociation rate constant of a complex, denoted as k_{off} , to the rate of its association rate constant, denoted k_{on} (so that $K_D = k_{\text{off}}/k_{\text{on}}$ and $K_A = k_{\text{on}}/k_{\text{off}}$). The off-rate k_{off} has units s^{-1} (where s is the SI unit notation of second). The on-rate k_{on} has units $M^{-1}s^{-1}$. The on-rate may vary between $10^2 M^{-1}s^{-1}$ to about $10^7 M^{-1}s^{-1}$, approaching the diffusion-limited association rate constant for bimolecular interactions. The off-rate is related to the half-life of a given molecular interaction by the relation $t_{1/2} = \ln(2)/k_{\text{off}}$. The off-rate may vary between $10^{-6} s^{-1}$ (near irreversible complex with a $t_{1/2}$ of multiple days) to $1 s^{-1}$ ($t_{1/2} = 0.69$ s).

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

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

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

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

It will also be clear to the skilled person that the measured K_D may correspond to the apparent K_D if the measuring process somehow influences the intrinsic binding affinity of the implied molecules for example by artifacts related to the coating on the biosensor of one molecule. Also, an apparent K_D may be measured if one molecule contains more than one recognition sites for the other molecule. In such situation the measured affinity may be affected by the avidity of the interaction by the two molecules. As will be clear to the skilled person, and as described on pages 53-56 of WO 08/020079, the dissociation constant may be the actual or apparent dissociation constant. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned on pages 53-56 of WO 08/020079.

The terms “epitope” and “antigenic determinant”, which can be used interchangeably, refer to the part of a macromolecule, such as a polypeptide or protein that is recognized by antigen-binding molecules, such as immunoglobulins, conventional antibodies, immunoglobulin single variable domains and/or polypeptides of the invention, and more particularly by the antigen-binding site of said molecules. Epitopes define the minimum binding site for an immunoglobulin, and thus represent the target of specificity of an immunoglobulin.

The part of an antigen-binding molecule (such as an immunoglobulin, a conventional antibody, an immunoglobulin single variable domain and/or a polypeptide of the invention) that recognizes the epitope is called a “paratope”.

A polypeptide (such as an immunoglobulin, an antibody, an immunoglobulin single variable domain, a polypeptide of the invention, or generally an antigen binding molecule or a fragment thereof) that can “bind (to)” or “specifically bind (to)”, that “has affinity for” and/or that “has specificity for” a certain epitope, antigen or protein (or for at least one part, fragment or epitope thereof) is said to be “against” or “directed against” said epitope, antigen or protein or is a “binding” molecule with respect to such epitope, antigen or protein, or is said to be “anti”-epitope, “anti”-antigen or “anti”-protein (*e.g.*, “anti”-CD123 or “anti”-TCR).

The term “specificity” has the meaning given to it in paragraph n) on pages 53-56 of WO 08/020079; and as mentioned therein refers to the number of different types of antigens or antigenic determinants to which a particular antigen-binding molecule or antigen-binding protein (such as an immunoglobulin single variable domain and/or a polypeptide of the invention) can bind. The specificity of an antigen-binding protein can be determined based on affinity and/or avidity, as described on pages 53-56 of WO 08/020079 (incorporated herein by reference), which also describes some preferred techniques for measuring binding between an antigen-binding molecule (such as an immunoglobulin single variable domain and/or polypeptide of the invention) and the pertinent antigen. Typically, antigen-binding proteins (such as the immunoglobulin single variable domains and/or polypeptides of the invention) will bind to their antigen with a dissociation constant (K_D) of 10^{-5} to 10^{-12} moles/liter or less, and preferably 10^{-7} to 10^{-12} moles/liter or less and more preferably 10^{-8} to 10^{-12} moles/liter (*i.e.* with an association constant (K_A) of 10^5 to 10^{12} liter/ moles or more, and preferably 10^7 to 10^{12} liter/moles or more and more preferably 10^8 to 10^{12} liter/moles). Any K_D value greater than 10^4 mol/liter (or any K_A value lower than 10^4 M^{-1}) is generally considered to indicate non-specific binding. Preferably, a monospecific polypeptide of the invention will bind to the desired antigen with an affinity less than 500 nM, preferably less than 200 nM, more preferably less than 10 nM, such as *e.g.*, between 10 and 5 nM, such as less than 10nM, less than 5nM, less than 3nM, less than 2nM, such as 10nM-1nM, 5nM-1nM or even less. Specific binding of an antigen-binding protein

to an antigen or antigenic determinant can be determined in any suitable manner known per se, including, for example, Scatchard analysis and/or competitive binding assays, such as radioimmunoassays (RIA), enzyme immunoassays (EIA) and sandwich competition assays, and the different variants thereof known per se in the art; as well as the other techniques mentioned herein.

As will be clear to the skilled person, and as described on pages 53-56 of WO 08/020079, the dissociation constant may be the actual or apparent dissociation constant. Methods for determining the dissociation constant will be clear to the skilled person, and for example include the techniques mentioned on pages 53-56 of WO 08/020079.

One approach that may be used to assess affinity is the 2-step ELISA (Enzyme-Linked Immunosorbent Assay) procedure of Friguet et al. (1985, J. Immunol. Methods 77: 305-19). This method establishes a solution phase binding equilibrium measurement and avoids possible artifacts relating to adsorption of one of the molecules on a support such as plastic.

However, the accurate measurement of K_D may be quite labor-intensive and as consequence, often apparent K_D values are determined to assess the binding strength of two molecules. It should be noted that as long all measurements are made in a consistent way (e.g. keeping the assay conditions unchanged) apparent K_D measurements can be used as an approximation of the true K_D and hence in the present document K_D and apparent K_D should be treated with equal importance or relevance.

Finally, it should be noted that in many situations the experienced scientist may judge it to be convenient to determine the binding affinity relative to some reference molecule. For example, to assess the binding strength between molecules A and B, one may e.g. use a reference molecule C that is known to bind to B and that is suitably labelled with a fluorophore or chromophore group or other chemical moiety, such as biotin for easy detection in an ELISA or FACS (Fluorescent activated cell sorting) or other format (the fluorophore for fluorescence detection, the chromophore for light absorption detection, the biotin for streptavidin-mediated ELISA detection). Typically, the reference molecule C is kept at a fixed concentration and the concentration of A is varied for a given concentration or amount of B. As a result an IC_{50} value is obtained corresponding to the concentration of A at which the signal measured for C in absence of A is halved. Provided $K_{D\text{ref}}$, the K_D of the reference molecule, is known, as well as the total concentration c_{ref} of the reference molecule, the apparent K_D for the interaction A-B can be obtained from following formula: $K_D = IC_{50} / (1 + c_{\text{ref}} / K_{D\text{ref}})$. Note that if $c_{\text{ref}} \ll K_{D\text{ref}}$, $K_D \approx IC_{50}$. Provided the measurement of the IC_{50} is performed in a consistent way (e.g. keeping c_{ref} fixed) for the binders that are compared, the strength or stability of a molecular interaction can be assessed by the IC_{50} and this measurement is judged as equivalent to K_D or to apparent K_D throughout this text.

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting a biological or biochemical function, e.g. a pharmacological effect. This quantitative measure indicates how much of the ISV (e.g. a Nanobody) (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor, chemotaxis, anaplasia, metastasis, invasiveness, etc.) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or IC_{50}). The IC_{50} of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist such as the ISVD (e.g. a Nanobody) of the invention on reversing agonist activity. IC_{50} values can be calculated for a given antagonist such as the ISVD (e.g. a Nanobody) of the invention by determining the concentration needed to inhibit half of the maximum biological response of the agonist.

The term half maximal effective concentration (EC_{50}) refers to the concentration of a compound which induces a response halfway between the baseline and maximum after a specified exposure time. In the present context it is used as a measure of a polypeptide's, ISV's (e.g. a Nanobody's) potency. The EC_{50} of a graded dose response curve represents the concentration of a compound where 50% of its maximal effect is observed. Concentration is preferably expressed in molar units.

In biological systems, small changes in ligand concentration typically result in rapid changes in response, following a sigmoidal function. The inflection point at which the increase in response with increasing ligand concentration begins to slow is the EC_{50} . This can be determined mathematically by derivation of the best-fit line. Relying on a graph for estimation is convenient in most cases. In case the EC_{50} is provided in the examples section, the experiments were designed to reflect the K_D as accurate as possible. In other words, the EC_{50} values may then be considered as K_D values.

It is also related to IC_{50} which is a measure of a compound's inhibition (50% inhibition). For competition binding assays and functional antagonist assays IC_{50} is the most common summary measure of the dose-response curve. For agonist/stimulator assays the most common summary measure is the EC_{50} .

The inhibitor constant, K_i , is an indication of how potent an inhibitor is; it is the concentration required to produce half maximum inhibition. The absolute inhibition constant K_i can be calculated by using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{\frac{[L]}{K_D} + 1}$$

in which [L] is the fixed concentration of the ligand.

An immunoglobulin single variable domain and/or polypeptide is said to be “specific for” a first target or antigen compared to a second target or antigen when it binds to the first antigen with an affinity (as described above, and suitably expressed as a K_D value, K_A value, k_{off} rate and/or k_{on} rate) that is at least 10 times, such as at least 100 times, and preferably at least 1000 times, and up to 10000 times or more better than the affinity with which the immunoglobulin single variable domain and/or polypeptide binds to the second target or antigen. For example, the immunoglobulin single variable domain and/or polypeptide may bind to the first target or antigen with a K_D value that is at least 10 times less, such as at least 100 times less, and preferably at least 1000 times less, such as 10000 times less or even less than that, than the K_D with which said immunoglobulin single variable domain and/or polypeptide binds to the second target or antigen. Preferably, when an immunoglobulin single variable domain and/or polypeptide is “specific for” a first target or antigen compared to a second target or antigen, it is directed against (as defined herein) said first target or antigen, but not directed against said second target or antigen.

An amino acid sequence, such as e.g., an immunoglobulin single variable domain or polypeptide according to the invention, is said to be “cross-reactive” for two different antigens or antigenic determinants (such as e.g., serum albumin from two different species of mammal, such as e.g., human serum albumin and cyno serum albumin, such as e.g., CD123 from different species of mammal, such as e.g., human CD123 and cyno CD123, such as e.g., TCR from different species of mammal, such as e.g., human TCR and cyno TCR) if it is specific for (as defined herein) both these different antigens or antigenic determinants.

The terms “(cross)-block”, “(cross)-blocked”, “(cross)-blocking”, “competitive binding”, “(cross)-compete”, “(cross)-competing” and “(cross)-competition” are used interchangeably herein to mean the ability of an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent to interfere with the binding of other immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or binding agents to a given target. The extent to which an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent is able to interfere with the binding of another to the target, and therefore whether it can be said to cross-block according to the invention, can be determined using competition binding assays. One particularly suitable quantitative cross-blocking assay is described in the Examples and includes e.g. a fluorescence-activated cell sorting (FACS) binding assay with CD123 expressed on cells. The extent of (cross)-blocking can be measured by the (reduced) channel fluorescence. Another suitable quantitative cross-blocking assay uses a Biacore instrument which can measure the extent of interactions using surface plasmon resonance technology. Another suitable quantitative cross-blocking assay uses an ELISA-based approach to measure competition between immunoglobulins,

antibodies, immunoglobulin single variable domains, polypeptides or other binding agents in terms of their binding to the target.

The following generally describes a suitable FACS assay for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent cross-blocks or is capable of cross-blocking according to the invention. It will be appreciated that the assay can be used with any of the immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or other binding agents described herein. The FACS instrument (e.g. FACSArray; Becton Dickinson) is operated in line with the manufacturer's recommendations.

To evaluate the “(cross)-blocking” or “(cross)-competition” between two binding agents (such as e.g., two immunoglobulin single variable domains and/or Nanobodies) for binding to CD123, a FACS competition experiment can be performed using cells (such as e.g., the endogenously CD123 expressing cell line MOLM-13 or Flp-In™-CHO cells overexpressing human CD123). Different detection reagents can be used including e.g. monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich, cat# F1804), monoclonal anti-C-myc antibody (Sigma-Aldrich, cat# WH0004609M2), monoclonal ANTI-HIS TAG antibody (Sigma-Aldrich, cat# SAB1305538), each labeled differently. A wide range of fluorophores can be used as labels in flow cytometry (such as e.g. PE (R-Phycoerythrin), 7-aminoactinomycin D (7-AAD), Acridine Orange, various forms of Alexa Fluor (such as e.g., Alexa647), Allophycocyanin (APC), AmCyan, Aminocoumarin, APC Cy5, APC Cy7, APC-H7, APC/Alexa Fluor 750, AsRed2, Azami-Green, Azurite, B ODIPY FL C5-ceramide, BCECF-AM, Bis-oxonol DiBAC2(3), BODIPY-FL, Calcein, Calcein AM, Carboxy-H2DCFDA, Cascade Blue, Cascade Yellow, Cell Tracker Green, Cerulean, CFSE, Chromomycin A3, CM-H2DCFDA, Cy2, Cy3, Cy3.5, Cy3B, Cy5, Cy5.5, Cy7, CyPet, DAF-FM DAF-FM diacetate, DAPI, DCFH (2'7'Dichlorodihydrofluorescein), DHR, Dihydrocalcein AM, Dihydrorhodamine, Dihydrothidium, DiLC1(5), DiOC6(3), DiOC7(3), dKeima-Red, DRAQ5, Dronpa-Green, various forms of DsRed dTomato, various forms of DyLight, *E.coli* BioParticles AF488, E2-Crimson, E2-Orange, EBFP2, ECFP, various forms of eFluor, EGFP, EGFP*, Emerald, eqFP650, eqFP670, ER-Tracker Blue-White DPX, Ethidium Bromide, Express2, EYFP, Fc OxyBurst Green, Fc OxyBurst Green 123, FITC, Fluo-3, Fluo-4, Fluorescein, Fura-2, Fura-Red, GFPuv, H2DCFDA, HcRed1, Hoechst Blue (33258), Hoechst Red (33342), Hydroxycoumarin, HyPer, Indo-1, Indo-1 Blue (Low Ca²⁺), Indo-1 Violet (High Ca²⁺), iRFP, J-Red, JC-1, JC-9, Katushka (TurboFP635), Katushka2 Kusabira-Orange, LDS 751, Lissamine Rhodamine B, various forms of Live/Dead, Lucifer yellow, Lucifer Yellow CH, Lyso Tracker Blue, Lyso Tracker Green, Lyso Tracker Red, mAmertine, Marina Blue, mBanana, mCFP, mCherry, mCitrine, Methoxycoumarin, mHoneyDew, Midoriishi-Cyan, Mithramycin, Mito Tracker Deep Red, Mito Tracker Green, Mito Tracker Orange, Mito Tracker Red, MitoFluor Green, mKate (TagFP635), mKate2, mKeima, mKeima-Red, mKO, mKOk, mNeptune, Monochlorobimane, mOrange,

mOrange2, mRaspberry, mPlum, mRFP1, mStrawberry, mTangerine, mTarquoise, mTFP1, mTFP1 (Teal), NBD, OxyBurst Green H2DCFDA, OxyBurst Green H2HFF BSA, Pacific Blue, PE (R-Phycoerythrin), PE Cy5, PE Cy5.5, PE Cy7, PE Texas Red, PE-Cy5 conjugates, PE-Cy7 conjugates, PerCP (Peridinin chlorophyll protein), PerCP Cy5.5, PhiYFP, PhiYFP-m, Propidium Iodide (PI), various forms of Qdot, Red 613, RFP Tomato, Rhod-2, S65A, S65C, S65L, S65T, Singlet Oxygen Sensor Green, Sirius, SNARF, Superfolder GFP, SYTOX Blue, SYTOX Green, SYTOX Orange, T-Sapphire, TagBFP, TagCFP, TagGFP, TagRFP, TagRFP657, TagYFP, tdTomato, Texas Red, Thiazole Orange, TMRE, TMRM, Topaz, TOTO-1, TO-PRO-1, TRITC, TRITC TruRed, TurboFP602, TurboFP635, TurboGFP, TurboRFP, TurboYFP, Venus, Vybrant CycleDye Violet, Wild Type GFP, X-Rhodamin, Y66F, Y66H, Y66W, YOYO-1, YPet, ZsGreen1, ZsYellow1, Zymosan A BioParticles AF488 (see more at: <http://www.thefcn.org/flow-fluorochromes>). Fluorophores, or simply “fluors”, are typically attached to the antibody (e.g. the immunoglobulin single variable domains, such as Nanobodies) that recognizes CD123 or to the antibody that is used as detection reagent. Various conjugated antibodies are available, such as (without being limiting) for example antibodies conjugated to Alexa Fluor®, DyLight®, Rhodamine, PE, FITC, and Cy3. Each fluorophore has a characteristic peak excitation and emission wavelength. The combination of labels which can be used will depend on the wavelength of the lamp(s) or laser(s) used to excite the fluorophore and on the detectors available.

To evaluate the competition between two test binding agents (termed A and B*) for binding to CD123, a dilution series of cold (without any label) binding agent A is added to (e.g. 100 000) cells together with the labeled binding agent B*. The concentration of B* in the test mix should be high enough to readily saturate the binding sites on CD123 expressed on the cells. The concentration of binding agent B* that saturates the binding sites for that binding agent on CD123 expressed on the cells can be determined with a titration series of B* on the CD123 expressing cells and determination of the EC₅₀ value for binding. In order to work at saturating concentration, binding agent B* can be used at 100x the EC₅₀ concentration.

After incubation of the cells with the mixture of A and B* and washing of the cells, read out can be performed on a FACS. First a gate is set on the intact cells as determined from the scatter profile and the total amount of channel fluorescence is recorded.

A separate solution of binding agent B* is also prepared. The binding agent in this solutions should be in the same buffer and at the same concentration as in the test mix (with binding agents A and B*). This separate solution is also added to the cells. After incubation and cells wash, read out can be performed on a FACS. First a gate is set on the intact cells as determined from the scatter profile and the total amount of channel fluorescence is recorded.

A reduction of fluorescence for the cells incubated with the mixture of A and B* compared to the fluorescence for the cells incubated with the separate solution of B* indicates that binding agent A (cross)-blocks binding by binding agent B* for binding to CD123 expressed on the cells.

A cross-blocking immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent according to the invention is one which will bind to the CD123 in the above FACS cross-blocking assay such that during the assay and in the presence of a second immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent the recorded fluorescence is between 80% and 0.1% (e.g. 80% to 4%) of the maximum fluorescence (measured for the separate labelled immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent), specifically between 75% and 0.1% (e.g. 75% to 4%) of the maximum fluorescence, and more specifically between 70% and 0.1% (e.g. 70% to 4%) of maximum fluorescence (as just defined above).

The competition between two test binding agents (termed A* and B*) for binding to CD123 can also be evaluated by adding both binding agents, each labeled with a different fluorophore, to the CD123 expressing cells. After incubation and cells wash, read out can be performed on a FACS. A gate is set for each fluorophore and the total amount of channel fluorescence is recorded. Reduction and/or absence of fluorescence of one of the fluorophore indicate (cross)-blocking by the binding agents for binding to CD123 expressed on the cells.

The following generally describes a suitable Biacore assay for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent cross-blocks or is capable of cross-blocking according to the invention. It will be appreciated that the assay can be used with any of the immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or other binding agents described herein. The Biacore instrument (for example the Biacore 3000) is operated in line with the manufacturer's recommendations. Thus in one cross-blocking assay, the target protein (e.g. CD123) is coupled to a CM5 Biacore chip using standard amine coupling chemistry to generate a surface that is coated with the target. Typically 200-800 resonance units of the target would be coupled to the chip (an amount that gives easily measurable levels of binding but that is readily saturable by the concentrations of test reagent being used). Two test binding agents (termed A* and B*) to be assessed for their ability to cross-block each other are mixed at a one to one molar ratio of binding sites in a suitable buffer to create the test mixture. When calculating the concentrations on a binding site basis the molecular weight of a binding agent is assumed to be the total molecular weight of the binding agent divided by the number of target binding sites on that binding agent. The concentration of each binding agent in the test mix should be high enough to readily saturate the binding sites for that binding agent on the target molecules

captured on the Biacore chip. The binding agents in the mixture are at the same molar concentration (on a binding basis) and that concentration would typically be between 1.00 and 1.5 micromolar (on a binding site basis). Separate solutions containing A* alone and B* alone are also prepared. A* and B* in these solutions should be in the same buffer and at the same concentration as in the test mix.

5 The test mixture is passed over the target-coated Biacore chip and the total amount of binding recorded. The chip is then treated in such a way as to remove the bound binding agents without damaging the chip-bound target. Typically this is done by treating the chip with 30 mM HCl for 60 seconds. The solution of A* alone is then passed over the target-coated surface and the amount of binding recorded. The chip is again treated to remove all of the bound binding agents without
10 damaging the chip-bound target. The solution of B* alone is then passed over the target-coated surface and the amount of binding recorded. The maximum theoretical binding of the mixture of A* and B* is next calculated, and is the sum of the binding of each binding agent when passed over the target surface alone. If the actual recorded binding of the mixture is less than this theoretical maximum then the two binding agents are said to cross-block each other. Thus, in general, a cross-
15 blocking immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent according to the invention is one which will bind to the target in the above Biacore cross-blocking assay such that during the assay and in the presence of a second immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent the recorded binding is between 80% and 0.1% (e.g. 80% to 4%) of the maximum theoretical binding, specifically
20 between 75% and 0.1% (e.g. 75% to 4%) of the maximum theoretical binding, and more specifically between 70% and 0.1% (e.g. 70% to 4%) of maximum theoretical binding (as just defined above) of the two immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or binding agents in combination. The Biacore assay described above is a primary assay used to determine if immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptide or
25 other binding agents cross-block each other according to the invention. On rare occasions particular immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or other binding agents may not bind to a target coupled via amine chemistry to a CM5 Biacore chip (this usually occurs when the relevant binding site on the target is masked or destroyed by the coupling to the chip). In such cases cross-blocking can be determined using a tagged version of the target, for
30 example a N-terminal His-tagged version. In this particular format, an anti-His antibody would be coupled to the Biacore chip and then the His-tagged target would be passed over the surface of the chip and captured by the anti-His antibody. The cross blocking analysis would be carried out essentially as described above, except that after each chip regeneration cycle, new His-tagged target would be loaded back onto the anti-His antibody coated surface. In addition to the example given
35 using N-terminal His-tagged target, C-terminal His-tagged target could alternatively be used.

Furthermore, various other tags and tag binding protein combinations that are known in the art could be used for such a cross-blocking analysis (e.g. HA tag with anti-HA antibodies; FLAG tag with anti-FLAG antibodies; biotin tag with streptavidin).

The following generally describes an ELISA assay for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent directed against a target (*e.g.*, CD123) cross-blocks or is capable of cross-blocking as defined herein. It will be appreciated that the assay can be used with any of the immunoglobulins, antibodies, immunoglobulin single variable domains, polypeptides or other binding agents described herein. The general principal of the assay is to have an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or binding agent that is directed against the target coated onto the wells of an ELISA plate. An excess amount of a second, potentially cross-blocking, anti-target immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent is added in solution (*i.e.* not bound to the ELISA plate). A limited amount of the target is then added to the wells. The coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent and the immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent in solution compete for binding of the limited number of target molecules. The plate is washed to remove excess target that has not been bound by the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent and to also remove the second, solution phase immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent as well as any complexes formed between the second, solution phase immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent and target. The amount of bound target is then measured using a reagent that is appropriate to detect the target. An immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent in solution that is able to cross-block the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent will be able to cause a decrease in the number of target molecules that the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent can bind relative to the number of target molecules that the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent can bind in the absence of the second, solution phase, immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent. In the instance where the first immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent, *e.g.*, an Ab-X, is chosen to be the immobilized immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent, it is coated onto the

wells of the ELISA plate, after which the plates are blocked with a suitable blocking solution to minimize non-specific binding of reagents that are subsequently added. An excess amount of the second immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent, i.e. Ab-Y, is then added to the ELISA plate such that the moles of Ab-Y target binding sites per well are at least 10 fold higher than the moles of Ab-X target binding sites that were used, per well, during the coating of the ELISA plate. Target is then added such that the moles of target added per well are at least 25-fold lower than the moles of Ab-X target binding sites that were used for coating each well. Following a suitable incubation period the ELISA plate is washed and a reagent for detecting the target is added to measure the amount of target specifically bound by the coated anti-target immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent (in this case Ab-X). The background signal for the assay is defined as the signal obtained in wells with the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent (in this case Ab-X), second solution phase immunoglobulin single variable domain, polypeptide or other binding agent (in this case Ab-Y), target buffer only (*i.e.*, without target) and target detection reagents. The positive control signal for the assay is defined as the signal obtained in wells with the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent (in this case Ab-X), second solution phase immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent buffer only (*i.e.*, without second solution phase immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent), target and target detection reagents. The ELISA assay may be run in such a manner so as to have the positive control signal be at least 6 times the background signal. To avoid any artefacts (e.g. significantly different affinities between Ab-X and Ab-Y for the target) resulting from the choice of which immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent to use as the coating immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent and which to use as the second (competitor) immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent, the cross-blocking assay may to be run in two formats: 1) format 1 is where Ab-X is the immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent that is coated onto the ELISA plate and Ab-Y is the competitor immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent that is in solution and 2) format 2 is where Ab-Y is the immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent that is coated onto the ELISA plate and Ab-X is the competitor immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent that is in solution. Ab-X and Ab-Y are defined as cross-blocking if, either in format 1 or in format 2, the solution phase anti-target immunoglobulin,

antibody, immunoglobulin single variable domain, polypeptide or other binding agent is able to cause a reduction of between 60% and 100%, specifically between 70% and 100%, and more specifically between 80% and 100%, of the target detection signal (*i.e.*, the amount of target bound by the coated immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent) as compared to the target detection signal obtained in the absence of the solution phase anti- target immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent (*i.e.*, the positive control wells).

Other methods for determining whether an immunoglobulin, antibody, immunoglobulin single variable domain, polypeptide or other binding agent directed against a target (cross)-blocks, is capable of (cross)-blocking, competitively binds or is (cross)-competitive as defined herein are described e.g. in Xiao-Chi Jia et al. (2004, Journal of Immunological Methods 288: 91–98), Miller et al. (2011, Journal of Immunological Methods 365: 118–125) and/or the methods described herein (see e.g. Example 16).

The term “CD123” as used herein refers to the α subunit of the interleukin 3 receptor (IL-3R α).

The term “TCR” as used herein refers to the T cell receptor, which consists of an TCR α and a TCR β chain. Both α and β chains of the TCR consist of a constant domain and a variable domain. The polypeptides and immunoglobulin single variable domains of the present invention bind to the constant domain of TCR.

The “half-life” of a polypeptide of the invention can generally be defined as described in paragraph o) on page 57 of WO 08/020079 and as mentioned therein refers to the time taken for the serum concentration of the polypeptide to be reduced by 50%, *in vivo*, for example due to degradation of the polypeptide and/or clearance or sequestration of the polypeptide by natural mechanisms. The *in vivo* half-life of a polypeptide of the invention can be determined in any manner known per se, such as by pharmacokinetic analysis. Suitable techniques will be clear to the person skilled in the art, and may for example generally be as described in paragraph o) on page 57 of WO 08/020079. As also mentioned in paragraph o) on page 57 of WO 08/020079, the half-life can be expressed using parameters such as the t_{1/2}-alpha, t_{1/2}-beta and the area under the curve (AUC). Reference is for example made to the standard handbooks, such as Kenneth et al (1986, Chemical Stability of Pharmaceuticals: A Handbook for Pharmacists, John Wiley & Sons Inc) and M Gibaldi and D Perron (1982, Pharmacokinetics, Marcel Dekker, 2nd Rev. Ed., 1982). The terms “increase in half-life” or “increased half-life” are also as defined in paragraph o) on page 57 of WO 08/020079 and in particular refer to an increase in the t_{1/2}-beta, either with or without an increase in the t_{1/2}-alpha and/or the AUC or both.

Unless indicated otherwise, the term “immunoglobulin” and “immunoglobulin sequence” - whether used herein to refer to a heavy chain antibody or to a conventional 4-chain antibody - is used as a general term to include both the full-size antibody, the individual chains thereof, as well as all parts, domains or fragments thereof (including but not limited to antigen-binding domains or fragments such as V_{HH} domains or V_H/V_L domains, respectively).

The term “domain” (of a polypeptide or protein) as used herein refers to a folded protein structure which has the ability to retain its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function of the remainder of the protein and/or of the domain.

The term “immunoglobulin domain” as used herein refers to a globular region of an antibody chain (such as *e.g.*, a chain of a conventional 4-chain antibody or of a heavy chain antibody), or to a polypeptide that essentially consists of such a globular region. Immunoglobulin domains are characterized in that they retain the immunoglobulin fold characteristic of antibody molecules, which consists of a two-layer sandwich of about seven antiparallel beta-strands arranged in two beta-sheets, optionally stabilized by a conserved disulphide bond.

The term “immunoglobulin variable domain” as used herein means an immunoglobulin domain essentially consisting of four “framework regions” which are referred to in the art and herein below as “framework region 1” or “FR1”; as “framework region 2” or “FR2”; as “framework region 3” or “FR3”; and as “framework region 4” or “FR4”, respectively; which framework regions are interrupted by three “complementarity determining regions” or “CDRs”, which are referred to in the art and herein below as “complementarity determining region 1” or “CDR1”; as “complementarity determining region 2” or “CDR2”; and as “complementarity determining region 3” or “CDR3”, respectively. Thus, the general structure or sequence of an immunoglobulin variable domain can be indicated as follows: FR1 - CDR1 - FR2 - CDR2 - FR3 - CDR3 - FR4. It is the immunoglobulin variable domain(s) that confers specificity to an antibody for the antigen by carrying the antigen-binding site.

The term “immunoglobulin single variable domain” or “ISV”, interchangeably used with “single variable domain”, defines molecules wherein the antigen binding site is present on, and formed by, a single immunoglobulin domain. This sets immunoglobulin single variable domains apart from “conventional” immunoglobulins or their fragments, 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 (VH) and a light chain variable domain (VL) interact to form an antigen binding site. In this case, the complementarity determining regions (CDRs) of both

VH and VL will contribute to the antigen binding site, i.e. a total of 6 CDRs will be involved in antigen binding site formation.

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

In contrast, immunoglobulin single variable domains are capable of specifically binding to an epitope of the antigen without pairing with an additional immunoglobulin variable domain. The binding site of an immunoglobulin single variable domain is formed by a single VH/VHH or VL domain. Hence, the antigen binding site of an immunoglobulin single variable domain is formed by no more than three CDRs.

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

In one aspect of the invention, the immunoglobulin single variable domains are heavy chain variable domain sequences (e.g., a VH-sequence); more specifically, the immunoglobulin single variable domains can be heavy chain variable domain sequences that are derived from a conventional four-chain antibody or heavy chain variable domain sequences that are derived from a heavy chain antibody.

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

In particular, the immunoglobulin single variable domain may be a Nanobody (as defined herein) or a suitable fragment thereof. [Note: Nanobody, Nanobodies and Nanoclone are registered trademarks

of Ablynx N.V.] For a general description of Nanobodies, reference is made to the further description below, as well as to the prior art cited herein, such as e.g. described in WO 08/020079 (page 16).

“VHH domains”, also known as VHHs, V_HH domains, VHH antibody fragments, and VHH antibodies, have originally been described as the antigen binding immunoglobulin (variable) domain of “heavy chain antibodies” (*i.e.*, of “antibodies devoid of light chains”; Hamers-Casterman et al. Nature 363: 446-448, 1993). The term “VHH 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” or “VH 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” or “VL domains”). For a further description of VHH’s and Nanobodies, reference is made to the review article by Muyldermans (2001, Reviews in Molecular Biotechnology 74: 277-302), as well as to the following patent applications, which are mentioned as general background art: WO 94/04678, WO 95/04079 and WO 96/34103 of the Vrije Universiteit Brussel; WO 94/25591, WO 99/37681, WO 00/40968, WO 00/43507, WO 00/65057, WO 01/40310, WO 01/44301, EP 1134231 and WO 02/48193 of Unilever; WO 97/49805, WO 01/21817, WO 03/035694, WO 03/054016 and WO 03/055527 of the Vlaams Instituut voor Biotechnologie (VIB); WO 03/050531 of Algonomics N.V. and Ablynx N.V.; WO 01/90190 by the National Research Council of Canada; WO 03/025020 (= EP 1433793) by the Institute of Antibodies; as well as WO 04/041867, WO 04/041862, WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858, WO 06/40153, WO 06/079372, WO 06/122786, WO 06/122787 and WO 06/122825, by Ablynx N.V. and the further published patent applications by Ablynx N.V. Reference is also made to the further prior art mentioned in these applications, and in particular to the list of references mentioned on pages 41-43 of the International application WO 06/040153, which list and references are incorporated herein by reference. As described in these references, Nanobodies (in particular VHH sequences and partially humanized Nanobodies) can in particular be characterized by the presence of one or more “Hallmark residues” in one or more of the framework sequences. A further description of the Nanobodies, including humanization and/or camelization of Nanobodies, as well as other modifications, parts or fragments, derivatives or “Nanobody fusions”, multivalent constructs (including some non-limiting examples of linker sequences) and different modifications to increase the half-life of the Nanobodies and their preparations can be found e.g. in WO 08/101985 and WO 08/142164. For a further general description of Nanobodies, reference is made to the prior art cited herein, such as *e.g.*, described in WO 08/020079 (page 16).

“Domain antibodies”, also known as “Dab”s, “Domain Antibodies”, and “dAbs” (the terms “Domain Antibodies” and “dAbs” being used as trademarks by the GlaxoSmithKline group of companies) have

been described in *e.g.*, EP 0368684, Ward et al. (1989, Nature 341: 544-546), Holt et al. (2003, Trends in Biotechnology 21: 484-490) and WO 03/002609 as well as for example WO 04/068820, WO 06/030220, WO 06/003388 and other published patent applications of Domantis Ltd. Domain antibodies essentially correspond to the VH or VL domains of non-camelid mammals, in particular human 4-chain antibodies. In order to bind an epitope as a single antigen binding domain, *i.e.*, without being paired with a VL or VH domain, respectively, specific selection for such antigen binding properties is required, *e.g.* by using libraries of human single VH or VL domain sequences. Domain antibodies have, like VHHs, a molecular weight of approximately 13 to approximately 16 kDa and, if derived from fully human sequences, do not require humanization for *e.g.* therapeutic use in humans.

It should also be noted that, although less preferred in the context of the present invention because they are not of mammalian origin, single variable domains can be derived from certain species of shark (for example, the so-called "IgNAR domains", see for example WO 05/18629).

Thus, in the meaning of the present invention, the term "immunoglobulin single variable domain" or "single variable domain" comprises polypeptides which are derived from a non-human source, preferably a camelid, preferably a camelid heavy chain antibody. They may be humanized, as previously described. Moreover, the term comprises polypeptides derived from non-camelid sources, *e.g.* mouse or human, which have been "camelized", as *e.g.*, described in Davies and Riechmann (1994, FEBS 339: 285-290; 1995, Biotechnol. 13: 475-479; 1996, Prot. Eng. 9: 531-537) and Riechmann and Muyldermans (1999, J. Immunol. Methods 231: 25-38).

The amino acid residues of a VHH domain are numbered according to the general numbering for V_H domains given by Kabat et al. ("Sequence of proteins of immunological interest", US Public Health Services, NIH Bethesda, MD, Publication No. 91), as applied to VHH domains from Camelids, as shown *e.g.*, in Figure 2 of Riechmann and Muyldermans (1999, J. Immunol. Methods 231: 25-38). Alternative methods for numbering the amino acid residues of V_H domains, which methods can also be applied in an analogous manner to VHH domains, are known in the art. However, in the present description, claims and figures, the numbering according to Kabat applied to VHH domains as described above will be followed, unless indicated otherwise.

It should be noted that - as is well known in the art for V_H domains and for VHH domains - the total number of amino acid residues in each of the CDRs may vary and may not correspond to the total number of amino acid residues indicated by the Kabat numbering (that is, one or more positions according to the Kabat numbering may not be occupied in the actual sequence, or the actual sequence may contain more amino acid residues than the number allowed for by the Kabat

numbering). This means that, generally, the numbering according to Kabat may or may not correspond to the actual numbering of the amino acid residues in the actual sequence. The total number of amino acid residues in a VH domain and a VHH 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.

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

In the present application, however, CDR sequences were determined according to Kontermann and Dübel (2010, Eds., 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 (according to Kabat numbering).

Immunoglobulin single variable domains such as Domain antibodies and Nanobodies (including VHH domains) can be subjected to humanization. In particular, humanized immunoglobulin single variable domains, such as Nanobodies (including VHH domains) may be immunoglobulin single variable domains that are as generally defined for in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, in at least one of the framework residues) that is and/or that corresponds to a humanizing substitution (as defined herein). Potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring V_{HH} sequence with the corresponding framework sequence of one or more closely related human V_H sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said V_{HH} sequence (in any manner known per se, as further described herein) and the resulting humanized V_{HH} sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) an immunoglobulin

single variable domain, such as a Nanobody (including VHH domains) may be partially humanized or fully humanized.

Immunoglobulin single variable domains such as Domain antibodies and Nanobodies (including VHH domains and humanized VHH domains), can also be subjected to affinity maturation by introducing one or more alterations in the amino acid sequence of one or more CDRs, which alterations result in an improved affinity of the resulting immunoglobulin single variable domain for its respective antigen, as compared to the respective parent molecule. Affinity-matured immunoglobulin single variable domain molecules of the invention may be prepared by methods known in the art, for example, as described by Marks et al. (1992, Biotechnology 10: 779-783), Barbas, et al. (1994, Proc. Nat. Acad. Sci, USA 91: 3809-3813), Shier et al. (1995, Gene 169: 147-155), Yelton et al. (Immunol. 155: 1994-2004,), Jackson et al. (J. Immunol. 154: 3310-9, 1995), Hawkins et al. (1995, J. Mol. Biol. 226: 889-896), Johnson and Hawkins (1996, Affinity maturation of antibodies using phage display, Oxford University Press).

The process of designing/selecting and/or preparing a polypeptide, starting from an immunoglobulin single variable domain such as a Domain antibody or a Nanobody, is also referred to herein as “formatting” said immunoglobulin single variable domain; and an immunoglobulin single variable domain that is made part of a polypeptide is said to be “formatted” or to be “in the format of” said polypeptide. Examples of ways in which an immunoglobulin single variable domain can be formatted and examples of such formats will be clear to the skilled person based on the disclosure herein; and such formatted immunoglobulin single variable domain form a further aspect of the invention.

For example, and without limitation, one or more immunoglobulin single variable domains may be used as a “binding unit”, “binding domain” or “building block” (these terms are used interchangeably) for the preparation of a polypeptide, which may optionally contain one or more further immunoglobulin single variable domains that can serve as a binding unit (*i.e.*, against the same or another epitope on CD123 and/or against one or more other antigens, proteins or targets than CD123, such as *e.g.*, TCR).

Monovalent polypeptides comprise or essentially consist of only one binding unit (such as *e.g.*, one immunoglobulin single variable domains). Polypeptides that comprise two or more binding units (such as *e.g.*, two or more immunoglobulin single variable domains) will also be referred to herein as “multivalent” polypeptides, and the binding units/immunoglobulin single variable domains present in such polypeptides will also be referred to herein as being in a “multivalent format”. For example a “bivalent” polypeptide may comprise two immunoglobulin single variable domains, optionally linked via a linker sequence, whereas a “trivalent” polypeptide may comprises three immunoglobulin single

variable domains, optionally linked via two linker sequences, whereas a “tetravalent” polypeptide may comprise four immunoglobulin single variable domains, optionally linked via three linker sequences, etc.

In a multivalent polypeptide, the two or more immunoglobulin single variable domains may be the same or different, and may be directed against the same antigen or antigenic determinant (for example against the same part(s) or epitope(s) or against different parts or epitopes) or may alternatively be directed against different antigens or antigenic determinants; or any suitable combination thereof. Polypeptides that contain at least two binding units (such as *e.g.*, at least two immunoglobulin single variable domains) in which at least one binding unit is directed against a first antigen (*i.e.*, CD123) and at least one binding unit is directed against a second antigen (*i.e.*, different from CD123) will also be referred to as “multispecific” polypeptides, and the binding units (such as *e.g.*, immunoglobulin single variable domains) present in such polypeptides will also be referred to herein as being in a “multispecific format”. Thus, for example, a “bispecific” polypeptide of the invention is a polypeptide that comprises at least one immunoglobulin single variable domain directed against a first antigen (*i.e.*, CD123) and one further immunoglobulin single variable domain directed against a second antigen (*i.e.*, different from CD123, such as *e.g.* TCR), whereas a “trispecific” polypeptide of the invention is a polypeptide that comprises at least one immunoglobulin single variable domain directed against a first antigen (*i.e.*, CD123), one further immunoglobulin single variable domain directed against a second antigen (*i.e.*, different from CD123, such as *e.g.*, TCR) and at least one further immunoglobulin single variable domain directed against a third antigen (*i.e.*, different from both CD123 and the second antigen); etc.

Polypeptides that are directed against one antigen will also be referred to as “monospecific” polypeptides. Such “monospecific” polypeptides may be monovalent polypeptides, containing only one binding unit (such as *e.g.*, one immunoglobulin single variable domain) directed against one antigen (*e.g.* TCR or CD123). Such “monospecific” polypeptides may also be multivalent polypeptides, containing two or more immunoglobulin single variable domains directed against the same antigen. Such “monospecific” multivalent polypeptides can be directed against the same part(s) or epitope(s) of the same antigen or against different parts or epitopes of the same antigen) (*e.g.* CD123).

Polypeptides that comprise two or more binding units directed against different parts or epitopes on the same antigen are also referred to as “multiparatopic” polypeptides. As such, “multiparatopic” polypeptides, such as *e.g.*, “biparatopic” polypeptides or “triparatopic” polypeptides, comprise or essentially consist of two or more binding units that each have a different paratope (as will be further described herein; see chapter on monospecific polypeptides of the invention).

Polypeptides of the invention

The present invention provides polypeptides that redirect T cells for killing of CD123 expressing cells. The ability of these polypeptides to exert this function arises from their multispecific format. The multispecific polypeptides provided by the present invention (referred to as “multispecific polypeptide(s) of the invention”) comprise one immunoglobulin single variable domain (ISV) that specifically binds T cell receptor (TCR) and one or more ISV that specifically bind CD123.

The invention also relates to monovalent polypeptides that may be used as a binding unit or building block in such a multispecific polypeptide of the invention. Accordingly, in one aspect, the invention provides ISVs that specifically bind TCR. In another aspect, the invention provides ISVs that specifically bind CD123. These monovalent polypeptides only bind to one antigen and will therefore be referred to as “monospecific polypeptide(s) of the invention”.

The ISVs that specifically bind CD123 may further be formatted to form multivalent polypeptides, which are also encompassed in the invention. Such multivalent polypeptides comprise two or more ISVs that specifically bind CD123. These multivalent polypeptides only bind one antigen (i.e. CD123) and will therefore also be referred to as “monospecific polypeptide(s) of the invention”.

The monospecific polypeptide(s) of the invention and multispecific polypeptide(s) of the invention are further described herein and are generally referred to as “polypeptide(s) of the invention”.

1. Monospecific polypeptides of the invention

1.1 Monospecific polypeptides that bind TCR

The present invention relates to a monospecific polypeptide that specifically binds TCR. Preferably, such monospecific polypeptide of the invention is monovalent. In a preferred aspect, the monospecific polypeptide is an immunoglobulin single variable domain, which will be referred to herein as “immunoglobulin single variable domain(s) of the invention” or “ISV(s) of the invention”.

The T cell receptor (also referred to herein as TCR) is a heterodimer that consists of a TCR α and a TCR β chain. Both α and β chains of the TCR consist of a constant domain and a variable domain. The polypeptides of the invention specifically bind to the constant domain of the TCR.

The T cell receptor forms part of the TCR complex. As used herein, the terms “TCR complex” or “TCR $\alpha\beta$ –CD3 complex” refers to the T cell receptor complex presented on the surface of T cells (see Kuhns et al. 2006, Immunity 24: 133-139). The TCR complex is composed of six different type I single-

spanning transmembrane proteins: the TCR α and TCR β chain that form the TCR heterodimer responsible for ligand recognition, and the non-covalently associated CD3 γ , CD3 δ , CD3 ϵ and ζ chains, which bear cytoplasmic sequence motifs that are phosphorylated upon receptor activation and recruit a large number of signalling components. The sequences for the human CD3 and the human TCR α/β constant domains are provided in Table A-8 (SEQ ID NOs: 70-75; cf. UniProt identifiers: CD3 delta: P04234, CD3 gamma: P09693, CD3 epsilon: P07766, CD3 zeta: P20963, TCR alpha: P01848 and TCR beta: related to P01850).

In one aspect, the present invention relates to a polypeptide as described herein, that binds to the constant domain of the T cell receptor α (TCR α) (SEQ ID NO: 74) and/or the constant domain of the T cell receptor β (TCR β) (SEQ ID NO: 75), or polymorphic variants or isoforms thereof.

Isoforms are alternative protein sequences that can be generated from the same gene by a single or by the combination of biological events such as alternative promoter usage, alternative splicing, alternative initiation and ribosomal frameshifting, all as known in the art.

Only after rigorous immunization, screening and selection methods, the present inventors were able to identify ISVs binding to the constant domains of TCR. A cluster of sequences, comprising 104 clones with similarities and differences in CDR1, CDR2 and CDR3 was identified (see Table A-5). A corresponding sequence alignment is provided (Table A-1).

Accordingly, the present invention relates to polypeptides that are ISVs chosen from the group consisting of SEQ ID NOs: 42 and 78-180 (cf. Table A-5). In a further aspect, the polypeptide is chosen from the group consisting of SEQ ID NOs: 42 and 78-180 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 42 and 78-180.

Accordingly, the present invention relates to a polypeptide that binds TCR and comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 has the amino acid sequence GX₁VX₂X₃X₄NX₅LX₆ in which X₁ is D, A, S, E or G, X₂ is H or Y, X₃ is K or L, X₄ is I or L, X₅ is F, I or V, and X₆ is G or S.

In a further aspect, the present invention relates to a polypeptide that binds TCR and that comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 has the amino acid sequence X₁IX₂IX₃DX₄X₅X₆ in which X₁ is H, T or R, X₂ is S, T or A, X₃ is G, S or A, X₄ is Q, D, E, T, A or V, X₅ is T, A or V and X₆ is D, A, Q, N, V or S.

In a further aspect, the present invention relates to a polypeptide that binds TCR and that comprises or (essentially) consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 has the amino acid sequence $X_1SRX_2X_3PYX_4Y$, in which X_1 is F, Y, G, L or K, X_2 is I or L, X_3 is Y or W, and X_4 is D, N or S.

- 5 Preferred CDR sequences for use in the polypeptides of the invention, as well as preferred combinations of CDR sequences, are depicted in Table A-5.

Accordingly, the present invention relates to a polypeptide, preferably an ISV, that specifically binds TCR and that comprises or essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- 10 i) CDR1 is chosen from the group consisting of:
- a) SEQ ID NOs: 181-191; or
 - b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a
- 15 higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- ii) CDR2 is chosen from the group consisting of:
- 20 c) SEQ ID NOs: 192-217; or
 - d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a
- 25 higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- iii) CDR3 is chosen from the group consisting of:
- e) SEQ ID NOs: 218-225; or
 - 30 f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a
- higher affinity compared to the binding by the ISV comprising the CDR3 without the

4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention relates to a polypeptide, preferably an ISV, in which:

i) CDR1 is chosen from the group consisting of:

5 a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

15 d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

25 f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

30 In particular, the present invention relates to a polypeptide, preferably an ISV, in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191, wherein the 4, 3, 2 or 1 amino acid(s)

difference are present at position 2, 4, 5, 6, 8 and/or 10 of the CDR1 (position 27, 29, 30, 31, 33 and/or 35 according to Kabat numbering); provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 3, 5, 7, 8 and/or 9 of the CDR2 (position 50, 52, 54, 56, 57 and/or 58 according to Kabat numbering); provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 4, 5 and/or 8 of the CDR3 (position 95, 98, 99 and/or 101 according to Kabat numbering); provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR1 is chosen from the group consisting of:

a) SEQ ID NO: 181; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein

- at position 2 the D has been changed into A, S, E or G;
- at position 4 the H has been changed into Y;

- at position 5 the K has been changed into L;
- at position 6 the I has been changed into L;
- at position 8 the F has been changed into I or V; and/or
- at position 10 the G has been changed into S;

5 provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR2 is
10 chosen from the group consisting of:

- a) SEQ ID NO: 192; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein
 - at position 1 the H has been changed into T or R;
 - 15 - at position 3 the S has been changed into T or A;
 - at position 5 the G has been changed into S or A;
 - at position 7 the Q has been changed into D, E, T, A or V;
 - at position 8 the T has been changed into A or V; and/or
 - at position 9 the D has been changed into A, Q, N, V or S;

20 provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR3 is
25 chosen from the group consisting of:

- a) SEQ ID NO: 218; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein
 - at position 1 the F has been changed into Y, L or G;
 - 30 - at position 4 the I has been changed into L;
 - at position 5 the Y has been changed into W; and/or
 - at position 8 the D has been changed into N or S;

provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by

the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention relates to a polypeptide, preferably an ISV, in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 181; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein
 - at position 2 the D has been changed into A, S, E or G;
 - at position 4 the H has been changed into Y;
 - at position 5 the K has been changed into L;
 - at position 6 the I has been changed into L;
 - at position 8 the F has been changed into I or V; and/or
 - at position 10 the G has been changed into S;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 192; or
- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein
 - at position 1 the H has been changed into T or R;
 - at position 3 the S has been changed into T or A;
 - at position 5 the G has been changed into S or A;
 - at position 7 the Q has been changed into D, E, T, A or V;
 - at position 8 the T has been changed into A or V; and/or
 - at position 9 the D has been changed into A, Q, N, V or S;

provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 218; or
- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein
 - at position 1 the F has been changed into Y, L or G;
 - at position 4 the I has been changed into L;
 - at position 5 the Y has been changed into W; and/or
 - at position 8 the D has been changed into N or S;

provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR1 is chosen from the group consisting of SEQ ID NOs: 181-191, CDR2 is chosen from the group consisting of SEQ ID NOs: 192-217, and CDR3 is chosen from the group consisting of SEQ ID NOs: 218-225.

Accordingly, in a preferred aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR1 is SEQ ID NO: 181, CDR2 is SEQ ID NO: 192, and CDR3 is SEQ ID NO: 218.

Generally, the combinations of CDRs listed in Table A-5 (i.e. those mentioned on the same line in Table A-5) are preferred. Thus, it is generally preferred that, when a CDR in an ISV is a CDR sequence mentioned in Table A-5 or suitably chosen from the group consisting of CDR sequences that have 4, 3, 2 or only 1 amino acid difference(s) with a CDR sequence listed in Table A-5, that at least one and preferably both of the other CDR's are suitably chosen from the CDR sequences that belong to the same combination in Table A-5 (i.e. mentioned on the same line in Table A-5) or are suitably chosen from the group consisting of CDR sequences that have 4, 3, 2 or only 1 amino acid difference(s) with the CDR sequence(s) belonging to the same combination.

The present invention also relates to a polypeptide, preferably an ISV, that cross-blocks the binding to TCR of at least one of the polypeptides as described herein and/or that is cross-blocked from binding to TCR by at least one of the polypeptides as described herein.

The polypeptides of the present invention specifically bind TCR on the surface of effector cells, such as T cells. In "monovalent" format, the monovalent polypeptides of the invention that bind TCR cause minimal to no T cell activation.

As used herein, the term "an effector cell" is a cell comprising a TCR complex, preferably an immune cell, such as a T cell, preferably a CD4⁺ T-helper cell (also known as CD4 cell, T-helper cell or T4 cell), more preferably a Cytotoxic T cell (also known as T_C cell, CTL or CD8⁺ T cells) or Natural Killer T cells

(NKT cells). In some aspects, the cell is present *in vivo*. In some aspects, the cell is present *in vitro*. The effector cell of the invention relates in particular to mammalian cells, preferably to primate cells, and even more preferably to human cells.

"T cell activation" as used herein refers to one or more cellular response(s) of a T cell, e.g. a cytotoxic T cell, such as selected from: proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, expression of activation markers, and redirected target cell lysis.

The monospecific polypeptide of the invention binds to the constant domain of the T cell receptor (TCR) with an average K_D value of between 100 nM and 10 pM, such as at an average K_D value of 90 nM or less, even more preferably at an average K_D value of 80 nM or less, such as less than 70, 60, 50, 40, 30, 20, 10, 5 nM or even less, such as less than 4, 3, 2, or 1 nM, such as less than 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20 pM, or even less, such as less than 10 pM. Preferably, the K_D is determined by Kinexa, BLI or SPR, for instance as determined by Proteon. For instance, said K_D is determined as set out in the Examples section.

The monospecific polypeptide of the invention binds to TCR with an EC50 value of between 100 nM and 1 pM, such as at an average EC50 value of 100 nM or less, even more preferably at an average EC50 value of 90 nM or less, such as less than 80, 70, 60, 50, 40, 30, 20, 10, 5 nM or even less, such as less than 4, 3, 2, or 1 nM or even less, such as less than 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5 pM, or even less, such as less than 4 pM. Said average EC50 is preferably determined by FACS, Biacore or ELISA, for instance, said EC50 is determined as set out in the Examples section.

It has been shown in the examples that the K_D correlates well with the EC50.

In a further aspect, the monospecific polypeptide as described herein, has an on rate constant (k_{on}) to (or for binding) TCR selected from the group consisting of at least about $10^2 \text{ M}^{-1}\text{s}^{-1}$, at least about $10^3 \text{ M}^{-1}\text{s}^{-1}$, at least about $10^4 \text{ M}^{-1}\text{s}^{-1}$, at least about $10^5 \text{ M}^{-1}\text{s}^{-1}$, at least about $10^6 \text{ M}^{-1}\text{s}^{-1}$, $10^7 \text{ M}^{-1}\text{s}^{-1}$, at least about $10^8 \text{ M}^{-1}\text{s}^{-1}$, at least about $10^9 \text{ M}^{-1}\text{s}^{-1}$, and at least about $10^{10} \text{ M}^{-1}\text{s}^{-1}$, preferably as measured by surface plasmon resonance or as performed in the examples section.

In a further aspect, the monospecific polypeptide as described herein, has an off rate constant (k_{off}) to (or for binding) TCR selected from the group consisting of at most about 10^{-3} s^{-1} , at most about 10^{-4} s^{-1} , at most about 10^{-5} s^{-1} , at most about 10^{-6} s^{-1} , at most about 10^{-7} s^{-1} , at most about 10^{-8} s^{-1} , at most about 10^{-9} s^{-1} , and at most about 10^{-10} s^{-1} , preferably as measured by surface plasmon resonance or as performed in the examples section.

The monospecific polypeptides and/or immunoglobulin single variable domains of the invention that bind TCR may have framework sequences that are preferably (a suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from

immunoglobulin framework sequences (for example, by sequence optimization such as humanization or camelization). For example, the framework sequences may be framework sequences derived from an immunoglobulin single variable domain such as 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). In one particularly preferred aspect, the framework sequences are either framework sequences that have been derived from a V_{HH} -sequence (in which said framework sequences may optionally have been partially or fully humanized) or are conventional V_H sequences that have been camelized.

The framework sequences may preferably be such that the monospecific polypeptide and/or immunoglobulin single variable domain is a Domain antibody (or an amino acid sequence that is suitable for use as a Domain antibody); a single domain antibody (or an amino acid that is suitable for use as a single domain antibody); a "dAb" (or an amino acid that is suitable for use as a dAb); a Nanobody; a V_{HH} ; a humanized V_{HH} ; a camelized V_H ; or a V_{HH} that has been obtained by affinity maturation. Again, suitable framework sequences will be clear to the skilled person, for example on the basis of the standard handbooks and the further disclosure and prior art mentioned herein.

In particular, the framework sequences present in the monospecific polypeptides of the invention may contain one or more of Hallmark residues (as defined in WO 08/020079 (Tables A-3 to A-8)), such that the monospecific polypeptide of the invention is a Nanobody. Some preferred, but non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein (see *e.g.*, Table A-5). Generally, Nanobodies (in particular V_{HH} s, partially or fully humanized V_{HH} s and camelized V_H s) can in particular be characterized by the presence of one or more "*Hallmark residues*" in one or more of the framework sequences (as *e.g.*, further described in WO 08/020079, page 61, line 24 to page 98, line 3).

More in particular, the invention provides polypeptides comprising or (essentially) consisting of at least one immunoglobulin single variable domain that is an amino acid 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 which:

- i) have at least 80%, more preferably 90%, even more preferably 95% amino acid identity with at least one of the amino acid sequences of SEQ ID NOs: 42 and 78-180 (see Table A-5), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded. In this respect, reference is

also made to Table A-5, which lists the framework 1 sequences (SEQ ID NOs: 226-250), framework 2 sequences (SEQ ID NOs: 251-276), framework 3 sequences (SEQ ID NOs: 277-319) and framework 4 sequences (SEQ ID NOs: 320-324) of the immunoglobulin single variable domains of SEQ ID NOs: 42 and 78-180 (see Table A-5); or

5 and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 to Table A-8 of WO 08/020079.

The present invention also provides a number of sequence optimized polypeptides and/or immunoglobulin single variable domains.

In particular, sequence optimized polypeptides and/or immunoglobulin single variable domains of the invention may be amino acid sequences that are as generally defined for immunoglobulin single variable domains in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, in at least one of the framework residues) that is and/or that corresponds to a humanizing substitution (as defined herein). Some preferred, but non-limiting humanizing substitutions (and suitable combinations thereof) will become clear to the skilled person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring VHH sequence with the corresponding framework sequence of one or more closely related human VH sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said VHH sequence (in any manner known per se, as further described herein) and the resulting humanized VHH sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) an immunoglobulin single variable domains may be partially humanized or fully humanized.

The present invention also relates to sequence optimized polypeptides and/or immunoglobulin single variable domains that may show improved expression and/or increased stability upon storage during stability studies. The sequence optimized polypeptides and/or ISVs of the present invention may show reduced pyroglutamate post-translational modification of the N-terminus and hence have increased product stability. In addition, the sequence optimized polypeptides and/or ISVs of the present invention may show other improved properties such as e.g. less immunogenicity, improved binding characteristics (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -

value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an EC_{50} value, as further described herein) for TCR, improved affinity and/or improved avidity for TCR.

Some particularly preferred sequence optimized immunoglobulin single variable domains of the invention are sequence optimized variants of the immunoglobulin single variable domains of SEQ ID
5 NOs: 42 and 78-180.

Thus, some other preferred immunoglobulin single variable domains of the invention are Nanobodies which can bind (as defined herein) to TCR and which:

i) are a sequence optimized variant of one of the immunoglobulin single variable domains of SEQ ID NOs: 42 and 78-180; and/or

10 ii) have at least 80% amino acid identity with at least one of the immunoglobulin single variable domains of SEQ ID NOs: 42 and 78-180 (see Table A-5), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded; In this respect, reference is also made to Table A-5, which lists the framework 1 sequences (SEQ ID NOs: 226-250), framework 2 sequences (SEQ ID
15 NOs: 251-276), framework 3 sequences (SEQ ID NOs: 277-319) and framework 4 sequences (SEQ ID NOs: 320-324) of the immunoglobulin single variable domains of SEQ ID NOs: 42 and 78-180 (see Table A-5);

and in which:

20 iii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 to Table A-8 of WO 08/020079.

The sequence optimized polypeptides and/or immunoglobulin single variable domains of the invention may also contain the specific mutations/amino acid residues described in the following co-
pending US provisional applications, all entitled "Improved immunoglobulin variable domains": US
25 61/994552 filed May 16, 2014; US 61/014,015 filed June 18, 2014; US 62/040,167 filed August 21, 2014; and US 62/047,560, filed September 8, 2014 (all assigned to Ablynx N.V.) as well as the International application WO 2015/173325 which was based on these provisional applications and which was published on November 19, 2015.

In particular, the sequence optimized polypeptides and/or immunoglobulin single variable domains
30 of the invention may suitably contain (i) a K or Q at position 112; or (ii) a K or Q at position 110 in combination with a V at position 11; or (iii) a T at position 89; or (iv) an L on position 89 with a K or Q at position 110; or (v) a V at position 11 and an L at position 89; or any suitable combination of (i) to (v).

As also described in said co-pending US provisional applications, when the polypeptides and/or immunoglobulin single variable domains of the invention contain the mutations according to one of (i) to (v) above (or a suitable combination thereof):

- the amino acid residue at position 11 is preferably chosen from L, V or K (and is most preferably V); and
- the amino acid residue at position 14 is preferably suitably chosen from A or P; and
- the amino acid residue at position 41 is preferably suitably chosen from A or P; and
- the amino acid residue at position 89 is preferably suitably chosen from T, V or L; and
- the amino acid residue at position 108 is preferably suitably chosen from Q or L; and
- the amino acid residue at position 110 is preferably suitably chosen from T, K or Q; and
- the amino acid residue at position 112 is preferably suitably chosen from S, K or Q.

As mentioned in said co-pending US provisional applications, said mutations are effective in preventing or reducing binding of so-called "pre-existing antibodies" to the polypeptides, immunoglobulin single variable domains and/or constructs of the invention. For this purpose, the polypeptides and/or immunoglobulin single variable domains of the invention may also contain (optionally in combination with said mutations) a C-terminal extension (X)_n (in which n is 1 to 10, preferably 1 to 5, such as 1, 2, 3, 4 or 5 (and preferably 1 or 2, such as 1); and each X is an (preferably naturally occurring) amino acid residue that is independently chosen, and preferably independently chosen from the group consisting of alanine (A), glycine (G), valine (V), leucine (L) or isoleucine (I)), for which reference is again made to said US provisional applications as well as to WO 12/175741. In particular, a polypeptide and/or immunoglobulin single variable domain of the invention may contain such a C-terminal extension when it forms the C-terminal end of a protein, polypeptide or other construct comprising the same (again, as further described in said US provisional applications as well as WO 12/175741).

Accordingly, the present invention relates to a polypeptide as described herein, further comprising a C-terminal extension (X)_n, in which n is 1 to 5, such as 1, 2, 3, 4 or 5, and in which X is a naturally occurring amino acid, preferably no cysteine.

These polypeptides of the invention, and in particular the immunoglobulin single variable domains comprising the CDR sequences of the invention are particularly suited for use as building block or binding unit for the preparation of multispecific polypeptides, such as the multispecific polypeptides of the invention.

Accordingly, the monospecific polypeptides of the invention that bind TCR can be in essentially isolated form (as defined herein), or they may form part of a protein or polypeptide, which may

comprise or essentially consist of one polypeptide or ISV that binds TCR and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers).

Accordingly, the present invention also relates to a protein or polypeptide that comprises or essentially consists of one monospecific polypeptide of the invention (or suitable fragments thereof). In a further aspect, the monospecific polypeptides of the invention that bind TCR may form part of a multispecific polypeptide, which may comprise or essentially consist of one ISV that binds TCR and which may optionally further comprise one or more further ISV that specifically binds another target, such as CD123, and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers).

The monospecific polypeptides of the invention are thus used as a binding unit or building block in such a protein or polypeptide, so as to provide a multispecific polypeptide of the invention, as described herein (for multispecific polypeptides containing one or more VHH domains and their preparation, reference is also made to Conrath et al. (2001, J. Biol. Chem. 276: 7346-7350), as well as to for example WO 96/34103, WO 99/23221 and WO 2010/115998). The present invention thus also relates to a polypeptide which is a monovalent construct comprising or essentially consisting of one monovalent polypeptide that binds TCR.

1.2 CD123 binding polypeptides

The present invention relates to a monospecific polypeptide that specifically binds CD123, preferably human and/or cyno CD123. In a preferred aspect, the monospecific polypeptide is an immunoglobulin single variable domain, also referred to herein as "immunoglobulin single variable domain(s) of the invention" or "ISV(s) of the invention".

CD123 is also known as the α subunit of the interleukin 3 receptor (IL-3R α). The sequences of the human CD123 and cyno CD123 are provided in Table A-8 (SEQ ID NOs: 68-69; cf. human CD123: NCBI RefSeq NP_002174 and cyno CD123: NCBI genbank no. EHH61867.1).

In one aspect, the present invention relates to a monospecific polypeptide as described herein, that binds to human CD123 (SEQ ID NO: 68).

The monospecific polypeptides that bind CD123 have been carefully selected for their specificity towards CD123. The polypeptides of the invention exhibit highly specific binding to CD123 upon formatting into a multispecific format of the invention (i.e. a format comprising one ISV that binds TCR and one or more ISVs that bind CD123). As such, off-target binding is avoided and target independent T cell activation is minimal, as further exemplified herein.

The inventors identified 2 clusters of Nanobodies (Example 12), that exhibited highly specific binding to CD123. Upon formatting of representatives of the clusters into a multispecific polypeptide of the invention (as further described), only minimal target-independent T cell activation was observed indicating the high specificity of the cluster representatives. Corresponding alignments are provided (see Table A-2 for the Nanobodies related to (family members of) Nanobody 56A10 (*i.e.*, Nanobodies belonging to the same family as Nanobody 56A10) and Table A-3 for the Nanobodies related to (family members of) Nanobody 55F03 (*i.e.* Nanobodies belonging to the same family as Nanobody 55F03)).

A “Nanobody family”, “V_{HH} family” or “family” as used in the present specification refers to a group of Nanobodies and/or V_{HH} sequences that have identical lengths (*i.e.* they have the same number of amino acids within their sequence) and of which the amino acid sequence between position 8 and position 106 (according to Kabat numbering) has an amino acid sequence identity of 89% or more.

Accordingly, the present invention relates to polypeptides, preferably ISVs, chosen from the group consisting of SEQ ID NOs: 1-10 (cf. Table A-4). In a further aspect, the polypeptide is chosen from the group consisting of SEQ ID NOs: 1-10 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-10.

Accordingly, the present invention relates to a polypeptide, preferably an ISV, that specifically binds CD123 and that comprises or essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same

or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

5 iii) CDR3 is chosen from the group consisting of:

 e) SEQ ID NOs: 21-25; or

 f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same
10 or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention relates to a polypeptide, preferably an ISV, that specifically binds CD123 and that comprises or essentially consists of 4 framework regions (FR1 to FR4,
15 respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

 i) CDR1 is chosen from the group consisting of:

 a) SEQ ID NOs: 11-16; or

 b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same
20 or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

25 ii) CDR2 is chosen from the group consisting of:

 c) SEQ ID NOs: 17-20; or

 d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same
30 or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

 iii) CDR3 is chosen from the group consisting of:

 e) SEQ ID NOs: 21-25; or

- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the polypeptide of the invention, preferably an ISV, comprises or essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or
b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 17-20; or
d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or

- f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the polypeptide comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the polypeptide of the invention, preferably an ISV, comprises or essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

- ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or
- f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the polypeptide comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

5

10 In one aspect, the polypeptides, preferably ISVs, of the invention may have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6 (cf. Table A-4). These polypeptides are referred to herein as "polypeptide(s) related to 56A10" or "ISV(s) related to 56A10".

15

Accordingly, the present invention relates to a polypeptide, preferably an ISV, in which CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 11; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein
 - at position 3 the T has been changed into S or P;
 - at position 6 the I has been changed into S;
 - at position 7 the N has been changed into D; and/or
 - at position 8 the D has been changed into V or A;

20

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

25

In a further aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR2 is SEQ ID NO: 17.

In a further aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR3 is chosen from the group consisting of:

30

- a) SEQ ID NO: 21; or
- b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein
 - at position 3 the P has been changed into A;

provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

5 Accordingly, the present invention relates to a polypeptide, preferably an ISV, in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NO: 11; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- 10
- at position 3 the T has been changed into S or P;
 - at position 6 the I has been changed into S;
 - at position 7 the N has been changed into D; and/or
 - at position 8 the D has been changed into V or A;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is SEQ ID NO: 17;

20 and

iii) CDR3 is chosen from the group consisting of:

c) SEQ ID NOs: 21; or

d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein

- 25
- at position 3 the P has been changed into A;

provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

30 In another aspect, the present invention relates to a polypeptide, preferably an ISV, as described herein, in which CDR1 is chosen from the group consisting of SEQ ID NOs: 11-15, CDR2 is SEQ ID NO: 17, and CDR3 is chosen from the group consisting of SEQ ID NOs: 21-22.

Accordingly, in a preferred aspect, the present invention relates to a polypeptide, preferably an ISV, as described herein, in which CDR1 is SEQ ID NO: 11, CDR2 is SEQ ID NO: 17, and CDR3 is SEQ ID NO: 21.

Accordingly, the present invention relates to polypeptides that are ISVs chosen from the group consisting of SEQ ID NOs: 1-6.

The polypeptides or ISVs related to 56A10 were selected for their exquisite specificity for CD123. Binding of the polypeptides of the invention can be measured in suitable binding assays, including but not limited a flow cytometry assay. In such flow cytometry assay, cells may be used that endogenously express CD123 (such as e.g. MOLM-13 or KG1a cells). Alternatively, cells may be used that are transfected to overexpress CD123 (such as e.g. CHO-K1 huCD123 or HEK293 cyno CD123). Suitable cell lines will become clear from the examples herein.

The polypeptide, preferably ISV, of the invention may bind to CD123 expressed on cells or CD123 expressing cells with an average EC50 value between 10nM and 100pM.

More specifically, the polypeptide, preferably ISV, of the invention binds to human CD123 expressed on MOLM-13 cells with an average EC50 value between 10 nM and 100 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, 3, 2, or 1 nM or even less, preferably as measured by flow cytometry.

The polypeptide, preferably ISV, of the invention binds to human CD123 expressed on CHO-K1 cells with an average EC50 value between 10 nM and 100 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, 3, 2, or 1 nM or even less, preferably as measured by flow cytometry.

The polypeptide, preferably ISV, of the invention binds to cyno CD123 expressed on HEK293 cells with an average EC50 value between 10 nM and 100 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, or 3 nM or even less, preferably as measured by flow cytometry.

Binding of the polypeptides, preferably ISVs, of the invention can also be measured by SPR.

As such, the polypeptide, preferably ISV, of the invention may bind to human CD123 with an average K_D value of between 10 nM and 100 pM, such as at an average K_D value of 5 nM or less, such as less than 4, 3 or 2 nM or even less, said K_D value preferably determined by surface plasmon resonance.

Accordingly, the present invention relates to a polypeptide or ISV as described herein, wherein said average K_D or EC50 is determined by flow cytometry or SPR, for instance said K_D or EC50 is determined as set out in the Examples section.

It has been shown in the examples that the K_D as measured in SPR correlates well with the EC50 as measured in flow cytometry.

In another aspect, the polypeptides of the invention may have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10 (cf. Table A-4). These polypeptides are referred to herein as "polypeptide(s) related to 55F03" or "ISV(s) related to 55F03".

- 5 Accordingly, the present invention relates to a polypeptide, preferably an ISV, in which CDR1 is SEQ ID NO: 16.

In a further aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR2 is chosen from the group consisting of:

- a) SEQ ID NO: 18; or
- 10 b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein
- at position 3 the Y has been changed into W;
 - at position 6 the N has been changed into S; and/or
 - at position 10 the Q has been changed into E;
- 15 provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

- In a further aspect, the present invention relates to a polypeptide, preferably an ISV, in which CDR3 is
- 20 chosen from the group consisting of:

- a) SEQ ID NO: 23; or
- b) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein
- at position 4 the E has been changed into R; and/or
 - 25 - at position 5 the T has been changed into D or Y;
- provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

- 30 Accordingly, the present invention relates to a polypeptide, preferably an ISV, in which:

- i) CDR1 is SEQ ID NO: 16;
- and
- ii) CDR2 is chosen from the group consisting of:
 - a) SEQ ID NO: 18; or

b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- at position 3 the Y has been changed into W;
- at position 6 the N has been changed into S; and/or
- at position 10 the Q has been changed into E;

provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

c) SEQ ID NOs: 23; or

d) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein

- at position 4 the E has been changed into R; and/or
- at position 5 the T has been changed into D or Y;

provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a polypeptide, preferably an ISV, as described herein, in which CDR1 is SEQ ID NO: 16, CDR2 is chosen from the group consisting of SEQ ID NOs: 18-20, and CDR3 is chosen from the group consisting of SEQ ID NOs: 23-25.

Accordingly, in a preferred aspect, the present invention relates to a polypeptide, preferably an ISV, as described herein, in which CDR1 is SEQ ID NO: 16, CDR2 is SEQ ID NO: 18, and CDR3 is SEQ ID NO: 23.

Preferred polypeptides and/or ISVs are chosen from the group consisting of SEQ ID NOs: 7-10.

The polypeptides or ISVs related to 55F03 were selected for their exquisite specificity for CD123. Binding of the polypeptides of the invention can be measured in suitable binding assays, including but not limited to a flow cytometry assay and SPR, as described herein.

The polypeptide, preferably ISV, of the invention may bind to CD123 expressed on cells or CD123 expressing cells with an average EC50 value between 10 μ M and 100 nM.

More specifically, the polypeptide, preferably ISV, of the invention binds to human CD123 expressed on MOLM-13 cells with an average EC50 value between 10 μ M and 100 nM, such as at an average EC50 value of 5 μ M or less, such as less than 4, 3, 2, or 1 μ M or even less, preferably as measured by flow cytometry.

- 5 The polypeptide, preferably ISV, of the invention binds to human CD123 expressed on CHO-K1 cells with an average EC50 value between 100 nM and 1 nM, such as at an average EC50 value of 50 nM or less, such as less than 40, 30, 20, or 10 nM or even less, such as less than 9, 8 or 7nM or even less, preferably as measured by flow cytometry.

10 The polypeptide, preferably ISV, of the invention binds to cyno CD123 expressed on HEK293 cells with an average EC50 value between 10 nM and 100 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, or 3 nM or even less, preferably as measured by flow cytometry.

In a further aspect, the present invention relates to a polypeptide or ISV that binds to human CD123 with an average K_D value of between 1 μ M and 10 nM, such as at an average K_D value of 500 nM or less, such as less than 400, 300 or 200 nM or even less, said K_D value preferably determined by
15 surface plasmon resonance.

Accordingly, the present invention relates to a polypeptide or ISV as described herein, wherein said average K_D or EC50 is determined by flow cytometry or SPR, for instance said K_D or EC50 is determined as set out in the Examples section.

It has been shown in the examples that the K_D , as measured in SPR, correlates well with the EC50, as
20 determined in a flow cytometry based assay using MOLM-13 cells.

Generally, the combinations of CDRs listed in Table A-4 (i.e. those mentioned on the same line in Table A-4) are preferred. Thus, it is generally preferred that, when a CDR in an ISV is a CDR sequence mentioned in Table A-4 or suitably chosen from the group consisting of CDR sequences that have 4, 3, 2 or only 1 amino acid difference(s) with a CDR sequence listed in Table A-4, that at least one and
25 preferably both of the other CDR's are suitably chosen from the CDR sequences that belong to the same combination in Table A-4 (i.e. mentioned on the same line in Table A-4) or are suitably chosen from the group consisting of CDR sequences that have 4, 3, 2 or only 1 amino acid difference(s) with the CDR sequence(s) belonging to the same combination. Representative polypeptides of the present invention having the CDRs described above are shown in Table A-4.

30 The present invention also relates to a polypeptide, preferably ISV, that specifically binds CD123 that cross-blocks the binding to CD123 of at least one of the polypeptides as described herein and/or selected from SEQ ID NOs: 1-10 and/or that is cross-blocked from binding to CD123 by at least one of the polypeptides as described herein and/or selected from SEQ ID NOs: 1-10.

The invention further relates to a monospecific polypeptide that comprises or (essentially) consists of two or more ISVs that bind CD123. In such a multivalent (monospecific) polypeptide, also referred to herein as “multivalent polypeptide(s) of the invention”, the two or more ISVs that bind CD123 may optionally be linked via one or more peptidic linkers, as further described herein.

- 5 Accordingly, the present invention relates to a polypeptide comprising two or more ISVs that specifically bind CD123, wherein the ISVs are chosen from the group of ISVs related to 56A10 or from the group of ISVs related to 55F03.

In a more specific aspect, the present invention relates to polypeptides comprising two ISVs that specifically bind CD123, wherein the ISVs are chosen from the group of ISVs related to 56A10 or from
10 group of ISVs related to 55F03.

In such a multivalent monospecific polypeptide of the invention, the two or more immunoglobulin single variable domains may be the same or different, and may be directed against the same antigenic determinant of CD123 (for example against the same part(s) or epitope(s) of CD123) or may alternatively be directed against different antigenic determinants of CD123 or against different parts
15 or epitopes of CD123; or any suitable combination thereof. For example, a bivalent polypeptide of the invention may comprise (a) two identical immunoglobulin single variable domains; (b) a first immunoglobulin single variable domain directed against a first antigenic determinant of CD123 and a second immunoglobulin single variable domain directed against the same antigenic determinant of CD123 which is different from the first immunoglobulin single variable domain; or (c) a first
20 immunoglobulin single variable domain directed against a first antigenic determinant of CD123 and a second immunoglobulin single variable domain directed against another antigenic determinant of CD123.

A trivalent polypeptide of the invention may be any of the above, further comprising (a) an identical immunoglobulin single variable domain; (b) a different immunoglobulin single variable domain
25 directed against the same antigenic determinant of CD123; or (c) a different immunoglobulin single variable domain directed against another antigenic determinant of CD123.

As such, in one aspect, the monospecific polypeptide of the invention may be a multiparatopic polypeptide, such as *e.g.*, a biparatopic polypeptide. The term "biparatopic" (antigen-)binding molecule or "biparatopic" polypeptide as used herein shall mean a polypeptide comprising at least
30 two (i.e. two or more) immunoglobulin single variable domains, wherein a “first” immunoglobulin single variable domain is directed against CD123 and a “second” immunoglobulin single variable domain is directed against CD123, and wherein these “first” and “second” immunoglobulin single variable domains have a different paratope. Accordingly, the biparatopic polypeptide comprises or

consists of two or more immunoglobulin single variable domains that are directed against CD123, wherein at least one "first" immunoglobulin single variable domain is directed against a first epitope on CD123 and at least one "second" immunoglobulin single variable domain is directed against a second epitope on CD123 different from the first epitope on CD123.

5 Accordingly, the present invention relates to polypeptides, wherein the two or more ISVs that specifically bind CD123 are biparatopic comprising a first ISV and a second ISV, wherein the first ISV binds to an epitope on CD123 that is different from the epitope on CD123 bound by the second ISV. Such polypeptide(s) are also referred to herein as "biparatopic polypeptide(s) of the invention".

10 In a further aspect, the present invention provides a (biparatopic) polypeptide as described herein, wherein the first ISV is selected from the group of ISVs related to 56A10 and the second ISV is selected from the group of ISVs related to 55F03.

In a further aspect, the present invention provides a polypeptide as described herein, wherein the second ISV is located N-terminally of the first ISV. Such a polypeptide comprises an ISV related to 55F03 N-terminally of an ISV related to 56A10.

15 In a further aspect, the present invention provides a polypeptide as described herein, wherein the second ISV is located C-terminally of the first ISV. Such a polypeptide comprises an ISV related to 55F03 C-terminally of an ISV related to 56A10.

The biparatopic polypeptides of the invention may have an improved affinity for binding to CD123 compared to the corresponding monovalent polypeptide, due to avid binding, also referred to as
20 "avidity".

Avidity is the affinity of the polypeptide, i.e. the ligand is able to bind via two (or more) pharmacophores (ISV) in which the multiple interactions synergize to enhance the "apparent" affinity. Avidity is the measure of the strength of binding between the polypeptide of the invention and the pertinent antigens or antigenic determinants. The polypeptide of the invention is able to
25 bind via its two (or more) building blocks, such as ISVs, to the at least two targets or antigenic determinants, in which the multiple interactions, e.g. the first building block or ISV binding to the first target or first antigenic determinant and the second building block or ISV binding to the second target or second antigenic determinant, synergize to enhance the "apparent" affinity. Avidity is related to both the affinity between an antigenic determinant and its antigen binding site on the
30 antigen-binding molecule and the number of pertinent binding sites present on the antigen-binding molecules. For example, and without limitation, polypeptides that contain two or more building blocks, such as ISVs directed against different targets on a cell or different antigenic determinants may (and usually will) bind with higher avidity than each of the individual monomers or individual

building blocks, such as, for instance, the monovalent ISVs, comprised in the polypeptides of the invention.

The monospecific polypeptides of the invention comprise or (essentially) consist of one or more immunoglobulin single variable domains. The framework sequences of these ISVs are preferably (a
5 suitable combination of) immunoglobulin framework sequences or framework sequences that have been derived from immunoglobulin framework sequences (for example, by sequence optimization such as humanization or camelization). For example, the framework sequences may be framework sequences derived from an immunoglobulin single variable domain such as 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). In one
10 particularly preferred aspect, the framework sequences are either framework sequences that have been derived from a V_{HH} -sequence (in which said framework sequences may optionally have been partially or fully humanized) or are conventional V_H sequences that have been camelized.

The framework sequences may preferably be such that the ISV encompassed in the monospecific polypeptide of the invention is a Domain antibody (or an amino acid sequence that is suitable for use
15 as a Domain antibody); a single domain antibody (or an amino acid that is suitable for use as a single domain antibody); a "dAb" (or an amino acid that is suitable for use as a dAb); a Nanobody; a V_{HH} ; a humanized V_{HH} ; a camelized V_H ; or a V_{HH} that has been obtained by affinity maturation. Again, suitable framework sequences will be clear to the skilled person, for example on the basis of the standard handbooks and the further disclosure and prior art mentioned herein.

20 In particular, the framework sequences present in the monospecific polypeptides of the invention may contain one or more of Hallmark residues (as defined in WO 08/020079 (Tables A-3 to A-8)), such that the monospecific polypeptide of the invention is a Nanobody. Some preferred, but non-limiting examples of (suitable combinations of) such framework sequences will become clear from the further disclosure herein (see *e.g.*, Table A-4). Generally, Nanobodies (in particular V_{HH} s, partially
25 or fully humanized V_{HH} s and camelized V_H s) can in particular be characterized by the presence of one or more "*Hallmark residues*" in one or more of the framework sequences (as *e.g.*, further described in WO 08/020079, page 61, line 24 to page 98, line 3).

More in particular, the invention provides polypeptides comprising at least one immunoglobulin single variable domain that is an amino acid sequence with the (general) structure

30 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 which:

- 5 i) have at least 80%, more preferably 90%, even more preferably 95% amino acid identity with at least one of the amino acid sequences of SEQ ID NOs: 1-10 (see Table A-4), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded. In this respect, reference is also made to Table A-4, which lists the framework 1 sequences (SEQ ID NOs: 26-29), framework 2 sequences (SEQ ID NOs: 30-33), framework 3 sequences (SEQ ID NOs: 34-39) and framework 4 sequences (SEQ ID NOs: 40-41) of the immunoglobulin single variable domains of SEQ ID NOs: 1-10 (see Table A-4); or

10 and in which:

- ii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 to Table A-8 of WO 08/020079.

15 The present invention also provides a number of sequence optimized polypeptides and/or immunoglobulin single variable domains.

In particular, sequence optimized polypeptides and/or immunoglobulin single variable domains of the invention may be amino acid sequences that are as generally defined for immunoglobulin single variable domains in the previous paragraphs, but in which at least one amino acid residue is present (and in particular, in at least one of the framework residues) that is and/or that corresponds to a humanizing substitution (as defined herein). Some preferred, but non-limiting humanizing substitutions (and suitable combinations thereof) will become clear to the skilled person based on the disclosure herein. In addition, or alternatively, other potentially useful humanizing substitutions can be ascertained by comparing the sequence of the framework regions of a naturally occurring VHH sequence with the corresponding framework sequence of one or more closely related human VH sequences, after which one or more of the potentially useful humanizing substitutions (or combinations thereof) thus determined can be introduced into said VHH sequence (in any manner known per se, as further described herein) and the resulting humanized VHH sequences can be tested for affinity for the target, for stability, for ease and level of expression, and/or for other desired properties. In this way, by means of a limited degree of trial and error, other suitable humanizing substitutions (or suitable combinations thereof) can be determined by the skilled person based on the disclosure herein. Also, based on the foregoing, (the framework regions of) an immunoglobulin single variable domains may be partially humanized or fully humanized.

The present invention also relates to sequence optimized polypeptides and/or immunoglobulin single variable domains that may show improved expression and/or increased stability upon storage during stability studies. The sequence optimized polypeptides and/or ISVs of the present invention may show reduced pyroglutamate post-translational modification of the N-terminus and hence have increased product stability. In addition, the sequence optimized polypeptides and/or ISVs of the present invention may show other improved properties such as e.g. less immunogenicity, improved binding characteristics (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an EC_{50} value, as further described herein) for CD123, improved affinity and/or improved avidity for CD123.

Some particularly preferred sequence optimized immunoglobulin single variable domains of the invention are sequence optimized variants of the immunoglobulin single variable domains of SEQ ID NOs: 1-10.

Thus, some other preferred immunoglobulin single variable domains of the invention are Nanobodies which can bind (as defined herein) to CD123 and which:

- i) are a sequence optimized variant of one of the immunoglobulin single variable domains of SEQ ID NOs: 1-10; and/or
 - ii) have at least 80% amino acid identity with at least one of the immunoglobulin single variable domains of SEQ ID NOs: 1-10 (see Table A-4), in which for the purposes of determining the degree of amino acid identity, the amino acid residues that form the CDR sequences are disregarded; In this respect, reference is also made to Table A-4, which lists the framework 1 sequences (SEQ ID NOs: 26-29), framework 2 sequences (SEQ ID NOs: 30-33), framework 3 sequences (SEQ ID NOs: 34-39) and framework 4 sequences (SEQ ID NOs: 40-41) of the immunoglobulin single variable domains of SEQ ID NOs: 1-10 (see Table A-4);
- and in which:
- iii) preferably one or more of the amino acid residues at positions 11, 37, 44, 45, 47, 83, 84, 103, 104 and 108 according to the Kabat numbering are chosen from the Hallmark residues mentioned in Table A-3 to Table A-8 of WO 08/020079.

The polypeptides and/or immunoglobulin single variable domains of the invention may also contain the specific mutations/amino acid residues described in the following co-pending US provisional applications, all entitled "Improved immunoglobulin variable domains": US 61/994552 filed May 16, 2014; US 61/014,015 filed June 18, 2014; US 62/040,167 filed August 21, 2014; and US 62/047,560, filed September 8, 2014 (all assigned to Ablynx N.V.) as well as the International application WO

2015/173325 which was based on these provisional applications and which was published on November 19, 2015.

In particular, the polypeptides and/or immunoglobulin single variable domains of the invention may suitably contain (i) a K or Q at position 112; or (ii) a K or Q at position 110 in combination with a V at position 11; or (iii) a T at position 89; or (iv) an L on position 89 with a K or Q at position 110; or (v) a V at position 11 and an L at position 89; or any suitable combination of (i) to (v).

As also described in said co-pending US provisional applications, when the polypeptide and/or immunoglobulin single variable domains of the invention contain the mutations according to one of (i) to (v) above (or a suitable combination thereof):

- the amino acid residue at position 11 is preferably chosen from L, V or K (and is most preferably V); and
- the amino acid residue at position 14 is preferably suitably chosen from A or P; and
- the amino acid residue at position 41 is preferably suitably chosen from A or P; and
- the amino acid residue at position 89 is preferably suitably chosen from T, V or L; and
- the amino acid residue at position 108 is preferably suitably chosen from Q or L; and
- the amino acid residue at position 110 is preferably suitably chosen from T, K or Q; and
- the amino acid residue at position 112 is preferably suitably chosen from S, K or Q.

As mentioned in said co-pending US provisional applications, said mutations are effective in preventing or reducing binding of so-called "pre-existing antibodies" to the polypeptides and/or immunoglobulin single variable domains, and/or constructs of the invention. For this purpose, the polypeptides and/or immunoglobulin single variable domains of the invention may also contain (optionally in combination with said mutations) a C-terminal extension (X)_n (in which n is 1 to 10, preferably 1 to 5, such as 1, 2, 3, 4 or 5 (and preferably 1 or 2, such as 1); and each X is an (preferably naturally occurring) amino acid residue that is independently chosen, and preferably independently chosen from the group consisting of alanine (A), glycine (G), valine (V), leucine (L) or isoleucine (I)), for which reference is again made to said US provisional applications as well as to WO 12/175741. In particular, a polypeptide and/or immunoglobulin single variable domain of the invention may contain such a C-terminal extension when it forms the C-terminal end of a protein, polypeptide or other construct comprising the same (again, as further described in said US provisional applications as well as WO 12/175741).

Accordingly, the present invention relates to a polypeptide as described herein, further comprising a C-terminal extension (X)_n, in which n is 1 to 5, such as 1, 2, 3, 4 or 5, and in which X is a naturally occurring amino acid, preferably no cysteine.

These polypeptides of the invention, and in particular the immunoglobulin single variable domains comprising the CDR sequences of the invention are particularly suited for use as building block or binding unit for the preparation of multivalent or multispecific polypeptides.

Accordingly, the monospecific polypeptides of the invention that bind CD123 can be in essentially isolated form (as defined herein), or they may form part of a protein or polypeptide, which may comprise or essentially consist of one or more ISV that bind CD123 and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers).

Accordingly, the present invention also relates to a protein or polypeptide that comprises or essentially consists of one or more monospecific polypeptide of the invention (or suitable fragments thereof). In a further aspect, the monospecific polypeptides of the invention that bind CD123 may form part of a multispecific polypeptide, which may comprise or essentially consist of one or more ISV that binds CD123 and which may optionally further comprise one ISV that specifically binds another target, such as *e.g.*, TCR, and which may optionally further comprise one or more further amino acid sequences (all optionally linked via one or more suitable linkers).

The monospecific polypeptides of the invention are thus used as a binding unit or building block in such a protein or polypeptide, so as to provide a multispecific polypeptide of the invention, as described herein (for multispecific polypeptides containing one or more VHH domains and their preparation, reference is also made to Conrath et al. (2001, J. Biol. Chem. 276: 7346-7350), as well as to for example WO 96/34103, WO 99/23221 and WO 2010/115998).

2. Multispecific polypeptides

The invention further relates to multispecific polypeptides comprising or (essentially) consisting of two or more building blocks (such as at least two monospecific polypeptides or ISVs of the invention), in which at least one building block is directed against a first antigen (*i.e.*, CD123) and at least one building block is directed against a second antigen (*i.e.*, TCR). These multispecific polypeptide are also referred to herein as “multispecific polypeptide(s) of the invention”. Preferred immunoglobulin single variable domains for use in these multispecific polypeptides of the invention are the monospecific polypeptides of the invention (as described earlier).

As described further herein, additional binding units, such as immunoglobulin single variable domains, having different antigen specificity (*i.e.*, different from CD123 and TCR) may be linked to the multispecific polypeptides of the invention. By combining immunoglobulin single variable domains of three or more specificities, trispecific, tetraspecific etc. constructs can be formed. These

multispecific polypeptide are also referred to herein as “(multispecific) polypeptide(s) of the invention” or “construct(s) of the invention”.

Thus, for example, a “bispecific polypeptide of the invention” is a polypeptide that comprises or (essentially) consists of at least one immunoglobulin single variable domain against a first antigen (i.e., CD123) and at least one further immunoglobulin single variable domain against a second antigen (i.e., TCR), whereas a “trispecific polypeptide of the invention” is a polypeptide that comprises or (essentially) consists of at least one immunoglobulin single variable domain against a first antigen (i.e., CD123), at least one further immunoglobulin single variable domain against a second antigen (i.e., TCR) and at least one further immunoglobulin single variable domain against a third antigen (i.e., different from CD123 and TCR), etc. The immunoglobulin single variable domains may optionally be linked via one or more peptidic linkers, as further described herein.

Accordingly, the present invention relates to polypeptides comprising or (essentially) consisting of one immunoglobulin single variable domain that specifically binds TCR and one or more ISV that specifically binds CD123. In a further aspect, the present invention also provides polypeptides comprising or (essentially) consisting of one immunoglobulin single variable domain that specifically binds TCR and two or more ISVs that specifically bind CD123. Some non-limiting examples of such multispecific polypeptides or constructs thereof will become clear from the further description herein.

It will be appreciated (as is also demonstrated in the Example section) that the ISV that specifically binds TCR and the one or more ISV that specifically bind CD123 can be positioned in any order in the polypeptide of the invention. More particularly, in one aspect, the ISV binding TCR is positioned N-terminally and the one or more ISV binding CD123 is positioned C-terminally. In another aspect, the one or more ISV binding CD123 is positioned N-terminally and the ISV binding TCR is positioned C-terminally. In another aspect, one or more ISV that bind CD123 is positioned N-terminally, the ISV that binds TCR is positioned centrally and one or more further ISV that bind CD123 is positioned C-terminally. In a preferred aspect, the invention relates to a polypeptide, wherein the ISV that specifically binds TCR is located at the N-terminus of the polypeptide.

In some aspects, the multispecific polypeptides of the invention comprise two or more ISVs that specifically bind CD123. In one aspect, the two or more ISVs that specifically bind CD123 bind to the same epitope on CD123. In one aspect, such multispecific polypeptides of the invention may comprise two or more ISVs related to 56A10. In another aspect, such polypeptides of the invention comprise two or more ISVs related to 55F03.

In a more preferred aspect, the two or more ISVs that specifically bind CD123 bind to a different epitope. Accordingly, the present invention relates to a multispecific polypeptide, wherein the two or more ISVs that specifically bind CD123 are biparatopic comprising a first ISV and a second ISV, wherein the first ISV binds to an epitope on CD123 that is different from the epitope on CD123 bound by the second ISV.

More specifically, the present invention relates to a multispecific polypeptide of the invention, wherein the first ISV that binds CD123 is selected from the ISVs related to 56A10 and the second ISV that binds CD123 is selected from the ISVs related to 55F03. As discussed earlier, these biparatopic polypeptides of the invention have an improved affinity for binding to CD123 compared to the monospecific polypeptides of the invention, due to avid binding, also referred to as avidity.

It will be appreciated (as is also demonstrated in the Example section) that the ISVs that bind CD123 can be positioned in any order in the multispecific polypeptide of the invention. More particularly, in one aspect, the second ISV (i.e., the ISV related to 55F03) is located N-terminally of the first ISV (i.e., the ISV related to 56A10). In another aspect, the second ISV (i.e., the ISV related to 55F03) is located C-terminally of the first ISV (i.e., the ISV related to 56A10). Some non-limiting examples of such multispecific constructs will become clear from the further description herein.

Typically, the multispecific polypeptides of the invention combine high affinity and high specificity antigen recognition on the target cell with T cell activation, resulting in an activation that is independent of the T cells' natural specificity.

A "target cell" as referred to herein, is a cell that presents a particular antigen (i.e., CD123) on its surface. In one aspect, the "target cell" is a cell that is characterized by overexpression of CD123. In a preferred aspect, such target cell is associated with a CD123 associated disease. In an even more preferred aspect, the target cell is a cancer cell that (over)expresses CD123. The term "cancer" refers to the pathological condition in mammals that is typically characterized by dysregulated cellular proliferation or survival.

"T cell activation" as used herein refers to one or more cellular response(s) of a T cell, e.g. a cytotoxic T cell, such as selected from: proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, expression of activation markers, and redirected target cell lysis. The term "cellular response(s)" as used herein, refers to a response of a cell as a result of intracellular signalling upon assembly of the TCR complex.

The mode of action of polypeptides that bind both to a cell surface molecule (such as e.g., CD123) on a target cell and to the T cell TCR is commonly known. Bringing a T cell in close vicinity to a target cell (such as e.g., a CD123 expressing cell), i.e., engaging said T cell and clustering of the TCR complex,

results in T cell activation and subsequent killing of the target cell by the T cell. In the present invention this process is exploited in fighting against a CD123 associated disease, such as a proliferative disease or an inflammatory condition. Generally, T cells are equipped with granules containing a deadly combination of pore-forming proteins, called perforins, and cell death-inducing proteases, called granzymes. Preferably, these proteins are delivered into target cells (such as e.g., CD123 expressing cells) via a cytolytic synapse that forms if T cells are in close vicinity with a target cell that is aimed to be killed. Normally, close vicinity between a T cell and a target cell is achieved by the T cell binding to an MHC/peptide complex using its matching T cell receptor. The polypeptides of the invention bring a T cell into such close vicinity to a target cell in the absence of the T cell receptor/MHC interaction.

Accordingly, the present invention relates to a multispecific polypeptide as described herein, wherein said polypeptide directs the T cell to the target cell. Accordingly, the polypeptide(s) of the present invention “redirect(s) T cells for killing of CD123 expressing cells”, which means that the polypeptide(s) of the invention bring(s) a T cell in such close proximity to a CD123 expressing cell that it is killed.

With one arm (an ISV that binds TCR), the multispecific polypeptide of the invention binds to the constant domain of the TCR subunit, a protein component of the signal-transducing complex of the T cell receptor on T cells. With the other arm (one or more ISV that binds CD123), the multispecific polypeptide binds to CD123 on target cells. Preferably, T cell activation is only seen when the multispecific polypeptides are presented to T cells at (the site of) CD123 expressing cells. Antigen dependence on target cells (i.e., CD123 expressing cells) for activation results in a favourable safety profile. The multispecific polypeptides of the invention exhibit highly specific binding to CD123. As such, off-target binding is avoided and target independent T cell activation is minimal, as exemplified herein. In one aspect, the multispecific polypeptides transiently tether T cells and target cells. Preferably, the multispecific polypeptide can induce resting polyclonal T cells, such as CD4⁺ and/or CD8⁺ T cells into activation, for highly potent redirected lysis of target cells (i.e., CD123 expressing cells). Preferably, the T cell is directed to a next target cell after lysis of the first target cell.

In one aspect, the present invention relates to a multispecific polypeptide as described herein, wherein said multispecific polypeptide induces T cell activation.

In a further aspect, the present invention relates to a multispecific polypeptide, wherein said T cell activation is independent from MHC recognition.

“T cell activation independent from MHC recognition” as used herein, refers to T cell activation that is independent of the binding of an MHC/peptide complex on a target cell to its matching T cell

receptor on a T cell. By bringing a T cell in close proximity to a target cell, the target cell will get killed. Normally, close vicinity between a T cell and a target cell is achieved by the T cell binding to an MHC/peptide complex using its matching T cell receptor. The multispecific polypeptides of the invention bring a T cell into such close vicinity to a target cell in the absence of the T cell receptor/MHC interaction. The multispecific polypeptides bind to CD123 on a target cell and are as such presented and bound to T cells, resulting in T cell activation and killing of the target cell.

Accordingly, in a further aspect, the present invention relates to a multispecific polypeptide, wherein said T cell activation depends on presenting said polypeptide bound to CD123 on a target cell to a T cell.

In a further aspect, the present invention relates to a multispecific polypeptide, wherein said T cell activation causes one or more cellular response by said T cell, wherein said cellular response is selected from the group consisting of proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, expression of activation markers and redirected target cell lysis.

Suitable assays to measure T cell activation are known in the art, for instance as described in WO 99/54440 or by Schlereth et al. (2005, Cancer Immunol. Immunother. 20: 1-12), or as exemplified in the examples or below.

Without being limited, T cell activation by the polypeptides of the invention can be measured by monitoring upregulation of CD69, CD25 and various cell adhesion molecules, de novo expression and/or release of cytokines (e.g., IFN- γ , TNF- α , IL-6, IL-2, IL-4 and IL-10), upregulation of granzyme and perforin expression, and/or cell proliferation, membrane blebbing, activation of procaspases 3 and/or 7, fragmentation of nuclear DNA and/or cleavage of caspase substrate poly (ADPribose) polymerase. Preferably, redirected lysis of target cells by the multispecific polypeptides is independent of T cell receptor specificity, presence of MHC class I and/or β 2 microglobulin, and/or of any co-stimulatory stimuli.

The polypeptides of the invention show redirected lysis *in vitro* with previously unstimulated (i.e. non activated) peripheral polyclonal CD8⁺- and CD4⁺-positive T cells, as exemplified further herein. The redirected lysis of target cells via the recruitment of T cells by the polypeptides of the invention involves cytolytic synapse formation and delivery of perforin and granzymes. Cell lysis by T cells has been described, e.g. by Atkinson and Bleackley (1995, Crit. Rev. Immunol 15(3-4): 359-384). Preferably, the polypeptide of the invention mediates killing of target cells, e.g. cancer cells, by stimulating T cells in pore formation and delivering pro-apoptotic components of cytotoxic T cell granules. Preferably, the engaged T cells are capable of serial target cell lysis. *In vitro*, with the

polypeptides of the invention, redirected lysis is seen at low picomolar concentrations, suggesting that very low numbers of the polypeptides of the invention need to be bound to target cells for triggering T cells. As demonstrated in the examples, the low effector to target cell ratio might be indicative for serial target cell lysis and demonstrated the high potency of the polypeptides of the invention.

As used herein, the term "potency" is a measure of the biological activity of an agent, such as a monospecific or multispecific polypeptide, ISV or Nanobody. Potency is a function of the amount of polypeptide of the invention required for its specific effect to occur. It is measured simply as the inverse of the IC_{50} for that polypeptide. For the multispecific polypeptides of the invention, it refers to the capacity of said polypeptide of the invention to induce T cell activation. Potency of an agent can be determined by any suitable method known in the art, such as for instance as described in the experimental section. Cell culture based potency assays are often the preferred format for determining biological activity since they measure the physiological response elicited by the agent and can generate results within a relatively short period of time. Various types of cell based assays, based on the mechanism of action of the product, can be used, including but not limited to proliferation assays, cytotoxicity assays, cell killing assays, reporter gene assays, cell surface receptor binding assays, and assays to measure induction/inhibition of functionally essential proteins or other signal molecules (such as phosphorylated proteins, enzymes, cytokines, cAMP and the like), T cell mediated tumour cell killing assay (for instance as set out in the Examples section), all well known in the art. Results from cell based potency assays can be expressed as "relative potency" as determined by comparison of the multispecific polypeptide of the invention to the response obtained for the corresponding reference monovalent ISV, e.g. a polypeptide comprising only one ISV or one Nanobody, optionally further comprising an irrelevant Nanobody (cf. experimental section).

The "efficacy" (of the polypeptide of the invention) measures the maximum strength of the effect itself, at saturating polypeptide concentrations. Efficacy indicates the maximum response achievable by the polypeptide of the invention. It refers to the ability of a polypeptide to produce the desired (therapeutic) effect.

In one aspect, the multispecific polypeptide of the invention activates T cells, resulting in killing of CD123 expressing cells (such as MOLM-13 or KG1a cells) with an average EC_{50} value between 10nM and 1pM, as determined in a flow cytometry based assay. (cf. Example 25)

More specifically, the polypeptide of the invention induces T cell activation, wherein said T cell activation causes killing of CD123 expressing cells (such as MOLM-13 cells) with an average EC_{50} value of between 1 nM and 1 pM, such as at an average EC_{50} value of 500 pM or less, such as less

than 400, 300, 200 or 100 pM or even less, such as less than 90, 80, 70, 60, 50, 40 or 30 pM or even less, said EC50 value for example determined in a flow cytometry based assay with TOPRO3 read-out using MOLM-13 cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

5 More specifically, the polypeptide of the invention induces T cell activation, wherein said T cell activation causes lysis of CD123 expressing cells (such as MOLM-13 cells) with an average lysis percentage of more than about 10%, such as 15%, 16%, 17%, 18%, 19% or 20% or even more, such as more than 25%, or even more than 30%, said lysis percentage for example determined in a flow cytometry based assay with TOPRO3 read-out using MOLM-13 cells as target cells and human T cells
10 as effector cells at an effector to target cell ratio of 10 to 1.

More specifically, the polypeptide of the invention induces T cell activation, wherein said T cell activation causes killing of CD123 expressing cells (such as KG1a cells) with an average EC50 value of between 10 nM and 10 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, 3, 2 or 1 nM or even less, such as less than 90, 80, 70 or 60 pM or even less, said EC50 value for example
15 determined in a flow cytometry based assay with TOPRO3 read-out using KG1a cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

More specifically, the polypeptide of the invention induces T cell activation, wherein said T cell activation causes lysis of CD123 expressing cells (such as KG1a cells) with an average lysis percentage of more than about 10%, such as 15%, 16%, 17% or 18% or even more, such as more than 24%, said
20 lysis percentage for example determined in a flow cytometry based assay with TOPRO3 read-out using KG1a cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

In another aspect, the multispecific polypeptides of the invention activate T cells and may as such induce cytokine secretion. Accordingly, the polypeptides cause IFN- γ or IL-6 secretion with an
25 average EC50 value of between 100 nM and 10 pM. (cf. Example 30)

More specifically, the polypeptide of the invention induces T cell activation, wherein said T cell activation causes IFN- γ secretion with an average EC50 value of between 100 nM and 10 pM, such as at an average EC50 value of 50 nM or less, such as less than 40, 30, 20, 10 or 9 nM or even less, such as less than 8, 7, 6, 5, 4, 3, 2 or 1 nM or even less, such as less than 500 pM or even less, such as less
30 than 400, 300, 200 or 100 pM or even less, said EC50 value for example determined in an ELISA based assay, as for example further explained in Example 30.

More specifically, the polypeptide of the invention induces T cell activation, wherein said T cell activation causes IL-6 secretion with an average EC50 value of between 100 nM and 10 pM, such as

at an average EC50 value of 50 nM or less, such as less than 40, 30, 20 or 10 nM or even less, such as less than 9, 8, 7, 6, 5, 4, 3, 2 or 1 nM or even less, such as less than 500pM or even less, such as less than 400, 300, 200 or 100 pM or even less, said EC50 value for example determined in an ELISA based assay, as for example further explained in Example 30.

- 5 In another aspect, the multispecific polypeptides of the invention cause depletion of plasmacytoid cells (pDCs) and basophils. (cf. Example 31)

Accordingly, the present invention relates to a polypeptide, wherein said T cell activation causes depletion of plasmacytoid cells (pDCs) and basophils.

- 10 In another aspect, the multispecific polypeptides of the invention may further cause T cell proliferation. (cf. Example 39)

Accordingly, the present invention relates to a polypeptide, wherein said T cell activation causes proliferation of said T cells.

- 15 The multispecific polypeptides of the invention comprise one or more ISV that specifically binds CD123, which has been carefully selected for their specificity. As such, the multispecific polypeptides of the invention exhibit highly specific binding to CD123, which enables them to kill CD123 expressing target cells. In contrast, only minimal killing was observed in the absence of CD123 expressing cells, which underscores the safety of the polypeptides of the invention.

Accordingly, in another aspect, the present invention relates to a polypeptide, wherein the T cell activation in the absence of CD123 positive cells is minimal. (cf. Example 36 to 38)

- 20 More specifically, the present invention relates to a polypeptide, wherein the T cell activation induced lysis of CD123 negative cells is no more than about 10%, such as 9% or less, such as 8, 7, or 6 % or even less, said lysis for example determined as average lysis percentage in a flow cytometry based assay with TOPRO3 read-out using CD123 negative cells, such as U-937 or NCI-H929 cells, as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

- 25 More specifically, the present invention relates to a polypeptide, which does not induce secretion of IFN- γ and IL-6 in the presence of CD123 negative cells, said secretion for example determined in an ELISA based assay.

- 30 The inventors observed that certain multispecific polypeptides of the invention, comprising a TCR binding ISV of the invention and one or more CD123 binding ISV of the invention, were particularly suited to redirect T cells for killing of CD123 expressing cells. With these multispecific polypeptides of the invention, activation of T cells was minimal in the absence of CD123 expressing cells.

Accordingly, the present invention relates to a multispecific polypeptide that redirects T cells for killing of CD123 expressing cells, comprising one immunoglobulin single variable domain (ISV) that specifically binds T cell receptor (TCR) and one or more ISV that specifically bind CD123, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 17-20; or
- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or
- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a further aspect, the present invention relates to a multispecific polypeptide, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 181-191; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the

4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

5 c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

15 e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

25 a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

30 c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or

- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In a particular aspect, the present invention relates to a multispecific polypeptide, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 181-191; or

- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 2, 4, 5, 6, 8 and/or 10 of the CDR1 (position 27, 29, 30, 31, 33 and/or 35 according to Kabat numbering); provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 192-217; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 3, 5, 7, 8 and/or 9 of the CDR2 (position 50, 52, 54, 56, 57 and/or 58 according to Kabat numbering); provided that the ISV

comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 4, 5 and/or 8 of the CDR3 (position 95, 98, 99 and/or 101 according to Kabat numbering); provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the ISV that specifically binds CD123 is as described further herein.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which

CDR1 is chosen from the group consisting of:

a) SEQ ID NO: 181; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein

- at position 2 the D has been changed into A, S, E or G;
- at position 4 the H has been changed into Y;
- at position 5 the K has been changed into L;
- at position 6 the I has been changed into L;
- at position 8 the F has been changed into I or V; and/or
- at position 10 the G has been changed into S;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 is chosen from the group consisting of:

- a) SEQ ID NO: 192; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein
 - at position 1 the H has been changed into T or R;
 - at position 3 the S has been changed into T or A;
 - at position 5 the G has been changed into S or A;
 - at position 7 the Q has been changed into D, E, T, A or V;
 - at position 8 the T has been changed into A or V; and/or
 - at position 9 the D has been changed into A, Q, N, V or S;

provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 is chosen from the group consisting of:

- a) SEQ ID NO: 218; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein
 - at position 1 the F has been changed into Y, L or G;
 - at position 4 the I has been changed into L;
 - at position 5 the Y has been changed into W; and/or
 - at position 8 the D has been changed into N or S;

provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention relates to a multispecific polypeptide as described above, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 181; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein
 - at position 2 the D has been changed into A, S, E or G;
 - at position 4 the H has been changed into Y;
 - at position 5 the K has been changed into L;
 - at position 6 the I has been changed into L;
 - at position 8 the F has been changed into I or V; and/or
 - at position 10 the G has been changed into S;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 192; or
- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein
 - at position 1 the H has been changed into T or R;
 - at position 3 the S has been changed into T or A;
 - at position 5 the G has been changed into S or A;
 - at position 7 the Q has been changed into D, E, T, A or V;
 - at position 8 the T has been changed into A or V; and/or
 - at position 9 the D has been changed into A, Q, N, V or S;

provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 218; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein

- at position 1 the F has been changed into Y, L or G;
- at position 4 the I has been changed into L;
- at position 5 the Y has been changed into W; and/or
- at position 8 the D has been changed into N or S;

provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the ISV that specifically binds CD123 is as described further herein.

In another aspect, the present invention relates to a multispecific polypeptide, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of SEQ ID NOs: 181-191, CDR2 is chosen from the group consisting of SEQ ID NOs: 192-217, and CDR3 is chosen from the group consisting of SEQ ID NOs: 218-225 and wherein the ISV that specifically binds CD123 is as described further herein.

Accordingly, the present invention relates to a multispecific polypeptide, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 181, CDR2 is SEQ ID NO: 192, and CDR3 is SEQ ID NO: 218 and wherein the ISV that specifically binds CD123 is as described further herein.

In a preferred aspect, the present invention relates to a multispecific polypeptide, wherein the ISV that specifically binds TCR is chosen from the group consisting of SEQ ID NOs: 42 and 78-180 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 42 and 78-180 and wherein the ISV that specifically binds CD123 is as described further herein.

Apart from the TCR binding ISV as described above, in the multispecific polypeptides of the invention, the one or more ISV that specifically bind CD123 are related to 56A10 and/or 55F03.

Accordingly, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the ISV comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the ISV comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In one aspect, the one or more ISV that specifically binds CD123 may be an ISV related to 56A10.

Accordingly, the present invention relates to a multispecific polypeptide as described above, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which

CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 11; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- at position 3 the T has been changed into S or P;
- at position 6 the I has been changed into S;
- at position 7 the N has been changed into D; and/or
- at position 8 the D has been changed into V or A;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 is SEQ ID NO: 17.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 is chosen from the group consisting of:

- a) SEQ ID NO: 21; or
- b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein

- at position 3 the P has been changed into A;

provided that the ISV comprising the CDR3 with 1 amino acid difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

Accordingly, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NO: 11; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- at position 3 the T has been changed into S or P;
- at position 6 the I has been changed into S;
- at position 7 the N has been changed into D; and/or
- at position 8 the D has been changed into V or A;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is SEQ ID NO: 17;

and

iii) CDR3 is chosen from the group consisting of:

c) SEQ ID NOs: 21; or

d) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein

- at position 3 the P has been changed into A;

provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of SEQ ID NOs: 11-15, CDR2 is SEQ ID NO: 17, and CDR3 is chosen from the group consisting of SEQ ID NOs: 21-22.

Accordingly, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 11, CDR2 is SEQ ID NO: 17, and CDR3 is SEQ ID NO: 21.

In a preferred aspect, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 is chosen from the group consisting of SEQ ID NOs: 1-6 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6.

Apart from the above, or in addition, the ISV that specifically binds CD123 may be an ISV related to 55F03.

Accordingly, the present invention also relates to a multispecific polypeptide as described above, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 is chosen from the group consisting of:

- a) SEQ ID NO: 18; or
- b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- at position 3 the Y has been changed into W;
- at position 6 the N has been changed into S; and/or
- at position 10 the Q has been changed into E;

provided that the ISV comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

In another aspect, the present invention relates to a multispecific polypeptide as described above, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 is chosen from the group consisting of:

- a) SEQ ID NO: 23; or
- b) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein

- at position 4 the E has been changed into R; and/or
- at position 5 the T has been changed into D or Y;

provided that the ISV comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

5 Accordingly, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is SEQ ID NO: 16;

10 and

ii) CDR2 is chosen from the group consisting of:

a) SEQ ID NO: 18; or

b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- 15
- at position 3 the Y has been changed into W;
 - at position 6 the N has been changed into S; and/or
 - at position 10 the Q has been changed into E;

provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

20

and

iii) CDR3 is chosen from the group consisting of:

c) SEQ ID NOs: 23; or

25

d) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein

- at position 4 the E has been changed into R; and/or
- at position 5 the T has been changed into D or Y;

provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

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In another aspect, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity

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determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16, CDR2 is chosen from the group consisting of SEQ ID NOs: 18-20, and CDR3 is chosen from the group consisting of SEQ ID NOs: 23-25.

Accordingly, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16, CDR2 is SEQ ID NO: 18, and CDR3 is SEQ ID NO: 23.

In a preferred aspect, the present invention relates to a multispecific polypeptide wherein the ISV that specifically binds TCR is as described herein and, wherein the one or more ISV that specifically bind CD123 is chosen from the group consisting of SEQ ID NOs: 7-10 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10.

As extensively described for the monospecific polypeptides, the immunoglobulin single variable domains present in the multispecific polypeptide of the invention may consist of a light chain variable domain sequence (e.g., a V_L -sequence) or of a heavy chain variable domain sequence (e.g., a V_H -sequence); they may consist of a heavy chain variable domain sequence that is derived from a conventional four-chain antibody or of a heavy chain variable domain sequence that is derived from heavy chain antibody. In a preferred aspect, they consist of a Domain antibody (or an amino acid that is suitable for use as a Domain antibody), of a single domain antibody (or an amino acid that is suitable for use as a single domain antibody), of a "dAb" (or an amino acid that is suitable for use as a dAb), of a Nanobody (including but not limited to a V_{HH}), a humanized VHH, a camelized VH, or of a VHH sequence that has been obtained by affinity maturation. The immunoglobulin single variable domains may consist of a partially or fully humanized Nanobody or a partially or fully humanized VHH. The immunoglobulin single variable domains may also contain mutations (as described herein) that are effective in preventing or reducing binding of so-called "pre-existing antibodies" to the immunoglobulin single variable domains and constructs of the invention. In a preferred aspect of the invention, the immunoglobulin single variable domains encompassed in the multispecific polypeptide of the invention are one or more monospecific polypeptides of the invention, as defined herein.

Preferred polypeptides of the invention may be chosen from the group consisting of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61 (cf. Table A-7). In a further aspect, the polypeptide is chosen from the group consisting of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61 or from polypeptides that have a

sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61.

The multispecific polypeptides of the invention may comprise one or more other groups, residues, moieties or binding units as to form "polypeptide(s) of the invention" or "construct(s) of the invention", as further described herein. For example, such a binding unit may be an amino acid sequence that increases the half-life (also referred to herein as "half-life extension" and "half-life extended construct") of the polypeptide. According to a specific, but non-limiting aspect of the invention, the polypeptides of the invention may thus contain, besides the one or more immunoglobulin single variable domains against CD123, and the one immunoglobulin single variable domain against TCR, at least one immunoglobulin single variable domain against serum albumin (such as human serum albumin). Accordingly, the present invention relates to a construct as described herein, wherein said binding unit that provides the polypeptide with an increased half-life is an immunoglobulin single variable domain that binds serum albumin. In a further aspect, the present invention relates to a construct as described herein, wherein said ISV that binds serum albumin essentially consists of a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH or a camelized VH.

In a preferred aspect, the ISV that binds serum albumin is selected from the group consisting of SEQ ID NOs 43 or 351 to 362.

In a preferred aspect, the ISVs are directly linked to each or linked to each other via a linker.

Preferred constructs of the invention may be chosen from the group of constructs consisting of SEQ ID NOs: 63-67 or constructs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 63-67.

In a preferred aspect, the construct is selected from the group consisting of SEQ ID NOs: 63-67.

Upon their administration, the half-life extended constructs of the invention will not be removed instantaneously by renal clearance. As such the half-life extension will contribute to a favourable PK profile. Accordingly, there will be no need for continuous intravenous infusion and, as such, patient compliance will be improved. In a specific aspect, the constructs of the present invention do not require continuous infusion.

Also as extensively described for the monospecific polypeptides, the multispecific polypeptides of the invention or constructs of the invention may further comprise mutations that are effective in preventing or reducing binding of so-called "pre-existing antibodies" to the polypeptides and constructs of the invention. For this purpose, the polypeptides and constructs of the invention may contain a C-terminal extension (X)_n (in which n is 1 to 10, preferably 1 to 5, such as 1, 2, 3, 4 or 5

(and preferably 1 or 2, such as 1); and each X is an (preferably naturally occurring) amino acid residue that is independently chosen, and preferably independently chosen from the group consisting of alanine (A), glycine (G), valine (V), leucine (L) or isoleucine (I)), as described herein.

Accordingly, the present invention relates to a polypeptide or construct of the invention, further comprising a C-terminal extension (X)_n, in which n is 1 to 5, such as 1, 2, 3, 4 or 5, and in which X is a naturally occurring amino acid, preferably no cysteine.

More specifically, the present invention relates to a polypeptide or construct, wherein said polypeptide or construct is chosen from the group consisting of SEQ ID NOs: 338-342.

10 A method for preparing the multivalent or multispecific polypeptides of the invention may comprise at least the steps of linking two or more immunoglobulin single variable domains, monovalent polypeptides and/or monospecific polypeptides of the invention and for example one or more linkers together in a suitable manner. The immunoglobulin single variable domains, monovalent polypeptides and/or monospecific polypeptides of the invention (and linkers) can be coupled by any
15 method known in the art and as further described herein. Preferred techniques include the linking of the nucleic acid sequences that encode the immunoglobulin single variable domains, monovalent polypeptides and/or monospecific polypeptides of the invention (and linkers) to prepare a genetic construct that expresses the multivalent or multispecific polypeptide. Techniques for linking amino acids or nucleic acids will be clear to the skilled person, and reference is again made to the standard
20 handbooks, such as Sambrook et al. and Ausubel et al., mentioned above, as well as the Examples below.

Accordingly, the present invention also relates to the use of an immunoglobulin single variable domain, monovalent polypeptide and/or monospecific polypeptide of the invention in preparing a multivalent polypeptide or multispecific polypeptide of the invention. The method for the
25 preparation of a multivalent or multispecific polypeptide will comprise the linking of one or more immunoglobulin single variable domains and/or polypeptides of the invention to at least one further immunoglobulin single variable domain, monovalent polypeptide and/or monospecific polypeptide of the invention, optionally via one or more linkers. The immunoglobulin single variable domain, monovalent polypeptide and/or monospecific polypeptide of the invention is then used as a binding
30 domain or binding unit in providing and/or preparing the multivalent or multispecific polypeptide comprising two (*e.g.*, in a bivalent polypeptide), three (*e.g.*, in a trivalent polypeptide) or more (*e.g.*, in a multivalent polypeptide) binding units. In this respect, the immunoglobulin single variable domain, the monovalent polypeptide and/or the monospecific polypeptide of the invention may be

used as a binding domain or binding unit in providing and/or preparing a multivalent or multispecific, such as bispecific or trispecific polypeptide comprising two, three or more binding units.

Accordingly, the present invention also relates to the use of an immunoglobulin single variable domain and/or particularly, a monovalent or monospecific polypeptide of the invention (as described
5 herein) in preparing a multivalent or multispecific polypeptide. The method for the preparation of the multivalent or multispecific polypeptide will comprise the linking of the immunoglobulin single variable domain, monovalent polypeptide and/or monospecific polypeptide of the invention to at least one further immunoglobulin single variable domain, monovalent polypeptide and/or monospecific polypeptide of the invention, optionally via one or more linkers (as further described
10 herein).

Constructs of the invention

The monospecific polypeptide of the invention and the multispecific polypeptide of the invention, may or may not further comprise one or more other groups, residues, moieties or binding units
15 (these monovalent polypeptides as well as multivalent polypeptides (with or without additional groups, residues, moieties or binding units) are all referred to as “construct(s) of the invention”). If present, such further groups, residues, moieties or binding units may or may not provide further functionality to the immunoglobulin single variable domain (and/or to the polypeptide in which it is present) and may or may not modify the properties of the immunoglobulin single variable domain.

For example, such further groups, residues, moieties or binding units may be one or more additional amino acid sequences, such that the polypeptide is a (fusion) protein or (fusion) polypeptide. In a preferred but non-limiting aspect, said one or more other groups, residues, moieties or binding units are immunoglobulins. Even more preferably, said one or more other groups, residues, moieties or binding units are immunoglobulin single variable domains chosen from the group consisting of
25 Domain antibodies, amino acids that are suitable for use as a Domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, “dAb”’s, amino acids that are suitable for use as a dAb, or Nanobodies (such as e.g. VHH, humanized VHH or a camelized VH).

As described above, additional binding units, such as immunoglobulin single variable domains having
30 different antigen specificity can be linked to form multispecific constructs. By combining immunoglobulin single variable domains of two or more specificities, bispecific, trispecific etc. constructs can be formed. For example, a polypeptide according to the invention may comprise a monospecific or multispecific polypeptide of the invention and one or more immunoglobulin single

variable domain(s) against another target (*i.e.*, different from CD123 or TCR). Such constructs and modifications thereof, which the skilled person can readily envisage, are all encompassed by the term “construct of the invention” as used herein.

In the constructs described above, the one, two or more immunoglobulin single variable domains and
5 the one or more groups, residues, moieties or binding units may be linked directly to each other and/or via one or more suitable linkers or spacers. For example, when the one or more groups, residues, moieties or binding units are amino acid sequences, the linkers may also be amino acid sequences, so that the resulting construct is a fusion (protein) or fusion (polypeptide).

The one or more further groups, residues, moieties or binding units may be any suitable and/or
10 desired amino acid sequences. The further amino acid sequences may or may not change, alter or otherwise influence the (biological) properties of the polypeptide of the invention, and may or may not add further functionality to the polypeptide of the invention. Preferably, the further amino acid sequence is such that it confers one or more desired properties or functionalities to the polypeptide of the invention.

15 Example of such amino acid sequences will be clear to the skilled person, and may generally comprise all amino acid sequences that are used in peptide fusions based on conventional antibodies and fragments thereof (including but not limited to ScFv's and single domain antibodies). Reference is for example made to the review by Holliger and Hudson (2005, Nature Biotechnology 23: 1126-1136).

For example, such an amino acid sequence may be an amino acid sequence that increases the half-
20 life, the solubility, or the absorption, reduces the immunogenicity or the toxicity, eliminates or attenuates undesirable side effects, and/or confers other advantageous properties to and/or reduces the undesired properties of the construct of the invention, compared to the polypeptide of the invention per se. Some non-limiting examples of such amino acid sequences are serum proteins, such as human serum albumin (see for example WO 00/27435) or haptenic molecules (for example
25 haptens that are recognized by circulating antibodies, see for example WO 98/22141).

In a specific, but non-limiting aspect of the invention, which will be further described herein, the construct of the invention may have an increased half-life in serum (as further described herein) compared to the immunoglobulin single variable domain or polypeptide from which they have been derived. For example, an immunoglobulin single variable domain or polypeptide of the invention may
30 be linked (chemically or otherwise) to one or more groups or moieties that extend the half-life (such as polyethylene glycol molecule (PEG)), so as to provide a derivative of the polypeptide of the invention with increased half-life.

In one specific aspect of the invention, a construct is prepared that has an increased half-life, compared to the corresponding polypeptide of the invention. Examples of constructs of the invention that comprise such half-life extending moieties for example include, without limitation, constructs in which the immunoglobulin single variable domains are suitably linked to one or more serum proteins or fragments thereof (such as (human) serum albumin or suitable fragments thereof) or to one or more binding units (such as, for example, Domain antibodies, amino acids that are suitable for use as a Domain antibody, single domain antibodies, amino acids that are suitable for use as a single domain antibody, "dAb"'s, amino acids that are suitable for use as a dAb, Nanobodies, VHHs, humanized VHHs or camelized VHHs) that can bind to serum proteins (such as serum albumin (such as human serum albumin)), serum immunoglobulins (such as IgG), transferrin or one of the other serum proteins listed in WO 04/003019; polypeptides in which the immunoglobulin single variable domain is linked to an Fc portion (such as a human Fc) or a suitable part or fragment thereof; or constructs in which the one or more immunoglobulin single variable domains are suitably linked to one or more small proteins or peptides that can bind to serum proteins (such as, without limitation, the proteins and peptides described in WO 91/01743, WO 01/45746 or WO 02/076489). Reference is also made to the dAb's described in WO 03/002609 and WO 04/003019 and to Harmsen et al. (2005, Vaccine 23: 4926-4942); to EP 0368684, as well as to WO 08/028977, WO 08/043821, WO 08/043822, WO 08/068280, WO 09/127691 and WO 11/095545 by Ablynx N.V..

According to a specific, but non-limiting aspect of the invention, the constructs of the invention may contain, besides the one or more immunoglobulin single variable domains against CD123, and/or the one immunoglobulin single variable domain against TCR, at least one immunoglobulin single variable domain that binds serum albumin (such as human serum albumin). Accordingly, the present invention relates to a construct as described herein, wherein said binding unit that provides the construct with an increased half-life is an immunoglobulin single variable domain that binds serum albumin. In a further aspect, the present invention relates to a construct as described herein, wherein said ISV that binds serum albumin essentially consists of a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH or a camelized VH.

The ISV that binds serum albumin may be any ISV as described in the art.

In one aspect, the immunoglobulin single variable domain that binds human serum albumin may be as generally described in the applications by Ablynx N.V. cited above (see for example WO 04/062551). Some preferred Nanobodies that provide for increased half-life and that can be used in the constructs of the invention include the Nanobodies ALB-1 to ALB-10 disclosed in WO 06/122787 (see Tables II and III), as well as the Nanobodies disclosed in WO 2012/175400 or WO 2015/173325 (e.g., SEQ ID NOs: 1-11 of WO 2012/175400, SEQ ID NO: 19 of WO 2015/173325) and Nanobodies

from the provisional applications US 62/256,841, US 62/335,746, US 62/349,294 and the corresponding International application WO 2017/085172 by Assignee entitled “Improved serum albumin binders” that invokes the priority of these three US provisional applications.”

In one aspect, the present invention relates to a polypeptide as described herein, wherein said ISV that binds serum albumin essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3 respectively), in which CDR1 is GFTFSSFGMS (SEQ ID NO: 363) or GFTFRSFGMS (SEQ ID NO: 364), CDR2 is SISGSGSDTL (SEQ ID NO: 365) and CDR3 is GGSLSR (SEQ ID NO: 366).

Some particularly preferred Nanobodies that provide for increased half-life and that can be used in the constructs of the invention include immunoglobulin single variable domains also referred to as Alb8, Alb23, Alb129, Alb132, Alb11, Alb11 (S112K)-A, Alb82, Alb82-A, Alb82-AA, Alb82-AAA, Alb82-G, Alb82-GG, Alb82-GGG (Table B-2).

Accordingly, the present invention relates to a construct as described herein, wherein said ISV that binds serum albumin is selected from the group consisting of SEQ ID NOs 43 or 351 to 362.

Table B-2: Immunoglobulin single variable domains that bind HSA for use in the constructs of the invention

ISV	SEQ ID NO	Sequence
Alb8	43	EVQLVESGGGLVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSD TLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LVT VSS
Alb23	351	EVQLLES GGGVLVQP GGS LRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGSD TLYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LVT VSS
Alb129	352	EVQLVESGGGVVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTATYYCTIGGSLRSSQGT LVT VSSA
Alb132	353	EVQLVESGGGVVQP GGS LRLSCAASGFTFRSFGMSWVRQAPGKGPEWVSSISGSGS DTLYADSVKGRFTISRDN SKNTLYLQMNSLRPEDTATYYCTIGGSLRSSQGT LVT VSS A
Alb11	354	EVQLVESGGGLVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSD TLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LVT VSS
Alb11 (S112K)-A	355	EVQLVESGGGLVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGSD TLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTAVYYCTIGGSLRSSQGT LVT VSSA
Alb82	356	EVQLVESGGGVVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LVT VSS
Alb82-A	357	EVQLVESGGGVVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LVT VSSA
Alb82-AA	358	EVQLVESGGGVVQP GNSLR LSCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDN AKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGT LVT VSSA A

ISV	SEQ ID NO	Sequence
Alb82-AAA	359	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTLLTVSSA AA
Alb82-G	360	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTLLTVSSG
Alb82-GG	361	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTLLTVSSG G
Alb82-GGG	362	EVQLVESGGGVVQPGNSLRSLCAASGFTFSSFGMSWVRQAPGKGLEWVSSISGSGS DTLYADSVKGRFTISRDNAKTTLYLQMNSLRPEDTALYYCTIGGSLRSSQGTLLTVSSG GG

Generally, the constructs of the invention with increased half-life preferably have a half-life that is at least 1.5 times, preferably at least 2 times, such as at least 5 times, for example at least 10 times or more than 20 times, greater than the half-life of the corresponding immunoglobulin single variable domain or polypeptide of the invention per se.

Generally, the constructs of the invention with increased half-life preferably have a half-life that is increased with more than 1 hours, preferably more than 2 hours, more preferably more than 6 hours, such as more than 12 hours, or even more than 24, 48 or 72 hours, compared to the half-life of the corresponding immunoglobulin single variable domain or polypeptide of the invention per se.

In another preferred, but non-limiting aspect, such constructs of the invention exhibit a serum half-life in human of at least about 12 hours, preferably at least 24 hours, more preferably at least 48 hours, even more preferably at least 72 hours or more. For example, constructs of the invention may have a half-life of at least 5 days (such as about 5 to 10 days), preferably at least 9 days (such as about 9 to 14 days), more preferably at least about 10 days (such as about 10 to 15 days), or at least about 11 days (such as about 11 to 16 days), more preferably at least about 12 days (such as about 12 to 18 days or more), or more than 14 days (such as about 14 to 19 days).

In the present invention, it was demonstrated that the inclusion of an albumin targeting binding unit in the construct as such did not have an essential impact on the obtained potency or efficacy. Although a minor loss of efficacy/potency was observed in the presence of HSA, the half-life extended TCR binding multispecific polypeptides were still potent in killing of CD123 expressing cells. Albumin-based drug delivery has been demonstrated to be useful for achieving improved cancer therapy, largely due to its passive target toward tumour via the enhanced permeability and retention effect and the increased demand for albumin by tumour cells as source of energy and amino acids.

According to one specific aspect, one or more polypeptides of the invention may be linked (optionally via a suitable linker or hinge region) to one or more constant domains (for example, 2 or 3 constant

domains that can be used as part of/to form an Fc portion), to an Fc portion and/or to one or more antibody parts, fragments or domains that confer one or more effector functions to the polypeptide of the invention and/or may confer the ability to bind to one or more Fc receptors. For example, for this purpose, and without being limited thereto, the one or more further amino acid sequences may

5 comprise one or more C_H2 and/or C_H3 domains of an antibody, such as from a heavy chain antibody (as described herein) and more preferably from a conventional human 4-chain antibody; and/or may form (part of) an Fc region, for example from IgG (e.g. from IgG1, IgG2, IgG3 or IgG4), from IgE or from another human Ig such as IgA, IgD or IgM. For example, WO 94/04678 describes heavy chain antibodies comprising a Camelid V_{HH} domain or a humanized derivative thereof (i.e. a Nanobody), in

10 which the Camelidae C_H2 and/or C_H3 domain have been replaced by human C_H2 and C_H3 domains, so as to provide an immunoglobulin that consists of 2 heavy chains each comprising a Nanobody and human C_H2 and C_H3 domains (but no C_H1 domain), which immunoglobulin has the effector function provided by the C_H2 and C_H3 domains and which immunoglobulin can function without the presence of any light chains. Other amino acid sequences that can be suitably linked to the polypeptides of the

15 invention so as to provide an effector function will be clear to the skilled person, and may be chosen on the basis of the desired effector function(s). Reference is for example made to WO 04/058820, WO 99/42077, WO 02/056910 and WO 05/017148, as well as the review by Holliger and Hudson, *supra*; and to WO 09/068628. Coupling of a polypeptide of the invention to an Fc portion may also lead to an increased half-life, compared to the corresponding polypeptide of the invention. For some

20 applications, the use of an Fc portion and/or of constant domains (*i.e.*, C_H2 and/or C_H3 domains) that confer increased half-life without any biologically significant effector function may also be suitable or even preferred. Other suitable constructs comprising one or more polypeptides of the invention and one or more constant domains with increased half-life *in vivo* will be clear to the skilled person, and may for example comprise polypeptides linked to a C_H3 domain, optionally via a linker sequence.

25 Generally, any fusion protein or derivatives with increased half-life will preferably have a molecular weight of more than 50 kDa, the cut-off value for renal absorption.

In another specific, but non-limiting, aspect, the polypeptides of the invention may be linked (optionally via a suitable linker or hinge region) to naturally occurring, synthetic or semi-synthetic constant domains (or analogs, variants, mutants, parts or fragments thereof) that have a reduced (or

30 essentially no) tendency to self-associate into dimers (*i.e.* compared to constant domains that naturally occur in conventional 4-chain antibodies). Such monomeric (*i.e.* not self-associating) Fc chain variants, or fragments thereof, will be clear to the skilled person. For example, Helm et al. (J. Biol. Chem. 271: 7494, 1996), describe monomeric Fc chain variants that can be used in the polypeptide chains of the invention.

Also, such monomeric Fc chain variants are preferably such that they are still capable of binding to the complement or the relevant Fc receptor(s) (depending on the Fc portion from which they are derived), and/or such that they still have some or all of the effector functions of the Fc portion from which they are derived (or at a reduced level still suitable for the intended use). Alternatively, in such a polypeptide chain of the invention, the monomeric Fc chain may be used to confer increased half-life upon the polypeptide chain, in which case the monomeric Fc chain may also have no or essentially no effector functions.

The further amino acid residues may or may not change, alter or otherwise influence other (biological) properties of the polypeptide of the invention and may or may not add further functionality to the polypeptide of the invention. For example, such amino acid residues:

a) can comprise an N-terminal Met residue, for example as result of expression in a heterologous host cell or host organism.

b) may form a signal sequence or leader sequence that directs secretion of the polypeptide from a host cell upon synthesis (for example to provide a pre-, pro- or prepro- form of the polypeptide of the invention, depending on the host cell used to express the polypeptide of the invention). Suitable secretory leader peptides will be clear to the skilled person, and may be as further described herein. Usually, such a leader sequence will be linked to the N-terminus of the polypeptide, although the invention in its broadest sense is not limited thereto;

c) may form a "tag", for example an amino acid sequence or residue that allows or facilitates the purification of the polypeptide, for example using affinity techniques directed against said sequence or residue. Thereafter, said sequence or residue may be removed (e.g. by chemical or enzymatical cleavage) to provide the polypeptide (for this purpose, the tag may optionally be linked to the amino acid sequence or polypeptide sequence via a cleavable linker sequence or contain a cleavable motif). Some preferred, but non-limiting examples of such residues are multiple histidine residues, glutathione residues and a myc-tag such as AAAEQKLISEEDLNGAA (SEQ ID NO: 367);

d) may be one or more amino acid residues that have been functionalized and/or that can serve as a site for attachment of functional groups. Suitable amino acid residues and functional groups will be clear to the skilled person and include, but are not limited to, the amino acid residues and functional groups mentioned herein for the derivatives of the polypeptides of the invention.

In the constructs of the invention, the two or more building blocks, ISVs or Nanobodies and the optionally one or more polypeptides, one or more other groups, drugs, agents, residues, moieties or

binding units may be directly linked to each other (as for example described in WO 99/23221) and/or may be linked to each other via one or more suitable spacers or linkers, or any combination thereof.

Suitable spacers or linkers for use in the constructs of the invention will be clear to the skilled person, and may generally be any linker or spacer used in the art to link amino acid sequences, and/or other groups, drugs, agents, residues, moieties or binding units. Preferably, said linker or spacer is suitable for use in constructing polypeptides and/or construct that are intended for pharmaceutical use.

Some particularly preferred spacers include the spacers and linkers that are used in the art to link antibody fragments or antibody domains. These include the linkers mentioned in the general background art cited above, as well as for example linkers that are used in the art to construct diabodies or ScFv fragments (in this respect, however, it should be noted that, whereas in diabodies and in ScFv fragments, the linker sequence used should have a length, a degree of flexibility and other properties that allow the pertinent V_H and V_L domains to come together to form the complete antigen-binding site, there is no particular limitation on the length or the flexibility of the linker used in the polypeptide of the invention, since each immunoglobulin single variable domain by itself forms a complete antigen-binding site).

For example, a linker may be a suitable amino acid sequence, and in particular amino acid sequences of between 1 and 50, preferably between 1 and 30, such as between 1 and 10 amino acid residues. Some preferred examples of such amino acid sequences include gly-ser linkers, for example of the type (gly_xser_y)_z, such as (for example (gly₄ser)₃ or (gly₃ser₂)₃), as described in WO 99/42077, and the GS30, GS15, GS9 and GS7 linkers described in the applications by Ablynx mentioned herein (see for example WO 06/040153 and WO 06/122825), as well as hinge-like regions such as the hinge regions of naturally occurring heavy chain antibodies or similar sequences (such as described in WO 94/04678).

Some other particularly preferred linkers are mentioned in Table B-3, of which 35GS (SEQ ID NO: 334) is particularly preferred.

Accordingly, the invention relates to polypeptides wherein the ISVs are linked to each other via a linker selected from the group consisting of SEQ ID NOs: 325 to 336.

Table B-3: Linkers

Linker	SEQ ID NO	Sequence
5GS	325	GGGGS
7GS	326	SGGSGGS
9GS	327	GGGGSGGGS
10GS	328	GGGGSGGGGS
15GS	329	GGGGSGGGSGGGGS

Linker	SEQ ID NO	Sequence
18GS	330	GGGGS GGGGS GGGGS GGGGS
20GS	331	GGGGS GGGGS GGGGS GGGGS
25GS	332	GGGGS GGGGS GGGGS GGGGS GGGGS
30GS	333	GGGGS GGGGS GGGGS GGGGS GGGGS
35GS	334	GGGGS GGGGS GGGGS GGGGS GGGGS
40GS	335	GGGGS GGGGS GGGGS GGGGS GGGGS
Poly-A	336	AAA

Other suitable linkers generally comprise organic compounds or polymers, in particular those suitable for use in proteins for pharmaceutical use. For instance, poly(ethyleneglycol) moieties have been used to link antibody domains, see for example WO 04/081026.

- 5 It is encompassed within the scope of the invention that the length, the degree of flexibility and/or other properties of the linker(s) used (although not critical, as it usually is for linkers used in ScFv fragments) may have some influence on the properties of the final polypeptide of the invention, including but not limited to the affinity, specificity or avidity for CD123 and/or TCR, or for one or more of the other antigens. Based on the disclosure herein, the skilled person will be able to
10 determine the optimal linker(s) for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

For example, in multivalent or multispecific polypeptides of the invention that comprise building blocks, ISVs or Nanobodies directed against a first and second target, the length and flexibility of the linker are preferably such that it allows each building block, ISV or Nanobody of the invention present
15 in the polypeptide to bind to its cognate target, e.g. the antigenic determinant on each of the targets. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linker(s) for use in a specific polypeptide of the invention, optionally after some limited routine experiments.

It is also within the scope of the invention that the linker(s) used confer one or more other
20 favourable properties or functionality to the polypeptides or constructs of the invention, and/or provide one or more sites for the formation of derivatives and/or for the attachment of functional groups (e.g., as described herein for the derivatives of the polypeptides of the invention). For example, linkers containing one or more charged amino acid residues can provide improved hydrophilic properties, whereas linkers that form or contain small epitopes or tags can be used for
25 the purposes of detection, identification and/or purification. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linkers for use in a specific polypeptide or construct of the invention, optionally after some limited routine experiments.

Finally, when two or more linkers are used in the polypeptides or constructs of the invention, these linkers may be the same or different. Again, based on the disclosure herein, the skilled person will be able to determine the optimal linkers for use in a specific polypeptide or construct of the invention, optionally after some limited routine experiments.

5 Usually, for ease of expression and production, a polypeptide or construct of the invention will be a linear polypeptide. However, the invention in its broadest sense is not limited thereto. For example, when a polypeptide of the invention comprises three or more amino acid sequences, ISVs or Nanobodies, it is possible to link them by use of a linker with three or more “arms”, which each “arm” being linked to an amino acid sequence, ISV or Nanobody, so as to provide a “star-shaped”
10 construct. It is also possible, although usually less preferred, to use circular constructs.

Accordingly, the present invention relates to a polypeptide as described herein, wherein said first ISV and said second ISV and possibly said third ISV and/or said ISV binding serum albumin are directly linked to each other or are linked via a linker.

Also encompassed in the present invention are constructs that comprise an immunoglobulin single
15 variable domain or polypeptide of the invention and further comprising tags or other functional moieties, *e.g.*, toxins, labels, radiochemicals, etc..

Alternatively, the additional groups, residues, moieties or binding units may for example be chemical groups, residues, moieties, which may or may not by themselves be biologically and/or pharmacologically active. For example, and without limitation, such groups may be linked to the two
20 or more immunoglobulin single variable domains or monovalent polypeptides so as to provide a “derivative” of the polypeptide of the invention.

Accordingly, the invention in its broadest sense also comprises derivatives of the polypeptides of the invention. Such derivatives can generally be obtained by modification, and in particular by chemical and/or biological (*e.g.*, enzymatical) modification, of the polypeptides of the invention and/or of one
25 or more of the amino acid residues that form a polypeptide of the invention.

Examples of such modifications, as well as examples of amino acid residues within the polypeptide sequences that can be modified in such a manner (*i.e.* either on the protein backbone but preferably on a side chain), methods and techniques that can be used to introduce such modifications and the potential uses and advantages of such modifications will be clear to the skilled person (see also Zangi
30 et al. 2013, Nat. biotechnol . 31: 898-907).

For example, such a modification may involve the introduction (*e.g.*, by covalent linking or in any other suitable manner) of one or more functional groups, residues or moieties into or onto the polypeptide of the invention, and in particular of one or more functional groups, residues or moieties

that confer one or more desired properties or functionalities to the polypeptide of the invention. Example of such functional groups will be clear to the skilled person.

For example, such modification may comprise the introduction (*e.g.*, by covalent binding or in any other suitable manner) of one or more functional groups that increase the half-life, the solubility and/or the absorption of the polypeptide of the invention, that reduce the immunogenicity and/or the toxicity of the polypeptide of the invention, that eliminate or attenuate any undesirable side effects of the polypeptide of the invention, and/or that confer other advantageous properties to and/or reduce the undesired properties of the polypeptide of the invention; or any combination of two or more of the foregoing. Examples of such functional groups and of techniques for introducing them will be clear to the skilled person, and can generally comprise all functional groups and techniques mentioned in the general background art cited hereinabove as well as the functional groups and techniques known per se for the modification of pharmaceutical proteins, and in particular for the modification of antibodies or antibody fragments (including ScFv's and single domain antibodies), for which reference is for example made to Remington (1980, Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA, 1980). Such functional groups may for example be linked directly (for example covalently) to a polypeptide of the invention, or optionally via a suitable linker or spacer, as will again be clear to the skilled person.

One specific example is a derivative polypeptide of the invention wherein the polypeptide of the invention has been chemically modified to increase the half-life thereof (for example, by means of pegylation). This is one of the most widely used techniques for increasing the half-life and/or reducing the immunogenicity of pharmaceutical proteins and comprises attachment of a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv's); reference is made to for example Chapman (2002, Nat. Biotechnol. 54: 531-545), Veronese and Harris (2003, Adv. Drug Deliv. Rev. 54: 453-456), Harris and Chess (2003, Nat. Rev. Drug. Discov. 2: 214-221) and WO 04/060965. Various reagents for pegylation of proteins are also commercially available, for example from Nektar Therapeutics, USA.

Preferably, site-directed pegylation is used, in particular via a cysteine-residue (see for example Yang et al. (2003, Protein Engineering 16: 761-770)). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a polypeptide of the invention, a polypeptide of the invention may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG

may be fused to the N- and/or C-terminus of a polypeptide of the invention, all using techniques of protein engineering known per se to the skilled person.

Preferably, for the polypeptides of the invention, a PEG is used with a molecular weight of more than 5000, such as more than 10,000 and less than 200,000, such as less than 100,000; for example in the
5 range of 20,000-80,000.

Another, usually less preferred modification comprises N-linked or O-linked glycosylation, usually as part of co-translational and/or post-translational modification, depending on the host cell used for expressing the polypeptide of the invention.

Yet another modification may comprise the introduction of one or more detectable labels or other
10 signal-generating groups or moieties, depending on the intended use of the labelled polypeptide of the invention. Suitable labels and techniques for attaching, using and detecting them will be clear to the skilled person, and for example include, but are not limited to, fluorescent labels (such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine and fluorescent metals such as ¹⁵²Eu or others metals from the
15 lanthanide series), phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, thermotropic acridinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes (such as ³H, ¹²⁵I, ³²P, ³⁵S, ¹⁴C, ⁵¹Cr, ³⁶Cl, ⁵⁷Co, ⁵⁸Co, ⁵⁹Fe, and ⁷⁵Se), metals, metals chelates or metallic cations (for example metallic cations such as ^{99m}Tc, ¹²³I, ¹¹¹In, ¹³¹I, ⁹⁷Ru, ⁶⁷Cu, ⁶⁷Ga, and ⁶⁸Ga or other metals or metallic cations that are particularly suited for use in
20 *in vivo*, *in vitro* or *in situ* diagnosis and imaging, such as (¹⁵⁷Gd, ⁵⁵Mn, ¹⁶²Dy, ⁵²Cr, and ⁵⁶Fe)), as well as chromophores and enzymes (such as malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate
25 dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels will be clear to the skilled person, and for example include moieties that can be detected using NMR or ESR spectroscopy.

Such labelled polypeptides of the invention may for example be used for *in vitro*, *in vivo* or *in situ* assays (including immunoassays known per se such as ELISA, RIA, EIA and other “sandwich assays”,
30 etc.) as well as *in vivo* diagnostic and imaging purposes, depending on the choice of the specific label.

As will be clear to the skilled person, another modification may involve the introduction of a chelating group, for example to chelate one of the metals or metallic cations referred to above. Suitable

chelating groups for example include, without limitation, diethyl-enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

Yet another modification may comprise the introduction of a functional group that is one part of a specific binding pair, such as the biotin-(strept)avidin binding pair. Such a functional group may be used to link the polypeptide of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a polypeptide of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated polypeptide of the invention may be used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may for example also be used to bind the polypeptide of the invention to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh (2000, Journal of Drug Targeting 8: 257). Such binding pairs may also be used to link a therapeutically active agent to the polypeptide of the invention.

Other potential chemical and enzymatical modifications will be clear to the skilled person. Such modifications may also be introduced for research purposes (e.g. to study function-activity relationships). Reference is for example made to Lundblad and Bradshaw (1997, Biotechnol. Appl. Biochem. 26: 143-151).

Preferably, the derivatives are such that they bind to CD123 and/or TCR, with an affinity (suitably measured and/or expressed as a K_D -value (actual or apparent), a K_A -value (actual or apparent), a k_{on} -rate and/or a k_{off} -rate, or alternatively as an IC_{50} value, as further described herein) that is as defined herein (i.e. as defined for the polypeptides of the invention).

Such polypeptides of the invention and derivatives thereof may also be in essentially isolated form (as defined herein).

The invention further relates to methods for preparing the polypeptides, nucleic acids, host cells, and compositions described herein.

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

The method for producing a polypeptide or construct (that is such that it can be obtained by expression of a nucleic acid encoding the same) of the invention may comprise the following steps:

- expressing, in a suitable host cell or host organism (also referred to herein as a “*host of the invention*”) or in another suitable expression system a nucleic acid that encodes said polypeptide or construct of the invention (also referred to herein as a “*nucleic acid of the invention*”),
optionally followed by:
 - isolating and/or purifying the polypeptide or construct of the invention thus obtained.

In particular, such a method may comprise the steps of:

- cultivating and/or maintaining a host of the invention under conditions that are such that said host of the invention expresses and/or produces at least one polypeptide or construct (that is such that it can be obtained by expression of a nucleic acid encoding the same) of the invention;
optionally followed by:
 - isolating and/or purifying the polypeptide or construct of the invention thus obtained.

Accordingly, the present invention also relates to a nucleic acid or nucleotide sequence that encodes a polypeptide or construct (that is such that it can be obtained by expression of a nucleic acid encoding the same) of the invention (also referred to as “*nucleic acid of the invention*”). A nucleic acid of the invention can be in the form of single or double stranded DNA or RNA, and is preferably in the form of double stranded DNA. For example, the nucleotide sequences of the invention may be genomic DNA, cDNA or synthetic DNA (such as DNA with a codon usage that has been specifically adapted for expression in the intended host cell or host organism).

According to one aspect of the invention, the nucleic acid of the invention is in essentially isolated form, as defined herein. The nucleic acid of the invention may also be in the form of, be present in and/or be part of a vector, such as for example a plasmid, cosmid or YAC, which again may be in essentially isolated form.

The nucleic acids of the invention can be prepared or obtained in a manner known per se, based on the information on the polypeptides or constructs (that are such that they can be obtained by expression of a nucleic acid encoding the same) of the invention given herein, and/or can be isolated from a suitable natural source. Also, as will be clear to the skilled person, to prepare a nucleic acid of the invention, also several nucleotide sequences, such as at least one nucleotide sequence encoding an immunoglobulin single variable domain of the invention and for example nucleic acids encoding one or more linkers can be linked together in a suitable manner.

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

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

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

In a preferred but non-limiting aspect, a genetic construct of the invention comprises

- a) at least one nucleic acid of the invention; operably connected to
- b) one or more regulatory elements, such as a promoter and optionally a suitable terminator; and optionally also
- c) one or more further elements of genetic constructs known per se;

in which the terms “regulatory element”, “promoter”, “terminator” and “operably connected” have their usual meaning in the art (as further described herein); and in which said “further elements” present in the genetic constructs may for example be 3′- or 5′-UTR sequences, leader sequences,

selection markers, expression markers/reporter genes, and/or elements that may facilitate or increase (the efficiency of) transformation or integration. These and other suitable elements for such genetic constructs will be clear to the skilled person, and may for instance depend upon the type of construct used; the intended host cell or host organism; the manner in which the nucleotide sequences of the invention of interest are to be expressed (e.g. via constitutive, transient or inducible expression); and/or the transformation technique to be used. For example, regulatory sequences, promoters and terminators known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

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

Preferably, the regulatory and further elements of the genetic constructs of the invention are such that they are capable of providing their intended biological function in the intended host cell or host organism.

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

Some particularly preferred promoters include, but are not limited to, promoters known per se for the expression in the host cells mentioned herein; and in particular promoters for the expression in the bacterial or yeast cells, such as those mentioned herein and/or those used in the Examples.

A selection marker should be such that it allows - *i.e.*, under appropriate selection conditions - host cells and/or host organisms that have been (successfully) transformed with the nucleotide sequence of the invention to be distinguished from host cells/organisms that have not been (successfully) transformed. Some preferred, but non-limiting examples of such markers are genes that provide resistance against antibiotics (such as kanamycin or ampicillin), genes that provide for temperature

resistance, or genes that allow the host cell or host organism to be maintained in the absence of certain factors, compounds and/or (food) components in the medium that are essential for survival of the non-transformed cells or organisms.

A leader sequence should be such that - in the intended host cell or host organism - it allows for the desired post-translational modifications and/or such that it directs the transcribed mRNA to a desired part or organelle of a cell. A leader sequence may also allow for secretion of the expression product from said cell. As such, the leader sequence may be any pro-, pre-, or prepro-sequence operable in the host cell or host organism. Leader sequences may not be required for expression in a bacterial cell. For example, leader sequences known per se for the expression and production of antibodies and antibody fragments (including but not limited to single domain antibodies and ScFv fragments) may be used in an essentially analogous manner.

An expression marker or reporter gene should be such that - in the host cell or host organism - it allows for detection of the expression of (a gene or nucleotide sequence present on) the genetic construct. An expression marker may optionally also allow for the localisation of the expressed product, *e.g.*, in a specific part or organelle of a cell and/or in (a) specific cell(s), tissue(s), organ(s) or part(s) of a multicellular organism. Such reporter genes may also be expressed as a protein fusion with the ISV, polypeptide or construct of the invention. Some preferred, but non-limiting examples include fluorescent proteins such as GFP.

Some preferred, but non-limiting examples of suitable promoters, terminator and further elements include those that can be used for the expression in the host cells mentioned herein; and in particular those that are suitable for expression in bacterial or yeast cells, such as those mentioned herein and/or those used in the Examples below. For some (further) non-limiting examples of the promoters, selection markers, leader sequences, expression markers and further elements that may be present/used in the genetic constructs of the invention - such as terminators, transcriptional and/or translational enhancers and/or integration factors - reference is made to the general handbooks such as Sambrook et al. and Ausubel et al. mentioned above, as well as to the examples that are given in WO 95/07463, WO 96/23810, WO 95/07463, WO 95/21191, WO 97/11094, WO 97/42320, WO 98/06737, WO 98/21355, US 7,207,410, US 5,693,492 and EP 1085089. Other examples will be clear to the skilled person. Reference is also made to the general background art cited above and the further references cited herein.

The genetic constructs of the invention may generally be provided by suitably linking the nucleotide sequence(s) of the invention to the one or more further elements described above, for example using

the techniques described in the general handbooks such as Sambrook et al. and Ausubel et al., mentioned above.

Often, the genetic constructs of the invention will be obtained by inserting a nucleotide sequence of the invention in a suitable (expression) vector known per se. Some preferred, but non-limiting
5 examples of suitable expression vectors are those used in the Examples below, as well as those mentioned herein.

The nucleic acids of the invention and/or the genetic constructs of the invention may be used to transform a host cell or host organism, *i.e.*, for expression and/or production of the polypeptide or construct (that is such that it can be obtained by expression of a nucleic acid encoding the same) of
10 the invention. The host is preferably a non-human host. Suitable hosts or host cells will be clear to the skilled person, and may for example be any suitable fungal, prokaryotic or eukaryotic cell or cell line or any suitable fungal, prokaryotic or (non-human) eukaryotic organism, for example:

- a bacterial strain, including but not limited to gram-negative strains such as strains of *Escherichia coli*; of *Proteus*, for example of *Proteus mirabilis*; of *Pseudomonas*, for example of
15 *Pseudomonas fluorescens*; and gram-positive strains such as strains of *Bacillus*, for example of *Bacillus subtilis* or of *Bacillus brevis*; of *Streptomyces*, for example of *Streptomyces lividans*; of *Staphylococcus*, for example of *Staphylococcus carnosus*; and of *Lactococcus*, for example of *Lactococcus lactis*;
- a fungal cell, including but not limited to cells from species of *Trichoderma*, for example from
20 *Trichoderma reesei*; of *Neurospora*, for example from *Neurospora crassa*; of *Sordaria*, for example from *Sordaria macrospora*; of *Aspergillus*, for example from *Aspergillus niger* or from *Aspergillus sojae*; or from other filamentous fungi;
- a yeast cell, including but not limited to cells from species of *Saccharomyces*, for example of *Saccharomyces cerevisiae*; of *Schizosaccharomyces*, for example of *Schizosaccharomyces pombe*;
25 of *Pichia*, for example of *Pichia pastoris* or of *Pichia methanolica*; of *Hansenula*, for example of *Hansenula polymorpha*; of *Kluyveromyces*, for example of *Kluyveromyces lactis*; of *Arxula*, for example of *Arxula adenivorans*; of *Yarrowia*, for example of *Yarrowia lipolytica*;
- an amphibian cell or cell line, such as *Xenopus oocytes*;
- an insect-derived cell or cell line, such as cells/cell lines derived from lepidoptera, including
30 but not limited to *Spodoptera* SF9 and Sf21 cells or cells/cell lines derived from *Drosophila*, such as Schneider and Kc cells;
- a plant or plant cell, for example in tobacco plants; and/or

- a mammalian cell or cell line, for example a cell or cell line derived from a human, a cell or a cell line from mammals including but not limited to CHO-cells, BHK-cells (for example BHK-21 cells) and human cells or cell lines such as HeLa, COS (for example COS-7) and PER.C6 cells;

as well as all other host cells or (non-human) hosts known per se for the expression and production of antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv fragments), which will be clear to the skilled person. Reference is also made to the general background art cited hereinabove, as well as to for example WO 94/29457; WO 96/34103; WO 99/42077; Frenken et al. (1998, Res Immunol. 149: 589-599); Riechmann and Muyldermans (1999), supra; van der Linden (2000, J. Biotechnol. 80: 261-270); Joosten et al. (2003, Microb. Cell Fact. 2: 1); Joosten et al. 2005, (Appl. Microbiol. Biotechnol. 66: 384-392); and the further references cited herein.

The polypeptides or constructs of the invention may also be expressed as so-called "intrabodies", as for example described in WO 94/02610, WO 95/22618 and US 7,004,940; WO 03/014960; in Cattaneo and Biocca (1997, Intracellular Antibodies: Development and Applications" Landes and Springer-Verlag); and in Kontermann (2004, Methods 34: 163-170).

The polypeptides or constructs of the invention can for example also be produced in the milk of transgenic mammals, for example in the milk of rabbits, cows, goats or sheep (see for example US 6,741,957, US 6,304,489 and US 6,849,992 for general techniques for introducing transgenes into mammals), in plants or parts of plants including but not limited to their leaves, flowers, fruits, seed, roots or tubers (for example in tobacco, maize, soybean or alfalfa) or in for example pupae of the silkworm *Bombix mori*.

Furthermore, the polypeptides or the constructs of the invention can also be expressed and/or produced in cell-free expression systems, and suitable examples of such systems will be clear to the skilled person. Some preferred, but non-limiting examples include expression in the wheat germ system; in rabbit reticulocyte lysates; or in the *E. coli* Zubay system.

Preferably, in the invention, an (*in vivo* or *in vitro*) expression system, such as a bacterial expression system, is used that provides the polypeptides or constructs of the invention in a form that is suitable for pharmaceutical use, and such expression systems will again be clear to the skilled person. As also will be clear to the skilled person, polypeptides or constructs of the invention suitable for pharmaceutical use can be prepared using techniques for peptide synthesis.

For production on industrial scale, preferred heterologous hosts for the (industrial) production of immunoglobulin single variable domains or immunoglobulin single variable domain-containing polypeptide therapeutics include strains of *E. coli*, *Pichia pastoris*, *S. cerevisiae* that are suitable for

large scale expression/production/fermentation, and in particular for large scale pharmaceutical expression/production/fermentation. Suitable examples of such strains will be clear to the skilled person. Such strains and production/expression systems are also made available by companies such as Biovitrum (Uppsala, Sweden).

- 5 Alternatively, mammalian cell lines, in particular Chinese hamster ovary (CHO) cells, can be used for large scale expression/production/fermentation, and in particular for large scale pharmaceutical expression/production/fermentation. Again, such expression/production systems are also made available by some of the companies mentioned above.

The choice of the specific expression system would depend in part on the requirement for certain
10 post-translational modifications, more specifically glycosylation. The production of an immunoglobulin single variable domain-containing recombinant protein for which glycosylation is desired or required would necessitate the use of mammalian expression hosts that have the ability to glycosylate the expressed protein. In this respect, it will be clear to the skilled person that the glycosylation pattern obtained (*i.e.*, the kind, number and position of residues attached) will depend
15 on the cell or cell line that is used for the expression. Preferably, either a human cell or cell line is used (*i.e.*, leading to a protein that essentially has a human glycosylation pattern) or another mammalian cell line is used that can provide a glycosylation pattern that is essentially and/or functionally the same as human glycosylation or at least mimics human glycosylation. Generally, prokaryotic hosts such as *E. coli* do not have the ability to glycosylate proteins, and the use of lower
20 eukaryotes such as yeast usually leads to a glycosylation pattern that differs from human glycosylation. Nevertheless, it should be understood that all the foregoing host cells and expression systems can be used in the invention, depending on the desired polypeptide or construct to be obtained.

Thus, according to one non-limiting aspect of the invention, the polypeptide or construct of the
25 invention is glycosylated. According to another non-limiting aspect of the invention, the polypeptide or construct of the invention is non-glycosylated.

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

- 30 According to another preferred, but non-limiting aspect of the invention, the polypeptide or construct of the invention is produced in a yeast cell, in particular a yeast cell suitable for large scale pharmaceutical production, such as cells of the species mentioned above.

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

5 When expression in a host cell is used to produce the polypeptides or constructs of the invention, the polypeptides or constructs of the invention can be produced either intracellularly (*e.g.*, in the cytosol, in the periplasma or in inclusion bodies) and then isolated from the host cells and optionally further purified; or can be produced extracellularly (*e.g.*, in the medium in which the host cells are cultured) and then isolated from the culture medium and optionally further purified. When
10 eukaryotic host cells are used, extracellular production is usually preferred since this considerably facilitates the further isolation and downstream processing of the polypeptides or constructs obtained. Bacterial cells such as the strains of *E. coli* mentioned above normally do not secrete proteins extracellularly, except for a few classes of proteins such as toxins and hemolysin, and secretory production in *E. coli* refers to the translocation of proteins across the inner membrane to
15 the periplasmic space. Periplasmic production provides several advantages over cytosolic production. For example, the N-terminal amino acid sequence of the secreted product can be identical to the natural gene product after cleavage of the secretion signal sequence by a specific signal peptidase. Also, there appears to be much less protease activity in the periplasm than in the cytoplasm. In addition, protein purification is simpler due to fewer contaminating proteins in the periplasm.
20 Another advantage is that correct disulfide bonds may form because the periplasm provides a more oxidative environment than the cytoplasm. Proteins overexpressed in *E. coli* are often found in insoluble aggregates, so-called inclusion bodies. These inclusion bodies may be located in the cytosol or in the periplasm; the recovery of biologically active proteins from these inclusion bodies requires a denaturation/refolding process. Many recombinant proteins, including therapeutic proteins, are
25 recovered from inclusion bodies. Alternatively, as will be clear to the skilled person, recombinant strains of bacteria that have been genetically modified so as to secrete a desired protein, and in particular a polypeptide or construct of the invention, can be used.

Thus, according to one non-limiting aspect of the invention, the polypeptide or construct of the invention is a polypeptide or construct that has been produced intracellularly and that has been
30 isolated from the host cell, and in particular from a bacterial cell or from an inclusion body in a bacterial cell. According to another non-limiting aspect of the invention, the polypeptide or construct of the invention is a polypeptide or construct that has been produced extracellularly, and that has been isolated from the medium in which the host cell is cultivated.

Some preferred, but non-limiting promoters for use with these host cells include:

- for expression in *E. coli*: lac promoter (and derivatives thereof such as the lacUV5 promoter); arabinose promoter; left- (PL) and rightward (PR) promoter of phage lambda; promoter of the trp operon; hybrid lac/trp promoters (tac and trc); T7-promoter (more specifically that of T7-phage gene 10) and other T-phage promoters; promoter of the Tn10 tetracycline resistance gene; engineered variants of the above promoters that include one or more copies of an extraneous regulatory operator sequence;

- for expression in *S. cerevisiae*: constitutive: ADH1 (alcohol dehydrogenase 1), ENO (enolase), CYC1 (cytochrome c iso-1), GAPDH (glyceraldehydes-3-phosphate dehydrogenase), PGK1 (phosphoglycerate kinase), PYK1 (pyruvate kinase); regulated: GAL1,10,7 (galactose metabolic enzymes), ADH2 (alcohol dehydrogenase 2), PHO5 (acid phosphatase), CUP1 (copper metallothionein); heterologous: CaMV (cauliflower mosaic virus 35S promoter);

- for expression in *Pichia pastoris*: the AOX1 promoter (alcohol oxidase I);

- for expression in mammalian cells: human cytomegalovirus (hCMV) immediate early enhancer/promoter; human cytomegalovirus (hCMV) immediate early promoter variant that contains two tetracycline operator sequences such that the promoter can be regulated by the Tet repressor; Herpes Simplex Virus thymidine kinase (TK) promoter; Rous Sarcoma Virus long terminal repeat (RSV LTR) enhancer/promoter; elongation factor 1 α (hEF-1 α) promoter from human, chimpanzee, mouse or rat; the SV40 early promoter; HIV-1 long terminal repeat promoter; β -actin promoter.

Some preferred, but non-limiting vectors for use with these host cells include:

- vectors for expression in mammalian cells: pMAMneo (Clontech), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593), pBPV-1 (8-2) (ATCC 37110), pDBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC37199), pRSVneo (ATCC37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460) and 1ZD35 (ATCC 37565), as well as viral-based expression systems, such as those based on adenovirus;

- vectors for expression in bacterial cells: pET vectors (Novagen) and pQE vectors (Qiagen);

- vectors for expression in yeast or other fungal cells: pYES2 (Invitrogen) and *Pichia* expression vectors (Invitrogen);

- vectors for expression in insect cells: pBlueBacII (Invitrogen) and other baculovirus vectors;

- vectors for expression in plants or plant cells: for example vectors based on cauliflower mosaic virus or tobacco mosaic virus, suitable strains of *Agrobacterium*, or Ti-plasmid based vectors.

Some preferred, but non-limiting secretory sequences for use with these host cells include:

- for use in bacterial cells such as *E. coli*: PelB, Bla, OmpA, OmpC, OmpF, OmpT, StII, PhoA, PhoE, MalE, Lpp, LamB, and the like; TAT signal peptide, hemolysin C-terminal secretion signal;
- for use in yeast: α -mating factor prepro-sequence, phosphatase (pho1), invertase (Suc), etc.;
- 5 - for use in mammalian cells: indigenous signal in case the target protein is of eukaryotic origin; murine Ig κ -chain V-J2-C signal peptide; etc.

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

10 After transformation, a step for detecting and selecting those host cells or host organisms that have been successfully transformed with the nucleotide sequence/genetic construct of the invention may be performed. This may for instance be a selection step based on a selectable marker present in the genetic construct of the invention or a step involving the detection of the polypeptide or construct of the invention, *e.g.*, using specific antibodies.

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

Preferably, these host cells or host organisms are such that they express, or are (at least) capable of expressing (*e.g.*, under suitable conditions), a polypeptide or construct of the invention (and in case of a host organism: in at least one cell, part, tissue or organ thereof). The invention also includes
20 further generations, progeny and/or offspring of the host cell or host organism of the invention, that may for instance be obtained by cell division or by sexual or asexual reproduction.

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

To produce/obtain expression of the polypeptides or constructs of the invention, the transformed host cell or transformed host organism may generally be kept, maintained and/or cultured under

conditions such that the (desired) polypeptide or construct of the invention is expressed/produced. Suitable conditions will be clear to the skilled person and will usually depend upon the host cell/host organism used, as well as on the regulatory elements that control the expression of the (relevant) nucleotide sequence of the invention. Again, reference is made to the handbooks and patent applications mentioned above in the paragraphs on the genetic constructs of the invention.

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

It will also be clear to the skilled person that the polypeptide or construct of the invention may (first) be generated in an immature form (as mentioned above), which may then be subjected to post-translational modification, depending on the host cell/host organism used. Also, the polypeptide or construct of the invention may be glycosylated, again depending on the host cell/host organism used.

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

Compositions of the invention

The invention further relates to a product or composition containing or comprising at least one polypeptide or construct of the invention, and/or at least one nucleic acid of the invention, and optionally one or more further components of such compositions known per se, *i.e.* depending on the intended use of the composition.

Generally, for pharmaceutical use, the polypeptides or constructs of the invention may be formulated as a pharmaceutical preparation or composition comprising at least one polypeptide or construct of the invention and at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally one or more further pharmaceutically active polypeptides and/or compounds. By means of non-limiting examples, such a formulation may be in a form suitable for oral administration, for parenteral administration (for example intravenous, intraperitoneal,

subcutaneous, intramuscular, intraluminal, intra-arterial or intrathecal administration), for topical administration, for administration by inhalation, by a skin patch, by an implant, by a suppository, etc, wherein the parenteral administration is preferred. Such suitable administration forms - which may be solid, semi-solid or liquid, depending on the manner of administration - as well as methods and carriers for use in the preparation thereof, will be clear to the skilled person, and are further described herein. Such a pharmaceutical preparation or composition will generally be referred to herein as a "pharmaceutical composition". A pharmaceutical preparation or composition for use in a non-human organism will generally be referred to herein as a "veterinary composition". Some preferred but non-limiting examples of such compositions will become clear from the further description herein.

Thus, in a further aspect, the invention relates to a pharmaceutical composition that contains at least one polypeptide or construct of the invention and at least one suitable carrier, diluent or excipient (*i.e.*, suitable for pharmaceutical use), and optionally one or more further active substances. In a particular aspect, the invention relates to a pharmaceutical composition that contains a polypeptide or construct of the invention selected from any of SEQ ID NOs: 1-10, 47, 49, 52, 53, 55, 56, 58-61, 63-67, and 338-342 and at least one suitable carrier, diluent or excipient (*i.e.* suitable for pharmaceutical use), and optionally one or more further active substances.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Generally, the polypeptides and constructs of the invention can be formulated and administered in any suitable manner known per se. Reference is for example made to the general background art cited above (and in particular to WO 04/041862, WO 04/041863, WO 04/041865, WO 04/041867 and WO 08/020079) as well as to the standard handbooks, such as Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Company, USA (1990), Remington, the Science and Practice of Pharmacy,

21st Ed., Lippincott Williams and Wilkins (2005); or the Handbook of Therapeutic Antibodies (S. Dubel, Ed.), Wiley, Weinheim, 2007 (see for example pages 252-255).

For example, the polypeptides or construct of the invention may be formulated and administered in any manner known per se for conventional antibodies and antibody fragments (including ScFv's and diabodies) and other pharmaceutically active proteins. Such formulations and methods for preparing the same will be clear to the skilled person, and for example include preparations suitable for parenteral administration (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular, intraluminal, intra-arterial or intrathecal administration).

Preparations for parenteral administration may for example be sterile solutions, suspensions, dispersions or emulsions that are suitable for infusion or injection. Suitable carriers or diluents for such preparations for example include, without limitation, those mentioned on page 143 of WO 08/020079. Usually, aqueous solutions or suspensions will be preferred.

The polypeptides or constructs of the invention may be administered intravenously or intraperitoneally by infusion or injection. Solutions of the polypeptides or constructs of the invention can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for parenteral administration comprise one or more immunoglobulin single variable domain, polypeptide or construct in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and non-aqueous carriers, which may be employed in the pharmaceutical compositions include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms upon the subject

compounds may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution, which in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microcapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly-(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions, which are compatible with body tissue.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage.

Sterile injectable solutions are prepared by incorporating the polypeptides or constructs of the invention in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

The amount of the polypeptides or constructs of the invention required for use in treatment will vary not only with the particular polypeptide or construct selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient

and will be ultimately at the discretion of the attendant physician or clinician. Also the dosage of the polypeptides or constructs of the invention will vary.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself
5 may be further divided, *e.g.*, into a number of discrete loosely spaced administrations.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W.,
10 ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

In another aspect, kits are provided comprising a polypeptide or construct of the invention, a nucleic acid of the invention, an expression vector of the invention, or a host or host cell of the invention. The kit may also comprise one or more vials containing the polypeptide or construct and instructions
15 for use. The kit may also contain means for administering the polypeptide or construct of the invention such as a syringe, infuser or the like.

Uses of the polypeptides, construct or compositions of the invention

The invention further relates to applications and uses of the polypeptides, constructs, nucleic acids,
20 host cells, and compositions described herein, as well as to methods for the prevention and/or treatment of CD123 associated diseases or conditions. Some preferred but non-limiting applications and uses will become clear from the further description herein.

The polypeptides, constructs, and compositions of the present invention can generally be used to activate T cells at (the site of) CD123 expressing cells; such as to lyse the CD123 expressing cells. The
25 simultaneous binding by the polypeptides and constructs of the present invention to TCR on T cells and CD123 on tumour cells induces the activation of the cells and the subsequent lysis (killing) of the CD123 expressing cells. When not bound to CD123 expressing cells, the polypeptides and constructs of the invention show hardly any T cell activation. As such, target-independent lysis (*i.e.*, lysis of cells without CD123 expression) by the polypeptides and constructs of the present invention is minimal.

Accordingly, in one aspect, the polypeptides, constructs and compositions of the present invention
30 cause lysis of CD123 expressing cells with an average lysis percentage of at least 10%, preferably at least 15%, such as at least 16%, 17%, 18%, 19% or 20% or more, such as 30% or more of compared to the number of CD123 expressing cells under the same conditions but without the presence of the

polypeptide or construct of the invention, measured in any suitable manner known per se, for example using one of the assays described herein (such as the redirected human T cell mediated killing flow-cytometry based assays, as described in the Example section).

Apart from this or at the same time, the T cell activation induced lysis of CD123 negative cells by the polypeptides, constructs and compositions of the present invention, is no more than about 10%, such as 9% or less, such as 8, 7, or 6 % or even less, of the number of CD123 negative cells under the same conditions but without the presence of the polypeptide or construct of the invention, measured in any suitable manner known per se, for example using one of the assays described herein (such as the redirected human T cell mediated killing flow-cytometry based assays, as described in the Example section).

This killing of CD123 expressing cells can be advantageous in diseases or conditions in which the presence of such CD123 expressing cells is abundant and/or not desired.

Accordingly, in one aspect, the present invention provides a polypeptide, construct or a composition, for use as a medicament.

In a further aspect, the present invention provides a polypeptide or construct of the invention or a composition comprising the same, for use in the prevention, treatment and/or amelioration of a CD123 associated disease or condition.

More particularly, the present invention provides a polypeptide or construct of the invention or a composition comprising the same, for use in the prevention, treatment and/or amelioration of a CD123 associated disease or condition, wherein the CD123 associated disease or condition is a proliferative disease or an inflammatory condition.

The invention also relates to a method for the prevention, treatment and/or amelioration of a CD123 associated disease or condition, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of a polypeptide or construct of the invention, and/or of a composition comprising the same.

In particular, the present invention relates to a method as described above, wherein the CD123 associated disease or condition is a proliferative disease or an inflammatory condition.

The inflammatory condition can be any inflammatory condition prevented, treated and/or ameliorated by killing of CD123 expressing cells.

In one aspect, the inflammatory condition is chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis.

Accordingly, the present invention relates to a polypeptide, construct or a composition for use in the prevention, treatment and/or amelioration of an inflammatory condition, wherein said inflammatory condition is chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis.

5 Accordingly, the present invention also relates to methods for the prevention, treatment and/or amelioration of an inflammatory condition, wherein said inflammatory condition is chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide or construct of the invention or a composition of the invention.

10 The proliferative disease can be any proliferative disease prevented, treated and/or ameliorated by killing of CD123 expressing cells.

In one aspect, said proliferative disease is cancer. Examples of cancers associated with CD123 overexpression will be clear to the skilled person based on the disclosure herein, and for example include (without being limiting) the following cancers: lymphomas (including Burkitt's lymphoma, 15 Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma.

Accordingly, the present invention relates to a polypeptide, construct or a composition for use in the 20 prevention, treatment and/or amelioration of cancer, wherein said cancer is chosen from the group consisting of lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple 25 myeloma.

Accordingly, the present invention also relates to methods for the prevention, treatment and/or amelioration of cancer, wherein said cancer is chosen from the group consisting of lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, 30 chronic lymphocytic leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma, said method comprising administering, to a subject in need thereof, a pharmaceutically active amount of at least one polypeptide or construct of the invention or a composition of the invention.

The invention also relates to the use of a polypeptide or construct of the invention, or a composition of the invention, for the manufacture of a medicament.

In a further aspect, the present invention relates to the use of a polypeptide or construct of the invention or a composition comprising the same, for the manufacture of a medicament for the prevention, treatment and/or amelioration of a CD123 associated disease or condition.

More particularly, the present invention relates to the use of a polypeptide or construct of the invention or a composition comprising the same, for the manufacture of a medicament for the prevention, treatment and/or amelioration of a CD123 associated disease or condition, wherein the CD123 associated disease or condition is a proliferative disease or an inflammatory condition.

In one aspect, the invention relates to the use of a polypeptide or construct of the invention, or a composition comprising the same, for the manufacture of a medicament for the prevention, treatment and/or amelioration of an inflammatory condition, wherein said inflammatory condition is chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis.

In another aspect, the invention relates to the use of a polypeptide or construct of the invention, or a composition comprising the same, for the manufacture of a medicament for the prevention, treatment and/or amelioration of a proliferative disease, wherein said proliferative disease is cancer. Examples of cancers associated with CD123 overexpression will be clear to the skilled person based on the disclosure herein, and for example include (without being limiting) the following cancers: lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma.

Accordingly, the present invention also relates the use of a polypeptide or construct of the invention, or a composition comprising the same, for the manufacture of a medicament for the prevention, treatment and/or amelioration of cancer, wherein said cancer is chosen from the group consisting of lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphocytic leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma.

In the context of the present invention, the term "prevention, treatment and/or amelioration" not only comprises preventing, treating and/or ameliorating the disease, but also generally comprises preventing the onset of the disease, slowing or reversing the progress of disease, preventing or

slowing the onset of one or more symptoms associated with the disease, reducing and/or alleviating one or more symptoms associated with the disease, reducing the severity and/or the duration of the disease and/or of any symptoms associated therewith and/or preventing a further increase in the severity of the disease and/or of any symptoms associated therewith, preventing, reducing or reversing any physiological damage caused by the disease, and generally any pharmacological action that is beneficial to the patient being treated.

As used interchangeably herein, the term "pharmaceutically effective amount" or "pharmaceutically active amount" refers to an amount that is sufficient to activate T cells in the presence of CD123 expressing cells. In the context of a CD123 associated disease, it refers to the amount of a polypeptide, construct or pharmaceutical composition alone, or in combination with another therapy, that provides a therapeutic benefit in the prevention, treatment and/or amelioration of the CD123 associated disease. Used in connection with an amount of a multispecific polypeptide or construct of the invention, the term can encompass an amount that improves overall therapy, reduces or avoids unwanted effects, or enhances the therapeutic efficacy of or synergies with another therapy.

As used herein, the term "therapy" refers to any protocol, method and/or agent that can be used in the treatment, prevention and/or management of a CD123 associated disease, e.g., an inflammatory condition or proliferative disease. In certain embodiments, the terms "therapies" and "therapy" refer to a biological therapy, supportive therapy, and/or other therapies useful in the treatment, prevention and/or management of a CD123 associated disease, e.g., an inflammatory condition or proliferative disease, or one or more symptoms thereof known to one of skill in the art such as medical personnel.

In another aspect, the invention relates to a method for immunotherapy, and in particular for passive immunotherapy, which method comprises administering, to a subject suffering from or at risk of a CD123 associated disease, a pharmaceutically active amount of a polypeptide or construct of the invention, and/or of a pharmaceutical composition comprising the same.

The subject to be treated may be any warm-blooded animal, but is in particular a mammal, and more in particular a human being. As will be clear to the skilled person, the subject to be treated will in particular be a person suffering from, or at risk of the diseases and conditions mentioned herein.

In general, the polypeptides or construct according to the invention and/or the compositions comprising the same can be administered in any suitable manner. For example (but not limited thereto) the polypeptides according to the invention and compositions comprising the same can be administered orally, parenterally (e.g., intravenously, intraperitoneally, subcutaneously,

intramuscularly, intraluminally, intra-arterially or intrathecally or via any other route of administration that circumvents the gastrointestinal tract), intranasally, transdermally, topically, by means of a suppository, by inhalation, again depending on the specific pharmaceutical formulation or composition to be used. The clinician will be able to select a suitable route of administration and a
5 suitable pharmaceutical formulation or composition to be used in such administration, depending on the disease or disorder to be prevented or treated and other factors well known to the clinician.

In a preferred aspect, the polypeptides or constructs of the invention or the compositions comprising the same are administered intravenously (e.g., (but not limited thereto), by infusion or a bolus) or subcutaneously.

10 The polypeptides or constructs of the invention and/or the compositions comprising the same are administered according to a regime of treatment that is suitable for preventing, treating and/or ameliorating a CD123 associated disease. The clinician will generally be able to determine a suitable treatment regimen, depending on factors such as the type of disease to be treated, the stage of the disease, the severity of the disease and/or the severity of the symptoms thereof, the specific
15 polypeptide or construct of the invention to be used, the specific route of administration and pharmaceutical formulation or composition to be used, the age, gender, weight, diet, general condition of the patient, and similar factors well known to the clinician.

Generally, the treatment regimen will comprise the administration of one or more polypeptides or constructs of the invention, or of one or more compositions comprising the same, in one or more
20 pharmaceutically effective amounts or doses. The specific amount(s) or doses to administered can be determined by the clinician, again based on the factors cited above.

Generally, for the prevention, treatment and/or amelioration of a CD123 associated disease and depending on the type of CD123 associated disease (e.g., a proliferative disorder (including cancer) or inflammatory condition) to be treated, the stage of the disease to be treated, the potency of the
25 polypeptide or construct of the invention to be used, the specific route of administration and the specific pharmaceutical formulation or composition used, the polypeptides of the invention will generally be administered in an amount between 1 gram and 1 microgram per kg body weight per day. The clinician will generally be able to determine a suitable daily dose, depending on the factors mentioned herein. It will also be clear that in specific cases, the clinician may choose to deviate from
30 these amounts, for example on the basis of the factors cited above and his expert judgment. Generally, some guidance on the amounts to be administered can be obtained from the amounts usually administered for comparable conventional antibodies or antibody fragments against the same

target via essentially the same route, taking into account however differences in affinity/avidity, efficacy, biodistribution, half-life and similar factors well known to the skilled person.

Usually, in the above method, a single polypeptide or construct of the invention will be used. It is however within the scope of the invention to use two or more polypeptides or constructs of the invention in combination.

The polypeptides or constructs of the invention, or compositions comprising the same may also be used in combination with one or more further pharmaceutically active compounds or principles, i.e. as a combined treatment regimen, which may or may not lead to a synergistic effect. Again, the clinician will be able to select such further compounds or principles, as well as a suitable combined treatment regimen, based on the factors cited above and his expert judgement.

In particular, the polypeptides, constructs and compositions of the invention may be used in combination with other pharmaceutically active compounds or principles that are or can be used for the prevention, treatment and/or amelioration of a CD123 associated disease (e.g., a proliferative disorder (including cancer) or inflammatory condition), as a result of which a synergistic effect may or may not be obtained. Examples of such compounds and principles, as well as routes, methods and pharmaceutical formulations or compositions for administering them will be clear to the clinician.

Examples of such compounds and principles, as well as routes, methods and pharmaceutical formulations or compositions for administering them will be clear to the clinician and include (without being limiting): Anthracyclines (daunorubicin, doxorubicin, idarubicin, mitoxantrone, rubidazole), Cytarabine (AML), haematopoietic growth factors, demethylating agents (such as decitabine or azacytidine), all-trans retinoic acid, arsenic trioxide, DNA methyltransferase inhibitors, Melphalan, Prednisone, Lenalidomide, Cyclophosphamide, Thalidomide, Dexamethasone, Bortezomib, fludarabine, corticosteroids, vincristine, rasburicase, L-Asparaginase, pegylated asparaginase, Cladribine, Pentostatin, Adriamycin, Bleomycin, Vinblastine, Dacarbazine; or any combination thereof.

When two or more substances or principles are to be used as part of a combined treatment regimen, they can be administered via the same route of administration or via different routes of administration, at essentially the same time or at different times (e.g. essentially simultaneously, consecutively, or according to an alternating regime). When the substances or principles are to be administered simultaneously via the same route of administration, they may be administered as different pharmaceutical formulations or compositions or part of a combined pharmaceutical formulation or composition, as will be clear to the skilled person.

Also, when two or more active substances or principles are to be used as part of a combined treatment regimen, each of the substances or principles may be administered in the same amount and according to the same regimen as used when the compound or principle is used on its own, and such combined use may or may not lead to a synergistic effect. However, when the combined use of the two or more active substances or principles leads to a synergistic effect, it may also be possible to reduce the amount of one, more or all of the substances or principles to be administered, while still achieving the desired therapeutic action. This may for example be useful for avoiding, limiting or reducing any unwanted side-effects that are associated with the use of one or more of the substances or principles when they are used in their usual amounts, while still obtaining the desired pharmaceutical or therapeutic effect.

The effectiveness of the treatment regimen used according to the invention may be determined and/or followed in any manner known per se for the disease or disorder involved, as will be clear to the clinician. The clinician will also be able, where appropriate and on a case-by-case basis, to change or modify a particular treatment regimen, so as to achieve the desired therapeutic effect, to avoid, limit or reduce unwanted side-effects, and/or to achieve an appropriate balance between achieving the desired therapeutic effect on the one hand and avoiding, limiting or reducing undesired side effects on the other hand.

Generally, the treatment regimen will be followed until the desired therapeutic effect is achieved and/or for as long as the desired therapeutic effect is to be maintained. Again, this can be determined by the clinician.

Further uses of the polypeptides or constructs, nucleic acids, genetic constructs and hosts and host cells of the invention will be clear to the skilled person based on the disclosure herein.

The aspects illustrated and discussed in this specification are intended only to teach those skilled in the art the best way known to the inventors to make and use the invention. Modifications and variations of the above-described aspects of the invention are possible without departing from the invention, as appreciated by those skilled in the art in light of the above teachings. It is therefore understood that, within the scope of the claims and their equivalents, the invention may be practiced otherwise than as specifically described.

The invention will now be further described by means of the following non-limiting preferred aspects, examples and figures.

The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference, in particular for the teaching that is referenced hereinabove.

5 **EXAMPLES**

Example 1: Material and methods related to TCR

1.1 *TCR $\alpha\beta$ /CD3 transfected cell lines*

Transient and stable CHO-K1 (ATCC: CCL-61), HEK293H (Life technologies, 11631-017), Llana (Fibroblast cells from llama Navel cord cells) cell lines with recombinant overexpression of all 6 chains of the full human T cell Receptor (TCR) complex were generated. For this, the coding sequences of the TCR alpha (α) and TCR beta (β) chain were cloned in a pcDNA3.1-derived vector, downstream of a CMV promotor and a 2A-like viral peptide sequence was inserted between both chains to induce ribosomal skipping during translation of the polyprotein. In the same vector, the coding sequences of the epsilon, delta, gamma and zeta chains of the CD3 complex were cloned downstream of an additional CMV promotor, also using 2A-like viral peptide sequences between the respective chains. In addition, a stable HEK293H clone with recombinant overexpression of the 4 chains of the human CD3 was generated as described above using a single gene vector.

The used sequences for the human CD3 and the human TCR α/β constant domains were derived from UniProtKB (CD3 delta: P04234, CD3 gamma: P09693, CD3 epsilon: P07766, CD3 zeta: P20963, TCR α : P01848 and TCR β : P01850; SEQ ID NOs: 70 to 75, respectively). The sequences for the human TCR α/β variable domains were derived from crystal structure sequences (PDB codes: 2IAN, 2XN9 and 3TOE) (human TCR α variable domains derived from 2IAN, 2XN9 and 3TOE with SEQ ID NOs: 343, 76 and 345, respectively; human TCR β variable domains derived from 2IAN, 2XN9 and 3TOE with SEQ ID NOs: 344, 77 and 346, respectively).

The cell surface expression of the human T cell receptor complex was confirmed by flow cytometry using a functional mouse IgG2b anti-human TCR α/β antibody, clone BW242/412 (Miltenyi, 130-098-219) and a functional mouse IgG2a anti-CD3 PE labelled antibody, clone OKT-3 (eBioscience, 12-0037) (Figure 1).

1.2 *Soluble recombinant TCR α/β proteins*

Soluble human and cynomolgus/rhesus monkey TCR α/β proteins were generated in house. The sequences for the extracellular part of the human TCR α/β constant domain were derived from UniProtKB (TCR α : P01848 and TCR β : P01850; SEQ ID NOs: 74 and 75, respectively). The human TCR

α/β variable domains were derived from crystal structure sequence (PDB code: 2XN9; SEQ ID NOs: 76 and 77, respectively for α and β chain).

The sequences for the extracellular part of the cynomolgus/rhesus monkey TCR α/β constant domains were derived from GenBank files EHH63463 and AEA41868 respectively (SEQ ID NOs: 347 and 348). The sequences for the cynomolgus/rhesus monkey TCR α/β variable domains were derived from AEA41865 and AEA41866 (SEQ ID NOs: 349 and 350, respectively for α and β chain).

The extracellular domains of human TCR α/β (2XN9) or cynomolgus/rhesus monkey TCR α/β were fused to a zipper protein coding sequence (O'Shea et al. 1993 Curr. Biol. 3(10): 658-667), produced by CHOK1SV cells (Lonza) using Lonza's GS Gene Expression System™ and subsequently purified.

Quality of the TCR α/β zipper proteins was assessed in an ELISA binding assay. Maxisorp 96-well ELISA plates (Nunc) were coated with 2 μ g/mL soluble recombinant human TCR α/β (2XN9)-zipper protein or soluble recombinant cynomolgus TCR α/β -zipper protein. After an overnight incubation, plates were washed and blocked with PBS + 1% casein for 1h at room temperature. Next, plates were incubated with serial dilutions of either a functional flag tagged Nanobody or the functional mouse IgG anti-non-human primate/Rat TCR α/β antibody, clone R73 (eBioscience, 16-5960) for 1h at room temperature while shaking, washed again and incubated with Monoclonal ANTI-FLAG M2-Peroxidase (HRP) (Sigma, A8592), respectively Peroxidase-Conjugated Rabbit Anti-Mouse Immunoglobulins (Dako, P0260). After 1h, TMB One Solution (Promega, G7431) was added. The reaction was stopped with 2M H₂SO₄ and the dose dependent binding was determined by measuring the OD at 450nm using the Tecan sunrise 4 (Figure 2).

Example 2: Immunization of llamas with TCR/CD3, cloning of the heavy chain-only antibody fragment repertoires and preparation of phages

2.1 Immunization

It was set out to generate heavy chain only antibodies in camelidae (e.g. llama and alpaca) against T cell receptor (TCR) α and/or β constant chains. Although the native T cell receptor complex consists of both CD3 (gamma, delta, epsilon and zeta) chains, as well as TCR α - and β -chains, it was hypothesized that the absence of CD3 chains would facilitate access to the constant domains of the TCR. Especially since the CD3 chains laterally surround, and limit access to the constant domains of the TCR α - and β -chains. Contrary to our experience with other targets, the obtaining of an immune response against TCR α - or β -chains was not as straight forward as expected.

In a final approach, after approval of the Ethical Committee (CRIA, LA1400575, Belgium- EC2012#1), the inventors attempted a complex immunization protocol with DNA encoding for T cell complex. In short, 3 additional llamas were immunized with a pVAX1-human TCR(2IAN)/CD3 (described in Example 1.1) plasmid vector (Invitrogen, Carlsbad, CA, USA) and with a pVAX1-human TCR α/β (2XN9)/CD3 (described in Example 1.1) plasmid vector (Invitrogen, Carlsbad, CA, USA) according to standard protocols. Two llamas received additionally 1 subcutaneous injection of primary human T cells. Human T cells were collected from Buffy Coat blood, from healthy volunteers (Blood bank Gent) using RosetteSep (StemCell Technologies, 15061) followed by enriching on Ficoll-PaqueTM PLUS (GE Healthcare, 17-1440-03) according to manufactures instructions and stored in liquid nitrogen. After thawing, cells were washed, and re-suspended in D-PBS from Gibco and kept on ice prior to injection.

2.2 Cloning of the heavy chain-only antibody fragment repertoires and preparation of phages

Per animal, blood samples were collected after the injection of one type of immunization antigen. From these blood samples, PBMC were prepared using Ficoll-Hypaque according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). For each immunized llama, libraries were constructed by pooling the total RNA isolated from samples originating from a certain subset of the immunization schedule, *i.e.* after one type of immunization antigen.

In short, the PCR-amplified VHH repertoire was cloned via specific restriction sites into a vector designed to facilitate phage display of the VHH library. The vector was derived from pUC119. In frame with the VHH coding sequence, the vector encodes a C-terminal 3xFLAG and His6 tag. Phages were prepared according to standard protocols (see for example WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858 and other prior art and applications filed by Ablynx N.V. cited herein).

Example 3: Selection of TCR/CD3 specific VHHs via phage display

The vast majority of selected VHHs were directed against the variable regions of either the TCR α or TCR β chain. Therefore different selection and counter-selection strategies had to be devised by the inventors.

In short, VHH repertoires obtained from all llamas and cloned as phage library were used in different selection strategies, applying a multiplicity of selection conditions. Selections using human TCR/CD3 transfected cell lines with the same variable domain as used during immunization resulted in only variable domain binders. Therefore, tools containing a different variable TCR α/β domain (transfected

cells (described in Example 1.1), soluble protein (described in Example 1.2), or human primary T cells (isolated as described in Example 2.1)) were used during selections and proved to be crucial in identification of constant domain binders. Additional variables during selections included the antigen presentation method (in solution when using cells or coated onto plates when proteins), the antigen concentration, the orthologue used (human or cynomolgus recombinant TCR α/β protein), and the number of selection rounds. All solid coated phase selections were done in Maxisorp 96-well plates (Nunc, Wiesbaden, Germany).

Selections were performed as follows: TCR α/β -CD3 antigen preparations for solid and solution phase selection formats were presented as described above at multiple concentrations. After 2h incubation with the phage libraries, followed by extensive washing, bound phages were eluted with trypsin (1 mg/mL) for 15 minutes. The trypsin protease activity was immediately neutralized by applying 0.8 mM protease inhibitor ABSF. As control, selections without antigen were performed in parallel.

Phage outputs were used to infect *E. coli* for analysis of individual VHH clones. Periplasmic extracts were prepared according to standard protocols (see for example WO 03/035694, WO 04/041865, WO 04/041863, WO 04/062551 and other prior art and applications filed by Ablynx N.V. cited herein).

Example 4: Screening, sequence analysis and purification

4.1 Screening for TCR/CD3 binding Nanobodies in a flow cytometry assay

Periplasmic extracts were screened for cell expressed TCR/CD3 binding using human TCR/CD3 transfected CHO-K1 or HEK293H cells and the respective CHO-K1 or HEK293H reference cell line in a mixed cell line setup. To this end, a large batch of the reference cell lines were labelled with 8 μ M PKH26 and frozen. 5×10^4 PKH labelled reference cells were mixed with 5×10^4 target cells and incubated with periplasmic extracts for 30min at 4°C, and washed 3 times. Next, cells were incubated with 1 μ g/ml monoclonal ANTI-FLAG® M2 antibody (Sigma-Aldrich, F1804) for 30 min at 4°C, washed again, and incubated for 30 min at 4°C with 5 μ g/ml Allophycocyanin (APC) AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch, 115-135-164). Samples were washed, resuspended in FACS Buffer (D-PBS from Gibco, with 10% FBS from Sigma and 0.05% sodium azide from Merck) and then analysed via a BD FACSArray. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 20,000 cells were counted during acquisition. Based on PKH26-SSC distribution, the PKH labelled parental population and the human TCR/CD3 unlabelled target population was selected. For these 2 populations the mean APC value was calculated.

4.2 *Screening for TCR/CD3 binding Nanobodies in a human T cell activation assay*

After several attempts, it turned out that activation of purified human T cells by antibodies or Nanobodies according to standard protocols, *i.e.* coated onto a 96 well plate, was not sensitive enough (data not shown).

5 In order to assess activity, a different assay was developed, based on bead coupled T cell activation. In short, Dynabeads® Goat Anti-Mouse IgG (ThermoFisher Scientific, 11033) were coated with monoclonal mouse ANTI-FLAG® M2 antibody (Sigma-Aldrich, F1804) (15µg/1E7beads). After an incubation period of 2h at 4°C, Dynabeads® were washed and incubated with 80µl periplasmic extract for 20 min at 4°C while shaking. Non-coupled Nanobodies were washed away before adding
 10 the bead complex together with soluble mouse anti-CD28 antibody (Pelicuster CD28 – Sanquin, M1650) to purified primary human T cells (isolated as described in Example 2.1). As control condition, non-stimulated human T cells were used. In brief, Dynabeads® Goat Anti-Mouse IgG (ThermoFisher Scientific, 11033) coupled to monoclonal mouse ANTI-FLAG® M2 antibodies were incubated in 80µl periplasmic extract containing irrelevant Nanobodies. After removal of the non-coupled Nanobodies
 15 during a wash step, the irrelevant Nanobody-bead complex was added to purified primary human T cells. After an incubation of 24h at 37° and 5% CO₂ the activation status of the human T cells was determined by measuring the CD69 expression level in flow cytometry using monoclonal mouse anti-human CD69PE (BD Biosciences, 557050).

20 4.3 *Sequence analysis of the obtained Nanobodies*

Nanobodies which scored positive in the flow cytometric binding screen and the T cell activation assay were sequenced.

The sequence analysis resulted in the identification of Nanobody T0170056G05 and different family members thereof, representing a total of 104 different clones (SEQ ID NOs: 42 and 78 to 180).

25 Corresponding alignment is provided (Table A-1).

The sequence variability of the CDRs of the family members against T0170056G05, is depicted in the tables below.

Table B-4

56G05	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3	4	5*	6	7	8	9	10
56G05 sequence	G	D	V	H	K	I	N	F	L	G
variations		A		Y	L	L		I		S
variations		S						V		
variations		E								
variations		G								

* in case position 5 is an L, then position 6 is also L

Table B-5

56G05	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
56G05 sequence	H	I	S	I	G	D	Q	T	D
variations	T		T		S		D	V	A
variations	R		A		A		E	A	Q
variations							T		N
variations							A		V
variations							V		S

5 Table B-6

56G05	CDR3								
Kabat numbering	95	96	97	98	99	100	100a	101	102
absolute numbering	1	2	3	4	5	6	7	8	9
56G05 sequence	F	S	R	I	Y	P	Y	D	Y
variations	Y			L	W			N	
variations	G							S	
variations	L								

4.4 Purification of monovalent Nanobodies

Two representative Nanobodies of the identified family were selected and expressed in *E. coli* TG1 as triple Flag, His6-tagged proteins. Expression was induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37°C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets. These extracts were used as starting material and Nanobodies were purified via IMAC and size exclusion chromatography (SEC).

The Nanobodies were purified to 95% purity as assessed via SDS-PAGE (data not shown).

Example 5: Binding of anti-TCR Nanobodies to human TCR/CD3 expressed on CHO-K1 cells and to purified primary human T cells

Dose-dependent binding of the purified monovalent anti-TCR Nanobodies to human TCR α/β (2XN9)/CD3 expressed on CHO-K1 cells and to purified primary human T cells was evaluated by flow cytometry. In brief, cells were harvested and transferred to a V-bottom 96-well plate (1×10^5 cells/well) and serial dilutions of Nanobodies (starting from 1 μ M) were allowed to associate for 30 minutes at 4°C in FACS buffer. Cells were washed three times by centrifugation and probed with 1 μ g/ml monoclonal mouse ANTI-FLAG® M2 antibodies (Sigma-Aldrich, F1804) for 30 minutes at 4°C, washed again, and incubated for 30 min at 4°C with 5 μ g/ml R-Phycoerythrin AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch 115-116-071). After incubation, cells were washed 3 times with FACS Buffer. Subsequently, cells were resuspended in FACS buffer supplemented with 5 nM TOPRO3 (Molecular Probes, T3605) to distinguish live from dead cells, which are removed during the gating procedure. Cells were analysed using a FACS Array flow cytometer (BD Biosciences) and Flowing Software. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 10000 cells were counted during acquisition. From this population the TOPRO+ cells (dead cells) were excluded and the median PE value was calculated.

The results are shown in Figure 3. The EC50 values obtained from the dose response curve are represented in Table C-1.

Table C-1: EC50 (M) of anti-TCR monovalent Nanobodies for binding CHO-K1 human TCR α/β (2XN9)-/CD3 cells and for binding purified primary T cells as determined in flow cytometry.

	CHO-K1 TCR α/β (2XN9)/CD3			Primary human T cells		
sample ID	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
T0170055A02	8.4E-09	7.2E-09	9.7E-09	9.1E-08	8.1E-08	1.0E-07
T0170056G05	8.9E-09	8.3E-09	9.4E-09	9.1E-08	8.3E-08	9.9E-08

The Nanobodies clearly bound to human TCR/CD3 expressed on CHO-K1 cells. The Nanobodies also bound to purified primary human T cells, although with slightly lower potency compared to the CHO-K1 human TCR α/β (2XN9)/CD3 cells.

5 Example 6: Determination of binding epitope

Binding of the purified monovalent anti-TCR Nanobodies to human TCR α/β (2IAN)/CD3 expressed on HEK293H cells was evaluated and compared with the binding to HEK293H cells transfected with human CD3 in flow cytometry, as outlined in Example 5. Dilution series of T0170055A02 and T0170056G05 starting from 1 μ M were applied to the cells. The parental HEK293H cell line was included as TCR/CD3 negative cell line.

The results are shown in Figure 4. The EC50 values obtained from the dose response curve are depicted in Table C-2.

Table C-2: EC50 (M) of anti-TCR monovalent Nanobodies for binding human TCR α/β (2IAN)/CD3 or human CD3 expressed on HEK293H cells, as determined in flow cytometry.

Sample ID	HEK293H wt		HEK293H CD3		HEK293H TCR/CD3	
	EC50	MCF at 1 μ M	EC50	MCF at 1 μ M	EC50	MCF at 1 μ M
T0170055A02	No fit	246	No fit	1194	5.5E-08	91229
T0170056G05	No fit	299	No fit	352	8.4E-08	86510

The Nanobodies clearly bound to human TCR(2IAN)/CD3 expressed on HEK293H but not to the HEK293H cells transfected with human CD3 only, nor to the HEK293H parental cell line. In conclusion, the 2 clones were specific for binding to human TCR α/β . No binding was observed to human CD3.

Example 7: Binding of anti-TCR Nanobodies to soluble recombinant human TCR α/β protein

20 7.1 Binding of anti-TCR Nanobodies to human T cell receptor protein in ELISA

Binding of the purified monovalent TCR Nanobodies to soluble recombinant human TCR α/β protein was evaluated in ELISA (as described in Example 1.2) using 2 μ g/ml directly coated soluble recombinant human TCR α/β protein.

The results are shown in Figure 5. The EC50 values obtained from the dose response curve are depicted in Table C-3.

Table C-3: EC₅₀ (M) of anti-TCR monovalent Nanobodies for binding soluble recombinant human TCR α/β (2XN9) protein, as determined in ELISA.

sample ID	EC ₅₀ (M)	95% LCI	95% UCI
T0170055A02	1.9E-09	1.7E-09	2.2E-09
T0170056G05	4.0E-09	3.5E-09	4.6E-09

The anti-TCR Nanobodies bound to soluble recombinant human TCR α/β protein.

5

7.2 Binding of anti-TCR Nanobodies to human T cell receptor protein in BLI

Binding affinities were measured using Bio-Layer Interferometry (BLI) on an Octet RED384 instrument (Pall ForteBio Corp.). Recombinant human soluble TCR α/β (2XN9)-zipper protein was covalently immobilized on amine-reactive sensors (ForteBio) via NHS/EDC coupling chemistry. For kinetic analysis, sensors were first dipped into running buffer (10mM Hepes, 150mM NaCl, 0.05% p20, pH7.4 from GE Healthcare Life Sciences) to determine baseline setting. Subsequently, sensors were dipped into wells containing different concentrations of purified Nanobodies (range between 1.4 nM and 1 mM) for the association step (180s) and transferred to wells containing running buffer for the dissociation (15 min) step. Affinity constants (KD) were calculated applying a 1:1 interaction model using the ForteBio Data Analysis software.

15

The results are depicted in Figure 6. The binding characteristics are listed in Table C-4.

Table C-4: Kinetic analysis of anti-TCR monovalent Nanobodies for binding soluble recombinant human TCR α/β (2XN9) protein as determined with the Octet RED384 instrument.

	human soluble TCR α/β (2XN9)-zipper protein		
sample ID	kon(1/Ms)	koff(1/s)	KD (M)
T0170055A02	4.9E+04	8.4E-04	1.7E-08
T0170056G05	5.0E+04	1.2E-03	2.4E-08

The binding affinities determined using BLI on human soluble TCR α/β (2XN9)-zipper protein showed correlation with the affinities determined on CHO-K1 human TCR α/β (2XN9)/CD3 cells in flow cytometry (cf. Example 5).

20

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Example 8: Binding of anti-TCR Nanobodies to recombinant cynomolgus soluble TCR α/β protein**8.1 Binding of anti-TCR Nanobodies to cynomolgus T cell receptor protein in ELISA**

Binding of purified monovalent anti-TCR Nanobodies to recombinant cynomolgus soluble TCR α/β protein was evaluated in ELISA (as described in Example 1.2) using 2 μ g/ml directly coated
 5 recombinant cynomolgus soluble TCR α/β zipper protein.

The EC50 values obtained from the dose response curve are depicted in Table C-5. An exemplary result is shown in Figure 7.

Table C-5: EC50 (M) of anti-TCR monovalent Nanobodies for binding to recombinant cynomolgus soluble TCR α/β -zipper protein as determined in ELISA.

sample ID	EC50 (M)	95% LCI	95% UCI
T0170055A02	1.6E-07	1.5E-07	1.7E-07
T0170056G05	7.7E-08	6.6E-08	9.1E-08

10 The results indicated that the anti-TCR Nanobodies bind to the recombinant cynomolgus soluble TCR α/β -zipper protein.

8.2 Binding of anti-TCR Nanobodies to cynomolgus T cell receptor protein in BLI

15 Binding affinities of the monovalent anti-TCR Nanobodies were measured using Bio-Layer Interferometry (BLI) on an Octet RED384 instrument (Pall ForteBio Corp.) essentially as described in Example 7.2 using recombinant cynomolgus soluble TCR α/β protein.

The results are depicted in Figure 8. The binding characteristics of the anti-TCR Nanobodies are listed in Table C-6.

20 **Table C-6: Kinetic analysis of anti-TCR monovalent Nanobodies for binding recombinant cynomolgus soluble TCR α/β -zipper protein as determined with the Octet RED384 instrument.**

sample ID	kon(1/Ms)	koff(1/s)	KD (M)
T0170055A02	1.1E+05	2.4E-02	2.1E-07
T0170056G05	1.1E+05	1.6E-02	1.5E-07

The Nanobodies bind to the recombinant cynomolgus soluble TCR α/β protein with a 10 fold lower affinity compared to recombinant human soluble TCR α/β (2XN9)-zipper protein.

Example 9: Determination of purified primary human T cell activation capacity

Functionality of purified monovalent anti-TCR Nanobodies was evaluated in the human T cell activation assay. Dynabeads® Goat Anti-Mouse IgG (ThermoFisher Scientific, 11033) were coated with monoclonal mouse ANTI-FLAG® M2 antibody (Sigma-Aldrich, F1804, 15µg/1E7beads). After an incubation period of 2h at 4°C, Dynabeads® were washed and incubated with a fixed (1µg) amount of purified Flag tagged Nanobody for 20 min at 4°C while shaking. Non-coupled Nanobodies were washed away before adding the bead complex together with soluble mouse anti-CD28 antibody (Peliccluster CD28 – Sanquin, M1650) to purified primary human T cells isolated (isolated as described in Example 2.1) from distinct healthy donors. In addition, the effect of monovalent TCR binding by the Nanobodies was evaluated by the incubation of the Nanobody with the purified primary human T cells without prior capture onto anti-mouse IgG Dynabeads®, in the presence of anti-CD28 antibody. The activation status of the purified primary human T cells was monitored by measuring the CD69 expression in flow cytometry using monoclonal mouse anti-human CD69PE (BD Biosciences, 557050) after an incubation of 24h at 37°C and 5% CO₂.

In conclusion, the anti-TCR Nanobodies showed clear CD69 upregulation after capturing onto anti-mouse IgG dynabeads. The irrelevant Nanobody did not show any CD69 upregulation (Figure 9A). In addition, none of the Nanobodies presented in solution were able to activate purified primary human T cells as measured by increased expression of CD69 (Figure 9B).

Example 10: Immunization of llamas with CD123, cloning of the heavy chain-only antibody fragment repertoires and preparation of phage**10.1 Immunization**

Three llamas were immunized, according to standard protocols, with recombinant His-tagged extracellular domain of human CD123 (R&D Systems, 301-R3/CF) via an intramuscular injection in the neck using Stimune as adjuvant (Cedi Diagnostics, Lelystad, The Netherlands).

Immune serum samples taken at day 35 were analysed for antigen-specific binding by ELISA to adsorbed hCD123. All llamas show an excellent IgG 1 mediated serum response, and a good to moderate heavy chain mediated response against hCD123.

10.2 Cloning of the heavy chain-only antibody fragment repertoires and preparation of phage

Per animal, 100 mL blood samples were collected four and eight days after the last injection of the immunization antigen. From these blood samples, PBMC were prepared using Ficoll-Hypaque

according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA). For each immunized llama, libraries were constructed by pooling the total RNA isolated from different blood samples.

In short, the PCR-amplified VHH repertoire was cloned via specific restriction sites into a phagemid vector designed to facilitate phage display of the VHH library. The vector was derived from pUC119. In frame with the VHH coding sequence, the vector encodes a C-terminal 3xFLAG and HIS6 tag. Phages were prepared according to standard protocols (see for example WO 04/041865, WO 04/041863, WO 04/062551, WO 05/044858 and other prior art and applications filed by Ablynx N.V. cited herein).

Example 11: Selection of CD123 specific VHHs via phage display

VHH repertoires obtained from all llamas and cloned as phagemid library were used in different selection strategies, applying a multiplicity of selection conditions. Variables included: i) the source of CD123 antigen (recombinant protein produced in human cells or full length protein overexpressed on cells), ii) antigen presentation (in solution when using biotinylated recombinant ectodomain, directly coated onto plates for non-biotinylated Fc-fused ectodomain), and iii) the antigen concentration.

In brief, HEK293T cells overexpressing CD123 (generated in house), biotinylated human CD123 (R&D Systems, 301-R3/CF, biotinylated in house) and plate-coated human CD123-Fc (Sino Biologicals, 10518-H08H) were incubated for 1h-2h with 2×10^{11} phage particles of the different libraries followed by extensive washing; bound phages were eluted with trypsin (1 mg/mL) for 15 minutes and then the protease activity was immediately neutralized by applying 0.8 mM protease inhibitor ABSF. As control, selections with parental cell line or without antigen were performed in parallel.

Phage outputs were used to infect *E. coli* for analysis of individual VHH clones. Periplasmic extracts were prepared according to standard protocols (see for example WO 03/035694, WO 04/041865, WO 04/041863, WO 04/062551 and other prior art and applications filed by Ablynx N.V. cited herein).

Example 12: Screening for CD123 binding Nanobodies

12.1 Screening in binding ELISA

Periplasmic extracts were screened in a binding ELISA on human CD123 (R&D Systems, 301-R3). To this end, a microtiter plate was coated with human CD123 (1 µg/ml) and incubated overnight at 4 °C. Plates were blocked for one hour at room temperature with 4% Marvel in PBS. The plates were washed with PBS-Tween. The periplasmic extracts (1/10 diluted in PBS with 2% Marvel) were

incubated for at least 1 hour at RT. Plates were washed with PBS-Tween, after which binding of VHH was detected with Monoclonal ANTI-FLAG M2-Peroxidase (HRP), (Sigma, A8592, 1/5000) in PBS with 1% Marvel. Staining was performed with the substrate esTMB (Nalgene) and the signals were measured after 15 minutes at 450 nm.

5

12.2 Screening in flow cytometry

Periplasmic extracts were screened in a flow cytometry assay on transient transfected HEK293T-hCD123 cells in 96-wells format. In addition, binding was assessed to endogeneously IL-3R expressing MOLM-13 cells, to confirm binding to IL-3R α in the presence of the IL-3R β partner in the heterodimeric receptor complex. To this end, the cells (1×10^5 cells/ well/ 0.1 mL) were incubated with the periplasmic extracts (1:10 dilutions) for 30 min at 4°C in FACS buffer (D-PBS from Invitrogen, with 10% FBS from Sigma and 0.05% sodium azide from Merck). Cells were washed 3 times, and incubated with 1 μ g/ml monoclonal ANTI-FLAG[®] M2 antibody (Sigma-Aldrich, F1804) for 30 min at 4°C, washed again, and incubated for 30 min at 4°C with goat anti-mouse RPE labelled antibody (Jackson ImmunoResearch, 115-116-071, 1:100). Samples were washed, incubated with TOPRO3 to stain for dead cells in FACS Buffer and fluorescence was assessed on a FACSArray device (BD).

15

12.3 Sequencing analysis of Nanobodies

Nanobodies which scored positive in the binding ELISA and the flow cytometry assay were sequenced. The sequence analysis resulted in the identification of Nanobodies A0110056A10 and A0110055F03 and different family members thereof. Corresponding alignments are provided in Table A-2 and Table A-3, respectively.

20

The sequence variability of the CDRs of the family members against A0110056A10, is depicted in the tables below.

Table B-7

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56A10	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3*	4	5	6**	7	8	9	10
56A10 sequence	G	I	T	S	K	I	N	D	M	G
variations			S			S	D	V		
variations			P					A		

* in case position 3 is an S, then position 7 is an D.

** in case position 6 is an I, then position 8 is an D.

Table B-8

56A10	CDR2								
Kabat numbering	50	51	52	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6	7	8	9
56A10 sequence variations	S	I	T	A	T	G	T	T	N

Table B-9

56A10	CDR3						
Kabat numbering	95	96	97	98	99	100	101
absolute numbering	1	2	3	4	5	6	7
56A10 sequence variations	F	P	P	I	S	N	F
			A				

- 5 The sequence variability of the CDRs of the family members against A011005F03, is depicted in the tables below.

Table B-10

55F03	CDR1									
Kabat numbering	26	27	28	29	30	31	32	33	34	35
absolute numbering	1	2	3*	4	5	6**	7	8	9	10
55F03 sequence variations	G	R	T	F	S	S	Y	V	M	G

Table B-11

55F03	CDR2									
Kabat numbering	50	51	52	52a	53	54	55	56	57	58
absolute numbering	1	2	3	4	5	6*	7	8	9	10
55F03 sequence variations	A	I	Y	W	S	N	G	K	T	Q
			W			S				E

* in case position 6 is an S, then position 10 is an E.

Table B-12

55F03	CDR3															
Kabat numbering	95	96	97	98	99	100	100a	100b	100c	100d	100e	100f	100g	100h	101	102
absolute numbering	1	2	3	4*	5	6	7	8	9	10	11	12	13	14	15	16
55F03 sequence	D	K	D	E	T	G	F	R	T	L	P	I	A	Y	D	Y
variations				R	D											
variations					Y											

* in case position 4 is an R, then position 5 is a D or Y.

5

12.4 Purification of monovalent Nanobodies

Representative Nanobodies for each family were selected and expressed in *E. coli* TG1 as triple Flag, His6-tagged proteins. Expression was induced by addition of 1 mM IPTG and allowed to continue for 4 hours at 37°C. After spinning the cell cultures, periplasmic extracts were prepared by freeze-thawing the pellets. These extracts were used as starting material and Nanobodies were purified via IMAC and size exclusion chromatography (SEC). The Nanobodies were purified to 95% purity as assessed via SDS-PAGE (data not shown).

10

Example 13: Additional cell lines for characterisation

13.1 CD123 transfected cell lines

Stable HEK293 Flp-In (Invitrogen, R750-07) and CHO Flp-In (Invitrogen, R758-07) cell lines with recombinant overexpression of CD123 were generated using the Flp-In™ site-directed recombination technology (Flp-In™ System For Generating Stable Mammalian Expression Cell Lines by Flp Recombinase-Mediated Integration (Invitrogen, K601001, K601002)). Hereby, DNA integration occurs at a specific genomic location at an FRT (Flp Recombination Target) site by the Flp recombinase (pOG44) derived from *Saccharomyces cerevisiae*. The Flp-In™ host cell line and expression plasmid (pcDNA5) both contain this FRT site, thereby allowing a single homologous DNA recombination. The sequence for human CD123 was derived from NCBI RefSeq NP_002174, the sequence of cynomolgus CD123 was derived from NCBI genbank no. EHH61867.1 (SEQ ID NOs: 68 and 69, respectively). The cell surface expression of human and cynomolgus CD123 was confirmed by flow cytometry using the mouse monoclonal anti-CD123 antibody (BD Biosciences, 554527) and the mouse IgG2a isotype control (BD Bioscience, 16-4724-85). In brief, cells (1x10⁵ cells/well) were harvested and transferred to a V-bottom 96-well plate (Greiner Bio-one, 651 180) and stained at 4°C with mouse monoclonal

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anti-CD123 antibody (0.25µg/ml) and with mouse IgG2a isotype control (0.25µg/ml). After 30 min of incubation, cells were pelleted by centrifugation and washed 3 times with FACS Buffer (D-PBS from Gibco with 10% FBS (Sigma, F7524) and 0.05% sodium azide (Acros organics, 190380050)). Next, cells were incubated with 5µg/ml R-Phycoerythrin AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch, 115-116-071) for 30 minutes at 4°C. After incubation, cells were washed 3 times with FACS Buffer. Subsequently, cells were resuspended in FACS buffer supplemented with 5 nM TOPRO3 (Molecular Probes, T3605) to distinguish live from dead cells. Cells were analysed using a FACS Array flow cytometer (BD Biosciences) and Flowing Software. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 10000 cells were counted during acquisition. From this population the TOPRO+ cells (dead cells) were excluded and the median PE value was calculated. The data are shown in Figure 10.

13.2 U937, MOLM-13, KG1a and NCI-H929 cell lines

The expression level of human CD123 on MOLM-13 (DSMZ, ACC-554), U-937 (ATCC®, CRL-1593.2™), KG1a (ATCC®, CCL246.1™) and NCI-H929 (DSMZ, ACC-163) was determined using the APC-labelled mouse monoclonal anti-CD123 antibody (BD Biosciences, 560087) and the APC-labelled isotype control (Biolegend, 400220) in flow cytometry. In brief, cells were harvested and suspended at a density of 1x10⁷ cells/ml in FACS buffer with 25µg human Fc block (BD Biosciences, 564220) and incubated for 10 min at RT. Next, cells were diluted to a cell concentration of 1x10⁶ cells/ml and transferred to a V-bottom 96-well plate (1x10⁵ cells/well). Cells were stained at 4°C with APC-labelled mouse monoclonal anti-CD123 antibody (diluted 10 times) and the APC-labelled isotype control (diluted 10 times). After 30 min of incubation, cells were washed 3 times and resuspended in FACS Buffer supplemented with 1µg/ml Propidium iodine (PI) (Sigma, P4170) for 30 min at 4°C and then analysed via a BD FACSCanto and Flowing software. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. 10000 cells were counted within P1. From this population the PI+ cells (dead cells) were excluded and the median APC value was calculated. The data are shown in Figure 11.

In addition, the number of receptors per cell was determined using the QIFIKIT (Dako, K0078) according to manufacturer's instructions. The data are shown in Table C-7.

Table C-7: Number of CD123 molecules per cell.

	MOLM-13	KG1a
CD123 molecules/ cell	6543	3353

Example 14: Binding of monovalent anti-CD123 Nanobodies to endogenously CD123 expressing cell lines

Dose-dependent binding of the purified monovalent anti-CD123 Nanobodies to endogenously CD123 expressing cell lines MOLM-13 and KG1a was evaluated by flow cytometry. In brief, cells were harvested and transferred to a V-bottom 96-well plate (1×10^5 cells/well) and serial dilutions of Nanobodies (starting from $1 \mu\text{M}$) were allowed to associate for 30 minutes at 4°C in FACS buffer. Cells were washed three times by centrifugation and probed with monoclonal mouse ANTI-FLAG® M2 antibody (Sigma-Aldrich, F1804) for 30 minutes at 4°C , washed again, and incubated for 30 min at 4°C with $5 \mu\text{g/ml}$ R-Phycoerythrin AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (Jackson Immunoresearch, 115-116-071). After incubation, cells were washed 3 times with FACS Buffer. Subsequently, cells were resuspended in FACS buffer supplemented with 5 nM TOPRO3 (Molecular Probes, T3605) to distinguish live from dead cells, which are removed during the gating procedure. Cells were analysed using a FACS Array flow cytometer (BD Biosciences) and Flowing Software. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 10000 cells were counted during acquisition. From this population the TOPRO+ cells (dead cells) were excluded and the median PE value was calculated.

Binding of the Nanobodies to MOLM-13 and KG1a is presented in Figure 12. The EC50 values obtained from the dose response curves are depicted in Table C-8.

Table C-8: EC50 (M) of monovalent anti-CD123 Nanobodies for binding to MOLM-13 and KG1a as determined in flow cytometry.

Sample ID	MOLM-13			KG1a		
	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
A0110056A10	$6.3\text{E-}10$	$3.8\text{E-}10$	$8.8\text{E-}10$	$5.0\text{E-}10$	$2.4\text{E-}10$	$7.6\text{E-}10$
A0110055F03	$>1\text{E-}07$			$>1\text{E-}07$		

There was binding of both Nanobodies to the CD123 endogenously expressing cells (MOLM-13, KG1a).

Example 15: Binding of monovalent anti-CD123 Nanobodies to CD123 on transfected cells

Dose-dependent binding of the purified monovalent anti-CD123 Nanobodies to human CD123 overexpressing CHO-K1 and cynomolgus CD123 overexpressing HEK293 cells was evaluated by flow cytometry.

To detect the binding of A0110055F03, cells were harvested and transferred to a V-bottom 96-well plate (1×10^5 cells/well). Serial dilutions of A0110055F03 (starting from 100nM) were allowed to

associate for 30 minutes at 4°C in FACS buffer. Cells were washed 3 times with FACS buffer by centrifugation and probed with 1µg/ml monoclonal mouse ANTI-FLAG® M2 antibody (Sigma-Aldrich, F1804) for 30 minutes at 4°C to detect bound Nanobody. Detection was done with 0.5µg/ml R-Phycoerythrin AffiniPure F(ab')₂ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch, 115-116-071) for 30 minutes at 4°C. Cells were washed and incubated with TOPRO3 to stain for dead cells, which are then removed during the gating procedure. The cells were then analysed via a BD FACSArray. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 10000 cells were counted during acquisition. From this population the TOPRO+ cells (dead cells) were excluded and the median PE value was calculated.

To detect the binding of A0110056A10, cells were harvested and transferred to a V-bottom 96-well plate (1x10⁵ cells/well). Serial dilutions of Alexa647-labelled A0110056A10 (starting from 100nM) were allowed to associate for 30 minutes at 4°C in FACS buffer. After 30 min of incubation, cells were pelleted by centrifugation and washed 3 times with FACS Buffer. Subsequently, cells were resuspended in FACS buffer supplemented with 1µg/ml Propidium iodine to distinguish live from dead cells. Cells were analysed using a FACS Array flow cytometer (BD Biosciences) and Flowing Software. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 10000 cells were counted during acquisition. From this population the PI+ cells (dead cells) were excluded and the median APC-value was calculated.

Binding of the Nanobodies to the CD123 transfected cell lines and reference cell line is presented in Figure 13 and Figure 14, for Nanobody A0110056A10 and A0110055F03 respectively. The EC50 values obtained from the dose response curves are depicted in and Table C-9.

Table C-9: EC50 (M) of monovalent anti-CD123 Nanobodies for binding to huCD123 and cyCD123 transfected cells as determined in flow cytometry.

Sample ID	HEK Flp-In cyCD123 transfected cells			CHO Flp-In huCD123 transfected cells		
	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
A0110056A10	2.48E-09	1.90E-09	3.20E-09	9.74E-10	8.50E-10	1.11E-09
A0110055F03	2.53E-09	2.04E-09	3.14E-09	5.94E-09	1.00E-09	3.52E-08

There was binding of both Nanobodies to the CD123 transfected cell lines. Both Nanobodies are human - cynomolgus CD123 cross-reactive.

Example 16: Nanobody competition for binding to CD123 expressed on cells in flow cytometry

To investigate whether the CD123 Nanobodies compete with each other, a Nanobody competition assay was set up. To this end, a large batch of A0110056A10 was labelled with Alexa647 and frozen. Next, cells were harvested and transferred to a V-bottom 96-well plate (1×10^5 cells/well) and mixed with a serial dilution of the Nanobodies and a fixed concentration of A0110056A10-Alexa647 (0.7nM for MOLM-13, 0.5nM for CHO Flp-In human CD123). The A0110056A10-Alexa647 concentrations used in the assay were below the EC50 value for binding of A0110056A10-Alexa647 to the respective cells (binding curves are depicted in Figure 15). After an incubation period of 90 min at 4°C, the binding of A0110056A10-Alexa647 was determined in flow cytometry. Thereto, cells were washed 3 times and resuspended in FACS Buffer supplemented with 1µg/ml Propidium iodine, incubated for 30 min at 4°C and then analysed via a BD FACSArray. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. 10000 cells were counted within P1. From this population the PI+ cells (dead cells) were excluded and the median APC value was calculated.

The results are presented in Figure 16. The IC50 values obtained from the dose response curves are depicted in Table C-10.

Table C-10: IC50 (M) of monovalent anti-CD123 Nanobodies in the A0110056A10 Nanobody competition assay.

Sample ID	MOLM-13			CHO Flp-In human CD123		
	IC50 (M)	95% LCI	95% UCI	IC50 (M)	95% LCI	95% UCI
A0110056A10	1.4E-09	1.1E-09	1.8E-09	6.7E-09	5.3E-09	8.5E-09
A0110055F03	>1E-07			/	/	/

The non-labelled A0110056A10 competed with A0110056A10-Alexa647 for binding to CD123 on MOLM-13 cells and to human CD123 expressed on transfected CHO Flp-In cells, as expected. Nanobody A0110055F03 did not compete with A0110056A10-Alexa647 for binding to CD123 on the human CD123 transfected CHO Flp-In cells. On the MOLM-13 cell line, A0110055F03 did only compete with A0110056A10-Alexa647 at the highest concentrations tested.

Example 17: Competition with mouse monoclonal anti-CD123 antibody (clone 7G3) for binding to CD123 expressed on cells in flow cytometry

To examine whether the anti-CD123 Nanobodies compete with the mouse monoclonal anti-CD123 antibody (clone 7G3) for binding to human CD123 on cells, a mouse monoclonal anti-CD123 antibody (clone 7G3) competition assay was performed using a flow cytometry based methodology as

described in Example 16. To this end, serial dilutions of Nanobodies and an EC30 concentration of the APC-labelled mouse monoclonal anti-CD123 antibody (clone 7G3) (BD Biosciences, 560087) were incubated for 90 min with the cells after which antibody binding was determined in flow cytometry. Binding curves of APC-labelled mouse monoclonal anti-CD123 antibody (clone 7G3) to MOLM-13 and human CD123 transfected CHO Flp-In cells are depicted in Figure 17.

The results from the competition experiments are presented in Figure 18. The IC50 values obtained from the dose response curves are depicted in Table C-11.

Table C-11: IC50 (M) of monovalent anti-CD123 Nanobodies in the mouse monoclonal anti-CD123 antibody (clone 7G3) competition assay.

Sample ID	MOLM-13			CHO Flp-In human CD123		
	IC50 (M)	95% LCI	95% UCI	IC50 (M)	95% LCI	95% UCI
A0110056A10	1.1E-09	1.1E-09	1.2E-09	6.5E-09	6.1E-09	6.9E-09
A0110055F03	4.8E-07	1.8E-07	1.2E-06			

Nanobody A0110056A10 showed competition with mouse monoclonal anti-CD123 antibody (clone 7G3) on the MOLM-13 and the human CD123 transfected CHO Flp-In cells; therefore the epitopes of mouse monoclonal anti-CD123 antibody (clone 7G3) and Nanobody A0110056A10 are at least partially overlapping. A0110055F03 competed with mouse monoclonal anti-CD123 antibody (clone 7G3) on the MOLM-13 cells. The absence of competition with mouse monoclonal anti-CD123 antibody (clone 7G3) on the human CD123 transfected CHO Flp-In cell line might be the result of the lower affinity of Nanobody A0110055F03 for human CD123.

Example 18: Competition with mouse monoclonal anti-CD123 antibody (clone 7G3) for binding to recombinant human CD123 in ELISA

To investigate whether the anti-CD123 Nanobodies compete with the mouse monoclonal anti-CD123 antibody (clone 7G3) for binding to recombinant human CD123 protein, a competition assay was performed using an ELISA based methodology. Briefly, mouse monoclonal anti-CD123 antibody (clone 7G3) (BD Biosciences, 554527) was coated at 1µg/ml in PBS. After an overnight incubation at 4°C, plates were blocked with casein (1% in PBS) at room temperature. Next, a serial dilution of the monovalent anti-CD123 Nanobodies and 4nM of in house biotinylated-CD123 recombinant protein (R&D Systems, 301-R3/CF) was added and incubated for 1h at room temperature in PBS + 0.1% Casein + 0.05 % Tween. The concentration of the in house biotinylated-CD123 recombinant protein was based on the EC30 value obtained from the binding of in house biotinylated-CD123 recombinant protein to the mouse monoclonal anti-CD123 antibody (clone 7G3) (binding curve is depicted in Figure 19). The non-coated mouse monoclonal anti-CD123 antibody, (clone 7G3) was taken along as

positive control, the irrelevant anti-egg lysozyme Nanobody cAbLys was taken along as negative control. The plates were washed with PBS + 0.05% Tween using the Tecan Hydrospeed washer and 7G3 associated biotinylated-CD123 was detected via 1µg/ml extravidine peroxidase (Sigma, E2886) in PBS + 0.1% Casein + 0.05 % Tween, followed by development with eTMB substrate. The reaction was stopped with 1M HCl and the absorption at OD450nm was measured using the Tecan Infinite M1000.

The results are presented in Figure 20. The IC₅₀ value obtained from the dose response curve are depicted in Table C-12.

Table C-12: IC₅₀ (M) of monovalent Nanobody A010056A10 in the 7G3 competition ELISA.

Sample ID	IC ₅₀ (M)	95% LCI	95% UCI
A0110056A10	3.1E-09	1.5E-09	6.5E-09

Competition was observed between A0110056A10 and the mouse monoclonal anti-CD123 antibody (clone 7G3) for binding to the recombinant human CD123 protein. A0110055F03 did not compete with mouse monoclonal anti-CD123 antibody (clone 7G3) for binding to the recombinant human CD123 protein.

Example 19: Binding of monovalent anti-CD123 Nanobodies to human CD123 protein (SPR)

Binding affinities of the purified CD123 specific Nanobodies for human CD123 were evaluated by means of an SPR based assay on a ProteOn XPR36 instrument. Thereto, recombinant CD123 (R&D Systems, 301-R3-025/CF) was immobilized on a CM5 chip via amine coupling, using EDC and NHS chemistry. Purified Nanobodies were injected for 2 minutes at different concentrations (between 4.2nM and 1000 nM) for kinetic analysis via a one-shot kinetics approach. Flow rate was 45 µl/min and ProteOn running buffer (PBS, pH 7.4, 0.005% Tween 20) was used as running buffer. The dissociation time of the 1000 nM sample was 15 min. Evaluation of the association/dissociation data was performed by fitting a 1:1 interaction model (Langmuir binding model).

Table C-13: Binding characteristics of monovalent anti-CD123 Nanobodies determined in Proteon using directly coated human CD123 protein.

Sample ID	ka (1/Ms)	kd (1/s)	KD (M)
A0110056A10	5.3E+05	7.9E-04	1.5E-09
A0110055F03	2.5E+04	3.9E-03	1.6E-07

The KD values of the monovalent anti-CD123 Nanobodies for binding to human CD123 correlated with the binding data on cells. A0110056A10 has a better affinity compared to the A0110055F03.

Example 20: Construction of CD123/TCR multispecific polypeptides and control polypeptides

In order to obtain polypeptides capable of engaging T cells, the CD123 Nanobodies were linked with the anti-TCR (T cell receptor) Nanobody T0170056G05 (SEQ ID NO: 42). The latter is a Nanobody specifically binding the TCR α/β constant domain. (see WO 2016/180969, entitled "T cell recruiting polypeptides based on TCR alpha/beta reactivity", filed on May 13, 2016 by Ablynx N.V)

The therapeutic activity of T cell engaging polypeptides can be improved by the simultaneous targeting of multiple epitopes on a tumour associated antigen. Not only can tumour cells create an escape mechanism by the down-regulation of targeted antigens within a therapy, but also by introducing (point-)mutations. Simultaneous targeting of multiple epitopes on an antigen is likely to reduce the probability of generating tumour escape variants. Furthermore, targeting multiple epitopes on a single antigen can increase the affinity of binding (avidity effect). For this multivalent tumour antigen targeting concept, the two Nanobodies reactive towards the CD123 antigen were linked with Nanobody T0170056G05 against the TCR/CD3 complex.

The specific order of the respective Nanobodies was varied within the format. The effector and tumour Nanobodies were genetically linked with a 35GS linker and subsequently expressed in the yeast *Pichia* according to standard protocols (multispecific polypeptides). In parallel, irrelevant polypeptides were generated by replacing one or both of the tumour reactive Nanobodies with the irrelevant anti-egg lysozyme Nanobody cAbLys or anti-RSV Nanobody RSV007B02(Q108L).

The generated polypeptides are listed in Table C-14.

Table C-14: Sample ID and description of multispecific polypeptides.

Sample ID	SEQ ID NO*	Description
T017000113	46	A0110055F03-35GS-cAbLys3(D1E,Q5V,A6E,Q108L)-35GS-T0170056G05-FLAG3-HIS6
T017000114	47	A0110055F03-35GS-A0110056A10-35GS-T0170056G05-FLAG3-HIS6
T017000115	48	A0110056A10-35GS-cAbLys3(D1E,Q5V,A6E,Q108L)-35GS-T0170056G05-FLAG3-HIS6
T017000116	49	A0110056A10-35GS-A0110055F03-35GS-T0170056G05-FLAG3-HIS6
T017000120	50	cAbLys3(D1E,Q5V,A6E,Q108L)-35GS-A0110055F03-35GS-T0170056G05-FLAG3-HIS6
T017000121	51	cAbLys3(D1E,Q5V,A6E,Q108L)-35GS-A0110056A10-35GS-T0170056G05-FLAG3-HIS6
T017000125	42	T0170056G05-HIS6
T017000126	52	A0110055F03(E1D)-35GS-A0110056A10-35GS-T0170056G05-A
T017000128	53	T0170056G05(E1D)-35GS-A0110056A10-A
T017000129	54	T0170056G05(E1D)-35GS-RSV007B02(Q108L)-A
T017000130	55	A0110056A10(E1D)-35GS-A0110055F03-35GS-T0170056G05-A
T017000131	56	A0110056A10(E1D)-35GS-T0170056G05-A

Sample ID	SEQ ID NO*	Description
T017000132	57	RSV007B02(E1D,Q108L)-35GS-T0170056G05-A
T017000134	58	A0110056A10(E1D)-35GS-T0170056G05-35GS-A0110055F03-A
T017000135	59	A0110055F03(E1D)-35GS-T0170056G05-35GS-A0110056A10-A
T017000138	60	T0170056G05(E1D)-35GS-A0110056A10-35GS-A0110055F03-A
T017000139	61	T0170056G05(E1D)-35GS-A0110055F03-35GS-A0110056A10-A

* SEQ ID NOs correspond to the sequences of the multispecific polypeptides without C-terminal tags or Ala-extension

Example 21: Competition between A0110056A10 and the multispecific CD123/TCR polypeptides for binding to CD123 expressed on cells in flow cytometry

The binding of the CD123/TCR multispecific polypeptides to human or cynomolgus CD123 was evaluated in the A0110056A10 competition assay as described in Example 16. Next to binding to the MOLM-13 and CHO Flp-In huCD123 cells, binding to cyno CD123 transfected HEK Flp-In cells was assessed.

To this end, a batch of A0110056A10 was labelled with Alexa647 and frozen. Next, cells were harvested and transferred to a V-bottom 96-well plate (1×10^5 cells/well) and mixed with a serial dilution of the multispecific binding polypeptides (starting from $1 \mu\text{M}$) and a fixed concentration of A0110056A10-Alexa647. The concentrations used in the assay (0.4 nM for MOLM-13, 0.9 nM for CHO Flp-In human CD123 and for HEK Flp-In cynomolgus CD123) were below the EC_{50} value for binding of A0110056A10-Alexa647 to the respective cells (binding curves are depicted in Figure 21). After an incubation period of 90 min at 4°C , the binding of A0110056A10-Alexa647 was determined in flow cytometry as described in Example 16. A0110056A10 and T017000129 were taken along as a positive and negative control, respectively.

The results are presented in Figure 22. The IC_{50} values obtained from the dose response curves are depicted in Table C-15 and Table C-16.

Table C-15: IC_{50} (M) of CD123/TCR multispecific polypeptides and controls in the A0110056A10 Nanobody competition assay on MOLM-13 cells.

Sample ID	MOLM-13		
	IC_{50} (M)	95% LCI	95% UCI
T017000121	2.6E-08	2.2E-08	3.1E-08
T017000128	3.1E-09	2.6E-09	3.6E-09
T017000138	3.5E-09	3.0E-09	4.1E-09
T017000139	3.7E-09	3.1E-09	4.4E-09
T017000116	1.8E-09	1.5E-09	2.2E-09
A0110056A10	1.1E-09	8.7E-10	1.3E-09

Table C-16: IC₅₀ (M) of CD123/TCR multispecific polypeptides and controls in the A0110056A10 Nanobody competition assay on CD123 transfected cells.

Sample ID	HEK Flp-In cyCD123 transfected cells			CHO Flp-In huCD123 transfected cells		
	IC ₅₀ (M)	95% LCI	95% UCI	IC ₅₀ (M)	95% LCI	95% UCI
T017000121	1.1E-07	9.6E-08	1.3E-07	2.3E-08	1.9E-08	2.7E-08
T017000128	3.1E-08	2.6E-08	3.6E-08	5.8E-09	5.4E-09	6.2E-09
T017000138	1.2E-08	1.0E-08	1.4E-08	3.6E-09	2.8E-09	4.6E-09
T017000139	1.1E-08	9.6E-09	1.3E-08	3.3E-09	2.7E-09	4.1E-09
T017000116	5.6E-09	4.8E-09	6.4E-09	1.6E-09	1.4E-09	1.9E-09
A0110056A10	7.0E-09	6.1E-09	8.1E-09	1.9E-09	1.7E-09	2.3E-09

All tested multispecific polypeptides showed binding to human and cynomolgus CD123 expressing cells confirming human cynomolgus CD123 crossreactivity. A small drop in affinity was observed for the polypeptides where the A0110056A10 is not at the N-terminal position.

Example 22: Competition between T0170056G05 and CD123/TCR multispecific polypeptides for binding to human TCR/CD3 expressed on cells in flow cytometry

The binding of the CD123/TCR multispecific polypeptides to human TCR/CD3 was evaluated in a T0170056G05 competition assay by flow cytometry. To this end, a large batch of T0170056G05 was labelled with biotin and frozen. Next, CHO-K1 human TCR/CD3 expressing cells were harvested and transferred to a V-bottom 96-well plate (1×10^5 cells/well) and mixed with a serial dilution of the multispecific binding polypeptides (starting from $1 \mu\text{M}$) and a fixed concentration of biotinylated T0170056G05 in FACS buffer. The concentration of biotinylated T0170056G05 used in the assay (30nM) was below the EC₅₀ value for binding to the cells (data not shown). After an incubation period of 90 min at 4°C, the binding of the biotinylated T0170056G05 Nanobody was determined in flow cytometry. There to, cells were washed 3 times and resuspended in streptavidin-PE (ebioscience, 12-4317-87, 1000 fold diluted) in FACS buffer and incubated for 30 min at 4°C. Afterwards, cells were washed 3 times and resuspended in FACS Buffer + $1 \mu\text{g/ml}$ TOPRO (Molecular Probes, T3605) for 30 min at 4°C and then analysed via a BD FACSCanto. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. 10000 cells were counted within P1. From this population the TOPRO+ cells (dead cells) were excluded and the median PE value was calculated. T0170056G05 was taken along as a positive control.

The results are presented in Figure 23. The IC₅₀ values obtained from the dose response curves are depicted in Table C-17.

Table C-17: IC₅₀ (M) of CD123/TCR multispecific polypeptides and control Nanobody in the T0170056G05 competition assay.

Sample ID	CHO-K1 huTCR/CD3		
	IC ₅₀ (M)	95% LCI	95% UCI
T017000138	5.9E-08	5.5E-08	6.4E-08
T017000139	4.9E-08	4.5E-08	5.2E-08
T017000129	1.8E-07	1.7E-07	1.9E-07
T017000128	5.7E-08	5.3E-08	6.1E-08
T017000116	>1E-07		
T0170056G05	5.5E-08	5.2E-08	5.9E-08

The binding of the CD123/TCR multispecific polypeptides to human TCR/CD3 expressed on cells was confirmed. A drop in affinity of the CD123/TCR multispecific polypeptide T017000116 versus the monovalent TCR Nanobody was observed due to the C-terminal position of the TCR Nanobody.

Example 23: Competition between T017000099 and the CD123/TCR multispecific polypeptides for binding to cynomolgus TCR/CD3 expressed on cells in flow cytometry

The binding of the CD123/TCR multispecific polypeptides to cyno TCR/CD3 was evaluated in a T01700099 (bivalent T0170056G01, SEQ ID NO: 337) competition assay in flow cytometry. To this end, HSC-F (JCRB, JCRB1164) cynomolgus TCR/CD3 expressing cells were harvested and transferred to a V-bottom 96-well plate (1x10⁵ cells/well) and mixed with a serial dilution of the CD123/TCR multispecific polypeptides (starting from 1μM) and 500nM of T01700099 in FACS buffer. After an incubation period of 90 min at 4°C, cells were washed 3 times with FACS buffer by centrifugation and probed with 1μg/ml monoclonal mouse ANTI-FLAG® M2 antibodies (Sigma-Aldrich, F1804) for 30 minutes at 4°C, to detect bound T01700099. Detection was done with 5μg/ml Allophycocyanin (APC) AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch, 115-135-164) for 30 minutes at 4°C. Cells were washed and incubated with Propidium Iodine to stain for dead cells, which are then removed during the gating procedure. The cells were then analysed via a BD FACSArray. First a P1 population which represented more than 80% of the total cell population was selected based on FSC-SSC distribution. In this gate, 10000 cells were counted during acquisition. From this population the PI+ cells (dead cells) were excluded and the median APC value was calculated. T017000125 was taken along as a positive control.

The results are presented in Figure 24. The IC₅₀ values obtained from the dose response curves are depicted in Table C-18.

Table C-18: IC₅₀ (M) of CD123/TCR multispecific polypeptides and control Nanobody in the T017000099 competition assay.

Sample ID	HSC-F		
	IC ₅₀ (M)	95% LCI	95% UCI
T017000125	3.7E-07	2.5E-07	5.3E-07
T017000128	6.3E-07	4.4E-07	8.8E-07
T017000129	5.2E-07	3.7E-07	7.2E-07
T017000138	4.4E-07	3.2E-07	6.2E-07
T017000139	6.4E-07	4.5E-07	9.2E-07

For all CD123/TCR multispecific polypeptides with the TCR binding Nanobody at the N-terminus,
 5 binding to cynomolgus TCR/CD3 was observed.

Example 24: Binding of monovalent Nanobodies and multispecific polypeptides to human CD123 protein (SPR)

Binding affinities for the CD123/TCR multispecific polypeptides were evaluated by means of an SPR based affinity determination on a ProteOn XPR36 instrument. Thereto, recombinant CD123 (R&D
 10 Systems, 301-R3-025/CF) was immobilized on a CM5 chip via amine coupling, using EDC and NHS chemistry. Purified Nanobodies were injected for 2 minutes at different concentrations (between 4.2nM and 1000 nM) via a one-shot kinetics approach for kinetic analysis. The dissociation time of the 1000 nM sample was 15 min. Flow rate was 45 µl/min and ProteOn running buffer (PBS, pH 7.4, 0.005% Tween 20) was used as running buffer. Evaluation of the association/dissociation data was
 15 performed by fitting a 1:1 interaction model (Langmuir binding model). The binding characteristics are listed in Table C-19.

Table C-19: Binding characteristics of multispecific polypeptides determined in Proteon using directly coated human CD123 protein

sample ID	ka (1/Ms)	kd (1/s)	KD (M)	
T017000120	1.5E+04	2.5E-03	1.7E-07	Values only indicative due to incomplete regeneration of previous polypeptide (T017000116)
T017000121	4.0E+04	8.6E-04	2.2E-08	-
T017000113	2.0E+04	3.2E-03	1.6E-07	
T017000115	3.3E+05	5.1E-04	1.5E-09	Values only indicative due to incomplete regeneration of previous polypeptide (T017000114)
T017000114	8.4E+04	1.9E-04	2.3E-09	
T017000116	2.4E+05	2.1E-04	8.8E-10	

The KD of the monovalent Nanobodies and multispecific polypeptides to human IL-3R α correlates with the binding data on cells. The polypeptide T017000116 containing the two IL-3R α building blocks had the best KD.

Example 25: Redirected human T cell mediated killing of CD123 target cells by CD123/TCR multispecific polypeptides in a flow cytometry based assay

In order to assess whether the CD123/TCR multispecific polypeptides were able to kill tumour cells, cytotoxicity assays were performed with isolated human T cells as effector cells.

Thereto, human T cells were collected from Buffy Coat blood from healthy volunteers (Blood bank Gent) using RosetteSep (StemCell Technologies, 15061) followed by enriching on Ficoll-PaqueTM PLUS (GE Healthcare, 17-1440-03) according to manufacturer's instructions. The quality and purity of the purified human T cells was checked with anti-CD3 (eBioscience, 12-0037-73), anti-CD8 (BDBiosciences, 555367), anti-CD4 (BD Biosciences, 345771), anti-CD45RO (BD Biosciences, 555493), anti-CD45RA (BDBiosciences, 550855), anti-CD19 (BDBiosciences, 555413), anti-CD25 (BDBiosciences, 557138) and anti-CD69 (BDBiosciences, 557050) fluorescently labelled antibodies in a flow cytometric assay. Cells were frozen in liquid nitrogen.

Human CD123 expressing MOLM-13 and KG1a cells were labelled with 8 μ M PKH-26 membrane dye using the PKH26 red fluorescent cell linker kit (Sigma, PKH26GL-1KT) according to manufacturer's instruction and used as target cells. 2.5x10⁵ effector (i.e. human primary T cells) and 2.5x10⁴ target cells (i.e. PKH-labelled MOLM-13 or KG1a cells) were co-incubated in 96-well V-bottom plates (effector versus target ratio of 10:1). For measurement of the concentration-dependent cell lysis, serial dilutions of the CD123/TCR multispecific polypeptides were added to the cells and incubated for 18 h in a 5% CO₂ atmosphere at 37°C. Nanobody A0110056A10 and polypeptides T017000129 and T017000132 were taken along as negative control. After incubation, cells were pelleted by centrifugation and washed with FACS buffer. Subsequently, cells were resuspended in FACS buffer supplemented with 5 nM TOPRO3 (Molecular Probes, T3605) to distinguish live from dead cells. Cells were analysed using a FACS Array flow cytometer (BD Biosciences). Per sample, a total sample volume of 80 μ l was acquired. Gating was set on PKH26 positive cells, and within this population the TOPRO3 positive cells were determined. T017000129, T017000132 and A0110056A10 were taken along as a negative control.

Exemplary results are shown in Figure 25 and Figure 26 for the MOLM-13 and KG1a cells, respectively. The EC₅₀ values are depicted in Table C-20 and Table C-21 for the MOLM-13 and KG1a cells, respectively.

Table C-20: EC50 (M) and % lysis of CD123/TCR multispecific polypeptides for redirected human T cell mediated killing of MOLM-13 cells in a flow cytometry based assay.

Sample ID	EC50 (M)	95% LCI	95% UCI	% lysis
T017000116	2.2E-10	1.1E-10	4.5E-10	17
T017000128	1.5E-10	9.4E-11	2.5E-10	20
T017000135	1.1E-11	8.1E-12	1.5E-11	33
T017000138	2.3E-11	1.5E-11	3.7E-11	22
T017000139	2E-11	1.4E-11	2.9E-11	29
T017000134	3E-10	1E-10	9.2E-10	9

Table C-21: EC50 (M) and % lysis of CD123/TCR multispecific polypeptides for redirected human T cell mediated killing of KG1a cells in a flow cytometry based assay.

Sample ID	EC50 (M)	95% LCI	95% UCI	% lysis
T017000114	2.4E-10	1.5E-10	3.6E-10	7
T017000116	4.2E-10	2.4E-10	7.3E-10	5
T017000128	8.7E-10	7.0E-10	1.1E-09	28
T017000135	5.1E-11	4.1E-11	6.5E-11	18
T017000138	2.8E-10	2.2E-10	3.4E-10	24
T017000139	1.1E-10	8.5E-11	1.3E-10	27

The CD123/TCR multispecific polypeptides induced human T cell mediated killing of CD123 positive cell lines. There was clear preference for the position of the TCR Nanobody in the multispecific polypeptide. In general, the polypeptides with the anti-TCR Nanobody at the N-terminal position showed best killing potential. The polypeptides T017000135, T017000138 and T017000139 with two CD123 reactive Nanobodies showed improved potency compared to polypeptide T017000128 with only one CD123 Nanobody. These results demonstrated that the CD123/TCR multispecific polypeptides can induce T cell mediated killing of tumour target positive cell lines and that targeting multiple epitopes on a single antigen improves functionality (avidity effect).

In addition, comparison of polypeptides T017000138 and T017000139, both trivalent polypeptides with the TCR reactive Nanobody at the N terminal position, showed that there is an impact of the orientation of the CD123 Nanobodies on potency and efficacy.

The monovalent CD123 building block and the irrelevant polypeptides containing the TCR building block did not induce any target cell killing, confirming the requirement of cross-linking the T cell and target cell with the multispecific CD123/TCR polypeptides to induce killing.

The results were confirmed using purified T cells from different donors (data not shown).

Example 26: Redirected cynomolgus T cell mediated killing of CD123 target cells by multispecific CD123/TCR polypeptides in a flow cytometry based assay

To confirm the human-cyno TCR cross-reactivity of the CD123/TCR multispecific polypeptides, the polypeptides were evaluated in a cynomolgus T cell mediated CD123 positive tumour cell killing assay. In brief, multispecific polypeptides were incubated with 2.5×10^4 PKH labelled target cells (i.e. MOLM-13 or KG1a cells) in the presence of 2.5×10^5 effector cells (i.e. cynomolgus primary T cells), corresponding to an effector cell to target cell ratio (E:T ratio) of 10 to 1, as described in Example 25. T cells were isolated by LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG, using the Pan T Cell Isolation Kit (MACS, 130-091-993). Nanobody A0110056A10 and polypeptides T017000129 and T017000132 were taken along as negative control.

Exemplary results are shown in Figure 27 and Figure 28 for the MOLM-13 and KG1a cells, respectively. The EC_{50} values are depicted in Table C-22 and Table C-23 for the MOLM-13 and KG1a cells, respectively.

Table C-22: EC_{50} (M) and % lysis of CD123/TCR multispecific polypeptides for redirected cyno T cell mediated killing of MOLM-13 cells in a flow cytometry based assay.

Sample ID	EC_{50} (M)	95% LCI	95% UCI	% lysis
T017000116	3.9E-10	2.4E-10	6.2E-10	15
T017000128	1.8E-10	1.5E-10	2.3E-10	50
T017000138	1.7E-11	1.3E-11	2.2E-11	47
T017000139	1.1E-11	8.9E-12	1.4E-11	55

Table C-23: EC_{50} (M) and % lysis of CD123/TCR multispecific polypeptides for redirected cyno T cell mediated killing of KG1a cells in a flow cytometry based assay.

Sample ID	EC_{50} (M)	95% LCI	95% UCI	% lysis
T017000114	1.9E-10	1.3E-10	2.8E-10	7
T017000116	3.7E-10	2.9E-10	4.7E-10	14
T017000128	3.2E-10	2.7E-10	3.7E-10	38
T017000135	2.4E-11	2.0E-11	2.9E-11	22
T017000138	8.8E-11	7.6E-11	1.0E-10	38
T017000139	2.6E-11	2.3E-11	3.0E-11	42

All CD123/TCR multispecific polypeptides, except for T017000134, induced cynomolgus T cell mediated killing of CD123 positive MOLM-13 or KG1a cell lines. polypeptides with the TCR reactive Nanobody at the N terminal position were most potent and efficacious. polypeptides T017000138 and T017000139, both trivalent polypeptides with two CD123 reactive Nanobodies showed improved potency compared to the polypeptide T017000128, which contains only one CD123 reactive Nanobody. The monovalent anti-CD123 Nanobody and the irrelevant polypeptides containing the

TCR building block did not induce any target cell killing, confirming the requirement of cross-linking the T cell and target cell with the multispecific CD123/TCR polypeptides to induce killing.

The results were confirmed using purified T cells from different cynomolgus monkeys (data not shown).

5 Example 27: Cynomolgus T cell activation by the CD123/TCR multispecific polypeptides during redirected cynomolgus T cell mediated killing of CD123 target cells

To monitor T cell activation following the treatment of the cynomolgus T cells and CD123 positive MOLM-13 cells with multispecific CD123/TCR polypeptides, the polypeptides were incubated for 72h at 37°C with 2.5×10^4 target cells in the presence of 2.5×10^5 primary T cells (E:T=10:1), as described in
 10 Example 26. Cynomolgus T cell activation was measured by monitoring the CD25 upregulation on the CD4/CD8 T cell population in flow cytometry.

Thereto, after the incubation of 72h, effector and target cells were collected by centrifugation and suspended in FACS buffer with 25µg/ml human Fc block (BD Bioscience, 564220) and incubated for 10 min at room temperature (RT). Next, cells were stained with monoclonal mouse anti-CD4-APC
 15 (Biolegend, 300505) (200-fold diluted), monoclonal mouse anti-CD8 APC (BDBiosciences, 555366) (50-fold diluted) and monoclonal anti-CD25 PE (BD Biosciences, 557138) (50-fold diluted) antibodies in FACS buffer for 30min at 4°C. After incubation, cells were pelleted by centrifugation and washed with FACS buffer. Subsequently, cells were resuspended in FACS buffer and analysed using a FACS Canto flow cytometer (BD Biosciences). Per sample, a total sample volume of 30µl was acquired. T
 20 cells were gated based on the SSC-APC plot. From this population the mean PE value was calculated.

The data is shown in Figure 29. The EC₅₀ value obtained for T017000139 is depicted in Table C-24.

Table C-24: EC₅₀ (M) of CD25 upregulation on cynomolgus T cell by T017000139 during the redirected cynomolgus T cell mediated killing of MOLM-13 cells in a flow cytometry based assay.

Sample ID	EC ₅₀ (M)	95% LCI	95% UCI
T017000139	6.0E-11	5.1E-11	7.1E-11

25 No CD25 upregulation was observed when T cells were incubated with CD123 positive MOLM-13 target cells and the monovalent TCR or CD123 binding building blocks T0170056G05, A0110055F03 or A0110056A10 or the irrelevant multivalent polypeptide T017000129. The data showed CD25 upregulation on cynomolgus primary T cells after incubation with CD123 positive MOLM-13 target cells and the T017000139 multispecific polypeptide.

MOLM-13 cells were killed in a dose-dependent manner (Figure 25, Table C-22), indicating that the multispecific CD123/TCR binding polypeptide induced T cell activation in the process of redirected killing.

Likewise, T cells were not activated when incubated with target cells and the monovalent building blocks and irrelevant multispecific polypeptide, indicating the requirement of cross-linking the T cell and target cell with the TCR/CD123 multispecific polypeptides to induce CD25 upregulation.

Example 28: Redirected human T cell mediated killing of CD123 transfected adherent target cells by multispecific CD123/TCR binding polypeptides in an xCELLigence based assay

The TCR/CD123 binding polypeptides were characterized for redirected human T cell mediated killing of human CD123 transfected adherent target cells in an xCELLigence based assay. In this assay, fluctuations in impedance induced by the adherence of target cells to the surface of an electrode are measured. T cells are non-adherent and therefore do not impact the impedance measurements. The xCELLigence instrument (Roche) quantifies the changes in electrical impedance, displaying them as a dimensionless parameter termed cell-index, which is directly proportional to the total area of tissue-culture well that is covered by cells. In brief, an xCELLigence station was placed in a 37°C incubator at 5% CO₂. 50 µl of assay medium was added to each well of E-plate 96 (ACEA Biosciences; 05 232 368 001) and a blank reading on the xCELLigence system was performed to measure background impedance in absence of cells. Subsequently, 2x10⁴ human CD123 transfected CHO Flp-In or CHO Flp-In reference cells were seeded onto the E-plate 96, and 50µl serial diluted multispecific polypeptide was added. After 30 min at RT, 50µl of human T cells (3x10⁵) was added per well to have an effector to target ratio of 15:1. The plate was placed in the xCELLigence station and impedance was measured every 15 min for 3 days. The data were analysed using a fixed time point indicated in the results.

The IC₅₀ values obtained in this assay are listed in Table C-25. The results are depicted in Figure 30, Figure 31 and Figure 32.

Table C-25: IC₅₀ (M) of T013700139 for redirected human T cell mediated killing of human CD123 transfected adherent target cells in an xCELLigence based assay, using an effector to target ratio of 15 to 1, analysed at 50h after seeding.

Sample ID	IC ₅₀ (M)	95% LCI	95% UCI
T017000139	2.4E-10	2.0E-10	2.8E-10

The obtained data confirmed the results obtained in the flow cytometry based killing assay, i.e. CD123/TCR multispecific polypeptides can induce human T cell mediated killing of CD123 positive cell lines (Figure 30) and no killing activity is observed in the absence of T cells (Figure 32). In addition,

only when the CD123 tumour target antigen was present T cell mediated killing was observed (see Figure 31 for absence of killing with reference cell line), indicating that the multispecific polypeptides are critically dependent on their target for induction of cytotoxicity.

The monovalent Nanobodies A0110056A10 and T0170056G05 and the irrelevant polypeptide T017000129 did not induce target cell killing, confirming the requirement of cross-linking the T cell and target cell with the multispecific CD123/TCR binding polypeptide to induce killing.

The results were confirmed using purified T cells from different donors (data not shown).

Example 29: Redirected cynomolgus T cell mediated killing of CD123 transfected adherent target cells by multispecific CD123/TCR binding polypeptides in an xCELLigence based assay

To confirm the cross-reactivity of the multispecific polypeptides, the polypeptides were evaluated in a redirected cynomolgus T cell mediated killing of cynomolgus CD123 transfected adherent target cells in an xCELLigence based assay as described in Example 28.

The IC₅₀ values obtained in this assay are listed in Table C-26. The results are depicted in Figure 33, Figure 34 and Figure 35.

Table C-26: IC₅₀ (M) of T013700139 for redirected cynomolgus T cell mediated killing of cynomolgus CD123 transfected adherent target cells in an xCELLigence based assay, using an effector to target ratio of 15 to 1, analysed at 80h after seeding.

Sample ID	IC ₅₀ (M)	95% LCI	95% UCI
T017000139	1.1E-11	2.7E-12	4.8E-11

CD123/TCR multispecific polypeptide T017000139 could induce cynomolgus T cell mediated killing of CD123 positive cell lines (Figure 33) and no killing activity was observed in the absence of T cells (Figure 35). In addition, only when the cynomolgus CD123 tumour target antigen was present T cell mediated killing was observed (Figure 34), indicating that the multispecific polypeptides are critically dependent on their target for induction of cytotoxicity.

The monovalent Nanobodies A0110056A10 and T0170056G05 and the irrelevant polypeptide T017000129 did not induce target cell killing, confirming the requirement of cross-linking the T cell and target cell with the multispecific CD123/TCR binding polypeptide to induce killing.

The human-cynomolgus CD123 and TCR cross-reactivity of the multispecific polypeptide T017000139 was confirmed in an xCELLigence based killing assay.

The results were confirmed using purified T cells from different donors (data not shown).

Example 30: Impact of multispecific CD123/TCR binding polypeptides on cytokine production during redirected killing

The induction of cytokine release was monitored during the human and cyno T cell mediated CD123 killing based on the xCELLigence assay. The release of the cytokine IFN- γ and IL-6 was measured by ELISA. Briefly, human CD123 transfected CHO-K1 cells (2×10^4 cells/well) were seeded in E-plate 96 in the presence of purified human or cynomolgus primary T cells (3×10^5 cells/well) with a serial dilution of multispecific TCR/CD123 binding polypeptides (starting at 125nM) or a fixed concentration (125nM) of irrelevant polypeptides, as described in Example 28. 72h after the addition of the human or cyno primary T cells/polypeptides to the plates, human IFN- γ and human IL-6 production by the human primary T cells and cynomolgus IFN- γ by the cynomolgus primary T cells in the supernatant was measured.

The release of human IL-6 was measured in ELISA using the Human IL-6 Quantikine ELISA Kit (R&D systems, D6050), according to the manufacturer's instructions. The release of IFN- γ was determined as follows: Maxisorp 96-well ELISA plates (Nunc) were coated with anti-human IFN- γ antibody (BDBiosciences, 551221) respectively anti-cynomolgus IFN- γ antibody (Biolegend, 507502). After overnight incubation, plates were washed and blocked with PBS + 2% BSA for 1h at room temperature. Next, plates were incubated with 100 μ l of the supernatants (2 fold diluted) and 1 μ g/ml biotinylated anti-IFN- γ antibody (BD Biosciences, 554550) for 2h 30 min while shaking, washed again and incubated with streptavidin-HRP (Dakocytomation, P0397). After 30 min, TMB One Solution (Promega, G7431) was added. The reaction was stopped with 2M H₂SO₄ and the polypeptide dose dependent production of IFN- γ was determined by measurement of the OD at 405nm using the Tecan sunrise 4.

The results for IFN- γ are shown in Figure 36. The results for IL-6 are shown in Figure 37. The EC50 values obtained in these assays are listed in Table C-27 and Table C-28.

Table C-27: EC50 (M) of the TCR/CD123 binding polypeptides for IFN- γ secretion during the redirected T cell mediated killing of CD123 transfected adherent target cells in the xCELLigence based assay.

Sample ID	human T cells			cynomolgus T cells		
	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
T017000128	8.6E-11	6.6E-11	1.1E-10	1.0E-10	3.7E-11	2.9E-10
T017000135	2.0E-09	1.3E-09	3.1E-09	/	/	/
T017000138	4.0E-10	2.3E-10	7.0E-10	8.7E-10	2.1E-10	3.6E-09
T017000139	1.0E-09	5.9E-10	1.9E-09	2.3E-09	7.8E-10	6.6E-09
T017000116	8.1E-09	4.6E-09	1.4E-08	/	/	/

Table C-28: EC50 (M) of the TCR/CD123 binding polypeptides for human IL-6 secretion during the redirected human T cell mediated killing of CD123 transfected adherent target cells in the xCELLigence based assay.

Sample ID	human T cells		
	EC50 (M)	95% LCI	95% UCI
T017000128	6.9E-11	3.8E-11	1.3E-10
T017000135	5.9E-10	3.0E-10	1.2E-09
T017000138	3.8E-11	1.8E-11	8.1E-11
T017000139	4.8E-10	2.6E-10	9.0E-10
T017000116	9.6E-09	5.6E-09	1.6E-08

Cytokine production was observed when the CD123 overexpressing CHO Flp-In cells and primary T cells were incubated with the CD123/TCR binding polypeptides. The irrelevant polypeptides T017000129 and T017000132 did not induce cytokine production.

Example 31: Redirected autologous T cell plasmacytoid dendritic cells (pDCs) and basophil depletion by multispecific CD123/TCR polypeptides in healthy PBMC

Cryopreserved peripheral blood mononucleocytes (PBMC) were thawed and washed with assay medium (RMPI 1640 + 10%FBS). 2×10^5 PBMCs were incubated with serial dilutions of multispecific polypeptides in 200µL assay medium in a 96-well V-bottom plate and incubated at 37°C in a 5% CO₂ incubator. At indicated time points, cells were stained at 4°C with monoclonal mouse anti-CD14-APC (Biolegend, 301808), anti-CD16-APC (Biolegend, 302012), anti-CD19-APC (Biolegend, 302212), anti-CD20-APC (Biolegend, 302312), anti-CD56-APC (Biolegend, 318310), anti-CD123-PE (Biolegend, 306006) and anti-HLA-DR-FITC (Biolegend, 307603) antibodies. In brief, cells were harvested and washed one time with FACS Buffer (D-PBS from Gibco, with 10% FBS from Sigma and 0.05% sodium azide from Merck) and resuspended in 25µL human BD Fc Block, 0.5µg/ml (BD Biosciences, 564220, 1000x diluted) for 10 minutes at RT. Next, 25µL of the antibodies were added and incubated for 30 min at 4°C according to the manufacturer's instructions. A separate well containing PBMC was resuspended in FACS buffer supplemented with 5 nM TOPRO3 (Molecular Probes, T3605) to distinguish live from dead cell population. Samples were washed 3 times, resuspended in FACS Buffer (D-PBS from Gibco, with 10% FBS from Sigma and 0.05% sodium azide from Merck) and then analysed via a FACS Canto (BD) cytometer equipped with FACS Diva software. Per sample, a total sample volume of 75µL was acquired. Data analysis was performed using FACS Diva and Flowing software.

Based on the well containing the TOPRO stain, gating was set to exclude the dead cells. Human and cynomolgus pDC and basophils were identified as the Lineage marker (CD16, CD20, CD19, CD56) negative / CD123 positive population.

The results are depicted in Figure 38.

Depletion of the human respectively cynomolgus CD123 positive population by the CD123/TCR multispecific polypeptides was observed after an incubation time of 5h. When the targeting Nanobody was replaced by an irrelevant Nanobody, no depletion of the CD123 positive population was observed, indicating the requirement of cross-linking the T cell and target cell with the TCR/CD123 multispecific polypeptides to induce depletion.

The assay was repeated using PBMC from 3 different human donors and PBMC from 2 different cynomolgus monkeys, confirming the functionality of the TCR/CD123 multispecific polypeptides.

Example 32: Redirected autologous human T cell monocyte depletion by multispecific CD123/TCR binding polypeptides in healthy human PBMC samples

To evaluate to depletion of monocytes, the assay was performed as described above, using Cryopreserved human PBMC that were thawed and washed with assay medium (RMPI 1640 + 10%FBS) and incubated with serial dilutions of multispecific polypeptide for either 5h or 24h. Staining of the cells was performed as described above. Monocytes were identified as the CD14+ population.

The results are depicted in Figure 39.

After 5h of incubation, no monocyte depletion was observed for the TCR/CD123 multispecific polypeptide and irrelevant Nanobodies. After 24h, monocyte depletion was observed for the TCR/CD123 multispecific polypeptides and not for the irrelevant polypeptides. The assay was repeated using PBMC from 3 different donors, confirming the functionality of the TCR/CD123 multispecific polypeptides.

Example 33: Human T cell activation by the multispecific CD123/TCR binding polypeptides during redirected T cell killing of autologous CD123 positive cells in healthy human PBMC samples.

To characterize T cell activation during the TCR/CD123 multispecific polypeptides mediated depletion process, the autologous PBMC assay was performed as described above and the activation status of human T cells was monitored by measurement of the upregulation of CD69 after 24h incubation. In brief, after the incubation time of 24h, cells were stained 30 min at 4°C with monoclonal mouse anti-CD3-FITC antibody (BD Biosciences, 555332) to identify the human T cells, and monoclonal mouse anti-human CD69-PE antibody (BD Biosciences, 557050) to evaluate T cell activation. Cells were washed 3 times, resuspended in FACS Buffer (D-PBS from Gibco, with 10% FBS from Sigma and 0.05% sodium azide from Merck) and then analysed via a FACS Canto (BD) cytometer equipped with FACS

Diva software. Per sample, a total sample volume of 75µl was acquired. Data analysis was performed using FACS Diva and Flowing software.

Exemplary results are shown in Figure 40.

The data showed dose dependent upregulation of CD69 on human CD3+ T cells when PBMC were incubated with the multispecific CD123/TCR binding polypeptides. Incubation with the monovalent Nanobodies or irrelevant polypeptides did not result in CD69 upregulation.

Example 34: Characterization of irrelevant polypeptides for redirected T cell mediated killing of CD123 target cells in a flow cytometry based assay

To evaluate the safety of the TCR Nanobody T0170056G05, an in depth analysis of the irrelevant polypeptides (the monovalent Nanobodies and the multivalent polypeptide T017000129) was performed in the redirected T cell mediated target killing assay using an E:T ratio of 10:1 as described in Example 25 and 26. Polypeptide T017000139 was taken along as positive control.

The results using the KG1a target cells are shown in Figure 41, the results using the MOLM-13 are shown in Figure 42. The EC50 values obtained are listed in Table C-29 and Table C-30.

Table C-29: EC50 (M) of T017000139 for redirected T cell mediated killing of CD123 positive KG1a cells in a flow cytometry based assay.

Sample ID	Human T cells			Cynomolgus T cells		
	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
T017000139	8.1E-11	7.4E-11	8.9E-11	1.7E-11	9.7E-12	3.1E-11

Table C-30: EC50 (M) of T017000139 for redirected T cell mediated killing of CD123 positive MOLM-13 cells in a flow cytometry based assay.

Experiment1

Sample ID	Cynomolgus T cells		
	EC50 (M)	95% LCI	95% UCI
T017000139	6E-11	5.1E-11	7.1E-11

Experiment2

Sample ID	Human T cells			Cynomolgus T cells		
	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
T017000139	3.0E-11	1.9E-11	4.7E-11	3.1E-11	1.9E-11	5.1E-11

The positive control T017000139 behaved as expected when using human and cynomolgus T cells. Neither the monovalent building blocks nor the irrelevant polypeptide T017000129 (CD123 building

blocks were replaced with an irrelevant building block) induced killing of CD123 positive cells, confirming the specificity of the TCR/CD123 multispecific polypeptides.

Example 35: Impact of multispecific CD123/TCR binding polypeptides on cytokine production during human redirected killing

- 5 The induction of cytokine release was monitored during the human T cell mediated CD123 killing assay based on the FACS based readout. The release of the human cytokine IFN- γ and IL-6 was measured by ELISA. Briefly, MOLM-13 or KG1a were seeded in V-bottom 96-well plate (2×10^4 cells/well) in the presence of purified human primary T cells (3×10^5 cells/well) with a serial dilution of multispecific TCR/CD123 binding polypeptides irrelevant polypeptides, as described in Example 25.
- 10 72h after the addition of the human primary T cells/polypeptides to the plates, human IFN- γ and IL-6 production by the human primary T cells in the supernatant was measured as described in Example 30.

The results are shown in Figures 43A, 43B and 43C. The EC50 values obtained in this assay are listed in Table C-31 and Table C-32.

- 15 **Table C-31: EC50 (M) of the TCR/CD123 binding polypeptides for human IFN- γ secretion during the redirected human T cell mediated killing of CD123 positive MOLM-13 or KG1a cells in the flow cytometry based assay.**

	MOLM-13			KG1a		
Sample ID	EC50 (M)	95% LCI	95% UCI	EC50 (M)	95% LCI	95% UCI
T017000139	3.4E-10	2.5E-10	4.6E-10	3.2E-11	1.9E-11	5.4E-11

Table C-32: EC50 (M) of the TCR/CD123 binding polypeptides for human IL-6 secretion during the redirected human T cell mediated killing of CD123 positive MOLM-13 cells in the flow cytometry based assay.

Sample ID	EC50 (M)	95% LCI	95% UCI
T017000139	2.3E-11	1.5E-11	3.5E-11

20

Cytokine production was observed when the MOLM-13 or KG1a cells and human primary T cells were incubated with the CD123/TCR binding polypeptides. The irrelevant polypeptide T017000129 did not induce cytokine production.

Example 36: Characterisation of target independent redirected human or cynomolgus effector T cell killing for multispecific CD123/TCR binding polypeptides in a flow cytometry based assay using CD123 negative cell lines

To evaluate the CD123 independent redirected killing of multispecific polypeptides, the CD123 negative U-937 and NCI-H929 cell lines were evaluated in a flow cytometry based killing assay. U-937 and NCI-H929 cells were labelled with 8 μ M PKH-26 membrane dye using the PKH26 red fluorescent cell linker kit (Sigma, PKH26GL-1KT) according to manufacturer's instruction and used as target cells. The assay was performed as described in Example 25 and 26 using primary human or cynomolgus T cells (E:T=10:1).

Exemplary results are shown in Figure 44 and Figure 45, for the NCI-H929 and U-937 cells, respectively.

The TCR/CD123 multispecific polypeptides and the irrelevant polypeptides showed only minimal T cell redirected U-937 killing activity (less than 6%), indicating that the multispecific polypeptides have good specificity for binding to CD123.

Example 37: Characterization of T cell activation for multispecific CD123/TCR binding polypeptides during redirected effector T cell killing assay using CD123 negative cell lines

To monitor T cell activation following the treatment of T cells and CD123 negative cells with multispecific CD123/TCR binding polypeptides, the polypeptides were incubated for 24h at 37°C with 2.5x10⁴ U-937 respectively NCI-H929 target cells in the presence of 2.5x10⁵ primary T cells (E:T=10:1), as described in Example 25 and 26. T cell activation was evaluated as described before by monitoring the CD25 upregulation after 72h of incubation on the CD4/CD8 T cell population was measured in flow cytometry as described in Example 27, using monoclonal mouse anti-CD4-APC (Biolegend, 300505), monoclonal mouse anti-CD8 APC (BD Biosciences, 555366) and monoclonal anti-CD25 (BD Biosciences, 557138) antibodies.

Exemplary results are shown in Figure 46.

Evaluation of the T cell activation after incubation with the multispecific polypeptides and the U-937 or NCI-929 CD123 negative cell lines showed only minimal upregulation of CD25 for any of the multispecific polypeptides. So, in the presence of CD123 negative target cells there was only minimal T cell activation or killing by the T cells.

Example 38: Characterisation of cytokine production for multispecific CD123/TCR binding polypeptides during human redirected killing

The aspecific induction of cytokine release was monitored during the human T cell mediated killing assay based on the FACS based readout. The release of the cytokine IFN- γ and IL-6 was measured by ELISA. Briefly, NCI-H929 were seeded in V-bottom 96-well plate (2×10^4 cells/well) in the presence of purified human primary T cells (3×10^5 cells/well) with a serial dilution of multispecific TCR/CD123 binding polypeptides or irrelevant polypeptides, as described in Example 25. 72h after the addition of the human primary T cells/polypeptides to the plates, IFN- γ and IL-6 production by the human primary T cells was measured in the supernatant as described in Example 30. The results are shown in Figure 47.

Cytokine production was not observed when CD123 negative NCI-H929 cell line and human primary T cells were incubated with the CD123/TCR binding polypeptides.

Example 39: Impact of multispecific CD123/TCR binding polypeptides on T cell proliferation during redirected killing

To investigate the effect of the multispecific CD123/TCR binding polypeptides on the proliferation of the human T cells, gamma-irradiated (100Gy) MOLM-13 cells were seeded in 96-well flat bottom microtiter plates (Greiner bio-one, 655 180, 2×10^4 cells/well) together with the multispecific polypeptides and the human primary T cells (2×10^5 cells/well) and incubated for 72 hours at 37°C in a humidified atmosphere of 5X CO₂ in air. Next, cells were pulsed for approximately 18 hours with 3H-thymidine (3H-Tdr, New England Nuclear, Boston, MA, 20 Ci/mM specific activities), harvested on glass fiber filter strips, and then counted by liquid scintillation counting.

Exemplary results are shown in Figure 48.

The CD123/TCR multispecific polypeptides induced T cell proliferation in a dose-dependent manner. No T cell proliferation was observed for the irrelevant polypeptide T017000129.

In parallel, the effect of the multispecific CD123/TCR binding polypeptides on the proliferation of the human T cells in the absence of target cells was evaluated. Thereto, the multispecific polypeptides and the human primary T cells (2×10^5 cells/well) were incubated for 72 hours at 37°C in a humidified atmosphere of 5X CO₂ in air. The proliferation was measured as described above. Data are shown in Figure 49.

Example 40: Lytic performance of pre-activated T cells versus non-activated T cells

To test the lytic performance of T cells in response to a multiple day-incubation period under stimulatory conditions, primary human T cells (isolated as described in Example 25) were thawed and pre-activated using Dynabeads® Human T-Activator CD3/CD28 (Gibco - Technologies, 11132D) using a T cell to beads ratio of 2:1. After 3 days, beads were replaced by fresh beads for an additional three days. Next, beads were removed and pre-activated and non-activated T cells were evaluated in a MOLM-13 target killing assay. In brief, non-activated or CD3/CD28 pre-activated primary T cells from the same donor were mixed with PKH labelled MOLM-13 cells at different E:T ratios (8:1, 2:1, 1:2, 1:4) and with serial dilutions of T017000114 or with PKH labelled KG1A cells at different E:T ratios (2:1, 1:2, 1:4, 1:8) and with serial dilutions of T017000139. Cytotoxicity readout after 24h of incubation was performed as described above in Example 25.

The results are shown in Figure 50 and Figure 51, for the MOLM-13 and KG1a cells respectively. The EC50 values obtained in this assay are listed in Table C-33 and Table C-34, for the MOLM-13 and KG1a cells respectively.

Table C-33: EC50 (M) and % lysis for T017000114 for redirected human T cell mediated killing of MOLM-13 cells in a flow cytometry based assay, using pre-activated and non-activated T cells.

Sample ID	non-activated T cells				pre-activated T cells			
	EC50 (M)	95% LCI	95% UCI	% lysis	EC50 (M)	95% LCI	95% UCI	% lysis
T017000114 E:T ratio 8:1	1.4E-09	2.4E-10	7.8E-09	12	2.4E-10	1.9E-10	3.2E-10	43
T017000114 E:T ratio 2:1	1.9E-09	6.2E-12	6.0E-07	10	6.8E-10	4.7E-10	9.9E-10	31
T017000114 E:T ratio 1:2					1.0E-09	4.0E-10	2.6E-09	18

Table C-34: EC50 (M) and % lysis for T017000139 for redirected human T cell mediated killing KG1a cells in a flow cytometry based assay, using pre-activated and non-activated T cells.

Sample ID	non-activated T cells				pre-activated T cells			
	EC50 (M)	95% LCI	95% UCI	% lysis	EC50 (M)	95% LCI	95% UCI	% lysis
T017000139 E:T ratio 2:1	1.4E-10	8.1E-11	2.5E-10	19	4.5E-11	2.2E-11	9.1E-11	43
T017000139 E:T ratio 1:2	7.7E-11	3.0E-12	2.0E-09	8	4.9E-11	2.2E-11	1.1E-10	20
T017000139 E:T ratio 1:4	9.2E-11	2.8E-12	3.0E-09	4	5.8E-11	3.1E-11	1.1E-10	13
T017000139 E:T ratio 1:8	3.4E-11	1.9E-13	6.1E-09	3	5.6E-11	2.3E-11	1.4E-10	8

Pre-activated T cells lysed the target cells more potently than the non-activated T cells at all E:T ratios tested. Pre-stimulation of effector cells with anti-CD3/anti-CD28 resulted in the higher lysis rates.

Example 41: Construction of half-life extended (HLE) multispecific CD123/TCR binding polypeptides and control polypeptides

5 ALB11 (SEQ ID NO: 43), a Nanobody binding to human serum albumin (HSA), was linked to the CD123/TCR binding polypeptides to increase the in vivo half-life of the formatted molecules (WO 06/122787). A number of formats were generated with the TCR α/β recruiting Nanobody at the N-terminus and the CD123 tumour targeting Nanobodies or the albumin targeting Nanobody at the C-terminus using a 35GS linker and expressed as indicated above. Irrelevant polypeptides were
10 generated by replacing the tumour antigen binding Nanobodies with an irrelevant anti-RSV Nanobody. An overview of the explored formats is shown in Table C-35.

Table C-35: Sample ID and description of HLE constructs.

Sample ID	SEQ ID NO*	Description
A022600009	62	T0170056G05-35GS-RSV007B02(Q108L)-35GS-ALB11
T017000142	63	T0170056G05(E1D)-35GS-A0110056A10-35GS-ALB11-A
T017000143	64	T0170056G05(E1D)-35GS-A0110056A10-35GS-A0110055F03-35GS-ALB11-A
T017000144	65	T0170056G05(E1D)-35GS-A0110056A10-35GS-ALB11-35GS-A0110055F03-A
T017000145	66	T0170056G05(E1D)-35GS-A0110055F03-35GS-A0110056A10-35GS-ALB11-A
T017000146	67	T0170056G05(E1D)-35GS-A0110055F03-35GS-ALB11-35GS-A0110056A10-A

* SEQ ID NOs correspond to the sequences of the multispecific polypeptides without C-terminal tags or Ala-extension
15

Example 42: Albumin binding properties of ALB11 in the multispecific recruitment polypeptides

The binding affinities of the half-life extended multispecific polypeptides to human, respectively cynomolgus serum albumin (SA) were measured by means of an SPR based affinity determination on a Biacore T100 instrument. Thereto, human (Sigma, A3782), respectively cynomolgus SA (produced in
20 house) was immobilized onto a CM5 chip via amine coupling, using EDC and NHS chemistry. TCR/CD123 binding polypeptides were injected for 2 minutes at different concentrations (between 6.2 and 500 nM) and allowed to dissociate for 15 min at a flow rate of 45 μ l/min. In between sample injections, the surfaces were regenerated with 10mM Glycine-HCl pH1.5. HBS-EP+ was used as running buffer. The kinetic constants were calculated from the sensorgrams using the BIAEvaluation

software with an algorithm using a single cycle kinetics 1:1 binding model. The affinity constant K_D was calculated from resulting association and dissociation rate constants k_a and k_d , and is shown in Table C-36.

Table C-36: Albumin binding properties of ALB11 in the HLE multispecific polypeptides.

	Human SA			Cynomolgus SA		
	k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)
T017000142	1.1E+05	7.2E-03	6.9E-08	9.0E+04	7.3E-03	8.1E-08
T017000143	1.6E+05	9.5E-03	5.9E-08	1.2E+05	9.4E-03	7.6E-08
T017000144	9.1E+04	7.8E-03	8.5E-08	7.4E+04	8.1E-03	1.1E-07
T017000145	1.1E+05	8.5E-03	7.6E-08	1.0E+05	9.9E-03	9.8E-08
T017000146	8.4E+04	8.5E-03	1.0E-07	6.8E+04	8.8E-03	1.3E-07
A022600009	1.1E+05	8.0E-03	7.5E-08	8.5E+04	8.0E-03	9.4E-08
ALB11	5.3E+05	1.6E-03	3.0E-09	5.1E+05	1.6E-03	3.2E-09

5

Formatting of the ALB11 building block into the multispecific recruitment polypeptides resulted in an allowable drop in affinity for binding to human and cynomolgus serum albumin.

Example 43: Redirected T cell mediated killing of MOLM-13 target cells by HLE CD123/TCR binding polypeptides in a flow cytometry based assay

10 Since the addition of the ALB11 Nanobody, and the binding to serum albumin (SA) might influence the potency of the polypeptides, the HLE CD123/TCR binding polypeptides were evaluated for redirected human and cynomolgus T cell mediated killing of CD123 positive MOLM-13 target cells based on flow cytometry assay as described in Example 25 and 26 in the absence or presence of 30 μ M SA.

15 The EC50 values obtained in this assay are listed in Table C-37. The results are depicted in Figure 52.

Table C-37: EC50 (M) and % lysis of HLE CD123/TCR binding polypeptides for redirected T cell mediated killing of MOLM-13 cells in a flow cytometry based assay using an E:T ratio of 10:1.

Sample ID	human T cells				cynomolgus T cells			
	% lysis	EC50 (M)	95% LCI	95% UCI	% lysis	EC50 (M)	95% LCI	95% UCI
T017000138	21	1.1E-10	8.8E-11	1.3E-10	54	1.5E-10	1.1E-10	2.1E-10
T017000144	23	2.7E-11	2.0E-11	3.6E-11	56	2.3E-11	1.7E-11	3.0E-11
T017000144+SA	24	2.6E-10	1.9E-10	3.5E-10	52	1.1E-10	9.3E-11	1.2E-10
T017000139	20	2.0E-11	9.3E-12	4.4E-11	56	2.9E-11	2.3E-11	3.5E-11
T017000146	21	2.5E-11			57	2.9E-11	2.3E-11	3.7E-11
T017000146 + SA	25	2.3E-10	1.7E-10	2.9E-10	44	1.6E-10	1.3E-10	1.9E-10

20 All the HLE multispecific CD123/TCR binding polypeptides showed dose dependent killing of the MOLM-13 cells both by human and by cynomolgus T cells. The inclusion of ALB11 in the polypeptide

did not decrease the potency. Upon addition of HSA or CSA, a small drop in potency was observed while the efficacy was not affected.

Example 44: Redirected T cell mediated killing of KG1a target cells by HLE CD123/TCR binding polypeptides in a flow cytometry based assay

- 5 The half-life extended TCR/CD123 binding polypeptides were also evaluated for redirected human and cynomolgus T cell mediated killing of CD123 KG1a target cells based on a flow cytometry assay as described in Example 24 and 25, in the absence or presence of 30 μ M serum albumin.

The EC₅₀ values obtained in this assay are listed in Table C-38. The results are depicted in Figure 53.

10 **Table C-38: EC₅₀ (M) and % lysis of HLE CD123/TCR binding polypeptides for redirected T cell mediated killing of KG1a cells in a flow cytometry based assay using an E:T ratio of 10:1.**

Sample ID	human T cells				cynomolgus T cells			
	% lysis	EC ₅₀ (M)	95% LCI	95% UCI	% lysis	EC ₅₀ (M)	95% LCI	95% UCI
T017000138	64	1.6E-10	1.3E-10	2.0E-10	21	2.8E-11	2.3E-11	3.4E-11
T017000143	62	1.9E-10	1.6E-10	2.3E-10	25	4.7E-11	2.9E-11	7.5E-11
T017000144	67	4.1E-11	3.4E-11	5.0E-11	28	6.3E-12	9.3E-13	4.3E-11
T017000143+SA	60	1.4E-09	1.0E-09	1.9E-09	25	8.6E-10	7.1E-10	1.0E-09
T017000144+SA	58	2.9E-10	2.4E-10	3.6E-10	29	6.6E-11	3.4E-11	1.3E-10
T017000139	67	8.1E-11	7.4E-11	8.9E-11	34	1.7E-11	9.7E-12	3.1E-11
T017000145	65	7.4E-11	6.4E-11	8.6E-11	35	1.6E-11	1.1E-11	2.4E-11
T017000146	64	6.6E-11	5.9E-11	7.4E-11	34	1.5E-11	7.2E-12	3.0E-11
T017000145+SA	51	5.9E-10	4.9E-10	7.2E-10	32	1.2E-10	7.4E-11	1.9E-10
T017000146+SA	60	6.8E-10	5.8E-10	8.0E-10	36	2.0E-10	1.1E-10	3.5E-10
T017000128	55	3.1E-10	3.0E-10	3.3E-10	32	2.8E-10	1.3E-10	6.1E-10
T017000142	59	6.8E-10	6.2E-10	7.5E-10	29	2.2E-10	1.4E-10	3.6E-10
T017000142+SA	48	3.1E-09	3.0E-09	3.3E-09	20	3.2E-10	2.9E-10	3.5E-10

- 15 All the HLE multispecific CD123/TCR binding polypeptides showed dose dependent killing of the KG1a cells both by human and by cynomolgus T cells. The inclusion of ALB11 in the polypeptide did not decrease the potency. Upon addition of HSA or CSA, a small drop in potency was observed while the efficacy was not affected.

Example 45: Impact of HLE multispecific CD123/TCR binding polypeptide T017000144 on cytokine production during redirected killing

The induction of cytokine release was monitored during human T cell mediated killing assay based on the FACS based readout. The release of IFN- γ and IL-6 was measured by ELISA. Briefly, MOLM-13 (2x10⁴ cells/well) were seeded in V-bottom 96-well plate in the presence of purified human primary T cells (3x10⁵ cells/well) with a serial dilution of multispecific TCR/CD123 binding polypeptides or irrelevant polypeptides, as described in Example 35. 72h after the addition of the human primary T cells/polypeptides to the plates, IFN- γ and IL-6 production by the human primary T cells in the supernatant was measured as described in Example 30.

The EC50 values obtained in this assay are listed in Table C-39 and Table C-40. The results are depicted in Figure 54.

Table C-39: EC50 (M) of the TCR/CD123 binding polypeptides for human IFN- γ secretion during the redirected human T cell mediated killing of CD123 positive MOLM-13 cells in the flow cytometry based assay.

Sample ID	EC50 (M)	95% LCI	95% UCI
T017000139	3.4E-10	2.5E-10	4.6E-10
T017000144	1.9E-10	1.3E-10	2.9E-10

Table C-40: EC50 (M) of the TCR/CD123 binding polypeptides for human IL-6 secretion during the redirected human T cell mediated killing of CD123 positive MOLM-13 cells in the flow cytometry based assay.

Sample ID	EC50 (M)	95% LCI	95% UCI
T017000139	2.3E-11	1.5E-11	3.5E-11
T017000144	1.6E-11	9.9E-12	2.7E-11

Cytokine production was observed when the MOLM-13 cells and human primary T cells were incubated with the HLE CD123/TCR binding polypeptide T017000144. The irrelevant polypeptide T017000129 did not induce cytokine production.

Example 46: Impact of HLE multispecific CD123/TCR binding polypeptides on T cell proliferation during redirected killing

To investigate the effect of the HLE multispecific CD123/TCR binding polypeptides on the proliferation of the human T cells, gamma-irradiated (100Gy) MOLM-13 cells were seeded in 96-well flat bottom microtiter plates (2 x 10⁴ cells/well) together with the HLE multispecific polypeptide T017000144 and the human primary T cells (2x10⁵ cells/well) and incubated for 72 hours at 37°C in a

humidified atmosphere of 5X CO₂ in air in the absence of SA. T017000129 was taken along as negative control. Next, cells were pulsed for approximately 18 hours with 3H-thymidine (3H-Tdr, New England Nuclear, Boston, MA, 20 Ci/mM specific activities), harvested on glass fiber filter strips, and then counted by liquid scintillation counting.

5 Exemplary results are shown in Figure 55.

The HLE CD123/TCR multispecific polypeptide T017000144 induced T cell proliferation in a dose-dependent manner. No T cell proliferation was observed for the irrelevant polypeptide T017000129.

Example 47: Redirected autologous T cell plasmacytoid dendritic cells (pDCs) and basophil depletion by HLE CD123/TCR multispecific polypeptides in healthy human PBMC samples and healthy cynomolgus PBMC

The HLE constructs were further evaluated in the human and cynomolgus autologous PBMC assay in the absence of SA, as described in Example 31. The depletion of CD123 positive cells (pDC: Lineage negative, CD123 positive, HLA-DR positive and basophils: Lineage negative, CD123 positive, HLA-DR negative) by multispecific polypeptides was evaluated after an incubation time of 5h.

15 The results are depicted in Figure 56 and Figure 57, for the human and cynomolgus PBMC respectively.

The HLE CD123/TCR multispecific polypeptides were able to deplete CD123+ pDCs and basophils within human and cynomolgus PBMC by redirected T cells. T017000144 was the most potent polypeptide. Polypeptide T017000142, composed of one TCR building block, ALB11 and only one CD123 building block did not show functionality in the PBMC assay after 5h. The human cynomolgus cross-reactivity of the HLE CD123/TCR multispecific polypeptides was confirmed in the autologous setting.

Example 48: Redirected autologous T cell monocyte depletion by HLE CD123/TCR multispecific polypeptides in healthy human PBMC samples

25 The depletion of monocytes (CD14+ cells) by the HLE multispecific polypeptides in an autologous human PBMC setting was evaluated after an incubation time of 24h in the absence of SA. The assay was performed as described in Example 32.

The results are depicted in Figure 58.

The HLE multispecific polypeptides were able to deplete CD123+ monocytes within human PBMC by redirected T cells. T017000144 was the most potent polypeptide. Polypeptide T017000142, composed of one TCR building block, ALB11 and only one CD123 building block showed functionality in the autologous monocyte depletion assay after 24h.

5 **Example 49: In vivo efficacy and safety in a non-human primate model**

In vivo efficacy and safety of non-HLE and HLE multispecific CD123/TCR binding Nanobodies are evaluated in a non-human primate model.

Animals treated with a reference compound are included as positive control. Treatment with non-HLE IRR/TCR binding polypeptides is used as specificity control for the CD123-targeting moiety of the multispecific polypeptides. The reference compound and the non-HLE multispecific CD123/TCR binding polypeptides are administered via continuous i.v. infusion after a 7-day NaCl infusion 'pre-treatment' of cynomolgus monkeys. The non-HLE multispecific CD123/TCR binding polypeptides are administered for 4 weeks as 4-day on/3-day off infusion at equimolar doses to the reference compound in a weekly dose escalation scheme according to Table C-41. The HLE multispecific CD123/TCR binding polypeptide is administered to cynomolgus monkeys via bolus i.v. injections on days 1, 2, 3, 8, 15, and 22 in a weekly dose escalation scheme according to Table C-41.

Table C-41: Treatment regimen.

Group	Compound	Dose levels (ng/kg/day)				Route of administration
		Test week 1	Test week 2	Test week 3	Test week 4	
1	Reference	D1-5: 100	D8-12: 300	D15-19: 600	D22-26: 1000	continuous 24h infusion
2	Irrelevant/TCR polypeptide	D1-5: 49.5	D8-12: 148.4	D15-19: 296.8	D22-26: 494.6	continuous 24h infusion
3	Non-HLE CD123/TCR polypeptide	D1-5: 74.0	D8-12: 222.1	D15-19: 444.3	D22-26: 740.4	continuous 24h infusion
4	HLE CD123/TCR polypeptide	D1: 0.6 D2: 0.4 D3: 0.34	D8: 2.21	D15: 4.42	D22: 7.13	i.v. bolus injection

T cell redistribution from the blood is monitored by measuring T cell subsets, as described in Table C-42, using flow cytometry and differential blood count on test days -7, d-4, d1 (pre-dose + 4 hrs post-dose), d4, d8 (pre-dose + 4 hrs post-dose), d11, d15 (pre-dose + 4 hrs post-dose), d18, d22 (pre-dose + 4 hrs post-dose), d25, d29, d32, and d36.

- 5 *In vivo* efficacy is assessed by evaluation of the percentage and number of CD123+ cells in PBMC, as detailed in Table C-43, using flow cytometry and differential blood count in the blood on test days -7, d-4, d1 (pre-dose + 4 hrs post-dose), d4, d8 (pre-dose + 4 hrs post-dose), d11, d15 (pre-dose + 4 hrs post-dose), d18, d22 (pre-dose + 4 hrs post-dose), d25, d29, d32, and d36.

- 10 Safety is assessed by evaluation of cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), TNF- α , TNF- β , IFN- γ) in the serum on test days -7, d-4, d1 (4 hrs post-dose), d4, d8 (4 hrs post-dose), d11, d15 (4 hrs post-dose), d18, d22 (4 hrs post-dose), d25, d29, d32, and d36.

Table C-42: Marker combinations for blood T cell phenotyping.

Marker	Cells	Unit
CD3+CD4+	T-helper cells (Th)	% of PBMC and cells/ μ L
CD3+CD8+	Cytotoxic T-cells (Tc)	% of PBMC and cells/ μ L
CD25+CD3+CD4+	activated Th	% of CD3+CD4+ and cells/ μ L
CD25+CD3+CD8+	activated Tc	% of CD3+CD8+ and cells/ μ L
PD1+CD3+CD4+	PD (programmed cell death protein) upregulation on Th	% of CD3+CD4+ and cells/ μ L
PD1+CD3+CD8+	PD upregulation on Tc	% of CD3+CD8+ and cells/ μ L
CD25+PD1+CD3+CD4+	PD upregulation on activated Th	% of CD3+CD4+CD25+ and cells/ μ L
CD25+PD1+CD3+CD8+	PD upregulation on activated Tc	% of CD3+CD8+CD25+ and cells/ μ L

Table C-43: Marker combinations for assessment of depletion of CD123+ cells.

Marker	Cells	Unit
CD14 ^{-low} CD123+	pDC (plamacytoid dendritic cells) based on CD123 marker and basophils and mDCs (myeloid dendritic cells)	% of PBMC and cells/ μ L
CD14 ^{-low} CD303+	pDC based on CD303 marker	% of PBMC and cells/ μ L
CD14 ^{-low} CD123+CD303+	overlap between CD123 and CD303	% of PBMC and cells/ μ L
CD14+	monocytes	% of PBMC and cells/ μ L
CD14+CD123+	CD123+ monocytes	% of CD14+and cells/ μ L
CD123+	absolute no. of target cells based on CD123	% of PBMC and cells/ μ L
CD303+	absolut no. of CD303 cells	% of PBMC and cells/ μ L

Serum samples for PK analysis are collected on days -7, d-4, d1 (pre-dose + 4 hrs post-dose), d4, d8 (pre-dose + 4 hrs post-dose), d11, d15 (pre-dose + 4 hrs post-dose), d18, d22 (pre-dose + 4 hrs post-dose), d25, d29, d32, and d36.

Serum samples for ADA analysis are collected on days -7, d1 (pre-dose), d8 (pre-dose), d15 (pre-dose), d22 (pre-dose), d29, and d36.

On day 4 and day 25, blood T cells are isolated from all animals and tested in an exhaustion test as described in Example 25.

On day 29, necropsy is performed on the group 2 animals. On day 36, necropsy is performed on all remaining animals.

Example 50: In vivo efficacy and safety in a non-human primate model – multispecific CD123/TCR binding Nanobody, experimental results

In vivo efficacy and safety of the multispecific CD123/TCR binding polypeptide T017000139 was evaluated in a non-human primate model.

Animals treated with the reference compound (MGD006, Macrogenics) were included as positive control. Treatment with the irrelevant/TCR binding polypeptide T017000129 was used as specificity control for the CD123-targeting moiety of the multispecific polypeptide. The reference compound, the irrelevant/TCR binding polypeptide and the multispecific CD123/TCR binding polypeptide were

administered via continuous i.v. infusion after a 7-day NaCl infusion 'pre-treatment' of cynomolgus monkeys. The irrelevant/TCR binding polypeptide and the multispecific CD123/TCR binding polypeptide were administered for 4 weeks as 4-day on/3-day off infusion at equimolar doses to the reference compound in a weekly dose escalation scheme according to Table C-44.

5 **Table C-44: Treatment regimen.**

Group	Compound	Dose levels (ng/kg/day)				Route of administration
		Test week 1 (D1-5)	Test week 2 (D8-12)	Test week 3 (D15-19)	Test week 4 (D22-26)	
1	Reference compound (MGD006)	100	300	600	1000	continuous 24h infusion
2	Irrelevant/TCR polypeptide (T017000129)	49.5	148.4	296.8	494.6	continuous 24h infusion
3	CD123/TCR polypeptide (T017000139)	74.0	222.1	444.3	740.4	continuous 24h infusion

T cell redistribution from the blood was monitored by measuring T cell subsets using flow cytometry on test days -7, d-4, d1 (pre-dose + 4 hrs post-dose), d4, d8 (pre-dose + 4 hrs post-dose), d11, d15 (pre-dose + 4 hrs post-dose), d18, d22 (pre-dose + 4 hrs post-dose), d25, d29, d32, and d36.

10 As shown in Figure 59, in the positive control group treated with the reference compound, the numbers of circulating CD4⁺CD3⁺ and CD8⁺CD3⁺ T cells fluctuated during the different dosing cycles, suggesting a trafficking and/or margination, rather than depletion. In contrast, treatment with CD123/TCR polypeptide or with the irrelevant/TCR polypeptide did not results in a strong fluctuation of CD4⁺CD3⁺ or CD8⁺CD3⁺ T cell numbers.

15 The circulating CD123⁺CD14⁻ cell numbers were explored as a pharmacodynamic endpoint to asses *in vivo* efficacy by measuring the number of CD123⁺CD14⁻ cells in PBMC using flow cytometry in the blood on test days -7, d-4, d1 (pre-dose + 4 hrs post-dose), d4, d8 (pre-dose + 4 hrs post-dose), d11, d15 (pre-dose + 4 hrs post-dose), d18, d22 (pre-dose + 4 hrs post-dose), d25, d29, d32, and d36.

20 The results are depicted in Figure 60. CD123⁺CD14⁻ cells were depleted in the animals treated with the reference compound MGD006 (positive control), although a loss of efficacy was observed towards the 4th dosing cycle. Treatment with CD123/TCR polypeptide caused a depletion of CD123⁺CD14⁻ cells in the blood already from the first dosing cycle, that persisted through the 4th and

final dosing cycle. In animals treated with the irrelevant/TCR polypeptide, no significant depletion of CD123⁺CD14⁻ cells was observed.

Next, the expression of PD-1 on circulating CD4⁺CD3⁺ T cells and CD8⁺CD3⁺ T cells was explored as a surrogate marker to assess T cell exhaustion in vivo. For this, PD-1 expression was measured in PBMC using flow cytometry in the blood on test days -7, d-4, d1 (pre-dose + 4 hrs post-dose), d4, d8 (pre-dose + 4 hrs post-dose), d11, d15 (pre-dose + 4 hrs post-dose), d18, d22 (pre-dose + 4 hrs post-dose), d25, d29, d32, and d36.

The results are depicted in Figure 61. PD-1 expression was strongly increased on the majority of CD4⁺CD3⁺ T cells and CD8⁺CD3⁺ T cells in the animals treated with the reference compound MGD006 (positive control) and remained on approximately half of the CD4⁺CD3⁺ T cells and CD8⁺CD3⁺ T cells after termination of dosing. In contrast, PD-1 expression remained at baseline upon treatment with CD123/TCR polypeptide or with the irrelevant/TCR polypeptide throughout the dosing cycles.

Safety was assessed by evaluation of cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), TNF- α , TNF- β , IFN- γ) in the serum on test days -7, d-4, d1 (4 hrs post-dose), d4, d8 (4 hrs post-dose), d11, d15 (4 hrs post-dose), d18, d22 (4 hrs post-dose), and d25.

The levels of the following cytokines remained below detection limit of the assay: IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12(p70), TNF- α , TNF- β and IFN- γ . Interleukin-6 was detected in low concentrations in the positive control group at the beginning of the first dosing cycle. This increase was transient and only in one animal. In the group treated with CD123/TCR polypeptide, one animal showed detectable IL-6 concentrations pre-dose and one animal showed a transient small increase at the beginning of the second dosing cycle, suggesting manipulative stress. In the group treated with irrelevant/TCR polypeptide, one animal showed a transient small increase in IL-6 in the third dosing cycle, again suggesting manipulative stress. The results from the IL-6 measurements are depicted in Figure 62.

Table A-1: Sequence alignment of TCR cluster A binders – part 1

SEQ ID NO:	Kabat #	1	10	20	30	40	50	60	70	78
42	T0170PMP056G05	:								
85	T0170PMP053D01	:								
136	T0170PMP067D01	:								
124	T0170PMP056F01	:								
152	T0170PMP069A06	:								
141	T0170PMP067F02	:								
142	T0170PMP068C03	:								
103	T0170PMP055E05	:								
96	T0170PMP055C02	:								
137	T0170PMP067D06	:								
105	T0170PMP055F03	:								
130	T0170PMP061A02	:								
122	T0170PMP056D11	:								
153	T0170PMP069B02	:								
172	T0170PMP070G02	:								
79	T0170PMP028B01	:								
81	T0170PMP028G06	:								
106	T0170PMP055F06	:								
78	T0170PMP027A05	:								
83	T0170PMP040C01	:								
80	T0170PMP028F10	:								
82	T0170PMP029F08	:								
90	T0170PMP055A08	:								
109	T0170PMP055G09	:								
145	T0170PMP068D05	:								
151	T0170PMP068F08	:								
171	T0170PMP070F11	:								
163	T0170PMP069E11	:								
175	T0170PMP084B07	:								
87	T0170PMP055A01	:								
88	T0170PMP055A02	:								
99	T0170PMP055D03	:								
101	T0170PMP055D10	:								
147	T0170PMP068E01	:								
177	T0170PMP084E03	:								

Table A-1: Sequence alignment of TCR cluster A binders – part 1 continued

		Kabat #	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	
	SEQ ID NO:	42																																		
	SEQ ID NO:	85																																		
5	SEQ ID NO:	136																																		
	SEQ ID NO:	124																																		
	SEQ ID NO:	152																																		
	SEQ ID NO:	141																																		
10	SEQ ID NO:	142																																		
	SEQ ID NO:	103																																		
	SEQ ID NO:	96																																		
	SEQ ID NO:	137																																		
	SEQ ID NO:	105																																		
15	SEQ ID NO:	130																																		
	SEQ ID NO:	122																																		
	SEQ ID NO:	153																																		
	SEQ ID NO:	172																																		
	SEQ ID NO:	79																																		
20	SEQ ID NO:	81																																		
	SEQ ID NO:	106																																		
	SEQ ID NO:	78																																		
	SEQ ID NO:	83																																		
	SEQ ID NO:	80																																		
25	SEQ ID NO:	82																																		
	SEQ ID NO:	90																																		
	SEQ ID NO:	109																																		
	SEQ ID NO:	145																																		
	SEQ ID NO:	151																																		
30	SEQ ID NO:	171																																		
	SEQ ID NO:	163																																		
	SEQ ID NO:	175																																		
	SEQ ID NO:	87																																		
	SEQ ID NO:	88																																		
35	SEQ ID NO:	99																																		
	SEQ ID NO:	101																																		
	SEQ ID NO:	147																																		
	SEQ ID NO:	177																																		

Table A-1: Sequence alignment of TCR cluster A binders – part 2

	Kabat #	1	10	20	30	40	50	60	70	79
SEQ ID NO: 42	T0170PMP056G05	:								
SEQ ID NO: 178	T0170PMP084E05	:								
SEQ ID NO: 107	T0170PMP055F08	:								
SEQ ID NO: 114	T0170PMP056C01	:								
SEQ ID NO: 126	T0170PMP056G02	:								
SEQ ID NO: 133	T0170PMP067A03	:								
SEQ ID NO: 135	T0170PMP067C09	:								
SEQ ID NO: 150	T0170PMP068F06	:								
SEQ ID NO: 158	T0170PMP069D02	:								
SEQ ID NO: 169	T0170PMP070D07	:								
SEQ ID NO: 176	T0170PMP084C02	:								
SEQ ID NO: 131	T0170PMP061B04	:								
SEQ ID NO: 159	T0170PMP069D07	:								
SEQ ID NO: 113	T0170PMP056B11	:								
SEQ ID NO: 180	T0170PMP084F10	:								
SEQ ID NO: 116	T0170PMP056C03	:								
SEQ ID NO: 156	T0170PMP069C04	:								
SEQ ID NO: 121	T0170PMP056D02	:								
SEQ ID NO: 132	T0170PMP067A01	:								
SEQ ID NO: 134	T0170PMP067B06	:								
SEQ ID NO: 164	T0170PMP069F05	:								
SEQ ID NO: 179	T0170PMP084F04	:								
SEQ ID NO: 98	T0170PMP055C10	:								
SEQ ID NO: 86	T0170PMP053E10	:								
SEQ ID NO: 92	T0170PMP055B01	:								
SEQ ID NO: 155	T0170PMP069C01	:								
SEQ ID NO: 138	T0170PMP067D09	:								
SEQ ID NO: 139	T0170PMP067E03	:								
SEQ ID NO: 120	T0170PMP056D01	:								
SEQ ID NO: 140	T0170PMP067E06	:								
SEQ ID NO: 162	T0170PMP069E09	:								
SEQ ID NO: 157	T0170PMP069C05	:								
SEQ ID NO: 166	T0170PMP070B08	:								
SEQ ID NO: 89	T0170PMP055A03	:								
SEQ ID NO: 118	T0170PMP056C07	:								

Table A-1: Sequence alignment of TCR cluster A binders – part 2 continued

		Kabat #	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112
	SEQ ID NO:	42																																		
	SEQ ID NO:	178																																		
5	SEQ ID NO:	107																																		
	SEQ ID NO:	114																																		
	SEQ ID NO:	126																																		
	SEQ ID NO:	133																																		
10	SEQ ID NO:	135																																		
	SEQ ID NO:	150																																		
	SEQ ID NO:	158																																		
	SEQ ID NO:	169																																		
	SEQ ID NO:	176																																		
15	SEQ ID NO:	131																																		
	SEQ ID NO:	159																																		
	SEQ ID NO:	113																																		
	SEQ ID NO:	180																																		
	SEQ ID NO:	116																																		
20	SEQ ID NO:	156																																		
	SEQ ID NO:	121																																		
	SEQ ID NO:	132																																		
	SEQ ID NO:	134																																		
	SEQ ID NO:	164																																		
25	SEQ ID NO:	179																																		
	SEQ ID NO:	98																																		
	SEQ ID NO:	86																																		
	SEQ ID NO:	92																																		
	SEQ ID NO:	155																																		
30	SEQ ID NO:	138																																		
	SEQ ID NO:	139																																		
	SEQ ID NO:	120																																		
	SEQ ID NO:	140																																		
	SEQ ID NO:	162																																		
35	SEQ ID NO:	157																																		
	SEQ ID NO:	166																																		
	SEQ ID NO:	89																																		
	SEQ ID NO:	118																																		

Table A-1: Sequence alignment of TCR cluster A binders – part 3

[illegible]

Table A-1: Sequence alignment of TCR cluster A binders – part 3 continued

		Kabat #	79		abc		90	100	110
	SEQ ID NO: 42	T0170PMP056G05	:		abc	:		100	110
	SEQ ID NO: 168	T0170PMP070C09	:		Y L Q M N S L K P E D T A V Y F C R A F S R I Y P D Y W G Q G T L I V T V S S	:		100	110
5	SEQ ID NO: 174	T0170PMP082B04	:	G.....	:	
	SEQ ID NO: 110	T0170PMP056A02	:	G.....	:	
	SEQ ID NO: 108	T0170PMP055G05	:	G.....	:	
	SEQ ID NO: 154	T0170PMP069B08	:	G.....	:	
10	SEQ ID NO: 97	T0170PMP055C06	:	N.....	:	
	SEQ ID NO: 100	T0170PMP055D06	:		F.....N.....	:	
	SEQ ID NO: 102	T0170PMP055E01	:	G.....	:	
	SEQ ID NO: 104	T0170PMP055F02	:	G.....	:	
	SEQ ID NO: 115	T0170PMP056C02	:	G.....	:	
15	SEQ ID NO: 173	T0170PMP070G06	:	G.....	:	
	SEQ ID NO: 165	T0170PMP069G08	:	G.....	:	
	SEQ ID NO: 170	T0170PMP070E07	:	G.....	:	
	SEQ ID NO: 146	T0170PMP068D07	:	G.....	:	
	SEQ ID NO: 127	T0170PMP056G11	:	G.....	:	
20	SEQ ID NO: 143	T0170PMP068C07	:	G.....	:	
	SEQ ID NO: 160	T0170PMP069E02	:	G.....	:	
	SEQ ID NO: 144	T0170PMP068C11	:	G.....	:	
	SEQ ID NO: 148	T0170PMP068E08	:	R.....	:	
	SEQ ID NO: 123	T0170PMP056E02	:	G.....	:	
25	SEQ ID NO: 112	T0170PMP056A10	:	R.....	:	
	SEQ ID NO: 119	T0170PMP056C10	:	R.....	:	
	SEQ ID NO: 93	T0170PMP055B02	:	R.....	:	
	SEQ ID NO: 111	T0170PMP056A08	:	R.....	:	
30	SEQ ID NO: 117	T0170PMP056C04	:	R.....	:	
	SEQ ID NO: 129	T0170PMP057D06	:	R.....	:	
	SEQ ID NO: 84	T0170PMP053A03	:	R.....	:	
	SEQ ID NO: 161	T0170PMP069E07	:	G.....	:	
	SEQ ID NO: 95	T0170PMP055B11	:	R.....	:	
35	SEQ ID NO: 125	T0170PMP056F08	:	R.....	:	
	SEQ ID NO: 167	T0170PMP070B09	:	N.....	:	
	SEQ ID NO: 149	T0170PMP068F04	:	R.....	:	
	SEQ ID NO: 91	T0170PMP055A10	:	R.....	:	
	SEQ ID NO: 94	T0170PMP055B03	:	R.....	:	
	SEQ ID NO: 128	T0170PMP057B02	:	L.....	:	

Table A-2: Sequence alignment of CD123 binding Nanobody A0110056A10 and family members thereof.

			1	10	20	30	40	50	60	70
	SEQ ID NO:	Kabat #	:							
5	1	A0110PMP056A10	:	EVQLVKSGGGLVQAGGSLRLSCAASGITSKINDMGWYRQTPGNVREWVASITATGTTNVRDSVKGRTISPDNAKSTVY						
	2	A0110PMP055A10	:	...E.....N..S..SDA.....K.....K.....N.....N...						
	3	A0110PMP055B11	:	...E.....N.....S.A.....K.....K.....N.....N...						
	3	A0110PMP055B12	:	...E.....N.....S.A.....K.....K.....N.....N...						
10	4	A0110PMP056D09	:	...E.....P.....F.....N.....N...						
	5	A0110PMP056G10	:	...E.....S.V.....K.....N.....N...						
	6	A0110PMP057B09	:	...E.....N..S..SDA.....K.....N.....N...						
	2	A0110PMP057D11	:	...E.....N..S..SDA.....K.....N.....N...						
			80	90	100	110				
		Kabat #	:		abc					
15	1	A0110PMP056A10	:	LQMNSLKPEDTTVYYCNTFFPPISNF-WGQGTLVTVSS						
	2	A0110PMP055A10	:A.....Q.....						
	3	A0110PMP055B11	:A.....Q.....						
	3	A0110PMP055B12	:A.....Q.....						
20	4	A0110PMP056D09	:-.....						
	5	A0110PMP056G10	:-.....						
	6	A0110PMP057B09	:A.....Q.....						
	2	A0110PMP057D11	:A.....Q.....						

Table A-3: Sequence alignment of CD123 binding Nanobody A0110055F03 and family members thereof.

			1	10	20	30	40	50	60	70
		Kabat #	:							
SEQ ID NO: 7	A0110PMP055F03	:	EVQLVESGGGLVQAGG	PLRLSCAASGR	TFSSYVMGWFRQ	APGKEREFVAAI	YWSNGK	TYTDSV	KGRFTISG	DNAKNTVY
5	SEQ ID NO: 8	A0110PMP055A04	:S.....S.....S.....S.....S.....L.....L.....
	SEQ ID NO: 9	A0110PMP056C03	:S.....S.....S.....S.....S.....E.....L.....
	SEQ ID NO: 10	A0110PMP056G01	:S.....S.....W.....S.....E.....L.....L.....
			80	90	100	110				
		Kabat #	:							
SEQ ID NO: 7	A0110PMP055F03	:	LQMNSLNPEDTAVY	YCVADKDET	GFRTLP	IAYDYWGQGT	QVT	VSS		
10	SEQ ID NO: 8	A0110PMP055A04	:RD.....RD.....RD.....RD.....RD.....		
	SEQ ID NO: 9	A0110PMP056C03	:RY.....RY.....RY.....RY.....RY.....		
	SEQ ID NO: 10	A0110PMP056G01	:N.....RD.....RD.....RD.....RD.....		

Table A-4: CDRs and framework sequences of CD123 binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. "SEQ" refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
1	A0110PMP 056A10	26	EVQLVKSGGGLVQ AGGSLRLSCAAS	11	GITSKIN DMG	30	WYRQTPGNY REWVA	17	SITATG TTN	34	YRDSVKGRFTISRDNAKSTV YLQMNSLKPEDTTVYYCNT	21	FPPISNF	40	WGQGTL VTVSS
2	A0110PMP 055A10 / A0110PMP 057D11	27	EVQLVESGGGLVQ AGGSLRLSCAAN	12	GISSKSD AMG	31	WYRQTPGKY REWVA	17	SITATG TTN	35	YRDSVKGRFTISRDNAKNTV YLQMNSLKPEDTTVYYCNT	22	FPAISNF	41	WGQGTQ VTVSS
3	A0110PMP 055B11 / A0110PMP 055B12	27	EVQLVESGGGLVQ AGGSLRLSCAAN	13	GITSKSN AMG	31	WYRQTPGKY REWVA	17	SITATG TTN	35	YRDSVKGRFTISRDNAKNTV YLQMNSLKPEDTTVYYCNT	22	FPAISNF	41	WGQGTQ VTVSS
4	A0110PMP 056D09	28	EVQLVESGGGLVQ AGGSLRLSCAAS	14	GIPSKIN DMG	32	WFRQTPGNY REWVA	17	SITATG TTN	35	YRDSVKGRFTISRDNAKNTV YLQMNSLKPEDTTVYYCNT	21	FPPISNF	40	WGQGTL VTVSS
5	A0110PMP 056G10	28	EVQLVESGGGLVQ AGGSLRLSCAAS	11	GITSKIN DMG	30	WYRQTPGNY REWVA	17	SITATG TTN	34	YRDSVKGRFTISRDNAKSTV YLQMNSLKPEDTTVYYCNT	21	FPPISNF	40	WGQGTL VTVSS
6	A0110PMP 057B09	28	EVQLVESGGGLVQ AGGSLRLSCAAS	15	GITSKSN VMG	31	WYRQTPGKY REWVA	17	SITATG TTN	35	YRDSVKGRFTISRDNAKNTV YLQMNSLKPEDTTVYYCNT	22	FPAISNF	41	WGQGTQ VTVSS
7	A0110PMP 055F03	29	EVQLVESGGGLVQ AGGPLRLSCAAS	16	GRTFSSY VMG	33	WFRQAPGKE REFVA	18	AIYWSN GKTQ	36	YTDSVKGRFTISGDNAKNTV YLQMNSLNPEDTAVYYCVA	23	DKDETGFRTL PIAYDY	41	WGQGTQ VTVSS
8	A0110PMP 055A04	28	EVQLVESGGGLVQ AGGSLRLSCAAS	16	GRTFSSY VMG	33	WFRQAPGKE REFVA	19	AIYWSS GKTE	37	YTDSVKGRFTLSGDNAKNTV YLQMNSLNPEDTAVYYCVA	24	DKDRDGFRTL PIAYDY	41	WGQGTQ VTVSS
9	A0110PMP 056C03	28	EVQLVESGGGLVQ AGGSLRLSCAAS	16	GRTFSSY VMG	33	WFRQAPGKE REFVA	19	AIYWSS GKTE	38	YTESVKGRFTLSGDNAKNTV YLQMNSLNPEDTAVYYCVA	25	DKDRYGFRTL PIAYDY	41	WGQGTQ VTVSS
10	A0110PMP 056G01	28	EVQLVESGGGLVQ AGGSLRLSCAAS	16	GRTFSSY VMG	33	WFRQAPGKE REFVA	20	AIWSS GKTE	39	YTDSVKGRFTLSGDNAKNTV YLOMNNLNPNEDTAVYYCVA	24	DKDRDGFRTL PIAYDY	41	WGQGTQ VTVSS

Table A-5 – part 1: CDRs and framework sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
42	T0170PMP 056G05	226	EVQLVESGGGLVQP GGSRLSCVAS	181	GDVHKIN FLG	251	WYRQAPGKE REKVA	192	HISIGD QTD	277	YADSAKGRFTISRDESKNMV YLQMNLSLKPEDTAVYFCRA	218	FSRIYPYDY	320	WGQGTQ VTVSS
78	T0170PMP 027A05	227	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	252	WYRQAPGKE RELVA	193	TITIGD TTD	278	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVHFCRA	219	GSRLYPYNY	321	WGQGTQ VTVSS
79	T0170PMP 028B01	227	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	253	WYRQAPGKE RGLVA	193	TITIGD TTD	279	YADYAKGRFTISRDEARNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGTQ VTVSS
80	T0170PMP 028F10	228	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	254	WYRQAPGKE REMA	194	TITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGTQ VTVSS
81	T0170PMP 028G06	227	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	252	WYRQAPGKE RELVA	193	TITIGD TTD	279	YADYAKGRFTISRDEARNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	320	WGQGTQ VTVSS
82	T0170PMP 029F08	227	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	254	WYRQAPGKE REMA	194	TITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	320	WGQGTQ VTVSS
83	T0170PMP 040C01	227	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	252	WYRQAPGKE RELVA	193	TITIGD TTD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGTQ VTVSS
84	T0170PMP 053A03	229	EVQLVESGGGLVQP GGSRLSCAVS	183	GSVHLLN FLG	254	WYRQAPGKE REMA	195	HITIGD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGTQ VTVSS
85	T0170PMP 053D01	230	EVQLVESGGGLVQP GGSRLSCAAS	184	GAHVHKIN FLG	255	WYRQAPGKE REMA	196	TITIGD DVD	282	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	320	WGQGTQ VTVSS
86	T0170PMP 053E10	231	EVQLVESGGGLVQP GGSRLSCRAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITIGD ATD	283	YAESAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	321	WGQGTQ VTVSS
87	T0170PMP 055A01	232	EVQLVESGGGLVQP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
88	T0170PMP 055A02	227	EVQLVESGGGLVQP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
89	T0170PMP 055A03	226	EVQLVESGGGLVQP GGSRLSCVAS	181	GDVHKIN FLG	251	WYRQAPGKE REKVA	198	HITIGD QAD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGTQ VTVSS
90	T0170PMP 055A08	233	EVQLVESGGGSVQVP GGSRLSCAAS	182	GSVHKIN FLG	254	WYRQAPGKE REMA	194	TITIGD ATD	284	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	320	WGQGTQ VTVSS
91	T0170PMP 055A10	227	EVQLVESGGGLVQP GGSRLSCAAS	183	GSVHLLN FLG	254	WYRQAPGKE REMA	199	HISIGD ATD	285	YAHFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIYPYDY	322	WGRGTQ VTVSS
92	T0170PMP 055B01	231	EVQLVESGGGLVQP GGSRLSCRAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	200	HITIGD ATV	283	YAESAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	321	WGQGTQ VTVSS

Table A-5 – part 2: CDRs and framework sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
93	T0170PMP 055B02	227	EVQLVESGGGLVQP GGSRLSCAAS	183	GSVHLIN FLG	257	WYRQCPGKE REMA	195	HIT1AD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNSLRPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGT VTSS
94	T0170PMP 055B03	234	EVQLVESGGGLVQP GGSRLPSCAAS	183	GSVHLIN FLG	254	WYRQAPGKE REMA	195	HIT1AD ATD	285	YAHFAKGRFTISRDEAKNMV YLQMNSLRPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGT VTSS
95	T0170PMP 055B11	227	EVQLVESGGGLVQP GGSRLSCAAS	183	GSVHLIN FLG	254	WYRQAPGKE REMA	195	HIT1AD ATD	285	YAHFAKGRFTISRDEAKNMV YLQMNSLRPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGT VTSS
96	T0170PMP 055C02	230	EVQLVESGGGLVQP GGSRLSCAAS	184	GAVHKIN FLG	255	WYRQTPGKE REMA	201	HIT1GD EVD	286	YADSAKGRFTISRDEAKNMV YLQMNLSLTPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGT VTSS
97	T0170PMP 055C06	235	EVQLVESGGGLVHP GGSRLSCAAS	181	GDVHKIN FLG	258	WHRQPPGKE REMA	202	HIT1GD VTD	287	YADSAKGRFTISRDEAKNMV YLQMNLSLTPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGT VTSS
98	T0170PMP 055C10	227	EVQLVESGGGLVQP GGSRLSCAAS	186	GDVHKIN VLG	256	WYRQAPAKE REMA	197	HIT1GD ATD	288	YADSAKGRFTISRDEAKNMV HLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	321	WGQGT VTSS
99	T0170PMP 055D03	227	EVQLVESGGGLVQP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	203	HIT1GD ATN	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGT VTSS
100	T0170PMP 055D06	227	EVQLVESGGGLVQP GGSRLSCAAS	181	GDVHKIN FLG	259	WHRQAPGKE REMA	202	HIT1GD VTD	289	YADSAKGRFTISRDEAKNMV FLQMNLSLKPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGT VTSS
101	T0170PMP 055D10	227	EVQLVESGGGLVQP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HIT1GD ATD	290	YAGSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGT VTSS
102	T0170PMP 055E01	227	EVQLVESGGGLVQP GGSRLSCAAS	187	GEVYKIN FLG	251	WYRQAPGKE REMA	204	HIT1AD VAD	291	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	321	WGQGT VTSS
103	T0170PMP 055E05	230	EVQLVESGGGLVQP GGSRLSCAAS	184	GAVHKIN FLG	260	WYRQAPGKE REMA	205	TIT1GD EVD	292	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGT VTSS
104	T0170PMP 055F02	227	EVQLVESGGGLVQP GGSRLSCAAS	187	GEVYKIN FLG	251	WYRQAPGKE REMA	204	HIT1AD VAD	291	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	320	WGQGT VTSS
105	T0170PMP 055F03	226	EVQLVESGGGLVQP GGSRLSCVAS	185	GDVHKIN ILG	251	WYRQAPGKE REMA	206	HIT1SD QTD	293	YAESAKGRFTISRDESKNMV YLQMNLSLKPEDTAVYLCRA	218	FSRIYPYDY	320	WGQGT VTSS
106	T0170PMP 055F06	227	EVQLVESGGGLVQP GGSRLSCAAS	182	GSVHKIN FLG	252	WYRQAPGKE RELVA	194	TIT1GD ATD	279	YADYAKGRFTISRDEARNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	320	WGQGT VTSS
107	T0170PMP 055F08	227	EVQLVESGGGLVQP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HIT1GD ATD	294	YADSAKGRFAISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGT VTSS
108	T0170PMP 055G05	227	EVQLVESGGGLVQP GGSRLSCAAS	181	GDVHKIN FLG	259	WHRQAPGKE REMA	197	HIT1GD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGT VTSS

Table A-5 – part 3: CDRs and framework sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
109	T0170PMP 055G09	233	EVQLVESGGGVQFP GSLRLSCAAS	182	GSVHKIN FLG	261	WYQAPGKE REVVA	194	TITIGD ATD	284	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	219	GSRLYPNY	321	WGQGTQ VTVSS
110	T0170PMP 056A02	236	EVQLVESGGGLVQFP GGSARLSCVAS	181	GDVHKIN FLG	251	WYQAPGKE REKVA	207	HITIGD QTD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	222	GSRIWPYDY	321	WGQGTQ VTVSS
111	T0170PMP 056A08	227	EVQLVESGGGLVQFP GSLRLSCAAS	183	GSVHLIN FLG	254	WYQAPGKE REMA	195	HITIAD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNLSLRPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGTQ VTVSS
112	T0170PMP 056A10	229	EVQLVESGGGLVQFP GSLRLSCAVS	183	GSVHLIN FLG	262	WYQAPGKE RGVVA	195	HITIAD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNLSLRPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGTQ VTVSS
113	T0170PMP 056B11	237	EVQLVESGGGLVQA GSLTLSCAAS	185	GDVHKIN ILG	256	WYQAPAKE REMA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	221	YSRIYPNY	320	WGQGTQ VTVSS
114	T0170PMP 056C01	227	EVQLVESGGGLVQFP GSLRLSCAAS	188	GGVHKIN ILG	256	WYQAPAKE REMA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	221	YSRIYPNY	320	WGQGTQ VTVSS
115	T0170PMP 056C02	227	EVQLVESGGGLVQFP GSLRLSCAAS	187	GEVYKIN FLG	251	WYQAPGKE REKVA	204	HITIAD VAD	295	YADFAQGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	222	GSRIWPYDY	321	WGQGTQ VTVSS
116	T0170PMP 056C03	227	EVQLVESGGGLVQFP GSLRLSCAAS	185	GDVHKIN ILG	256	WYQAPAKE REMA	208	HITIGD TTD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	221	YSRIYPNY	321	WGQGTQ VTVSS
117	T0170PMP 056C04	227	EVQLVESGGGLVQFP GSLRLSCAAS	183	GSVHLIN FLG	254	WYQAPGKE REMA	195	HITIAD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNLSLRPEDTAVYFCRA	220	GSRIYPYDY	323	WGHGTL VTVSS
118	T0170PMP 056C07	226	EVQLVESGGGLVQFP GSLRLSCVAS	181	GDVHKIN FLG	251	WYQAPGKE REKVA	198	HITIGD QAD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	220	GSRIYPYDY	324	WGRGTL VTVSS
119	T0170PMP 056C10	229	EVQLVESGGGLVQFP GSLRLSCAVS	183	GSVHLIN FLG	263	WYQAPGKE REMT	195	HITIAD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNLSLRPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGTQ VTVSS
120	T0170PMP 056D01	238	EVQLVESGGDIVQFP GSLRLSCAAS	181	GDVHKIN FLG	254	WYQAPGKE REMA	195	HITIAD ATD	296	YAEFAKGRFTISRDEPKNMV HLQMNLSLPEDTAVYLCRA	223	GSRIYPNY	321	WGQGTQ VTVSS
121	T0170PMP 056D02	227	EVQLVESGGGLVQFP GSLRLSCAAS	185	GDVHKIN ILG	256	WYQAPAKE REMA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	221	YSRIYPNY	322	WGRGTQ VTVSS
122	T0170PMP 056D11	226	EVQLVESGGGLVQFP GSLRLSCVAS	185	GDVHKIN ILG	251	WYQAPGKE REKVA	209	RISISD QTD	293	YAESAKGRFTISRDESKNMV YLQMNLSLPEDTAVYLCRA	218	FSRIYPYDY	320	WGQGTQ VTVSS
123	T0170PMP 056E02	239	EVQLVESGGGLVQFP EGSLRLSCAAS	187	GEVYKIN FLG	251	WYQAPGKE REKVA	204	HITIAD VAD	297	YADFAKGRFTISRDEAKNMV YLQMNLSLPEDTAVYFCRA	222	GSRIWPYDY	320	WGQGTQ VTVSS
124	T0170PMP 056F01	240	EVQLVESGGGLVQFP GSLKLPCAAS	184	GAHVHKIN FLG	255	WYRQPEKE REMA	205	TITIGD EVD	282	YADSAKGRFTISRDEAKNMV YLQMTSLKPEDTAVYVCRA	219	GSRLYPNY	320	WGQGTQ VTVSS

Table A-5 – part 4: CDRs and framework sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
125	T0170PMP 056F08	241	EVQLVESGGGLVQP GSGISLSCAAS	183	GSVHLLN FLG	254	WYRQAPGKE REMA	195	HIT1AD ATD	285	YAHFAKGRFTISRDEAKNMV YLQMNSLRPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGTQ VTVSS
126	T0170PMP 056G02	242	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HIT1GD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
127	T0170PMP 056G11	227	EVQLVESGGGLVQP GSLRLSCAAS	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	210	HIT1AD AAD	298	YADFAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	222	GSRIWPYDY	321	WGQGTQ VTVSS
128	T0170PMP 057B02	243	EVQLVESGGGLVQP GSLRLSCAAS	189	GSVYKIN FLS	264	WYRQAPGHE RELVA	211	TIT1GD AAD	299	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGTQ VTVSS
129	T0170PMP 057D06	227	EVQLVESGGGLVQP GSLRLSCAAS	183	GSVHLLN FLG	254	WYRQAPGKE REMA	195	HIT1AD ATD	281	YSHFAKGRFTISRDEAKNMV YLQMNSLRPEDTAVYFCRA	220	GSRIYPYDY	321	WGQGTQ VTVSS
130	T0170PMP 061A02	226	EVQLVESGGGLVQP GSLRLSCVAS	185	GDVHKIN ILG	251	WYRQAPGKE REKVA	212	HIA1SD QTD	293	YAESAKGRFTISRDESKNMV YLQMNSLKPEDTAVYLCRA	218	FSRIYPYDY	320	WGQGTQ VTVSS
131	T0170PMP 061B04	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HIT1GD ATD	300	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
132	T0170PMP 067A01	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	265	WYRQAPAKE RGMVA	197	HIT1GD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	221	YSRIYPYNY	321	WGQGTQ VTVSS
133	T0170PMP 067A03	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	266	WYRQAPAKE HEMVA	197	HIT1GD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
134	T0170PMP 067B06	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	267	WYRQAPARE REMA	197	HIT1GD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	221	YSRIYPYNY	321	WGQGTQ VTVSS
135	T0170PMP 067C09	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HIT1GD ATD	301	YADSAKGRFTISRDEAKNMV YLQMNSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
136	T0170PMP 067D01	230	EVQLVESGGGLVQP GSLKLSCAAS	184	GAHVHIN FLG	255	WYRQTPKE REMA	205	TIT1GD EVD	282	YADSAKGRFTISRDEAKNMV YLQMTSLKPEDTAVYVCRA	219	GSRLYPYNY	320	WGQGTQ VTVSS
137	T0170PMP 067D06	230	EVQLVESGGGLVQP GSLKLSCAAS	184	GAHVHIN FLG	260	WYRQAPEKE REMA	205	TIT1GD EVD	302	YADSAKGRFTISRDEAKNMV YLQMTSLKPEDTAVYFCRA	223	GSRIYPYNY	321	WGQGTQ VTVSS
138	T0170PMP 067D09	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	213	HIT1GD ATS	303	YAGSAKGRFTISRDEAKNMV YLQNLNLPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
139	T0170PMP 067E03	227	EVQLVESGGGLVQP GSLRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	213	HIT1GD ATS	304	YADSAKGRFTISRDEAKNMV YLQNLNLPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
140	T0170PMP 067E06	238	EVQLVESGGDLVQP GSLRLSCAAS	181	GDVHKIN FLG	254	WYRQAPGKE REMA	195	HIT1AD ATD	305	YAEFAKGRFTISRDEPKNMV YLQMNSLKPEDTAVYLCRA	223	GSRIYPYNY	321	WGQGTQ VTVSS

Table A-5 – part 5: CDRs and framework sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
141	T0170PMP 067F02	230	EVQLVESGGGLVQ P184	184	GA VHKIN FLG	255	WYRQTPKE REMA	205	TTITGD EVD	306	YAHSAKGRFTISRDEAKNMV YLQMTSLKPEDTAVYVCRA	219	GSRLYPNY	321	WGQGTQ VTSS
142	T0170PMP 068C03	230	EVQLVESGGGLVQ P184	184	GA VHKIN FLG	255	WYRQTPKE REMA	214	TTITGD EVA	282	YADSAKGRFTISRDEAKNMV YLQMTSLKPEDTAVYVCRA	219	GSRLYPNY	321	WGQGTQ VTSS
143	T0170PMP 068C07	227	EVQLVESGGGLVQ P187	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	210	HITITAD AAD	291	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	321	WGQGTQ VTSS
144	T0170PMP 068C11	227	EVQLVESGGGLVQ P187	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	210	HITITAD AAD	291	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIWPYDY	321	WGQGTQ VTSS
145	T0170PMP 068D05	244	EVQLVESGGGSVQ P182	182	GSVHKIN FLG	254	WYRQAPGKE REMA	194	TTITGD ATD	284	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPNY	321	WGQGTQ VTSS
146	T0170PMP 068D07	227	EVQLVESGGGLVQ P187	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	204	HITITAD VAD	307	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	320	WGQGTQ VTSS
147	T0170PMP 068E01	245	EVQLVESGGGLVQ P185	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITITGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPNY	320	WGQGTQ VTSS
148	T0170PMP 068E08	227	EVQLVESGGGLVQ P187	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	210	HITITAD AAD	308	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	321	WGQGTQ VTSS
149	T0170PMP 068F04	227	EVQLVESGGGLVQ P183	183	GSVHLLN FLG	254	WYRQAPGKE REMA	215	HITITAD VTD	309	YSYFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIWPYDY	321	WGQGTQ VTSS
150	T0170PMP 068F06	227	EVQLVESGGGLVQ P185	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITITGD ATD	310	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPNY	320	WGQGTQ VTSS
151	T0170PMP 068F08	233	EVQLVESGGGSVQ P182	182	GSVHKIN FLG	268	WYRQAPGKE RGMA	194	TTITGD ATD	284	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPNY	321	WGQGTQ VTSS
152	T0170PMP 069A06	230	EVQLVESGGGLVQ P184	184	GA VHKIN FLG	255	WYRQTPKE REMA	205	TTITGD EVD	311	YEDSAKGRFTISRDEAKNMV YLQMTGLKPEDTAVYVCRA	219	GSRLYPNY	320	WGQGTQ VTSS
153	T0170PMP 069B02	246	EVQLVESGGGLVQ P185	185	GDVHKIN ILG	251	WYRQAPGKE REKVA	206	HITITSD QTD	293	YAESAKGRFTISRDESKNMV YLQMNLSLKPEDTAVYLCRA	218	FSRIYPYDY	321	WGQGTQ VTSS
154	T0170PMP 069B08	227	EVQLVESGGGLVQ P190	190	GDVYKIN FLG	259	WHRQAPGKE REKVA	197	HITITGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIWPYDY	320	WGQGTQ VTSS
155	T0170PMP 069C01	231	EVQLVESGGGLVQ P185	185	GDVHKIN ILG	269	WYRQAPAKE REMA	197	HITITGD ATD	283	YAESAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPNY	321	WGQGTQ VTSS
156	T0170PMP 069C04	227	EVQLVESGGGLVQ P185	185	GDVHKIN ILG	270	WYRQAPAKG REMA	197	HITITGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPNY	321	WGQGTQ VTSS

Table A-5 – part 6: CDRs and framework sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ	FR1	SEQ	CDR1	SEQ	FR2	SEQ	CDR2	SEQ	FR3	SEQ	CDR3	SEQ	FR4
157	T0170PMP 069C05	238	EVQLVESGGDLVQPP GGSRLSCAAS	181	GDVHKIN FLG	254	WYRQAPGKE REMA	195	HITIGD ATD	305	YAEFAKGRFTISRDEPKNMV YLQMNLSLKPEDTAVYLCRA	224	GSRIYPYSY	321	WGQGTQ VTVSS
158	T0170PMP 069D02	247	EVQLVESGGGMVQPP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
159	T0170PMP 069D07	227	EVQLVESGGGLVQPP GGSRLSCAAS	185	GDVHKIN ILG	271	WYRQVPAKE REMA	197	HITIGD ATD	300	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
160	T0170PMP 069E02	227	EVQLVESGGGLVQPP GGSRLSCAAS	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	210	HITIGD AAD	291	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	320	WGQGTQ VTVSS
161	T0170PMP 069E07	227	EVQLVESGGGLVQPP GGSRLSCAAS	183	GSVHLIN FLG	254	WYRQAPGKE REMA	195	HITIGD ATD	312	YSHFAKGRFTISRDEAKNMV YLQMNGLRPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGTQ VTVSS
162	T0170PMP 069E09	227	EVQLVESGGGLVQPP GGSRLSCAAS	181	GDVHKIN FLG	254	WYRQAPGKE REMA	195	HITIGD ATD	305	YAEFAKGRFTISRDEPKNMV YLQMNLSLKPEDTAVYLCRA	223	GSRIYPYNY	321	WGQGTQ VTVSS
163	T0170PMP 069E11	227	EVQLVESGGGLVQPP GGSRLSCAAS	191	GEVHKIN ILG	272	WYRQCPGKE RDMVA	194	TITIGD ATD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	225	LSRLYPYNY	320	WGQGTQ VTVSS
164	T0170PMP 069F05	227	EVQLVESGGGLVQPP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITIGD ATD	313	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYLCRA	221	YSRIYPYNY	321	WGQGTQ VTVSS
165	T0170PMP 069G08	227	EVQLVESGGGLVQPP GGSRLSCAAS	187	GEVYKIN FLG	273	WYRQAPGKE REKVA	204	HITIGD VAD	291	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	320	WGQGTQ VTVSS
166	T0170PMP 070B08	238	EVQLVESGGDLVQPP GGSRLSCAAS	181	GDVHKIN FLG	254	WYRQAPGKE REMA	195	HITIGD ATD	314	YAEFAKGRFTISRDEPKNMV YLQMNLSLKPDVDTAVYLCRA	223	GSRIYPYNY	320	WGQGTQ VTVSS
167	T0170PMP 070B09	227	EVQLVESGGGLVQPP GGSRLSCAAS	183	GSVHLIN FLG	251	WYRQAPGKE REKVA	195	HITIGD ATD	315	YSHFAKGRFTISRDEAKNMV YLQMNILRPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGTQ VTVSS
168	T0170PMP 070C09	248	EVQLVESGGGLVQPP GGSRLSCVAS	181	GDVHKIN FLG	251	WYRQAPGKE REKVA	198	HITIGD QAD	280	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	220	GSRIYPYDY	320	WGQGTQ VTVSS
169	T0170PMP 070D07	227	EVQLVESGGGLVQPP GGSRLSCAAS	185	GDVHKIN ILG	256	WYRQAPAKE REMA	197	HITIGD ATD	316	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDAAVYFCRA	221	YSRIYPYNY	320	WGQGTQ VTVSS
170	T0170PMP 070E07	227	EVQLVESGGGLVQPP GGSRLSCAAS	187	GEVYKIN FLG	251	WYRQAPGKE REKVA	204	HITIGD VAD	298	YADFAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	222	GSRIWPYDY	320	WGQGTQ VTVSS
171	T0170PMP 070F11	233	EVQLVESGGSVQPP GGSRLSCAAS	182	GSVHKIN FLG	274	WYRQAPGKE REMA	194	TITIGD ATD	284	YADSAKGRFTISRDEAKNMV YLQMNLSLKPEDTAVYFCRA	219	GSRLYPYNY	321	WGQGTQ VTVSS
172	T0170PMP 070G02	226	EVQLVESGGGLVQPP GGSRLSCVAS	185	GDVHKIN ILG	251	WYRQAPGKE REKVA	206	HITIGD QTD	317	YAESAKGRFTISRDESKNMV YLQMNLSLKPEDAAVYLCRA	218	FSRIYPYDY	321	WGQGTQ VTVSS

Table A-5 – part 7: CDRs and framework sequences of TCR binding sequences of TCR binding building blocks, plus preferred combinations as provided in formula I, namely FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. “SEQ” refers to the given SEQ ID NO. The first column refers to the SEQ ID NO of the complete ISV, i.e. FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. CDR1, CDR2 and CDR3 were determined according to Kontermann, 2010.

SEQ	Nanobody	SEQ FR1	SEQ	CDR1	SEQ FR2	SEQ CDR2	SEQ FR3	SEQ	CDR3	SEQ FR4	
173	T0170PMP 070G06	EVQLVESGGGLVQFP187	GEVYKIN FLG	251	WYRQAPGKE REKVA	204	HIT1AD VAD	291	YADFAKGRFTISRDEAKNMV	321	WGQGTQ
		GGSLRPSCAAS							YLQMSLKPEDTAVYFCRA		VTVSS
174	T0170PMP 082B04	EVQLVESGGGLVQFP181	GDVHKIN FLG	251	WYRQAPGKE REKVA	216	HIT1AD QAD	280	YADSAKGRFTISRDEAKNMV	320	WGQGTQ
		GGSLRPSCVAS							YLQMSLKPEDTAVYFCRA		VTVSS
175	T0170PMP 084B07	EVQLVESGGGLVQFP191	GEVHKIN ILG	275	WYRQAPGKE RDMVA	217	TITIGD ETQ	280	YADSAKGRFTISRDEAKNMV	320	WGQGTQ
		GGSLRLSCAAS							YLQMSLKPEDTAVYFCRA		VTVSS
176	T0170PMP 084C02	EVQLVESGGGLVQFP185	GDVHKIN ILG	256	WYRQAPAKE REMVA	197	HITIGD ATD	318	YADSAKGRFTISRDEAKNMV	320	WGQGTQ
		GGSLRLSCAAS							YLQMSLKPEDTAVYFCRA		VTVSS
177	T0170PMP 084E03	EVQLVESGGGLVQFP185	GDVHKIN ILG	256	WYRQAPAKE REMVA	197	HITIGD ATD	319	YADSAKGRFTISRDEAKNMV	320	WGQGTQ
		GGSLRLSCAAS							YLQMSLKPEDTAVYFCRA		VTVSS
178	T0170PMP 084E05	EVQLVESGGGLVQFP185	GDVHKIN ILG	276	WYRQAPAKE REMVA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV	320	WGQGTQ
		GGSLRLSCAAS							YLQMSLKPEDTAVYFCRA		VTVSS
179	T0170PMP 084F04	EVQLVESGGGLVQFP185	GDVHKIN ILG	276	WYRQAPAKE REMVA	197	HITIGD ATD	300	YADSAKGRFTISRDEAKNMV	321	WGQGTQ
		GGSLRLSCAAS							YLQMSLKPEDTAAVFCRA		VTVSS
180	T0170PMP 084F10	EVQLVESGGGWVQA185	GDVHKIN ILG	256	WYRQAPAKE REMVA	197	HITIGD ATD	280	YADSAKGRFTISRDEAKNMV	320	WGQGTQ
		GGSLRLSCAAS							YLQMSLKPEDTAVYFCRA		VTVSS

Table A-6: Sequences of control Nanobodies. “SEQ” refers to a given SEQ ID NO; “ID” refers to identification name; “Sequence” denotes amino acid sequence

SEQ	ID	Sequence
44	RSV7B2 (Q10 8L)	EVQLVESGGGLVQAGDSLRSLSCAASGRFTSSYAMGWFRQAPGKEREFVAAISWSDGSTYYADSVKGRFTISRDNAKNTVYQLQMNSLKPEDTAVYYCAADLTSTNPGSYIYIWAYDYWGQGLTVTVSS
45	cAbLys3 D1E, Q5V, A6 E, Q108L)	EVQLVESGGGSVQAGGSLRSLSCAASGYTIGPYCMGWFRQAPGKEREGVAAINMGGGITYYADSVKGRFTISRDNAKNTVYLLMNSLEPEDTAVYYCAADSTIYASYECGHGLSTGGYGYDSWGQGLTVTVSS

Table A-7 – part 1: Sequences of multispecific polypeptides. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence.

[illegible]

Table A-7 – part 2: Sequences of multispecific polypeptides. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence.

[illegible]

Table A-7 – part 3: Sequences of multispecific polypeptides. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence.

[illegible]

Table A-7 – part 4: Sequences of multispecific polypeptides. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence.

[illegible]

Table A-7 – part 5: Sequences of multispecific polypeptides. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence.

[illegible]

Table A-8 – part 1: Sequences of CD123 and components of TCR complex. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence

SEQ	ID	Sequence
68	Human CD123 (NP_002174)	MVLLWLTLLLPCLLQTKEDPNPPIITNLRMKAKAQQLTWDLNRNVTDIECVKDADYSMPAVNNNSYQFGAISLCEVTNNTVTVRVANPPFSTWILF PENSGKPWGAENLTWHDVDFLSCSWAVGPGAPADVQDLYLVNANRRQQVECLHYKTDAGTRIGRFDITSRSSGSSSHILVRGRSAAFG IPCTDKFVFFSQIEILTPPNMTAKCNKTHSFHWKMRSHFNKFRYELQIKRMQPVITEQVRDRTSFQLNPGTYTVQIRARERVYEFLSAWSTP QRFECQEEGANTRAWRTSLLIALGTLTLLALVCFVICRRYLMQRLFPRI PHMKDPIGDSFQNDKLVVWEAGKAGLEECLECLVTEVVVQKT
69	Cyno CD123 (EHH61867.1)	MTLLWLTLLLVATPCLLRTEKEDPNAPIRNLRMKEKAQQQLMWDLNRNVTDVEIKGTDYSMPAMNNNSYQFGAISLCEVTNNTVTVRVASPPFSTWILF PENSGTPRAGAENLTWHDVDFLSCSWVVGPAAPADVQDLYLVNPNNSHEQYRCLHYKTDARGTQIGCRFDDIAPLSRGSQSSSHILVRGRSAAVS IPCTDKFVFFSQIERLTPPNMTGECNETHSFHWKMKSHFNKFRYELRIQKRMQPVITEQVRDRTSFQLNPGTYTVQIRARETVYEFLSAWSTP QRFECQEEGASSRAWRTSLLIALGTLTLLALCVFELICRRYLMQRLFPRI PHMKDPIGDTFOQDKLVVWEAGKAGLEECLECLVSEVVVEKT
70	Human CD3 delta (P04234)	MEHSTFLSGVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTGTLTLLSDITRLDLGKRILDPRIYRCNGTDIYKDKESTVQVHYRMCQSC VELDPAITVAGIIVTDVIATLLALGVFCFAGHETGRLSGAADTQALLRNDQVYQPLRRDRDDAQYSHLGGNWARNK
71	Human CD3 gamma (P09693)	MEQKGGLAVLILAILLLQGTLAQSIKGNHLVKVYDYQEDGSGVLLTCDAAEAKNITWFKDGKMI GFLTEDKKKWNILGNSNAKDPGRMYQCKGSGQNKSKP LQVYYRMCQNCIELNAATISGFLFAEIVSIFVLAVGVYFIAGQDGVQRASRDKQTLLPNDQLYQPLKDRDDQYSHLQGNQLRRN
72	Human CD3 epsilon (P077 66)	MQSGTHWRVLGCLLSVGWVGQDNEEMGGITQTPYKVISGTTVILTCPOYPGSEITLWQHNKNI GGDEDDKNIGSDEDHLSLKEFSELEQSGYY VCYPRGSKPEDANFYLYLRARVCNCEMMDVM SVATIVIVDICTGGLLLLLVYWSKNRKA KAKPVTRGAGAGGRQGRQGNKERPPVPVNPDPYEPPIR KGQRLYSGLNQRI
73	Human CD3 zeta (P20963)	MKWKALFTAAI LQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAYQQGQNLVNEILNLRREEYDVLDKRRGRDPEM GGKPPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR
74	Human TCR alpha constant domain (P01848)	PNIQNPDPVYQLRDSKSSDKSVCLFTDFDSQTNVVSQSKSDVYITDKTVLDMRSMDFKSN SAVWSNKSDFACANAFNNSIIPEDTFFFPESPSSC DVKLVEKSFETDTNLNFQNL SVIGFRILLKLVAGFNLLMTLRWSS
75	Human TCR beta constant domain	EDLNKVFPEVAVFEPSEAEISHTQKATLVCLATGFFPDHVELSWVNGKEVHSGVSTDPQPLKEQPALNDSRYCLSSRLRVSATFWQNP RNHFRC QVQFYGLSENDEWTQDRAKPVQTIVSAEAWGRADCFTSVSYQQGVL SATILYEILLGKATLYAVLSALVLMAMVKRKDF
76	Human TCR alpha variable domain derived from 2XN9	QLLEQSPQFLSIQEGENLTVYCNSSSVFSSLQWYRQEPGEGPVLLVTVVTGGEVKKLKRLLTFQFGDARKDSSLHTTAAQPGDTGLYLCAGAGSQGN LIFGKGTKLSVK

Table A-8 – part 2: Sequences of CD123 and components of TCR complex. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence

SEQ	ID	Sequence
77	Human TCR beta variable domain derived from 2XN9	DGGITQSPKYLFRKEGQNVTLSCQEQLNHDAMYWRQDPGGRLRIYYSQIVNDFQKGDIAEGYSVSRKKESFPLTFTVTSAQKNPTAFYLCASSR SSYEQYFGPGTRLTVT
343	Human TCR alpha variable domain derived from 2IAN	IQVEQSPDILLQEGANSTLRCNFSVNNLQWFHQNPWGQLINLFYIPSGTKQNGRLSATTVATERYSLLYISSSQTTDSGVYFCAALIQGAQKL VFGQGTRLTIN
344	Human TCR beta variable domain derived from 2IAN	NAGVTQTPKFRILKIGQSMTLQCTQDMNHNMYWYRQDPGMGLKLIYYSVGAGITDKGEVPNGYNVSRSTTFDPLRLLELAAPSQTSVYFCASTYH GTGYFGEGSWLTVV
345	Human TCR alpha variable domain derived from 3TOE	GDAKTTQPNMESNEEPPVHLPCHNSTISGTDYIHWYRQLPSQGPEYVIHGLTSNVNNRMASLAIAEDRKSSLLILHRATLRLDAAVYVCTVYGGAT NKLIFGTGTLAVQ
346	Human TCR beta variable domain derived from 3TOE	VVSQHPSWVIKSGTSVKIECRSLDFQATTMFWYRQFPKQSLMLMATSNEGSKATYEQGVEKDKFLINHASLTLSTLTLVTSAHPEPDSFFYICSARG GSYNSPLHFGNGTRLTVT
347	Cyno TCR alpha constant domain	PYIQNPDPVYQLRGSKSNDTSVCLFTDFEDSVNVSQSKDSVHITDKTVLDMRSMDFKSNGAVWWSNKSDEFACTSAFKDSVIPADTFFFPFESSC

Table A-8 – part 3: Sequences of CD123 and components of TCR complex. "SEQ" refers to the given SEQ ID NO; "ID" refers to identification name; "Sequence" denotes amino acid sequence

SEQ	ID	Sequence
348	Rhesus TCR beta constant domain	EDLKKVFPKVAVFEPSEAEISHTQKATLVCLATGFYPDHSVWVNGKEVHSGVSTDPQPLKEQPALEDSESRSLRVSATFWHNPRNHFR QVQFYGLSEDDWTEDRDKPITQKISAEAWGRADC
349	Rhesus TCR alpha variable domain	QQIMQIPQYQHVGEGEDFTTYCNSSTTILSNIQWYKQRPGGHPVFLIMLVKSGEVKKQKRLLIFQFGEAKKNSSLHITATQTQTTDVGYFCATTGVNNL FFGTGTRLTVL
350	Rhesus TCR beta variable domain	AGPVNAGVTQTTPKFQVLKTGQSMTLQCAQDMNHDYMYWYRQDPGMGLRLIHYSVGEGSTEKGEVPDGYNVTRSNTEDEPLRLESAAPSQTSVYFCA SSYWTGRSYEQYFGPGTRLTVI

CLAIMS

1. A polypeptide that redirects T cells for killing of CD123 expressing cells, comprising one immunoglobulin single variable domain (ISV) that specifically binds T cell receptor (TCR) and one or more ISV that specifically bind CD123, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 17-20; or
- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or
- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

2. The polypeptide according to claim 1, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 181-191; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 192-217; or
- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 218-225; or
- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the

4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

5 c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

15 f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

3. The polypeptide according to any of claims 1 or 2, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

25 i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 181-191; or

30 b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 181-191, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 2, 4, 5, 6, 8 and/or 10 of the CDR1 (position 27, 29, 30, 31, 33 and/or 35 according to Kabat numbering); provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 192-217; or

d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 192-217, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 3, 5, 7, 8 and/or 9 of the CDR2 (position 50, 52, 54, 56, 57 and/or 58 according to Kabat numbering); provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 218-225; or

f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 218-225, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 1, 4, 5 and/or 8 of the CDR3 (position 95, 98, 99 and/or 101 according to Kabat numbering); provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

4. The polypeptide according to any of claims 1 to 3, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

a) SEQ ID NO: 181; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 181, wherein

- at position 2 the D has been changed into A, S, E or G;
- at position 4 the H has been changed into Y;
- at position 5 the K has been changed into L;
- at position 6 the I has been changed into L;

- at position 8 the F has been changed into I or V; and/or
- at position 10 the G has been changed into S;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

- 5
- 10
5. The polypeptide according to any of claims 1 to 4, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 is chosen from the group consisting of:

- a) SEQ ID NO: 192; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 192, wherein

- 15
- at position 1 the H has been changed into T or R;
 - at position 3 the S has been changed into T or A;
 - at position 5 the G has been changed into S or A;
 - at position 7 the Q has been changed into D, E, T, A or V;
 - at position 8 the T has been changed into A or V; and/or
 - at position 9 the D has been changed into A, Q, N, V or S;
- 20

provided that the ISV comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

- 25
6. The polypeptide according to any of claims 1 to 5, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 is chosen from the group consisting of:

- 30
- a) SEQ ID NO: 218; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 218, wherein

- at position 1 the F has been changed into Y, L or G;
- at position 4 the I has been changed into L;

- at position 5 the Y has been changed into W; and/or
- at position 8 the D has been changed into N or S;

provided that the ISV comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds TCR with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

7. The polypeptide according to any of claims 1 to 6, wherein the ISV that specifically binds TCR essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 181, CDR2 is SEQ ID NO: 192, and CDR3 is SEQ ID NO: 218.

8. The polypeptide according to any of claims 1 to 7, wherein the ISV that specifically binds TCR is chosen from the group consisting of SEQ ID NOs: 42 and 78-180 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 42 and 78-180.

9. The polypeptide according to any of claims 1 to 8, wherein the ISV that specifically binds TCR is located at the N-terminus of the polypeptide.

10. The polypeptide according to any of claims 1 to 9, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or
- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the ISV comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the ISV comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

11. The polypeptide according to any of claims 1 to 10, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is chosen from the group consisting of:

a) SEQ ID NO: 11; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- at position 3 the T has been changed into S or P;
- at position 6 the I has been changed into S;
- at position 7 the N has been changed into D; and/or
- at position 8 the D has been changed into V or A;

provided that the ISV comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV

comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

12. The polypeptide according to any of claims 1 to 11, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 is SEQ ID NO: 17.

13. The polypeptide according to any of claims 1 to 12, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 is chosen from the group consisting of:

a) SEQ ID NO: 21; or

b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of SEQ ID NO: 21, wherein

- at position 3 the P has been changed into A;

provided that the ISV comprising the CDR3 with 1 amino acid difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

14. The polypeptide according to any of claims 1 to 13, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 11, CDR2 is SEQ ID NO: 17, and CDR3 is SEQ ID NO: 21.

15. The polypeptide according to any of claims 1 to 14, wherein the one or more ISV that specifically bind CD123 is chosen from the group consisting of SEQ ID NOs: 1-6 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6.

16. The polypeptide according to any of claims 1 to 15, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively)

and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16.

17. The polypeptide according to any of claims 1 to 16, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR2 is chosen from the group consisting of:

a) SEQ ID NO: 18; or

b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- at position 3 the Y has been changed into W;
- at position 6 the N has been changed into S; and/or
- at position 10 the Q has been changed into E;

provided that the ISV comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

18. The polypeptide according to any of claims 1 to 17, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR3 is chosen from the group consisting of:

a) SEQ ID NO: 23; or

b) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein

- at position 4 the E has been changed into R; and/or
- at position 5 the T has been changed into D or Y;

provided that the ISV comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the ISV comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

19. The polypeptide according to any of claims 1 to 18, wherein the one or more ISV that specifically bind CD123 essentially consists of 4 framework regions (FR1 to FR4, respectively)

and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which CDR1 is SEQ ID NO: 16, CDR2 is SEQ ID NO: 18, and CDR3 is SEQ ID NO: 23.

20. The polypeptide according to any of claims 1 to 19, wherein the one or more ISV that specifically bind CD123 is chosen from the group consisting of SEQ ID NOs: 7-10 or from ISVs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10.

21. The polypeptide according to any of claims 1 to 20, comprising two or more ISVs that specifically bind CD123.

22. The polypeptide according to claim 21, wherein the two or more ISVs that specifically bind CD123 are biparatopic, comprising a first ISV and a second ISV, wherein the first ISV binds to an epitope on CD123 that is different from the epitope on CD123 bound by the second ISV.

23. The polypeptide according to claim 22, wherein the first ISV is selected from the ISVs according to any of claims 41 to 48 and wherein the second ISV is selected from the ISVs according to any of claims 49 to 56.

24. The polypeptide according to claim 23, wherein the second ISV is located N-terminally of the first ISV.

25. The polypeptide according to claim 23, wherein the second ISV is located C-terminally of the first ISV.

26. The polypeptide according to any of claims 1 to 25, wherein the ISV that specifically binds TCR and the one or more ISV that specifically bind CD123 essentially consist of a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH, a camelized VH or a VHH which has been obtained by affinity maturation.

27. The polypeptide according to any of claims 1 to 26, wherein said polypeptide is chosen from the group consisting of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61.

28. The polypeptide according to any of claims 1 to 27, wherein said polypeptide is chosen from the group consisting of SEQ ID NOs: 47, 49, 52, 53, 55, 56 and 58-61.

5 29. The polypeptide according to any of claims 1 to 28, wherein said polypeptide induces T cell activation.

30. The polypeptide according to claim 29, wherein said T cell activation is independent from MHC recognition.

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31. The polypeptide according to any of claims 29 or 30, wherein said T cell activation depends on presenting said polypeptide bound to CD123 on a target cell to a T cell.

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32. The polypeptide according to any of claims 29 to 31, wherein said T cell activation causes one or more cellular response by said T cell, wherein said cellular response is selected from the group consisting of proliferation, differentiation, cytokine secretion, cytotoxic effector molecule release, cytotoxic activity, expression of activation markers, and redirected target cell lysis.

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33. The polypeptide according to any of claims 29 to 32, wherein said T cell activation causes killing of CD123 expressing cells with an average EC50 value of between 1 nM and 1 pM, such as at an average EC50 value of 500 pM or less, such as less than 400, 300, 200 or 100 pM or even less, such as less than 90, 80, 70, 60, 50, 40 or 30 pM or even less, said EC50 value preferably determined in a flow cytometry based assay with TOPRO3 read-out using MOLM-13 cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

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34. The polypeptide according to any of claims 29 to 33, wherein said T cell activation causes lysis of CD123 expressing cells with an average lysis percentage of more than about 10%, such as 15%, 16%, 17%, 18%, 19% or 20% or even more, such as more than 25%, or even more than 30%, said lysis percentage preferably determined in a flow cytometry based assay with TOPRO3 read-out using MOLM-13 cells as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

35. The polypeptide according to any of claims 29 to 34, wherein said T cell activation causes IFN- γ secretion with an average EC50 value of between 100 nM and 10 pM, such as at an average EC50 value of 50 nM or less, such as less than 40, 30, 20, 10 or 9 nM or even less, such as less than 8, 7, 6, 5, 4, 3, 2 or 1 nM or even less, such as less than 500 pM or even less, such as less than 400, 300, 200 or 100 pM or even less, said EC50 value preferably determined in an ELISA based assay.

36. The polypeptide according to any of claims 29 to 35, wherein said T cell activation causes proliferation of said T cells.

37. The polypeptide according to any of claims 29 or 36, wherein the T cell activation in the absence of CD123 positive cells is minimal.

38. The polypeptide according to any of claims 29 to 37, wherein the T cell activation induced lysis of CD123 negative cells is no more than about 10%, such as 9% or less, such as 8, 7, or 6 % or even less, said lysis preferably determined as average lysis percentage in a flow cytometry based assay with TOPRO3 read-out using CD123 negative cells, such as U-937 or NCI-H929 cells, as target cells and human T cells as effector cells at an effector to target cell ratio of 10 to 1.

39. A polypeptide that is an ISV that specifically binds CD123 and that comprises or essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

i) CDR1 is chosen from the group consisting of:

a) SEQ ID NOs: 11-16; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16; provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

ii) CDR2 is chosen from the group consisting of:

c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20; provided that the polypeptide comprising the CDR2 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- iii) CDR3 is chosen from the group consisting of:

- e) SEQ ID NOs: 21-25; or

- f) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25; provided that the polypeptide comprising the CDR3 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

40. The polypeptide according to claim 39, that comprises or essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementarity determining regions (CDR1 to CDR3, respectively), in which:

- i) CDR1 is chosen from the group consisting of:

- a) SEQ ID NOs: 11-16; or

- b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 11-16, wherein the 4, 3, 2 or 1 amino acid(s) difference are present at position 3, 6, 7 and/or 8 of the CDR1 (position 28, 31, 32 and/or 33 according to Kabat numbering); provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

- ii) CDR2 is chosen from the group consisting of:

- c) SEQ ID NOs: 17-20; or

- d) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 17-20, wherein the 3, 2 or 1 amino acid(s)

difference are present at position 3, 6 and/or 10 of the CDR2 (position 52, 54 and/or 58 according to Kabat numbering); provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance;

and/or

iii) CDR3 is chosen from the group consisting of:

e) SEQ ID NOs: 21-25; or

f) amino acid sequences that have 3, 2 or 1 amino acid(s) difference with the amino acid sequence of one of SEQ ID NOs: 21-25, wherein the 3, 2 or 1 amino acid(s) difference are present at position 3, 4 and/or 5 of the CDR3 (position 97, 98 and/or 99 according to Kabat numbering); provided that the polypeptide comprising the CDR3 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

41. The polypeptide according to any of claims 39 or 40, in which CDR1 is chosen from the group consisting of:

a) SEQ ID NO: 11; or

b) amino acid sequences that have 4, 3, 2 or 1 amino acid(s) difference with the amino acid sequence of SEQ ID NO: 11, wherein

- at position 3 the T has been changed into S or P;
- at position 6 the I has been changed into S;
- at position 7 the N has been changed into D; and/or
- at position 8 the D has been changed into V or A;

provided that the polypeptide comprising the CDR1 with 4, 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR1 without the 4, 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

42. The polypeptide according to any of claims 39 to 41, in which CDR2 is SEQ ID NO: 17.

43. The polypeptide according to any of claims 39 to 42, in which CDR3 is chosen from the group consisting of:

a) SEQ ID NO: 21; or

b) amino acid sequences that have 1 amino acid difference with the amino acid sequence of

SEQ ID NO: 21, wherein

- at position 3 the P has been changed into A;

provided that the polypeptide comprising the CDR3 with 1 amino acid difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 1 amino acid difference, said affinity as measured by surface plasmon resonance.

44. The polypeptide according to any of claims 39 to 43, in which CDR1 is SEQ ID NO: 11, CDR2 is SEQ ID NO: 17, and CDR3 is SEQ ID NO: 21.

45. The polypeptide according to any of claims 39 to 44, wherein the polypeptide is chosen from the group consisting of SEQ ID NOs: 1-6 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 1-6.

46. The polypeptide according to any of claims 39 to 45, wherein the polypeptide is chosen from the group consisting of SEQ ID NOs: 1-6.

47. The polypeptide according to any of claims 41 to 46, wherein the polypeptide binds to human CD123 expressed on MOLM-13 cells with an average EC50 value between 10 nM and 100 pM, such as at an average EC50 value of 5 nM or less, such as less than 4, 3, 2, or 1 nM or even less, preferably as measured by flow cytometry.

48. The polypeptide according to any of claims 41 to 47, wherein the polypeptide binds to human CD123 with an average K_D value of between 10 nM and 100 pM, such as at an average K_D value of 5 nM or less, such as less than 4, 3 or 2 nM or even less, said K_D value preferably determined by surface plasmon resonance.

49. The polypeptide according to any of claims 39 or 40, in which CDR1 is SEQ ID NO: 16.

50. The polypeptide according to any of claims 39, 40 or 49, in which CDR2 is chosen from the group consisting of:

a) SEQ ID NO: 18; or

b) amino acid sequences that have 3, 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 18, wherein

- at position 3 the Y has been changed into W;
- at position 6 the N has been changed into S; and/or
- at position 10 the Q has been changed into E;

provided that the polypeptide comprising the CDR2 with 3, 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR2 without the 3, 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

51. The polypeptide according to any of claims 39, 40, 49 or 50, in which CDR3 is chosen from the group consisting of:

a) SEQ ID NO: 23; or

b) amino acid sequences that have 2 or 1 amino acid difference with the amino acid sequence of SEQ ID NO: 23, wherein

- at position 4 the E has been changed into R; and/or
- at position 5 the T has been changed into D or Y;

provided that the polypeptide comprising the CDR3 with 2 or 1 amino acid(s) difference binds CD123 with about the same or a higher affinity compared to the binding by the polypeptide comprising the CDR3 without the 2 or 1 amino acid(s) difference, said affinity as measured by surface plasmon resonance.

52. The polypeptide according to any of claims 39, 40 or 49 to 51, in which CDR1 is SEQ ID NO: 16, CDR2 is SEQ ID NO: 18, and CDR3 is SEQ ID NO: 23.

53. The polypeptide according to any of claims 39, 40 or 49 to 52, wherein the polypeptide is chosen from the group consisting of SEQ ID NOs: 7-10 or from polypeptides that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 7-10.

54. The polypeptide according to any of claims 39, 40 or 49 to 53, wherein the polypeptide is chosen from the group consisting of SEQ ID NOs: 7-10.
55. The polypeptide according to any of claims 49 to 54, wherein the polypeptide binds to human
5 CD123 expressed on MOLM-13 cells with an average EC50 value between 10 μ M and 100 nM, such as at an average EC50 value of 5 μ M or less, such as less than 4, 3, 2, or 1 μ M or even less, preferably as measured by flow cytometry.
56. The polypeptide according to any of claims 49 to 55, wherein the polypeptide binds to human
10 CD123 with an average K_D value of between 1 μ M and 10 nM, such as at an average K_D value of 500 nM or less, such as less than 400, 300 or 200 nM or even less, said K_D value preferably determined by surface plasmon resonance.
57. A polypeptide that specifically binds CD123 and that cross-blocks the binding to CD123 of at
15 least one of the polypeptides according to any of claims 39 to 56 and/or selected from SEQ ID NOs: 1-10.
58. A polypeptide that specifically binds CD123 and that is cross-blocked from binding to CD123 by
20 at least one of the polypeptides according to any of claims 39 to 56 and/or selected from SEQ ID NOs: 1-10.
59. The polypeptide according to any of claims 39 to 58, wherein the polypeptide essentially consists of a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH, a camelized VH or a VHH which has been obtained by affinity maturation.
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60. A polypeptide comprising two or more ISVs that specifically bind CD123, wherein the ISVs are chosen from the group of ISVs according to any of claims 39 to 59.
61. The polypeptide according to claim 60, comprising two ISVs that specifically bind CD123,
30 wherein the ISVs are chosen from the group of ISVs according to any of claims 39 to 56.
62. The polypeptide according to any of claims 60 or 61, wherein the two or more ISVs that specifically bind CD123 are biparatopic comprising a first ISV and a second ISV, wherein the

first ISV binds to an epitope on CD123 that is different from the epitope on CD123 bound by the second ISV.

63. The polypeptide according to claim 62, wherein the first ISV is selected from the ISVs according to any of claims 41 to 48 and wherein the second ISV is selected from the ISVs according to any of claims 49 to 56.

64. The polypeptide according to claim 63, wherein the second ISV is located N-terminally of the first ISV.

65. The polypeptide according to claim 63, wherein the second ISV is located C-terminally of the first ISV.

66. The polypeptide according to any of claims 1 to 38 and 60 to 65, wherein the ISVs are directly linked to each other or linked to each other via a linker.

67. The polypeptide according to claim 66, in which the linker is selected from the group consisting of SEQ ID NOs: 325 to 336.

68. A construct comprising a polypeptide according to any of claims 1 to 67, and further comprising one or more other groups, residues, moieties or binding units, optionally linked via one or more peptidic linkers.

69. The construct according to claim 68, in which said one or more other groups, residues, moieties or binding units provide the construct with increased half-life, compared to the corresponding polypeptide according to any of claims 1 to 67.

70. The construct according to claim 69, in which said one or more other groups, residues, moieties or binding units that provide the construct 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.

71. The construct according to any of claims 69 or 70, in which said one or more other groups, residues, moieties or binding units that provide the construct with increased half-life is chosen from the group consisting of serum albumin (such as human serum albumin) or a serum immunoglobulin (such as IgG).

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72. The construct according to any of claims 69 or 70, in which said one or more other binding units that provide the construct 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).

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73. The construct according to claim 70, wherein said one or more other binding units that provide the construct with increased half-life is an ISV that binds serum albumin.

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74. The construct according to claim 73, wherein said ISV that binds serum albumin essentially consist of a single domain antibody, a dAb, a Nanobody, a VHH, a humanized VHH or a camelized VH.

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75. The construct according to claim 73 or 74, wherein said ISV that binds serum albumin essentially consists of 4 framework regions (FR1 to FR4, respectively) and 3 complementary determining regions (CDR1 to CDR3, respectively), in which CDR1 is GFTFSSFGMS (SEQ ID NO: 363) or GFTFRSFGMS (SEQ ID NO: 364), CDR2 is SISGSGSDDL (SEQ ID NO: 365) and CDR3 is GGSLSR (SEQ ID NO: 366).

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76. The construct according to claim 75, wherein said ISV that binds serum albumin is selected from the group consisting of SEQ ID NOs: 43 and 351 to 362.

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77. The construct according to any of claims 68 to 76, wherein said construct is chosen from the group consisting of SEQ ID NOs: 63-67 or constructs that have a sequence identity of more than 80%, more than 85%, more than 90%, more than 95%, or even more than 99% with one of SEQ ID NOs: 63-67.

78. The construct according to any of claims 68 to 77, comprising a polypeptide selected from the group consisting of SEQ ID NOs: 63-67.

79. A construct comprising the polypeptide according to any of claims 1 to 67 or the construct according to any of claims 68 to 78 and further comprising a C-terminal extension (X)_n, in which n is 1 to 5, such as 1, 2, 3, 4 or 5, and in which X is a naturally occurring amino acid, preferably no cysteine.

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80. The construct according to claim 79, wherein said construct is chosen from the group consisting of SEQ ID NOs: 338-342.

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81. A nucleic acid encoding the polypeptide according to any of claims 1 to 67 or the construct according to any of claims 68 to 80 that is such that it can be obtained by expression of a nucleic acid encoding the same.

82. The nucleic acid according to claim 81, which is in the form of a genetic construct.

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83. An expression vector comprising the nucleic acid according to claim 81 and/or the genetic construct according to claim 82.

84. A host or host cell comprising the nucleic acid according to claim 81, the genetic construct according to claim 82, or the expression vector according to claim 83.

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85. A method for the production of the polypeptide according to any of claims 1 to 67 or the construct according to any of claims 68 to 80 that is such that it can be obtained by expression of a nucleic acid encoding the same, said method at least comprising the steps of:

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- a) expressing, in a suitable host cell or host organism or in another suitable expression system, the nucleic acid according to claim 81; optionally followed by
- b) isolating and/or purifying the polypeptide according to any of claims 1 to 67 or the construct according to any of claims 68 to 80.

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86. A composition comprising at least one polypeptide according to any of claims 1 to 67, or a construct according to any of claims 68 to 80, or a nucleic acid according to claim 81.

87. The composition according to claim 86, which is a pharmaceutical composition.

88. The composition according to claim 87, which further comprises at least one pharmaceutically acceptable carrier, diluent or excipient and/or adjuvant, and optionally comprises one or more further pharmaceutically active polypeptides and/or compounds.

5 89. The polypeptide according to any of claims 1 to 67, the construct according to any of claims 68 to 80, or the composition according to any of claims 87 or 88, for use as a medicament.

90. The polypeptide according to any of claims 1 to 67, the construct according to any of claims 68 to 80, or the composition according to any of claims 87 or 88, for use in the prevention,
10 treatment and/or amelioration of a CD123 associated disease or condition.

91. The polypeptide, construct, or the composition for use according to claim 90, wherein said CD123 associated disease or condition is a proliferative disease or an inflammatory condition.

15 92. A method for the prevention, treatment and/or amelioration of a CD123 associated disease or condition, comprising the step of administering to a subject in need thereof a pharmaceutically active amount of the polypeptide according to any of claims 1 to 67, the construct according to any of claims 68 to 80, or the composition according to any of claims 87 or 88.

20 93. The method according to claim 92, wherein said CD123 associated disease or condition is a proliferative disease or an inflammatory condition.

94. The polypeptide or composition for use according to claim 91 or the method according to claim 93, wherein said proliferative disease is cancer.

25 95. The polypeptide or composition for use or the method according to claim 94, wherein said cancer is chosen from the group consisting of lymphomas (including Burkitt's lymphoma, Hodgkin's lymphoma and non-Hodgkin's lymphoma), leukemias (including acute myeloid leukemia, chronic myeloid leukemia, acute B lymphoblastic leukemia, chronic lymphoid
30 leukemia and hairy cell leukemia), myelodysplastic syndrome, blastic plasmacytoid dendritic cell neoplasm, systemic mastocytosis and multiple myeloma.

96. The polypeptide or composition for use according to claim 91 or the method according to claim 93, wherein said inflammatory condition is chosen from the group consisting of Autoimmune Lupus (SLE), allergy, asthma and rheumatoid arthritis.
- 5 97. The polypeptide or composition for use according to any of claims 89 to 91 and 94 to 96 or the method according to any of claims 92 to 96, wherein the treatment is a combination treatment.
- 10 98. A kit comprising the polypeptide according any of claims 1 to 67, the construct according to any of claims 68 to 80, the nucleic acid according to claim 81, the expression vector according to claim 83, or the host or host cell according to claim 84.

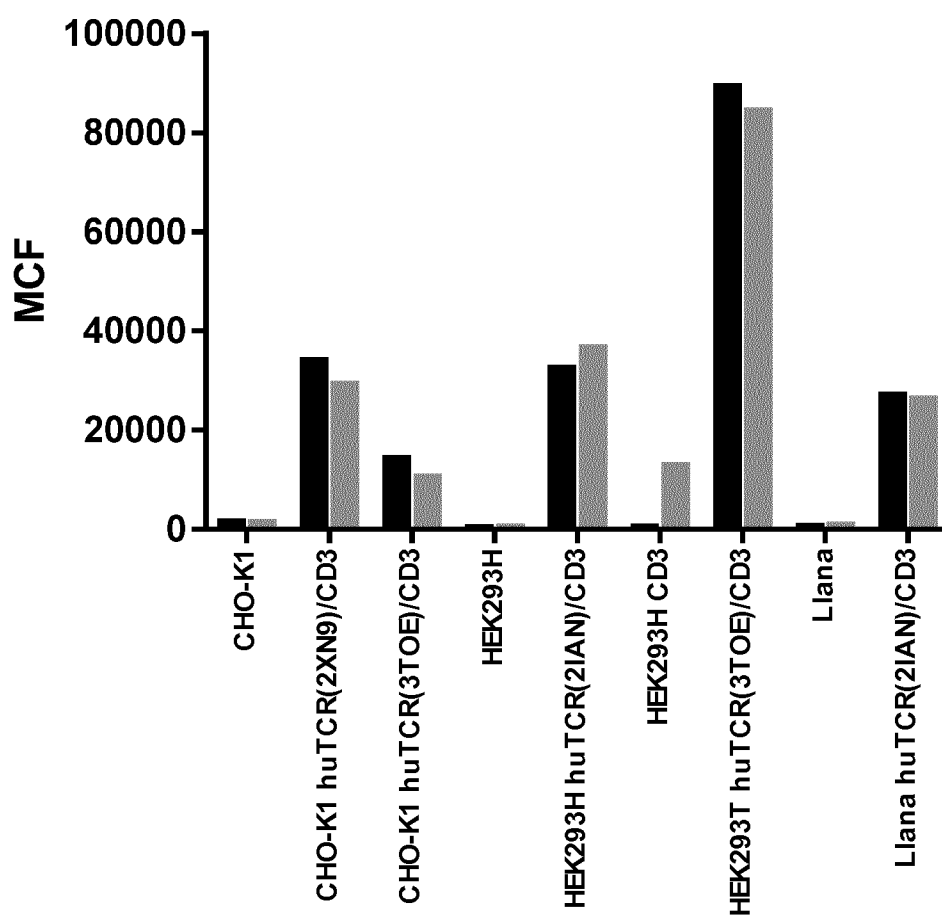
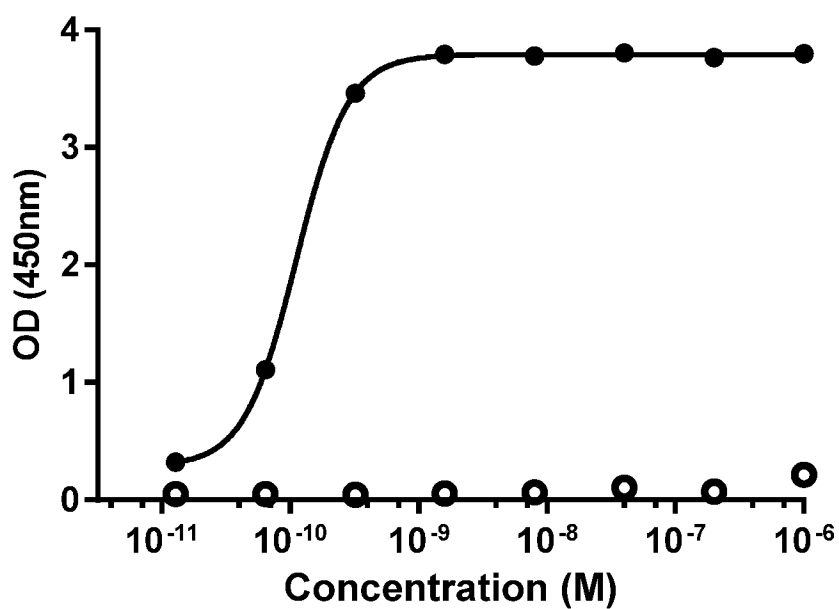
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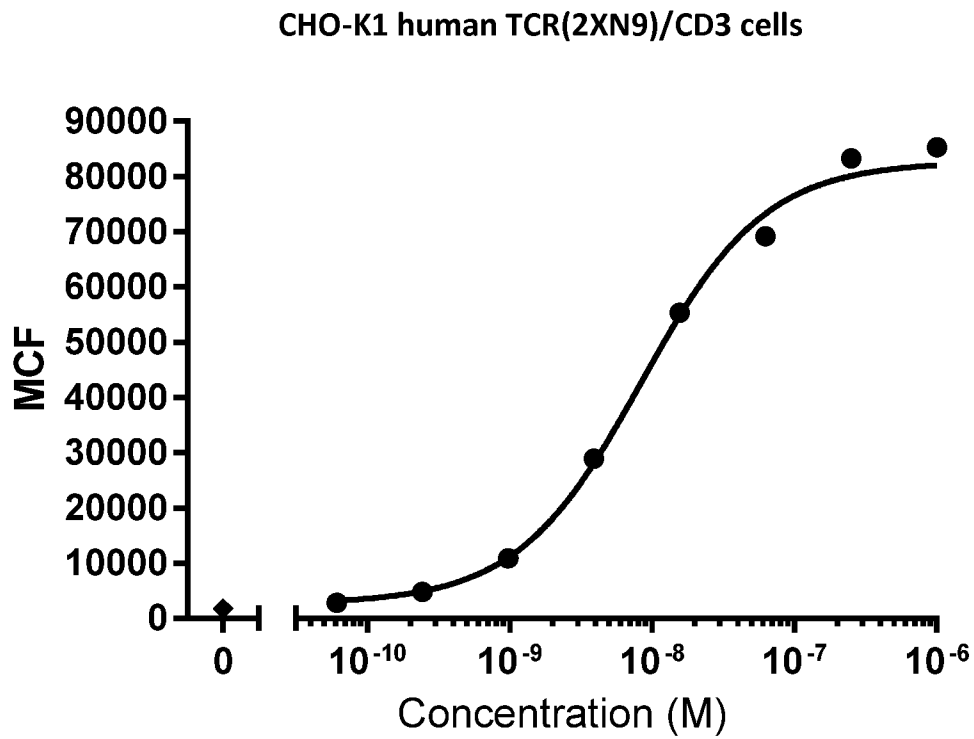
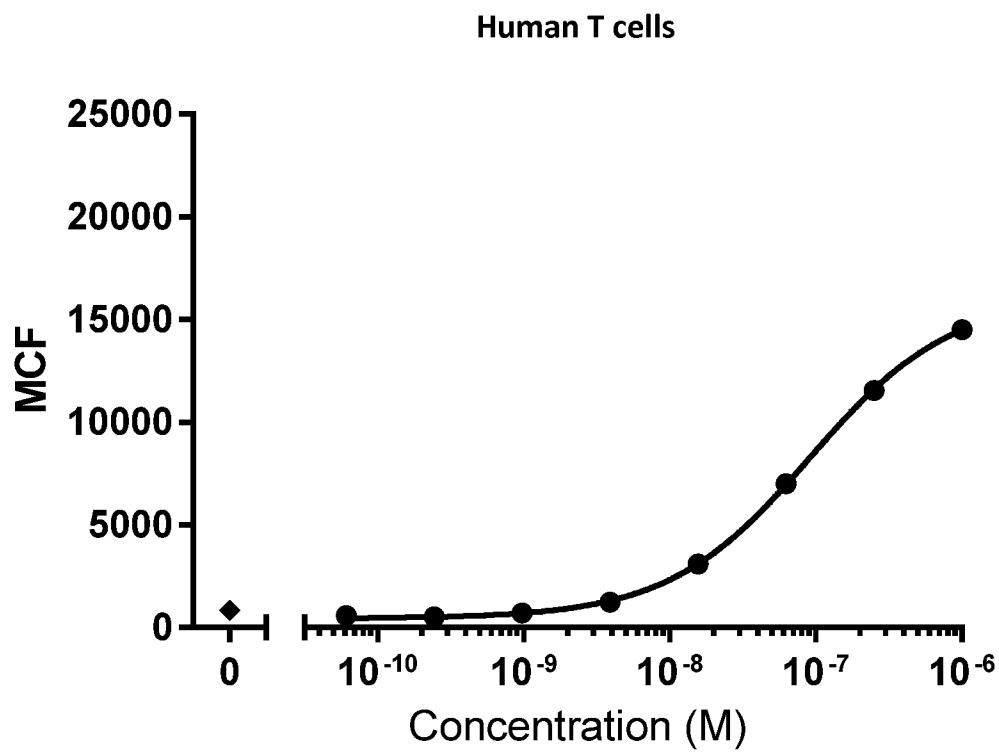
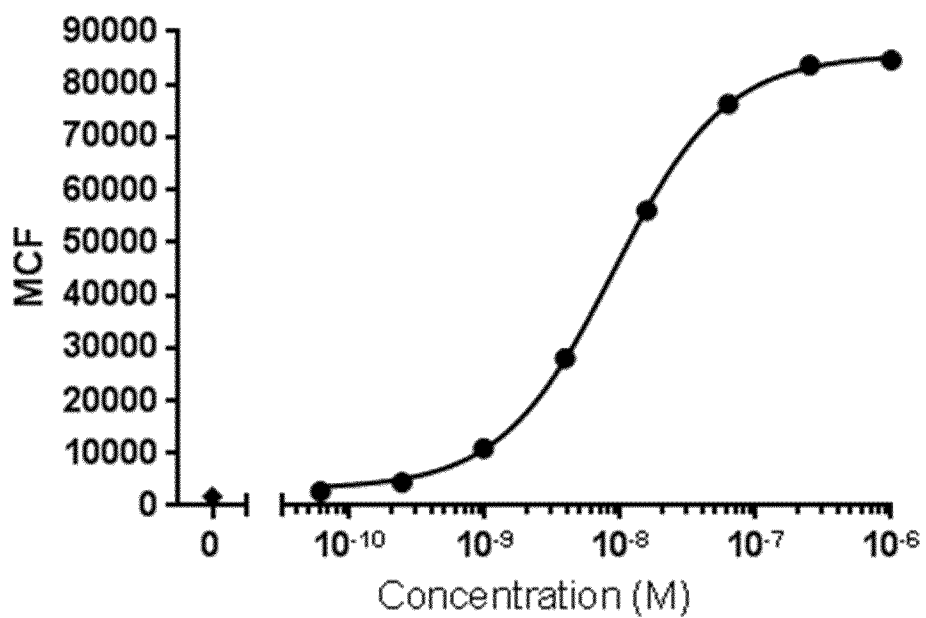
Figure 3:**A.****B.**

Figure 3 cont':

C.

CHO-K1 human TCR(2XN9)/CD3 cells



D.

Human T cells

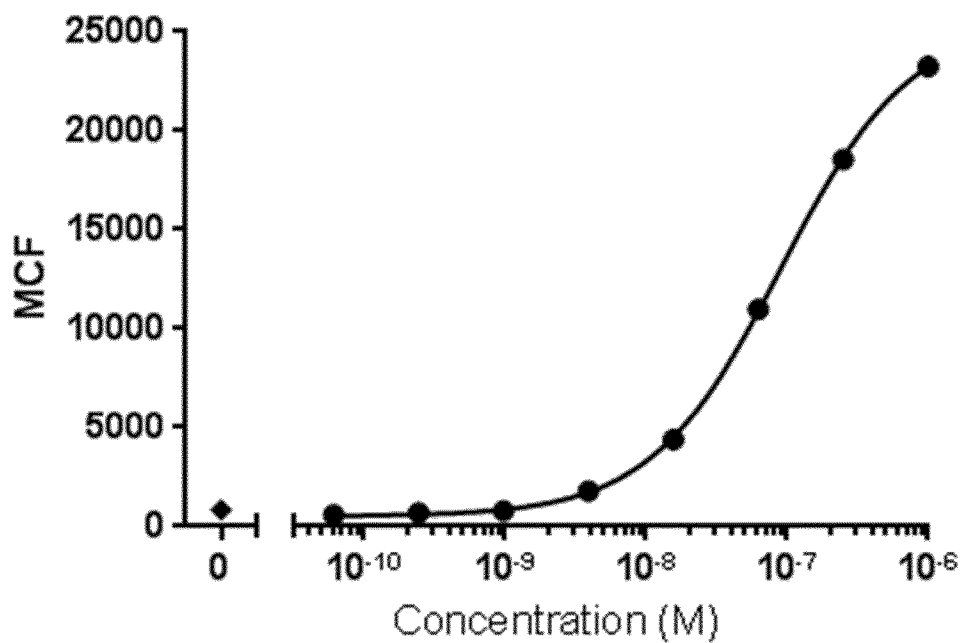


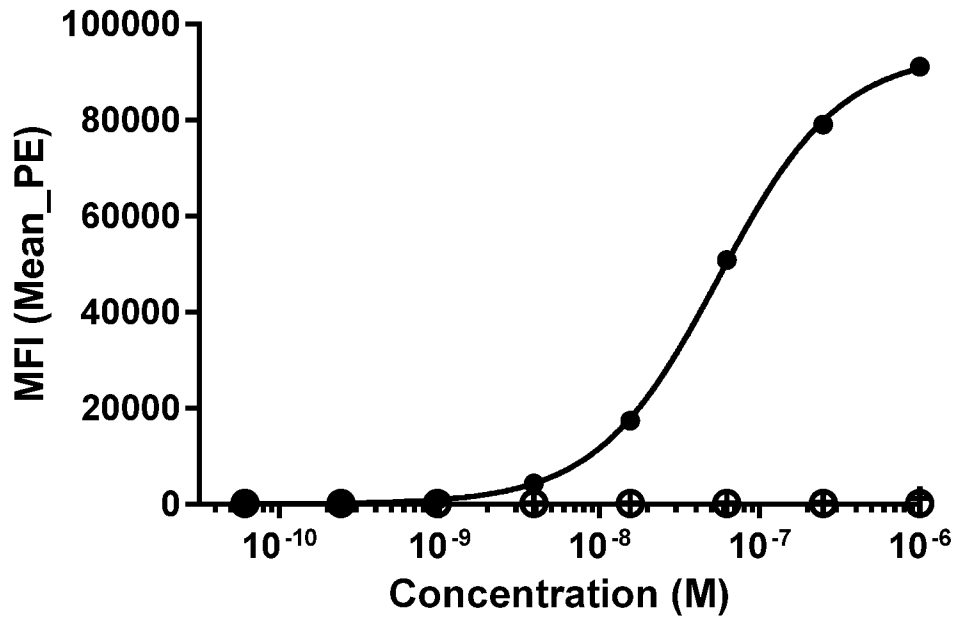
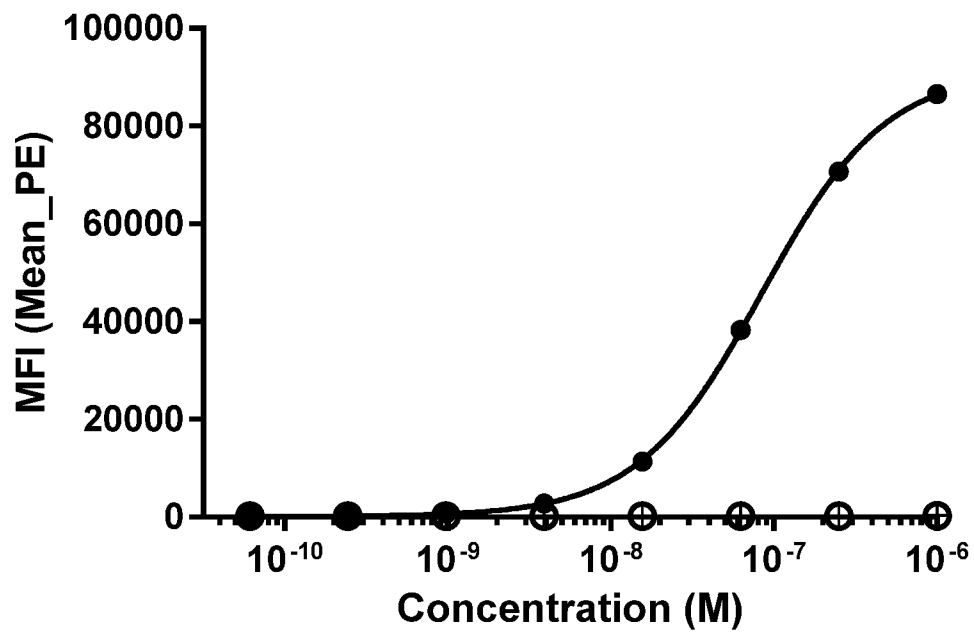
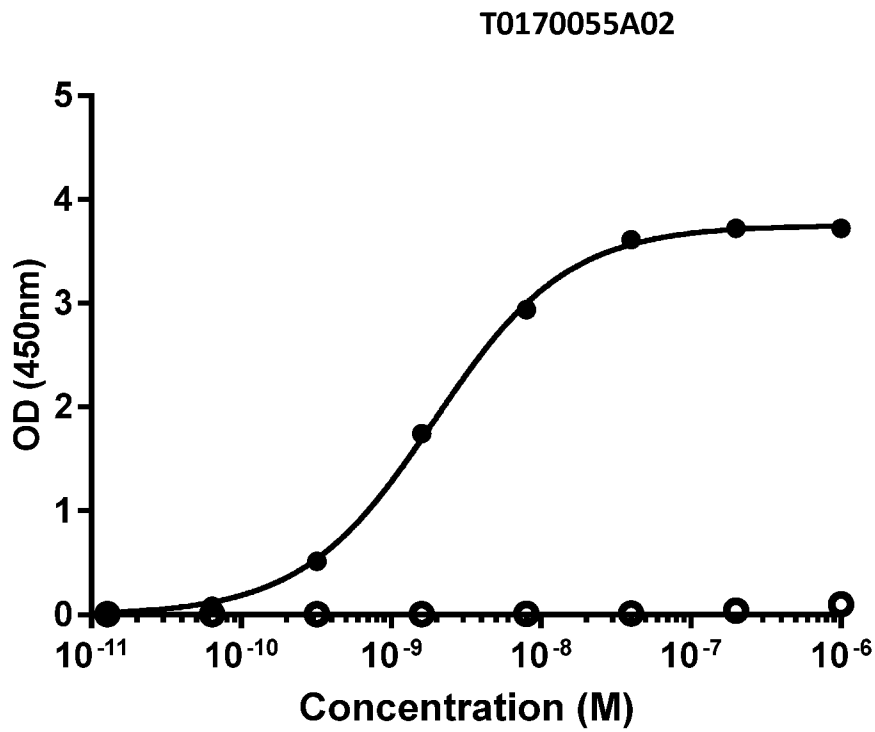
Figure 4:**A.****T0170055A02****B.****T0170056G05**

Figure 5

A.



B.

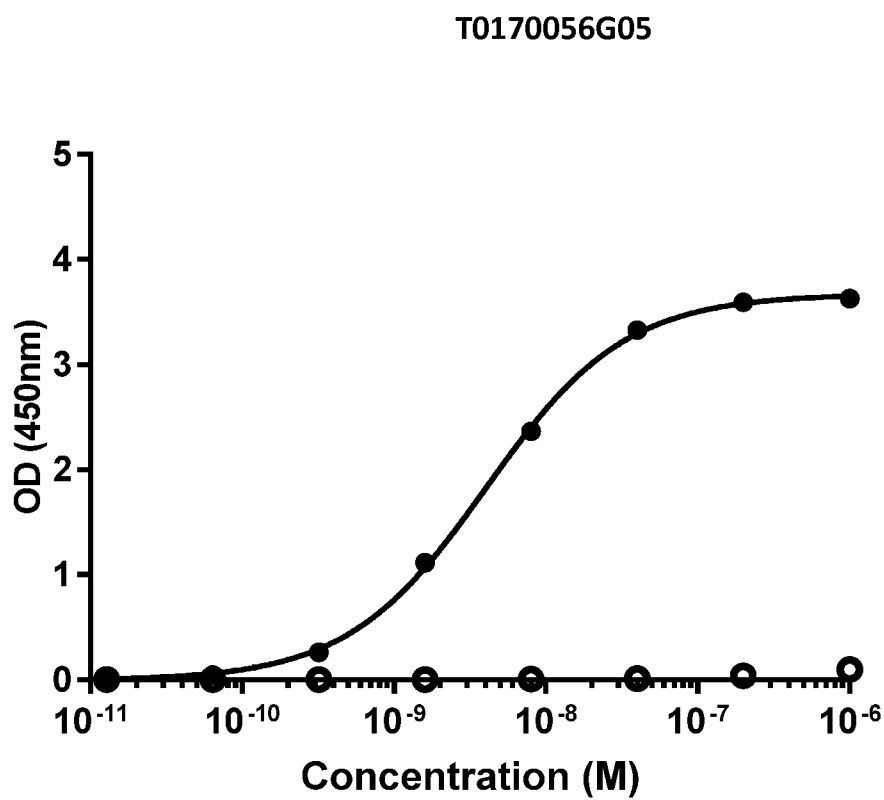


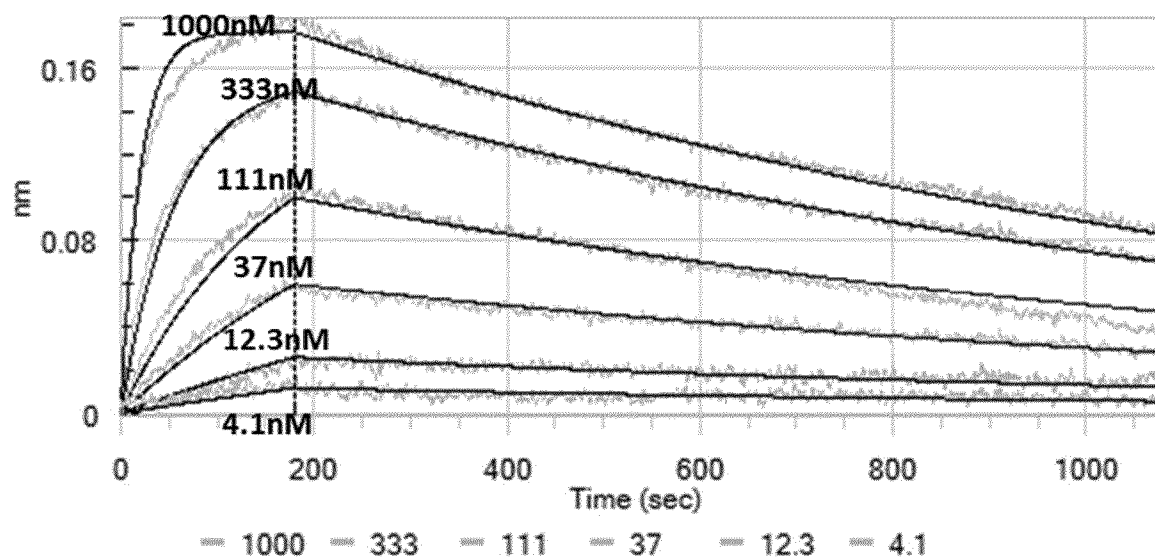
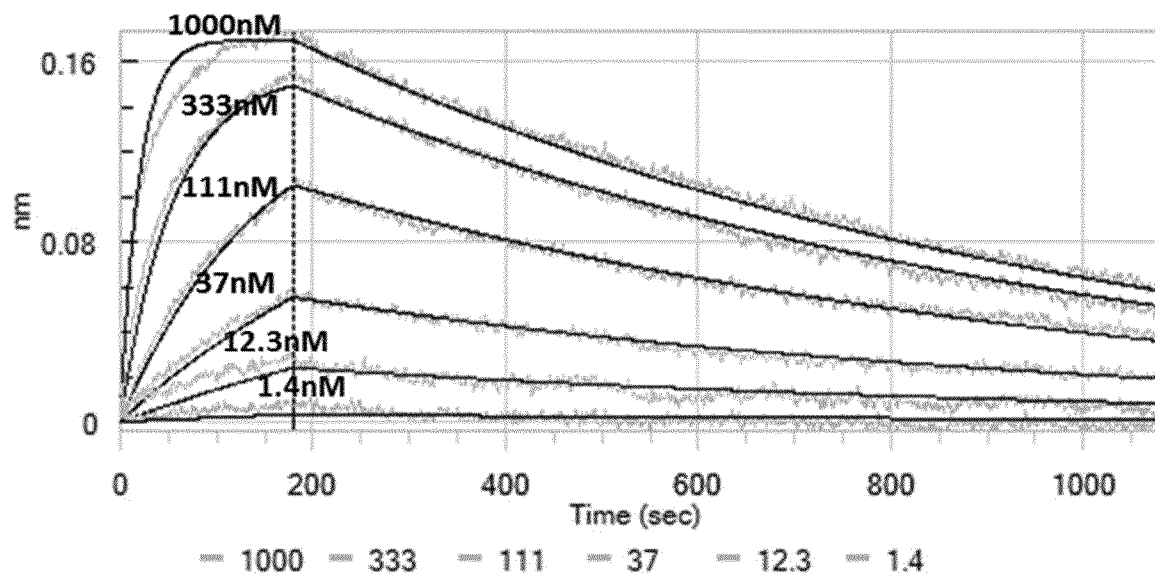
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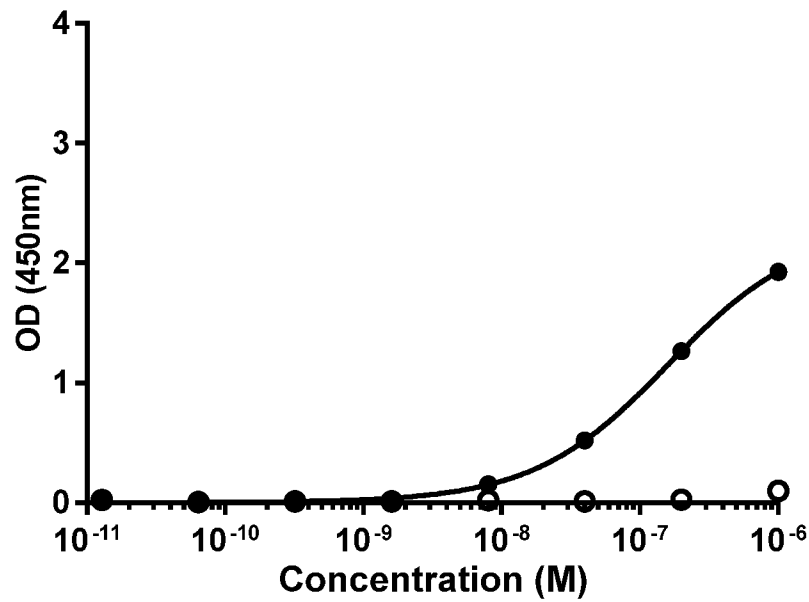
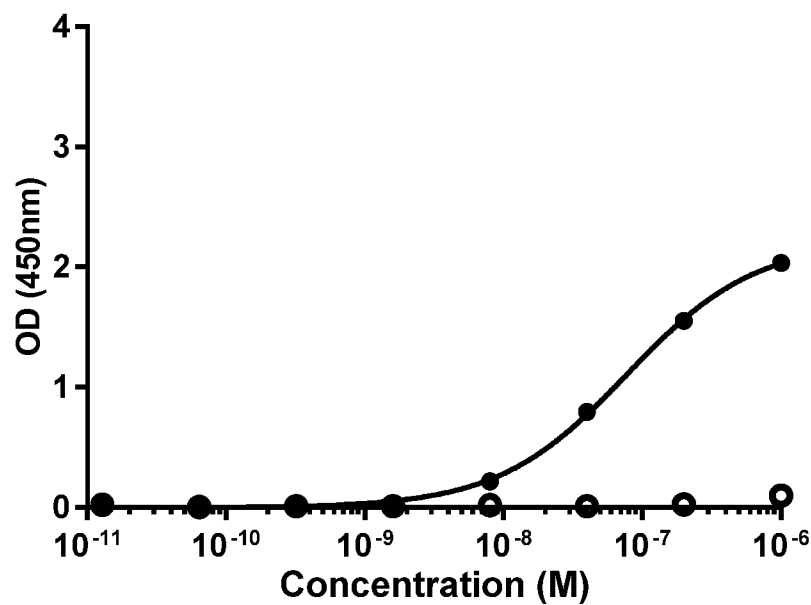
Figure 7:**A.****T0170055A02****B.****T0170056G05**

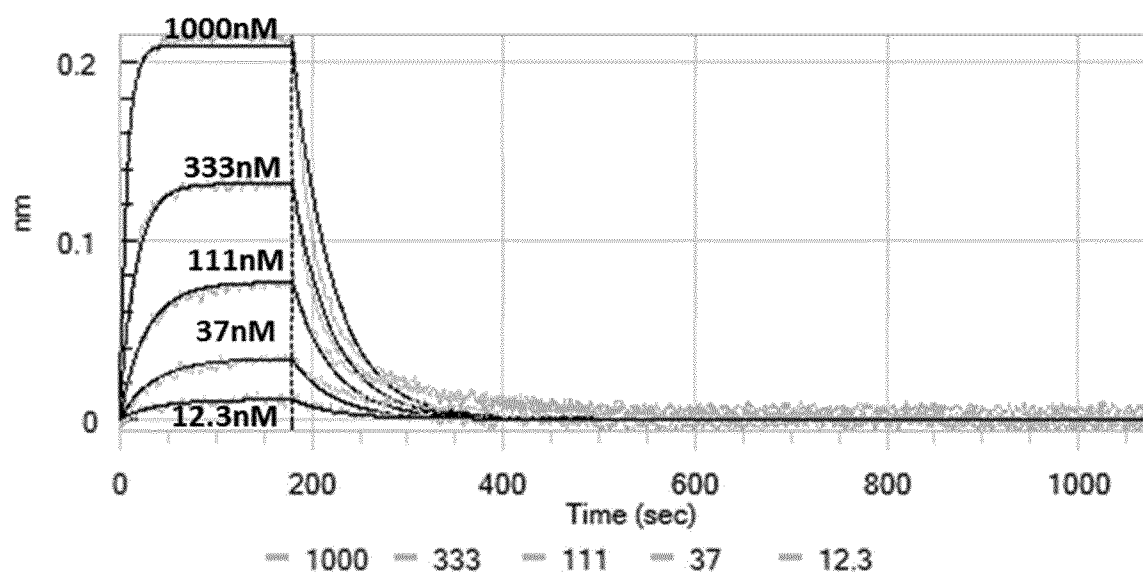
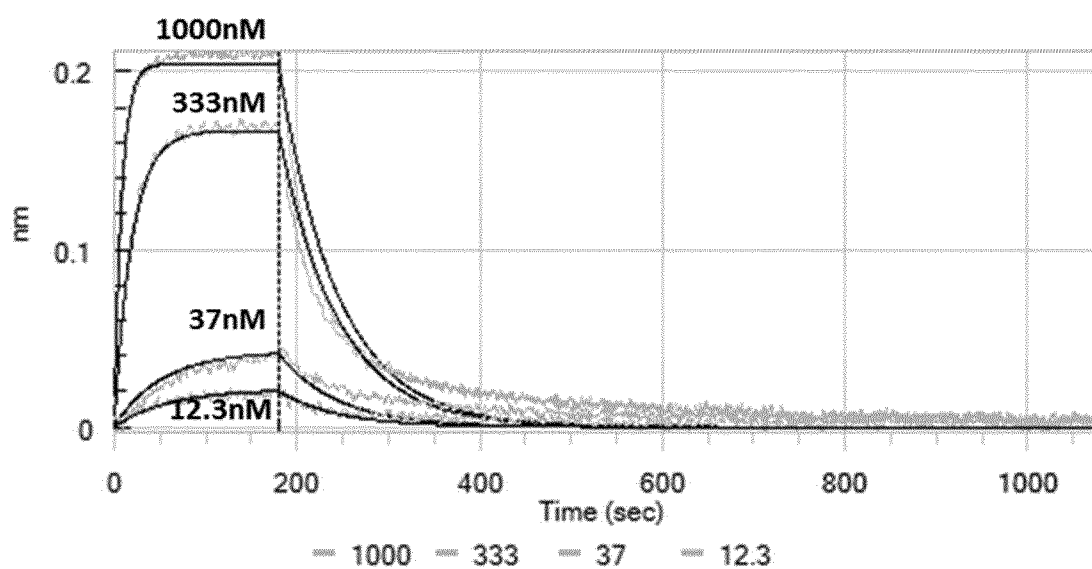
Figure 8:**A.****B.**

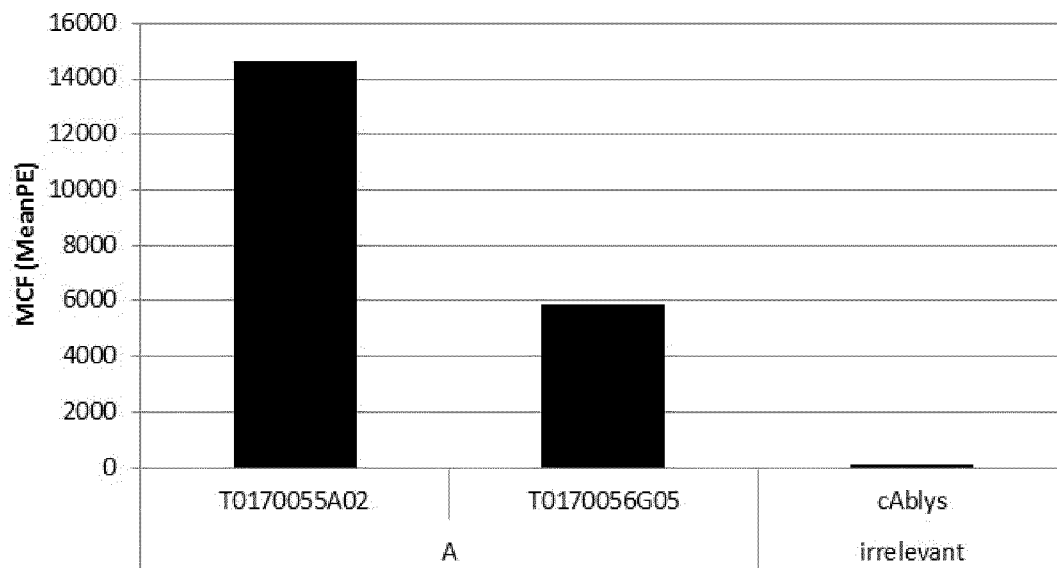
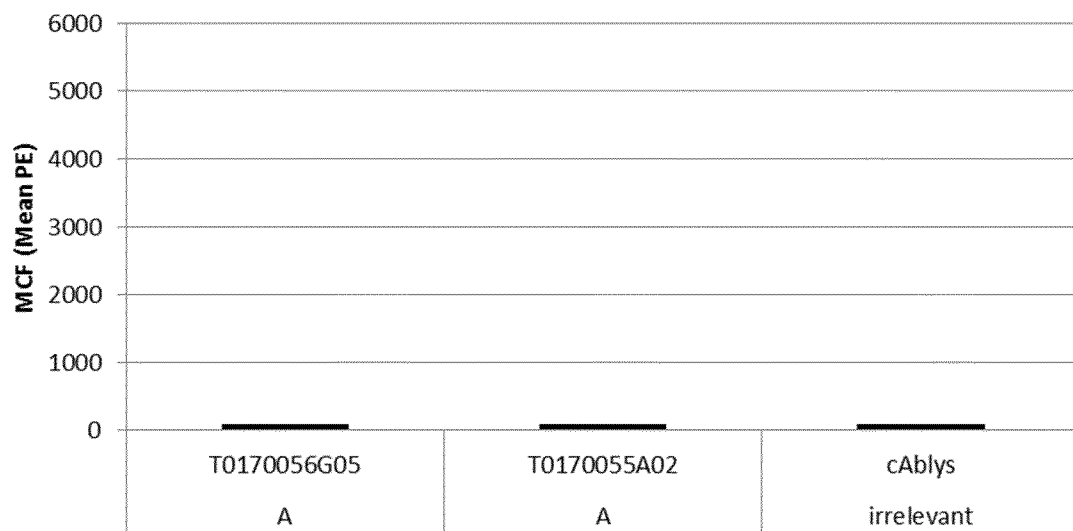
Figure 9:**A.****B.**

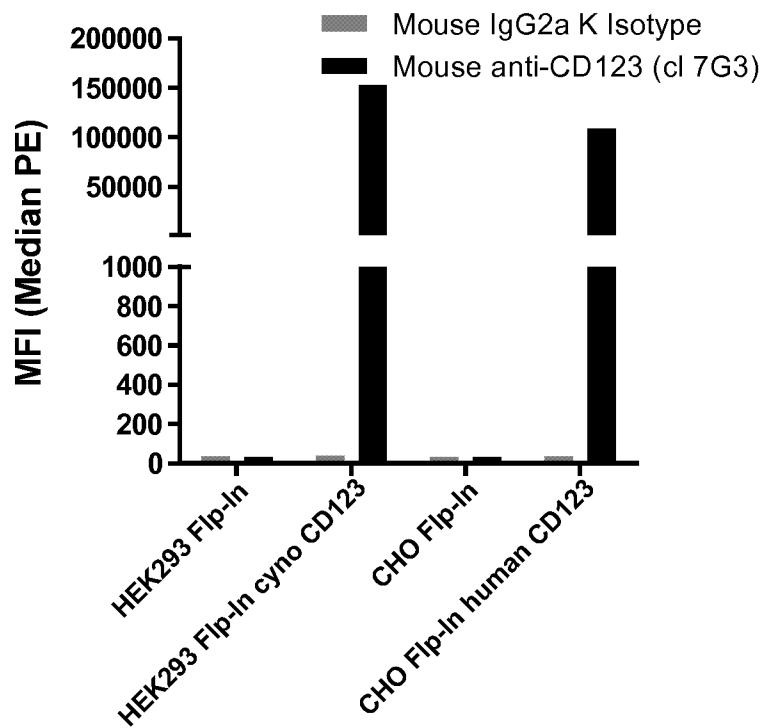
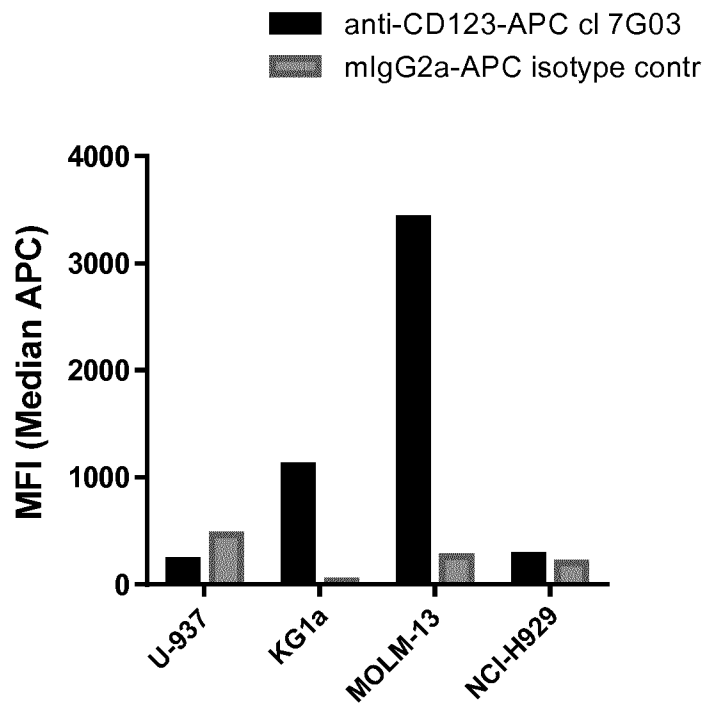
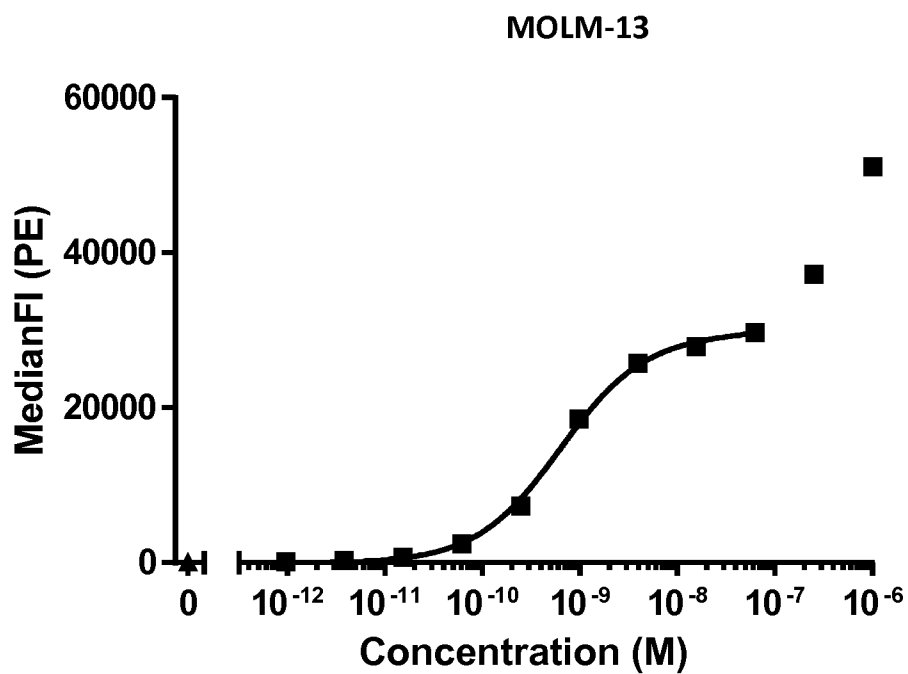
Figure 10:**Figure 11:**

Figure 12:

A.



B.

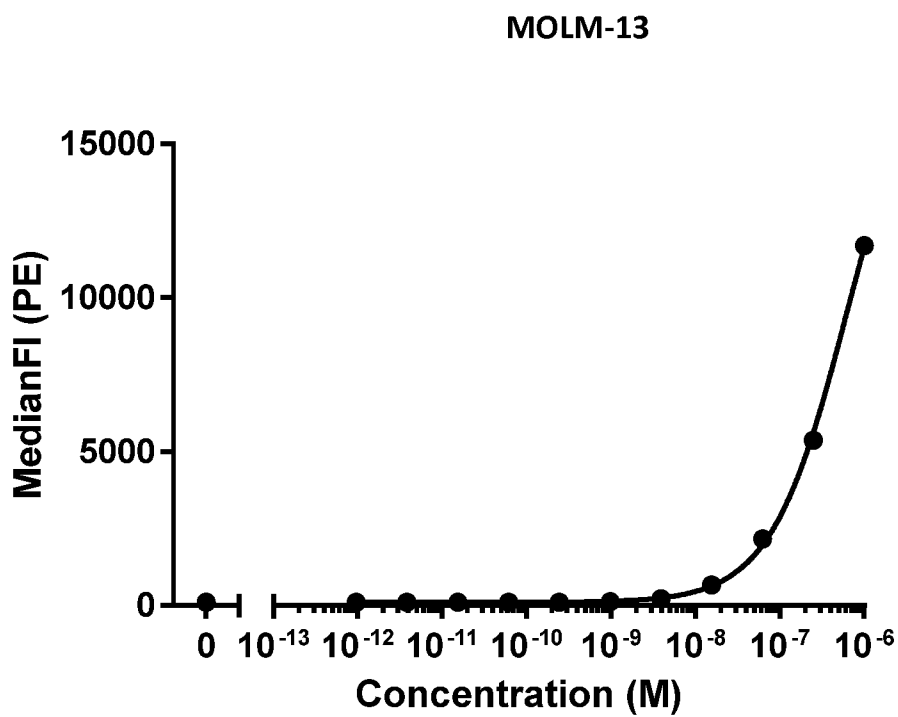
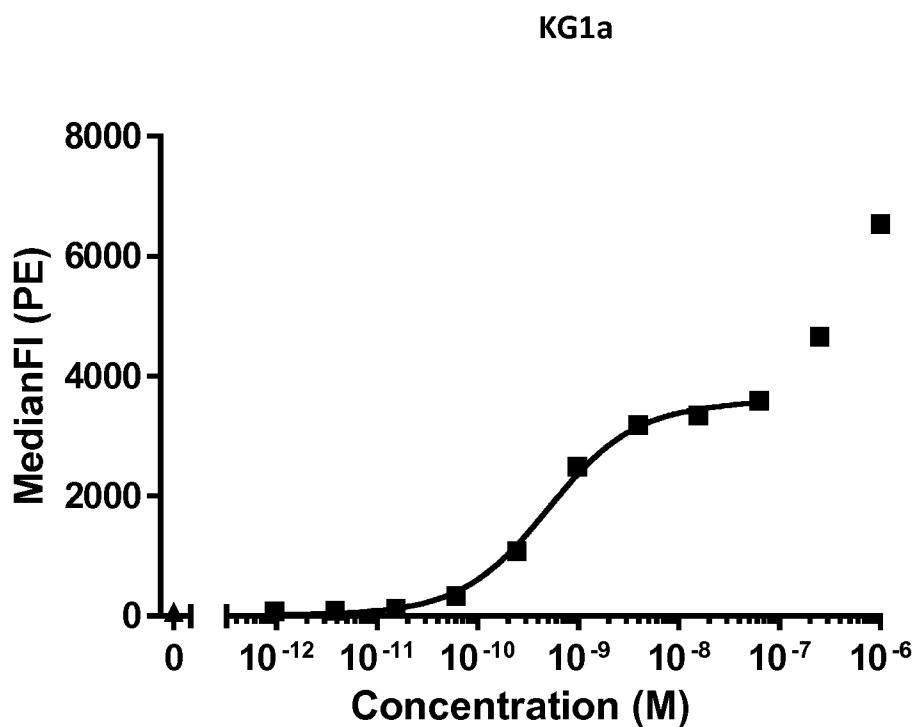


Figure 12 cont':

C.



D.

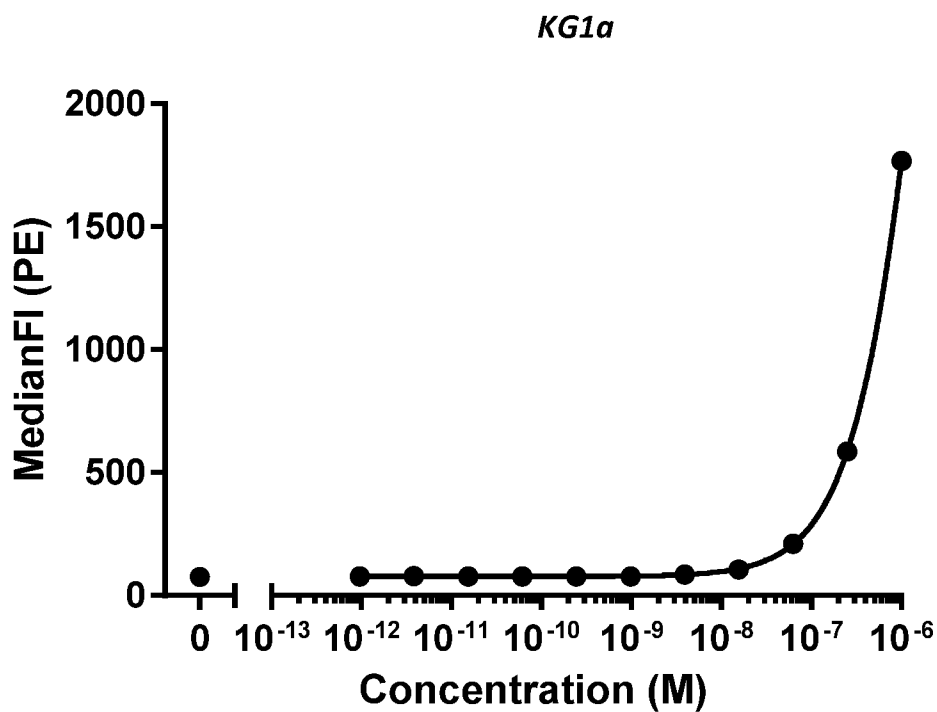


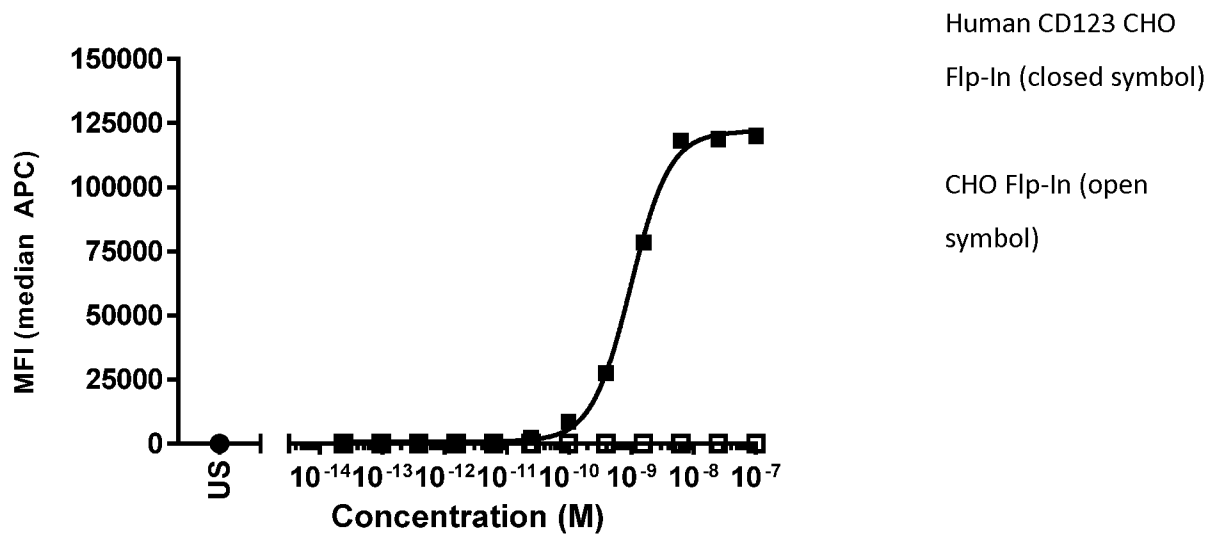
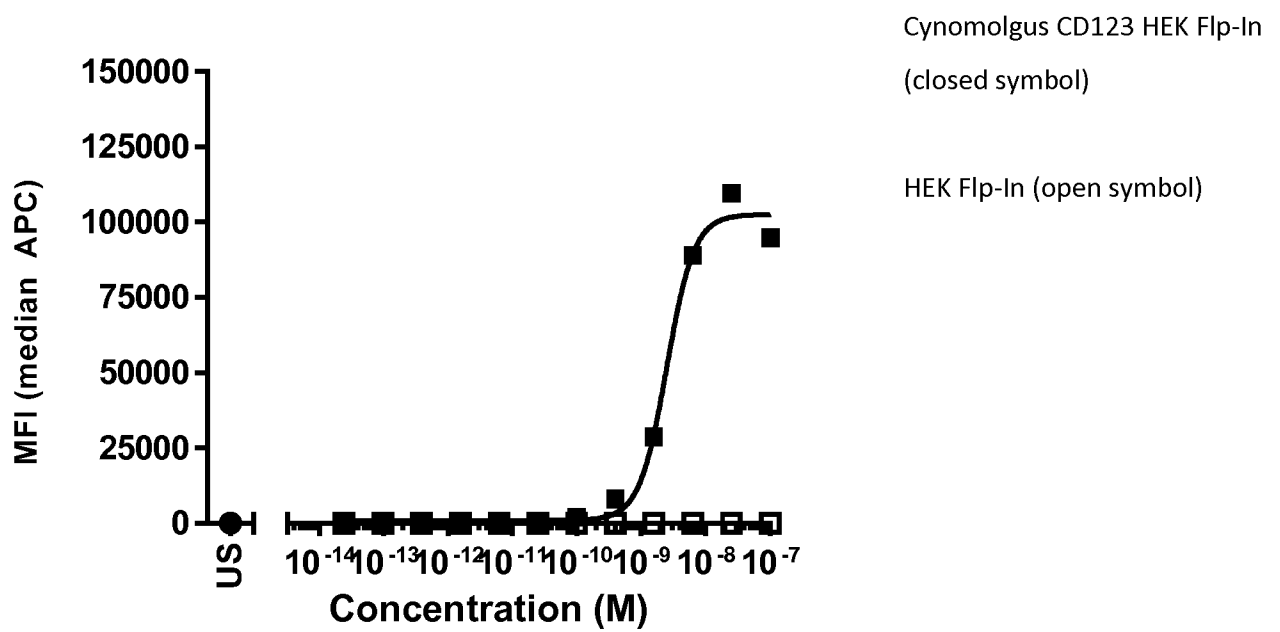
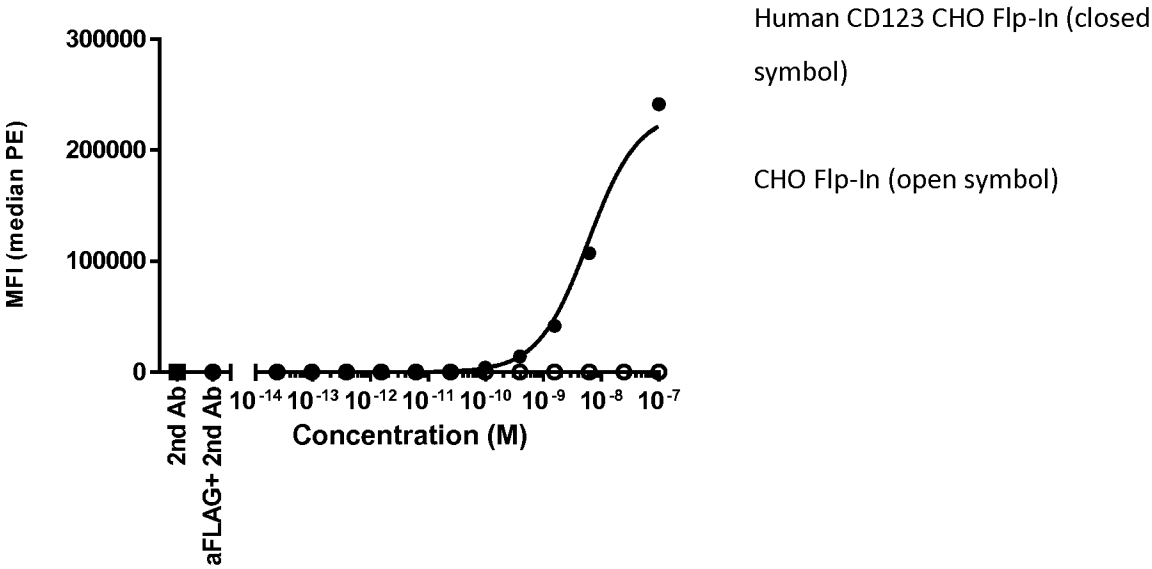
Figure 13:**A.****B.**

Figure 14:

A.



B.

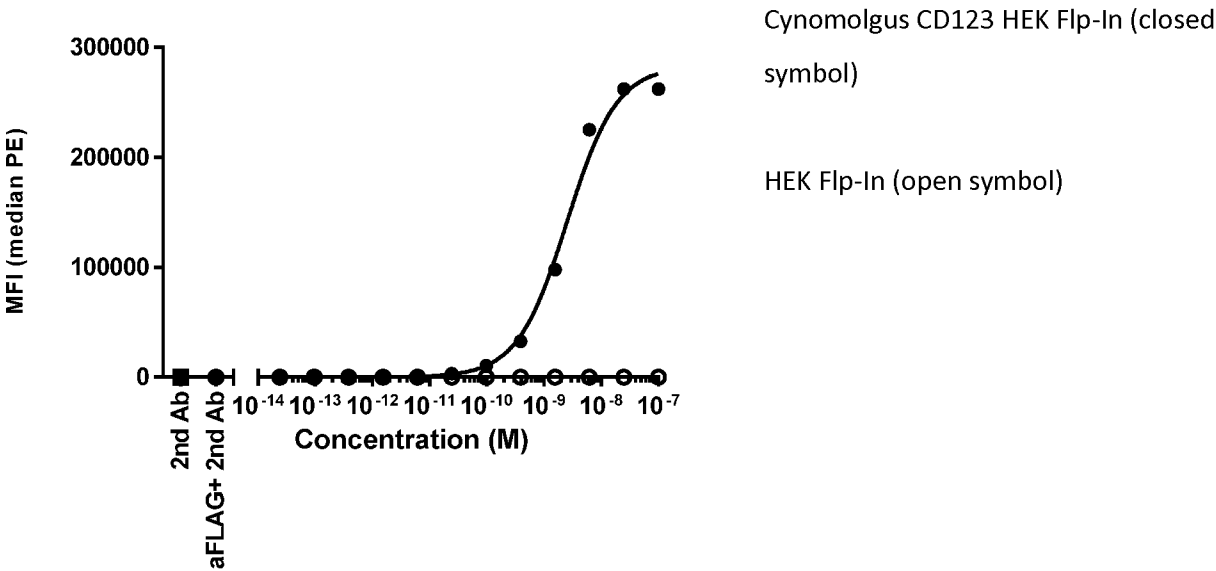
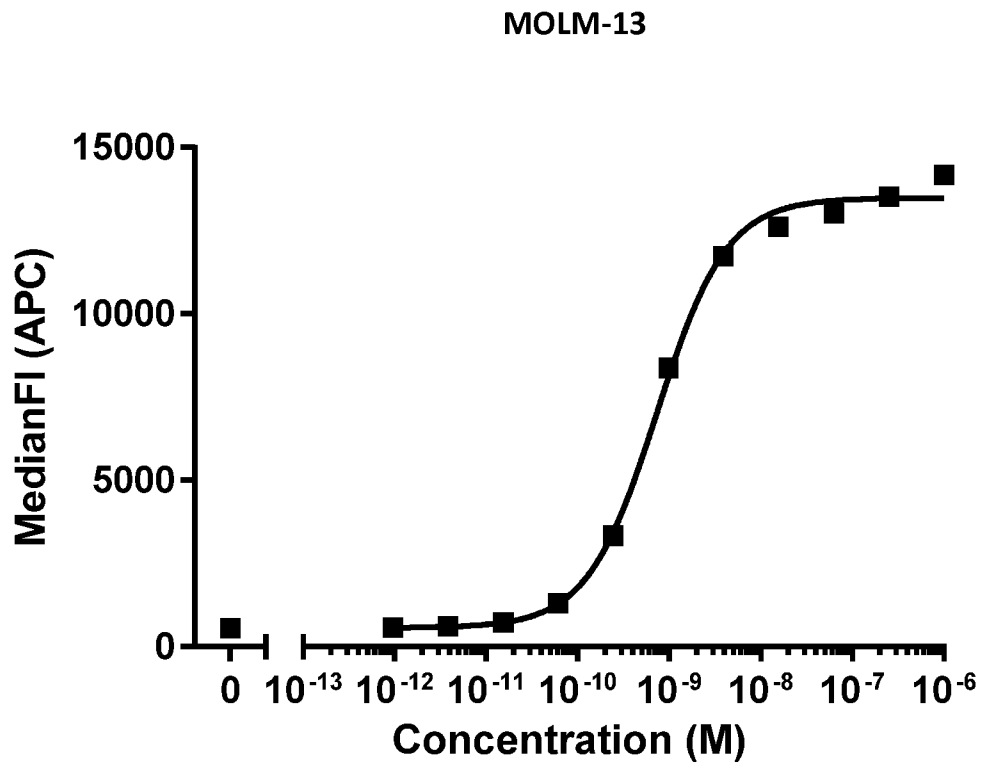


Figure 15:

A.



B.

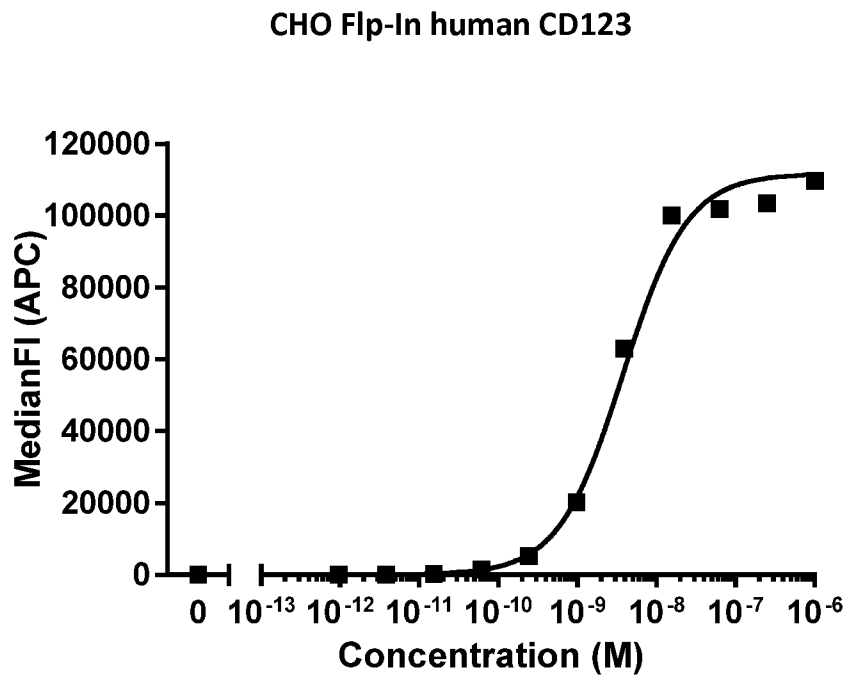


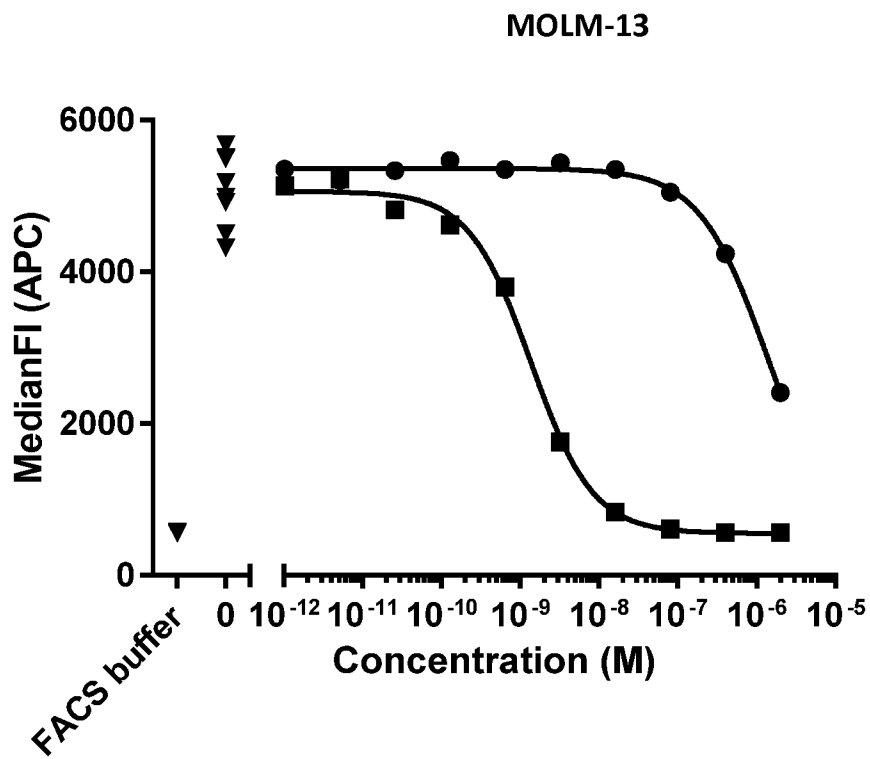
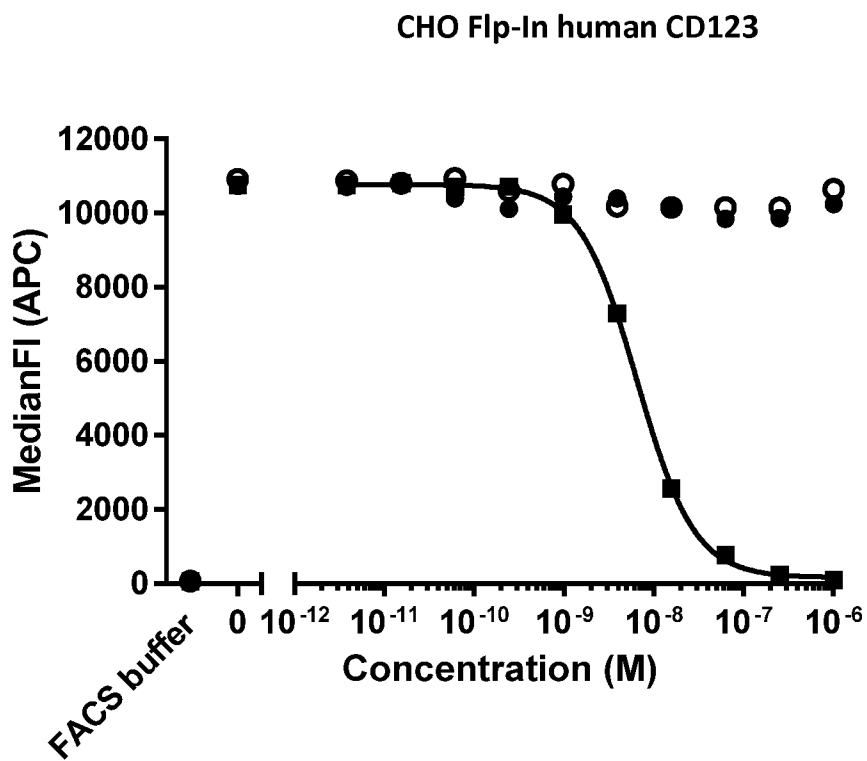
Figure 16:**A.****B.**

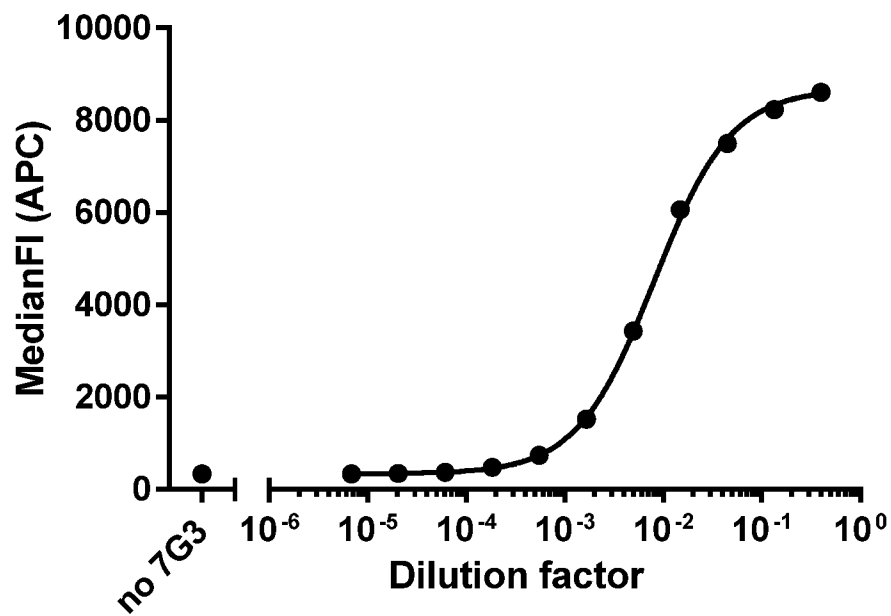
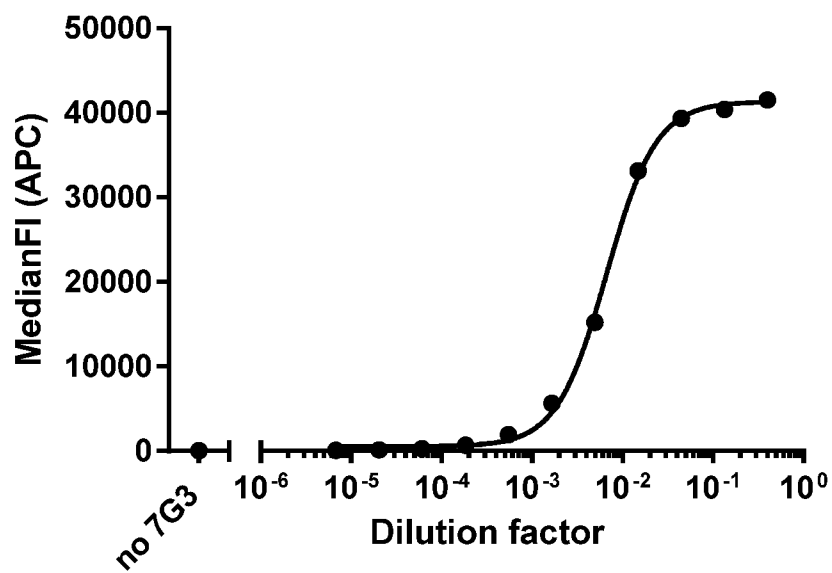
Figure 17:**A.****MOLM-13****B.****CHO Flp-In human CD123**

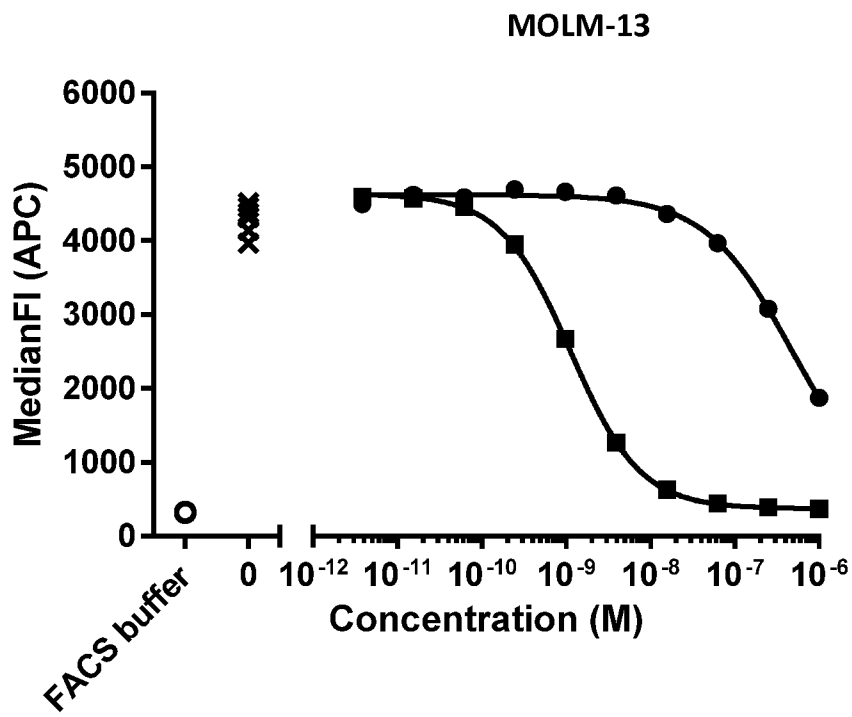
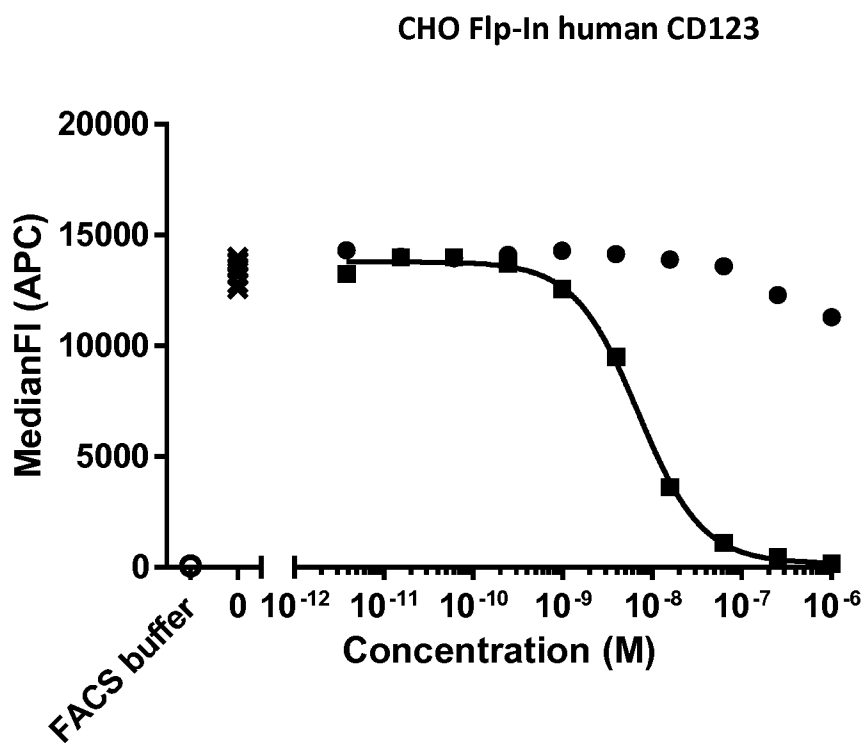
Figure 18:**A.****B.**

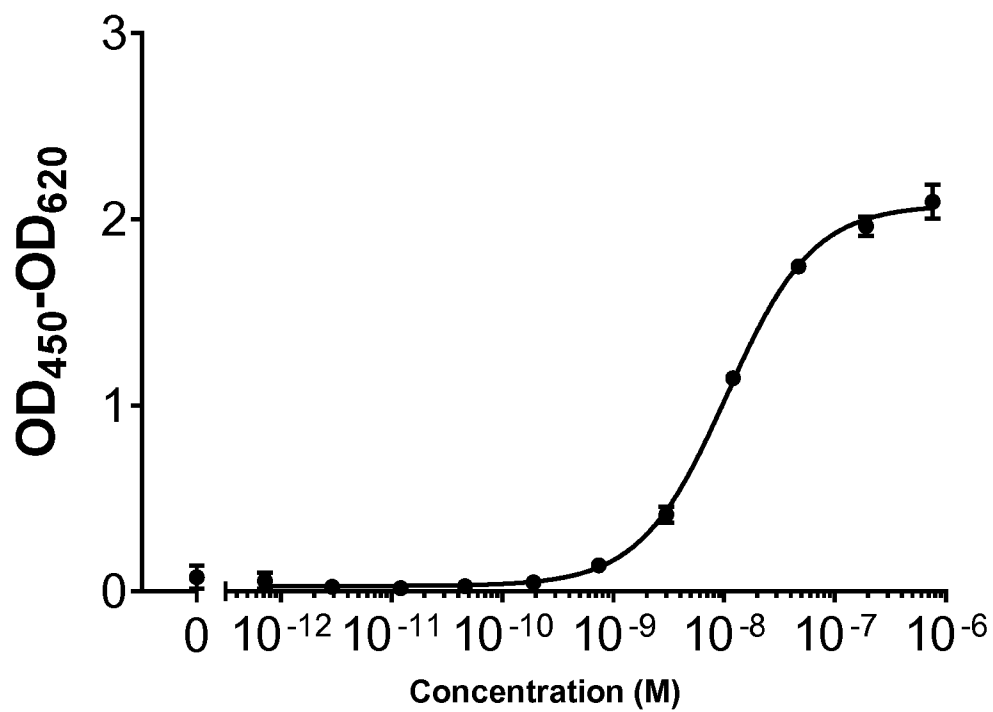
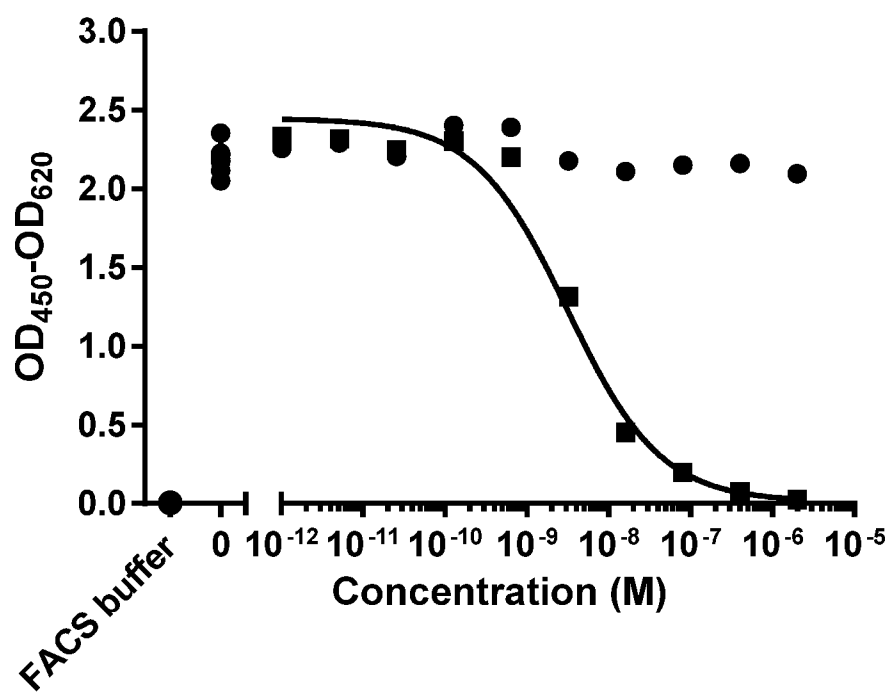
Figure 19:

Figure 20:

A.



B.

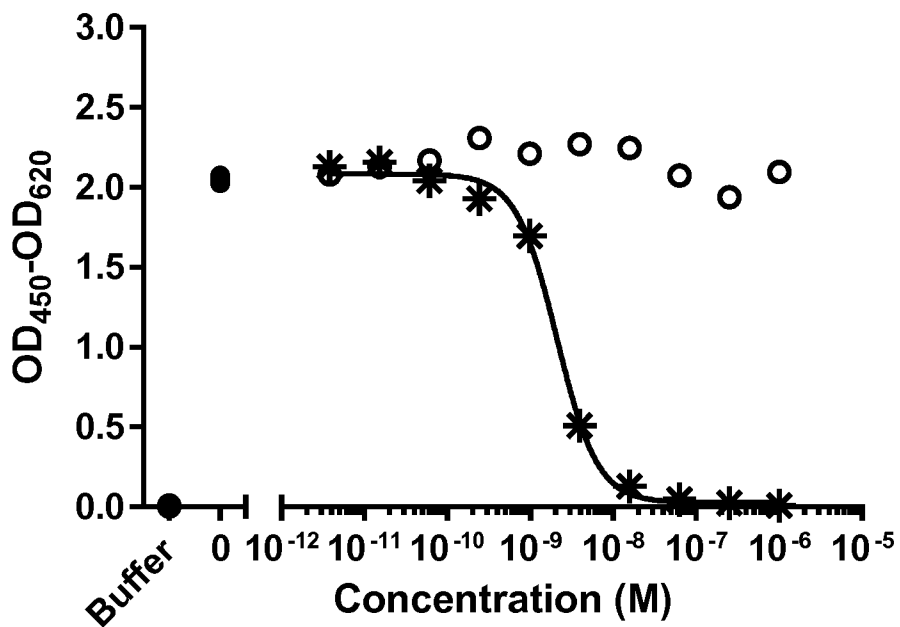
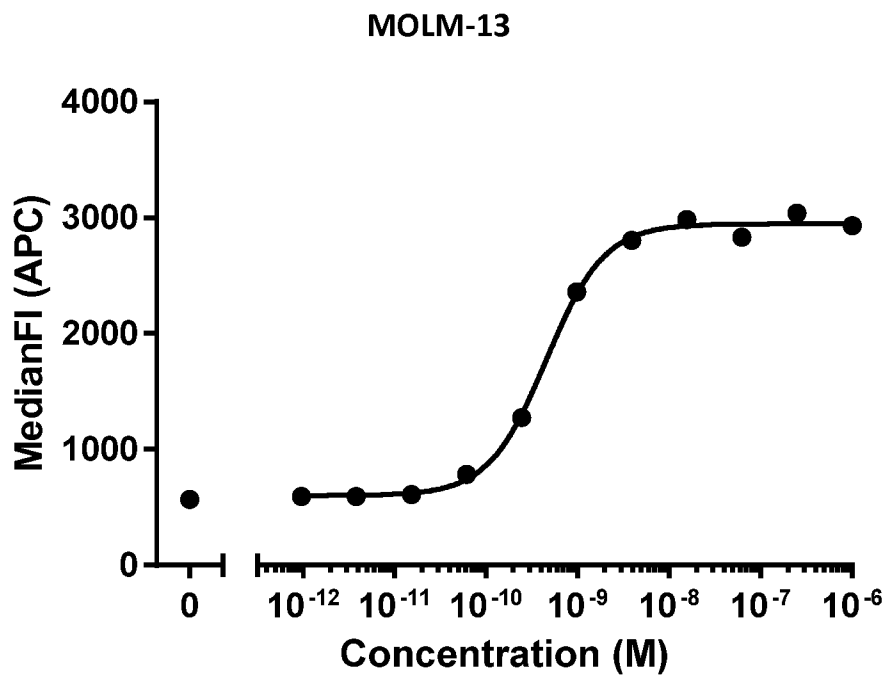
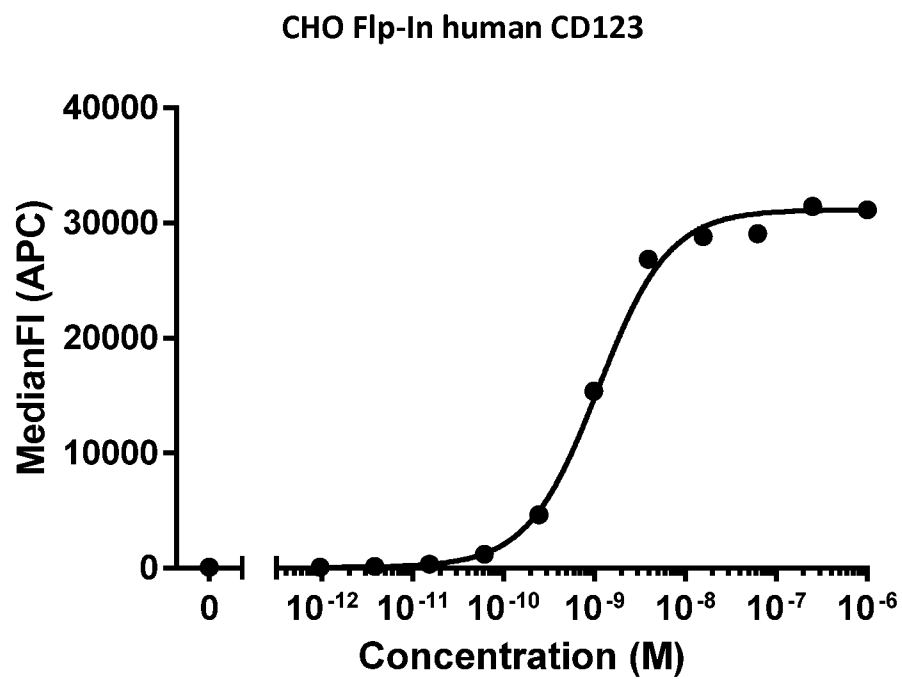


Figure 21:**A.****B.**

C.

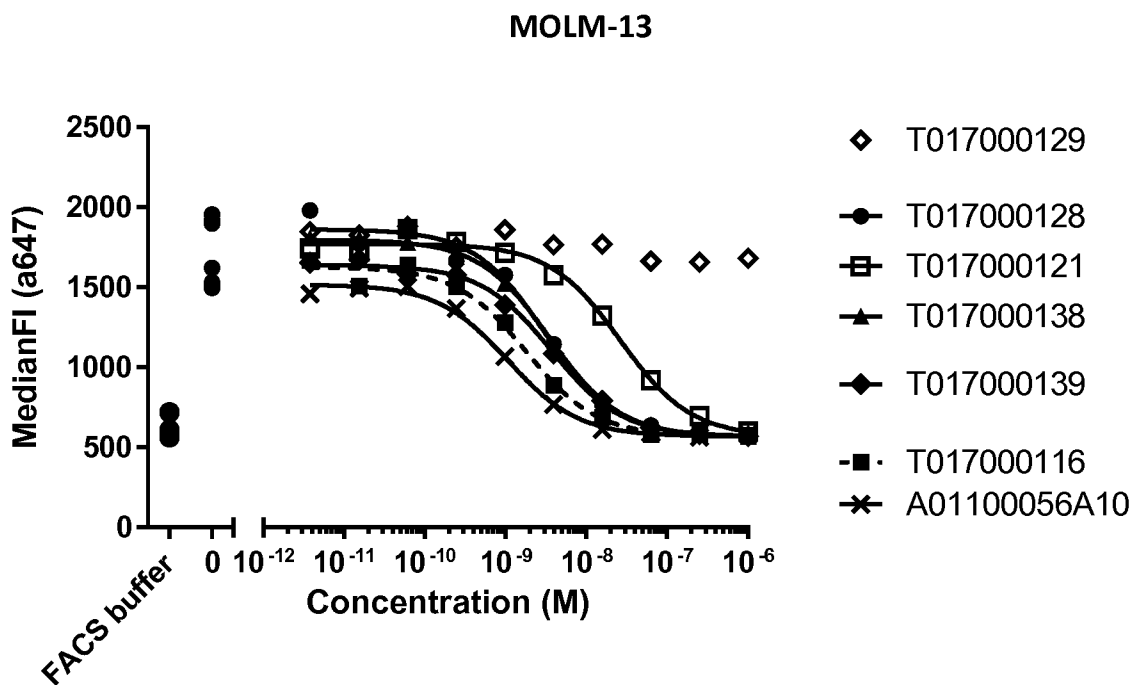


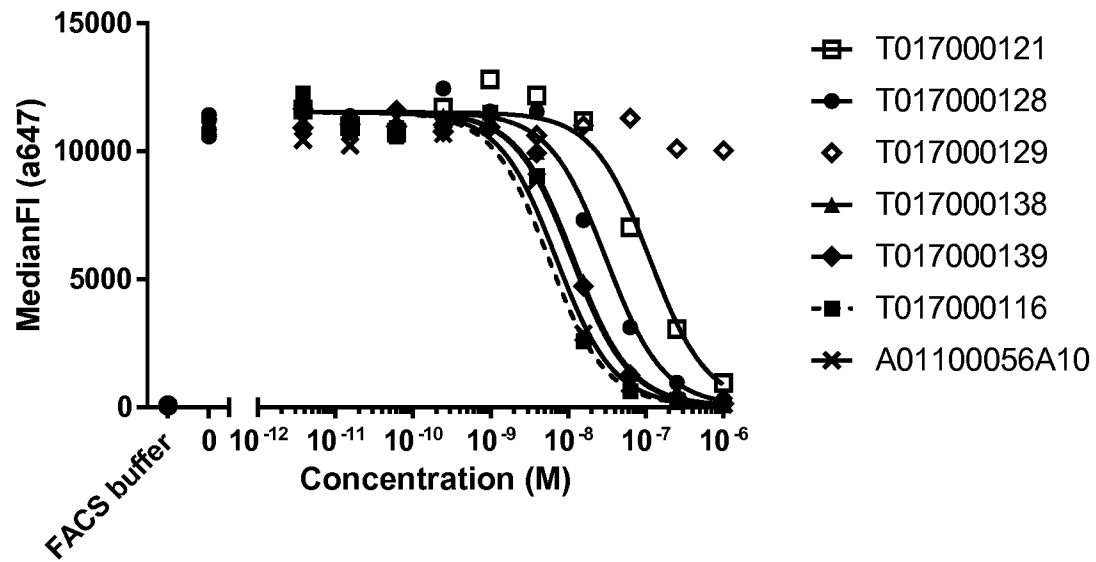
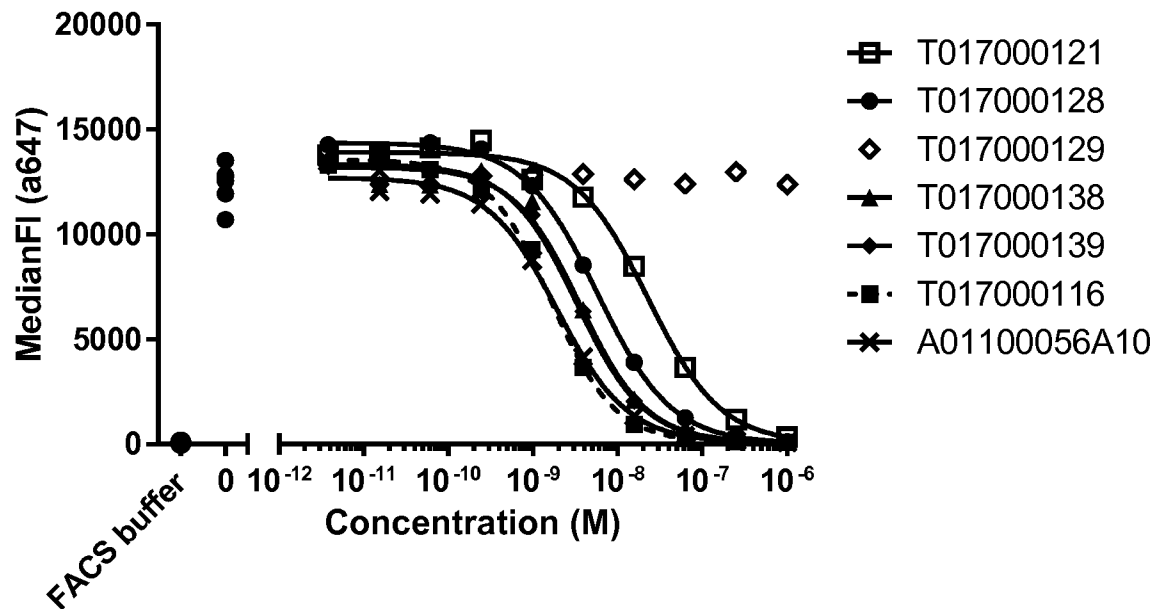
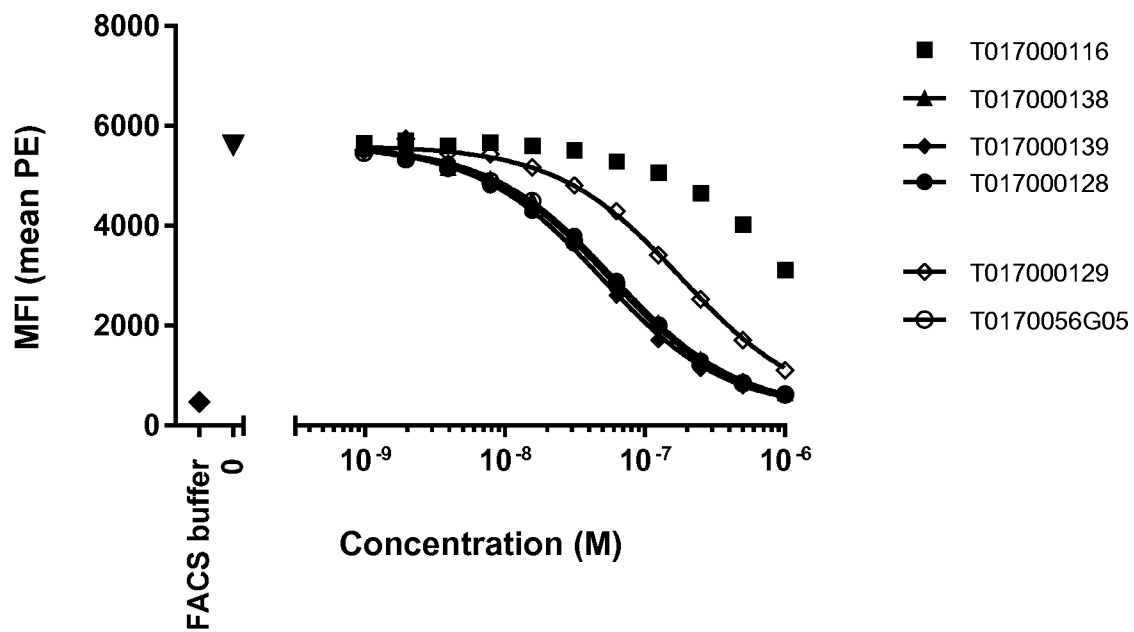
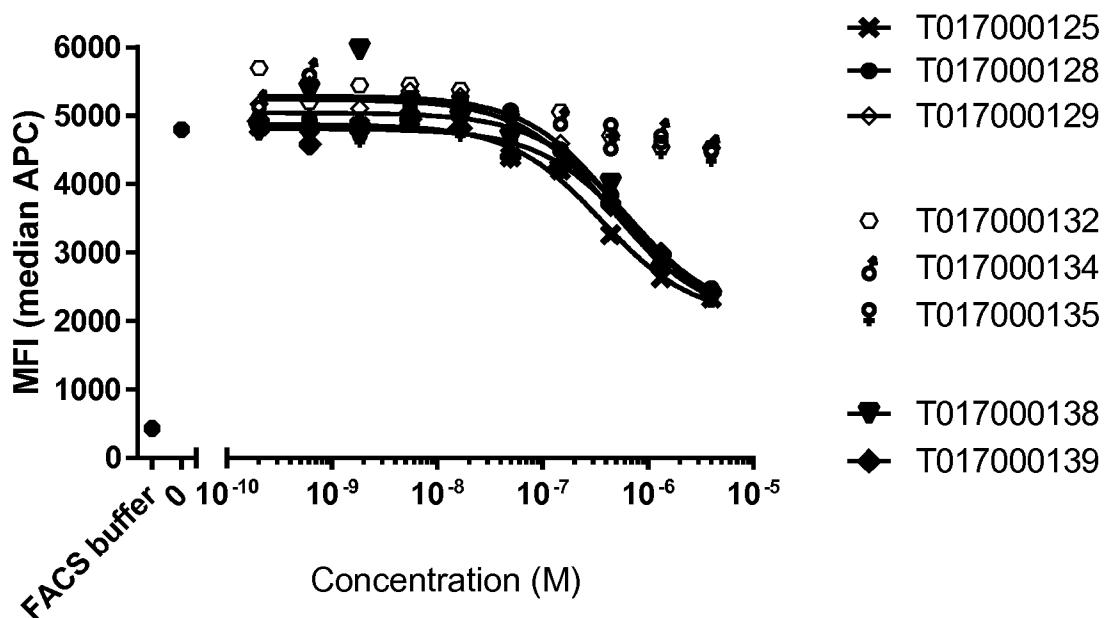
Figure 22 cont':**B.****HEK293 Flp-In cynomolgus CD123****C.****CHO Flp-In human CD123**

Figure 23:**Figure 24:**

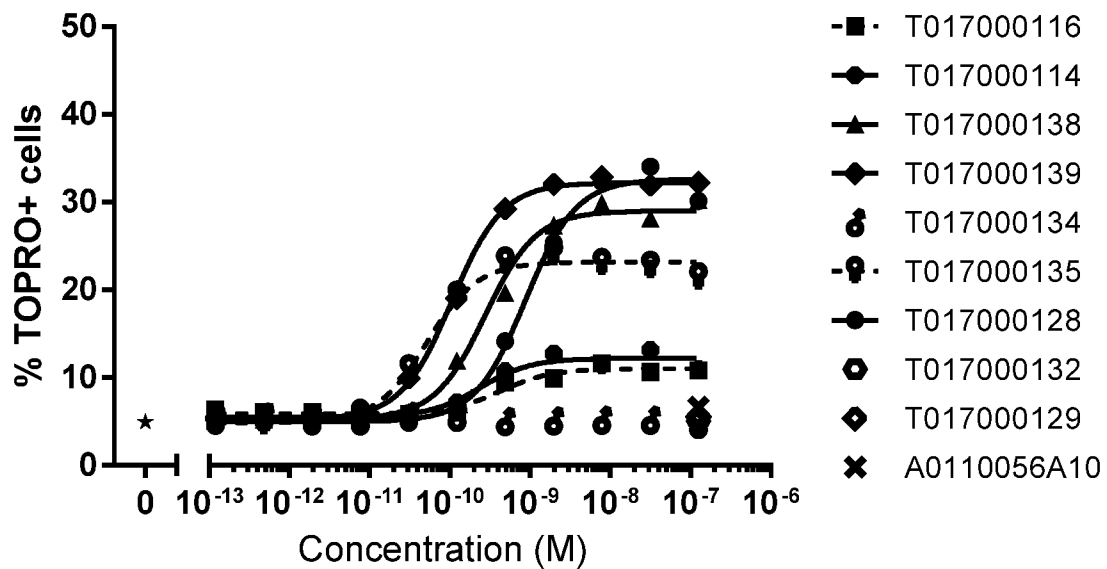
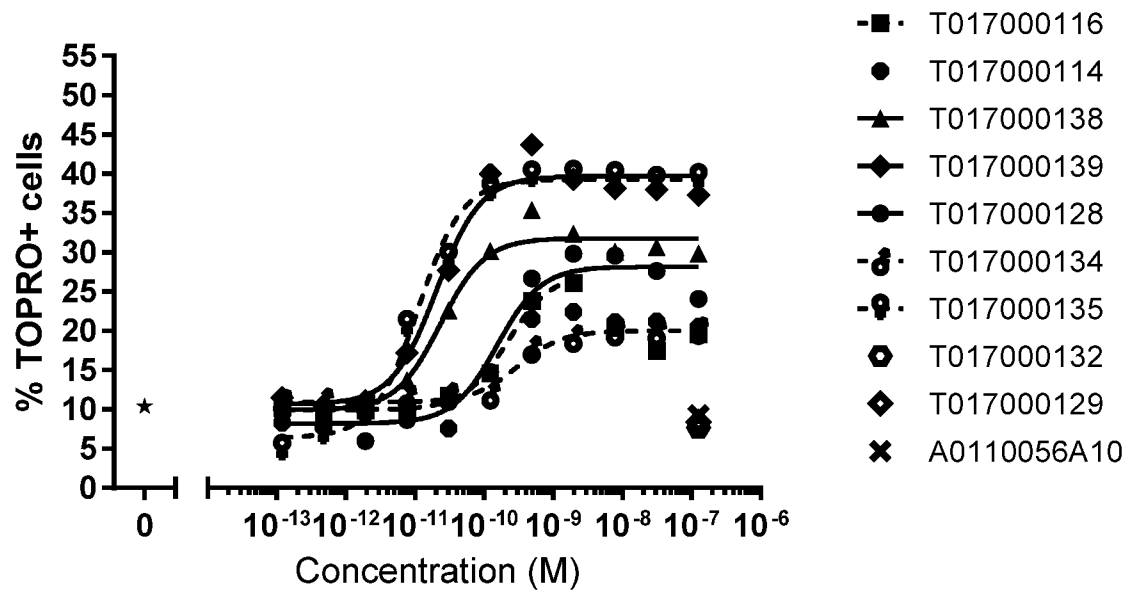


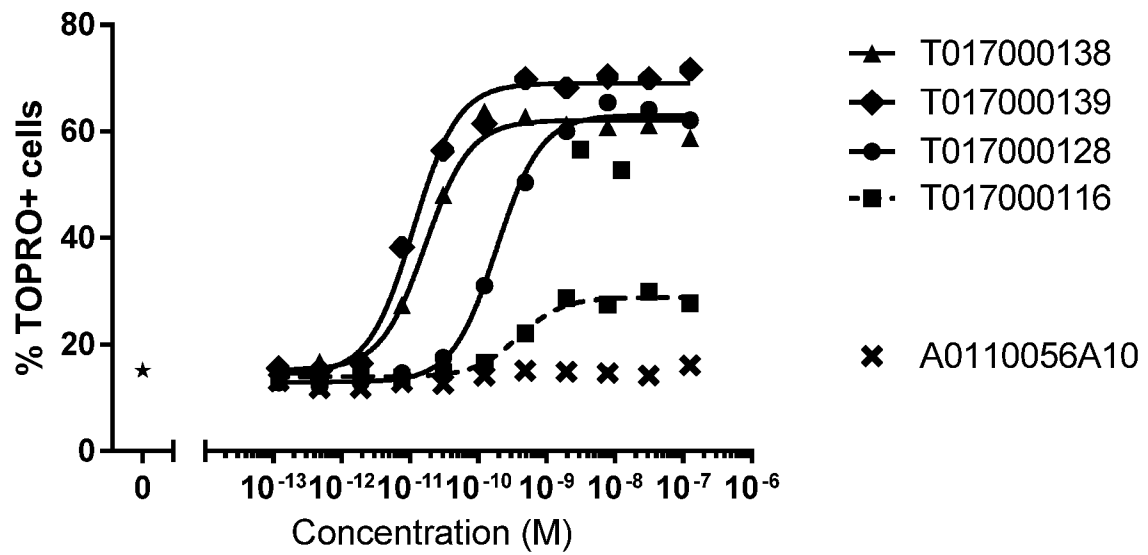
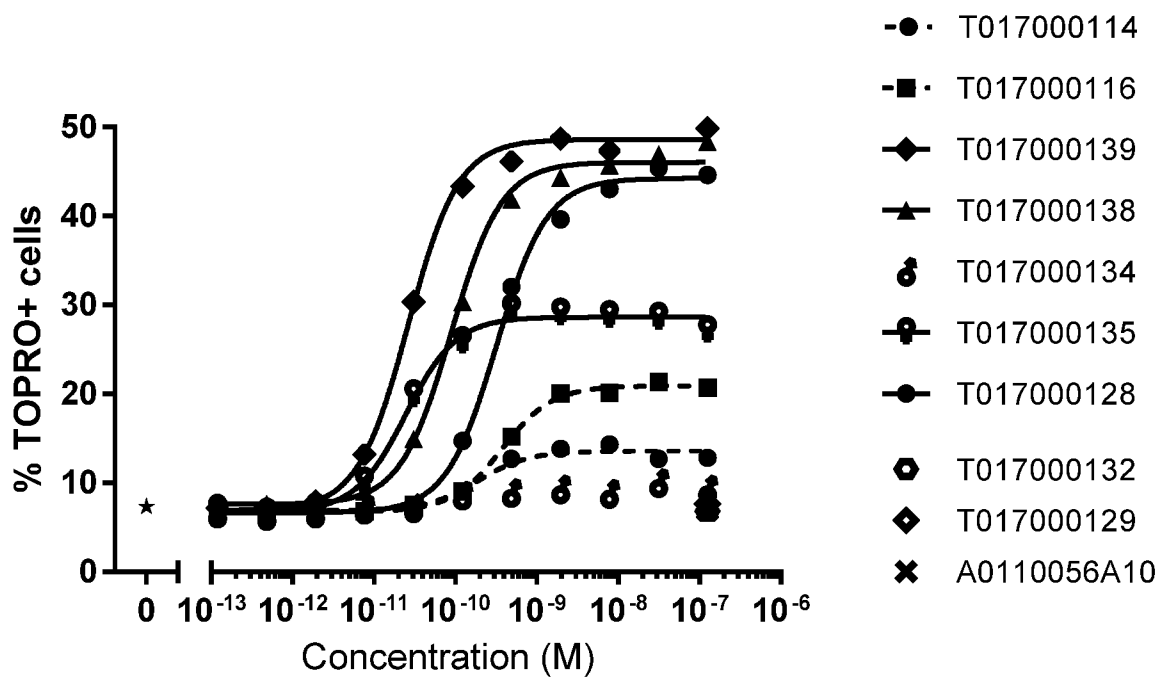
Figure 27:**Figure 28:**

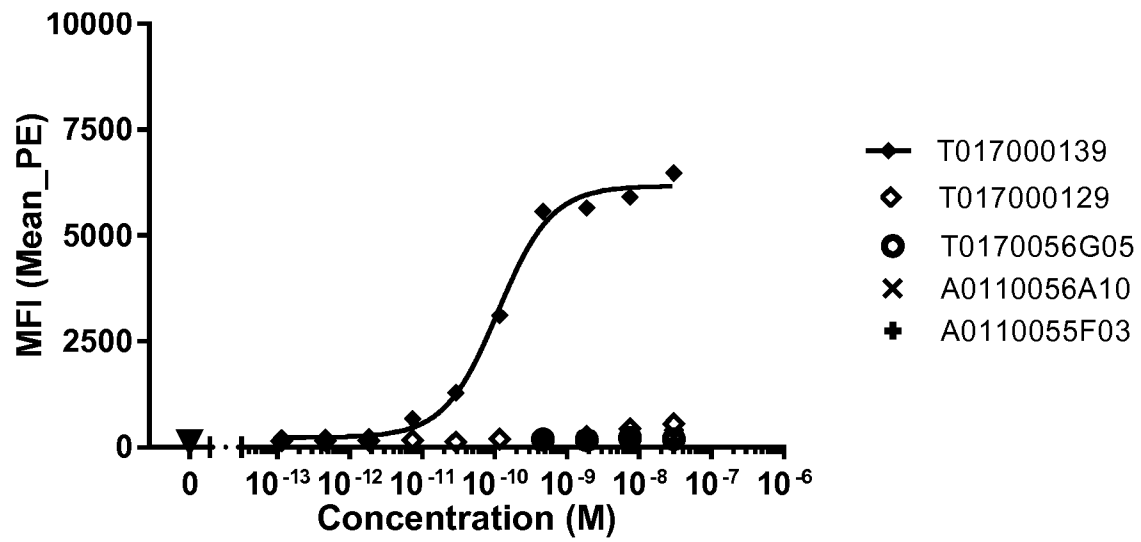
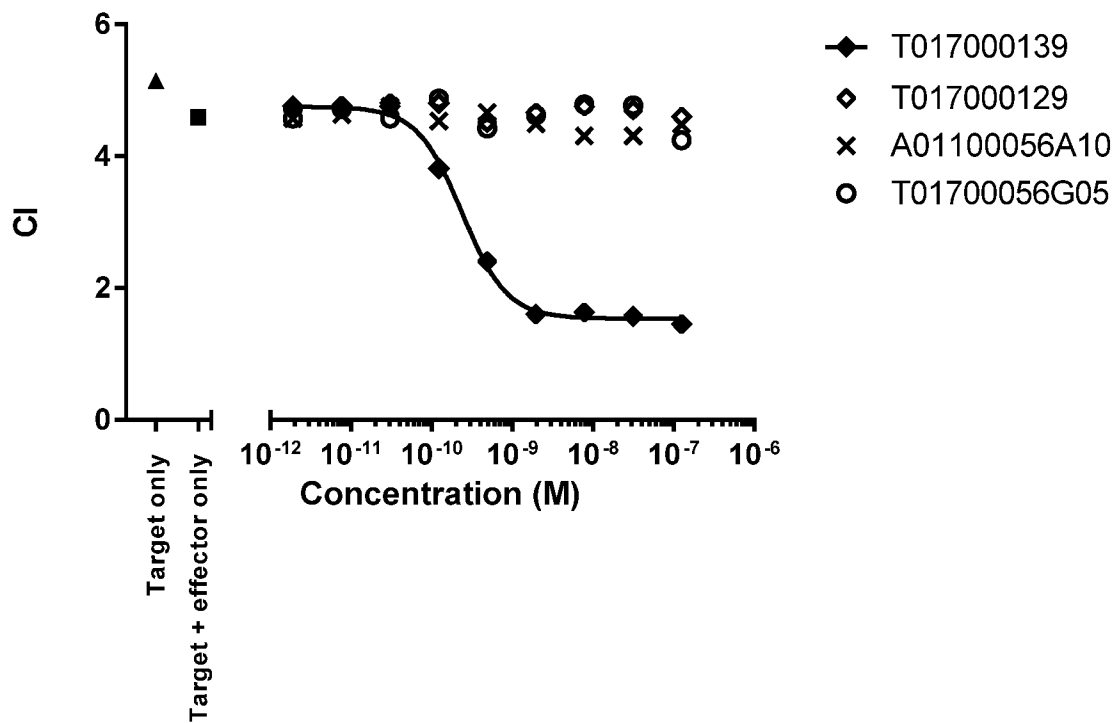
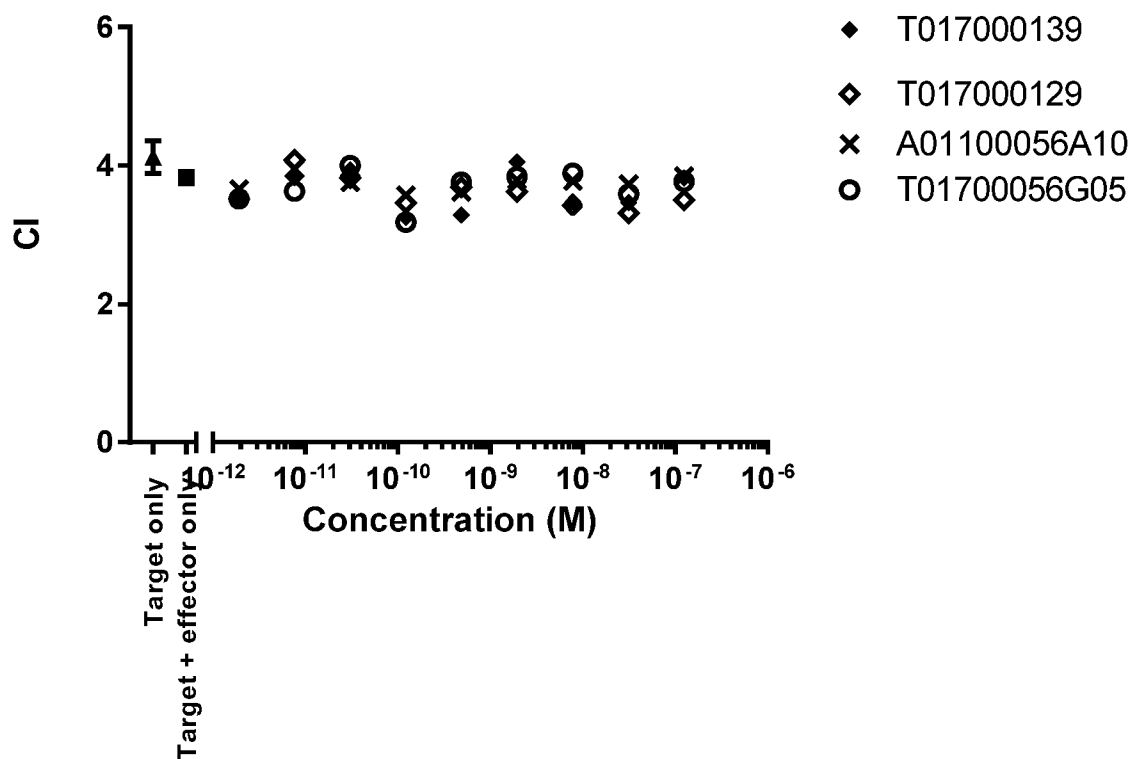
Figure 29:**Figure 30:**

Figure 31:**Figure 32:**

A.

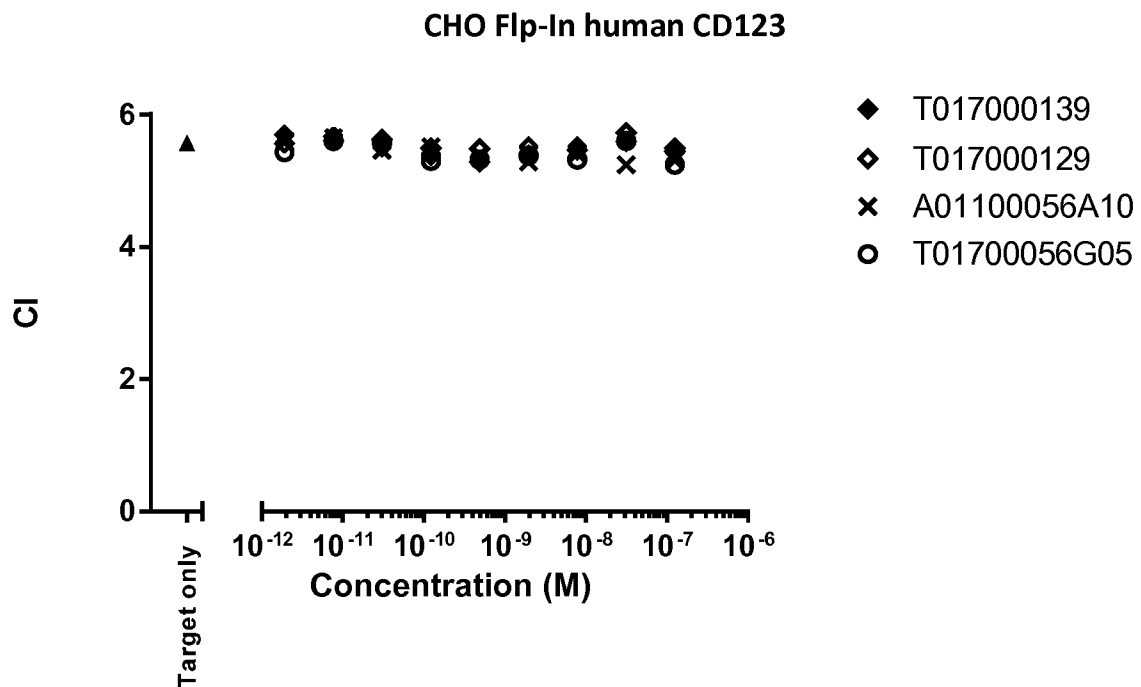


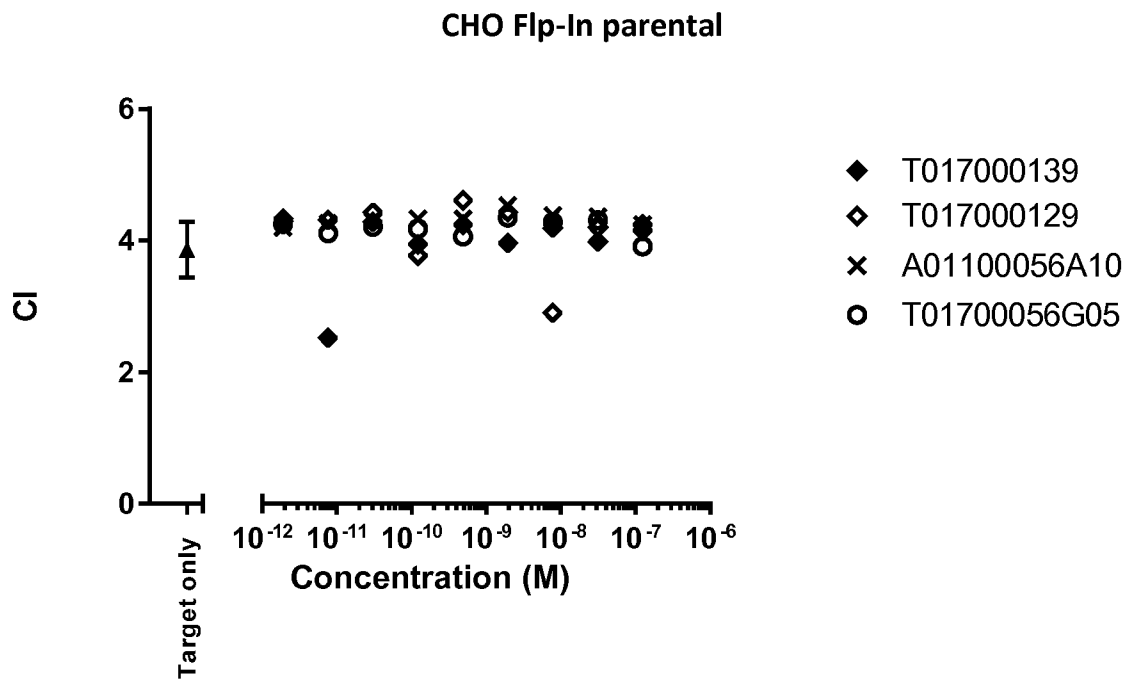
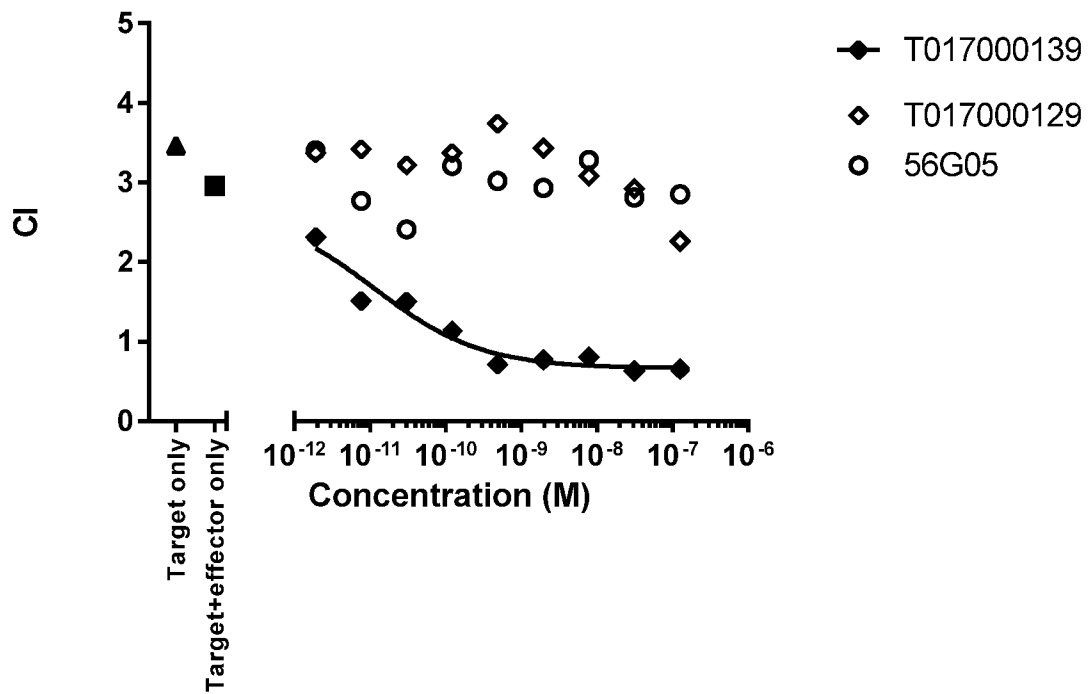
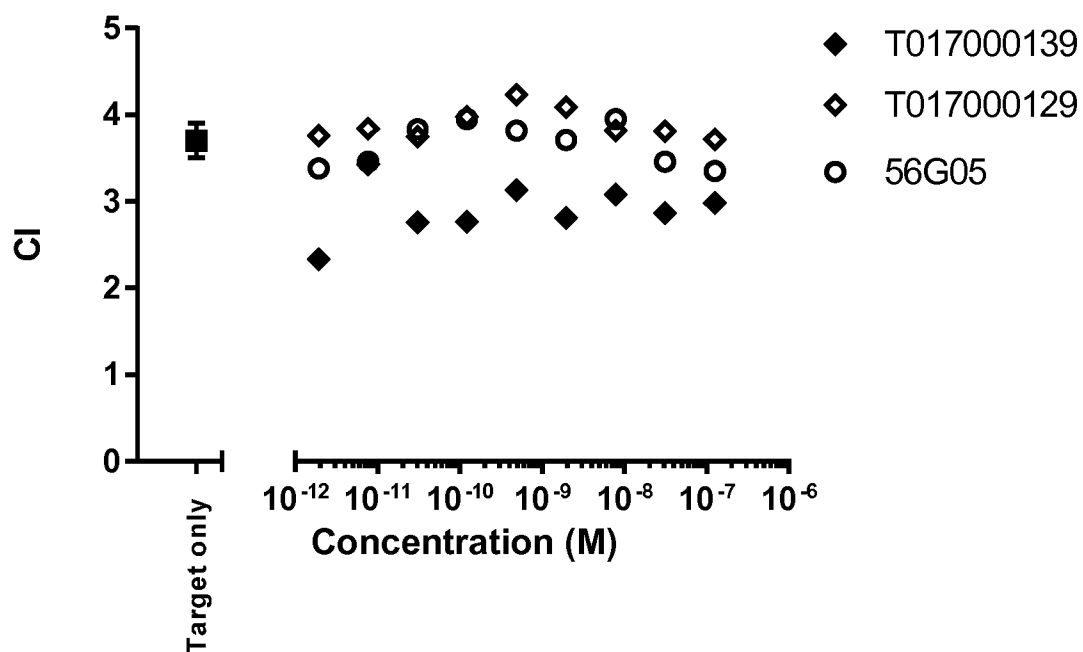
Figure 32 cont':**B.****Figure 33:**

Figure 34:**Figure 35:**

A.

HEK Flp-In cynomolgus CD123

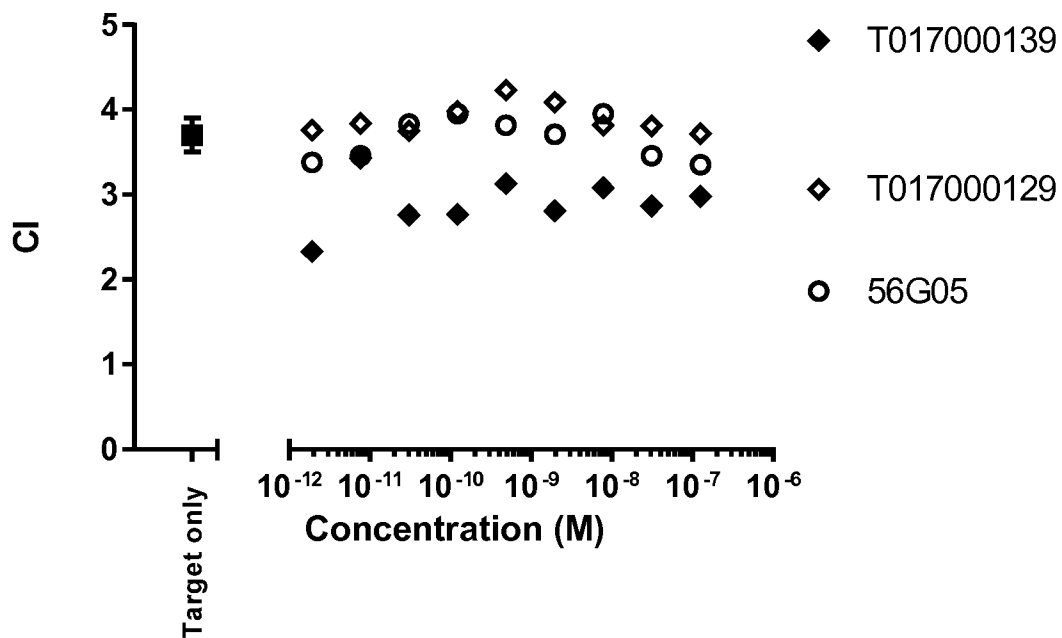


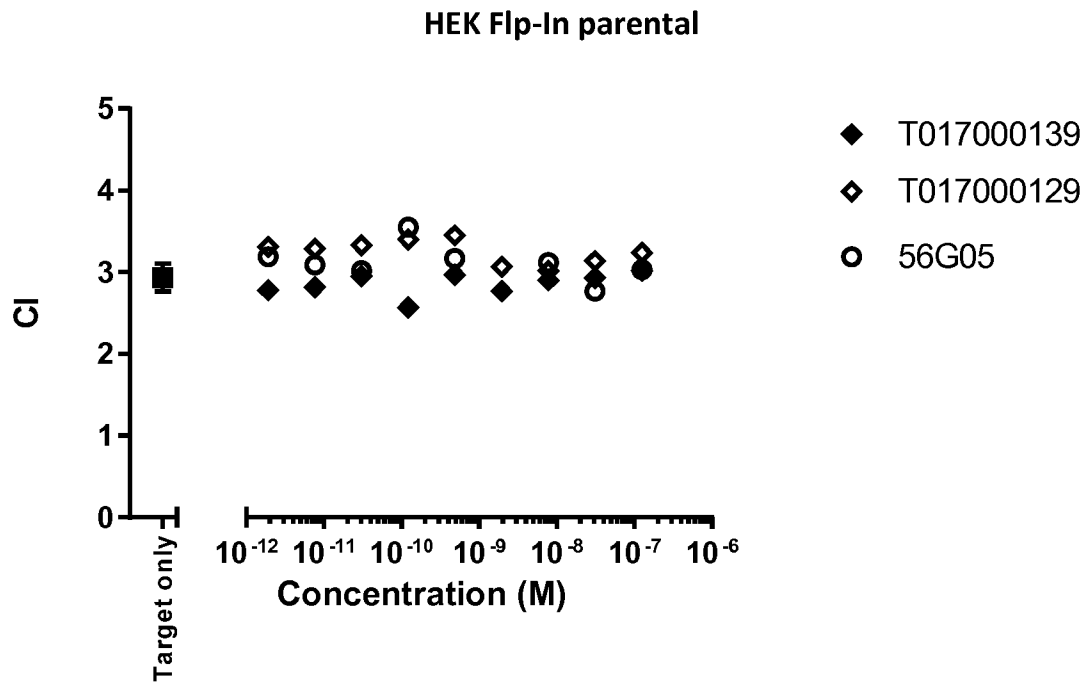
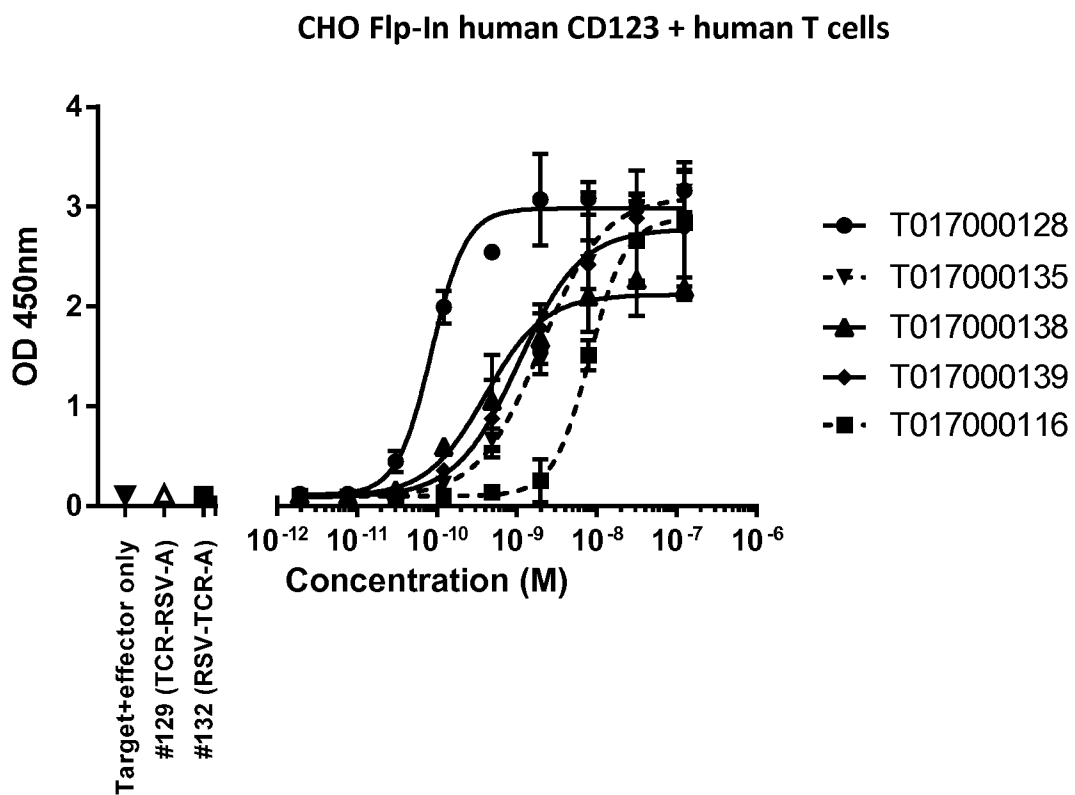
Figure 35 cont':**B.****Figure 36:****A.**

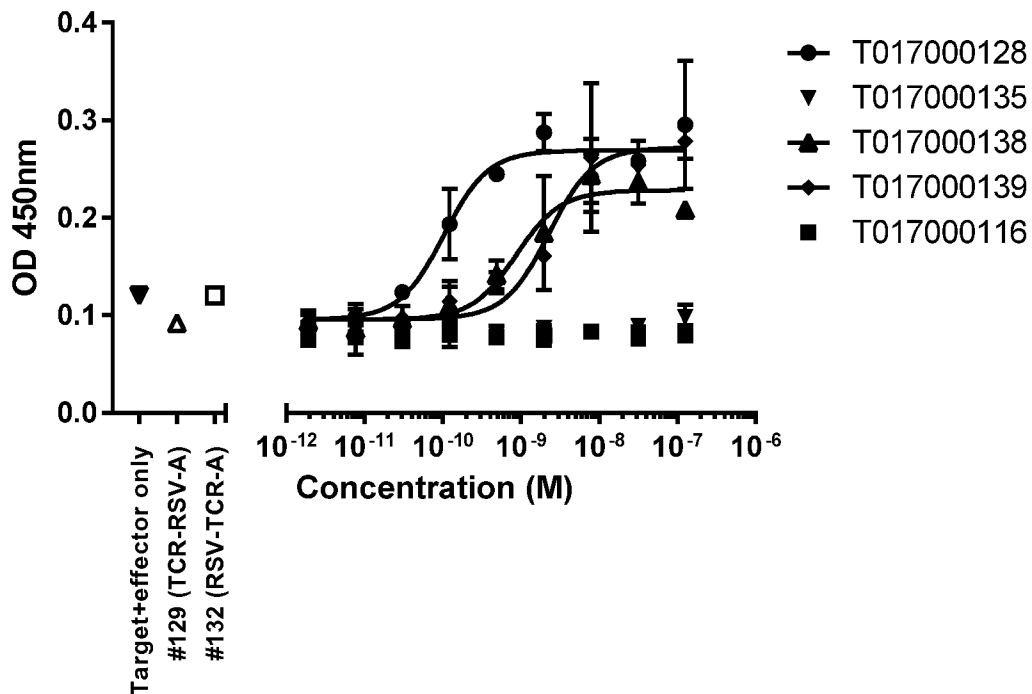
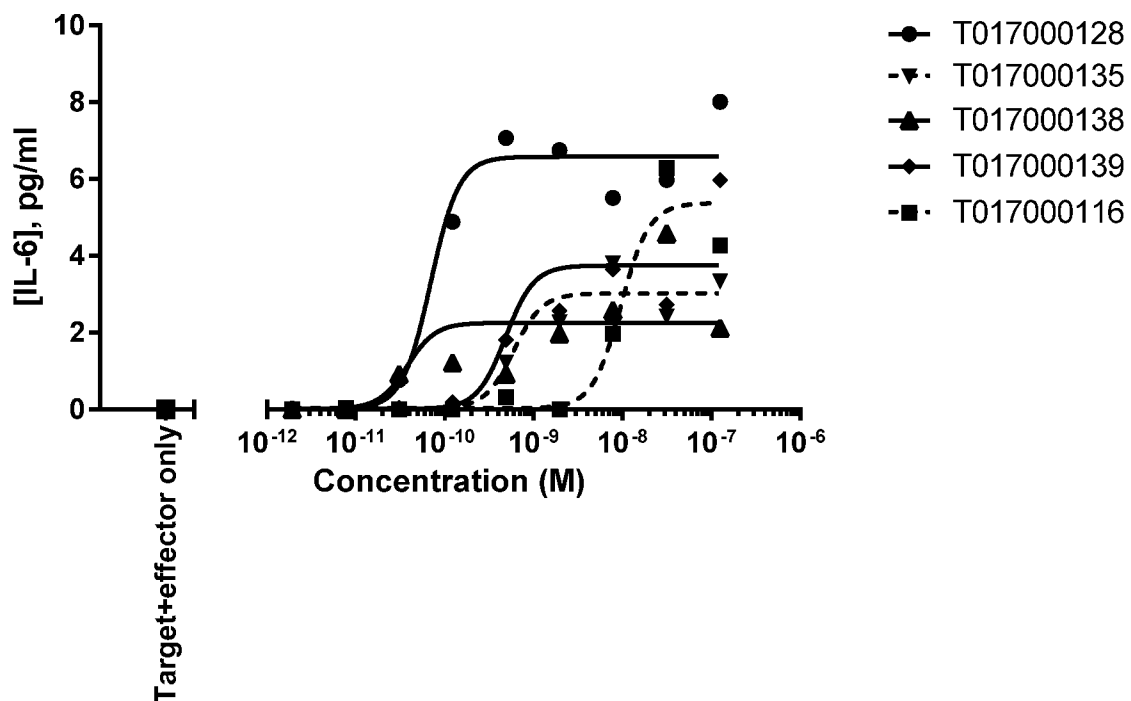
Figure 36 cont':**B.****CHO Flp-In human CD123 + cyno T cells****Figure 37:**

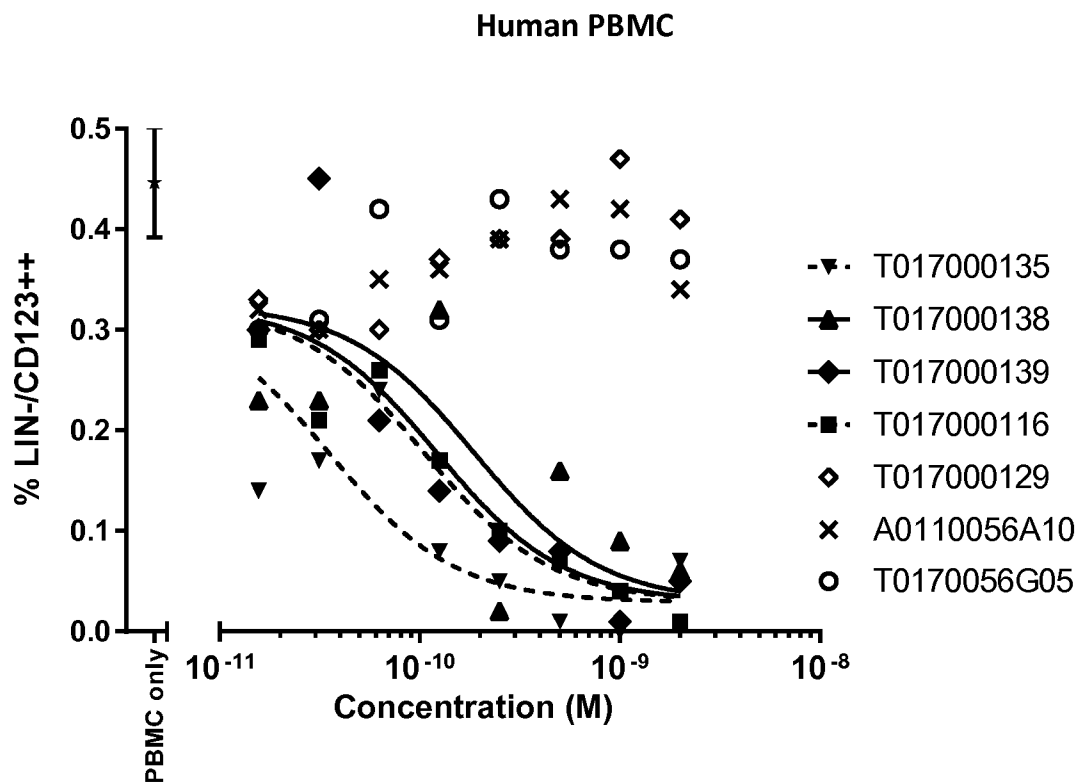
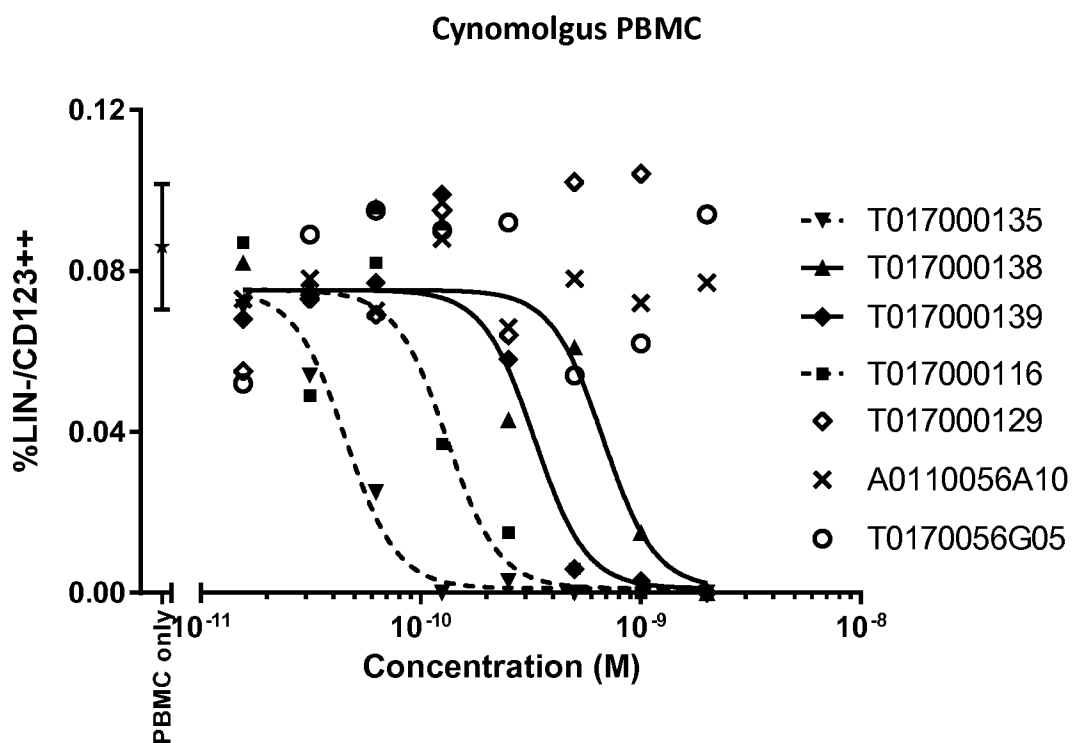
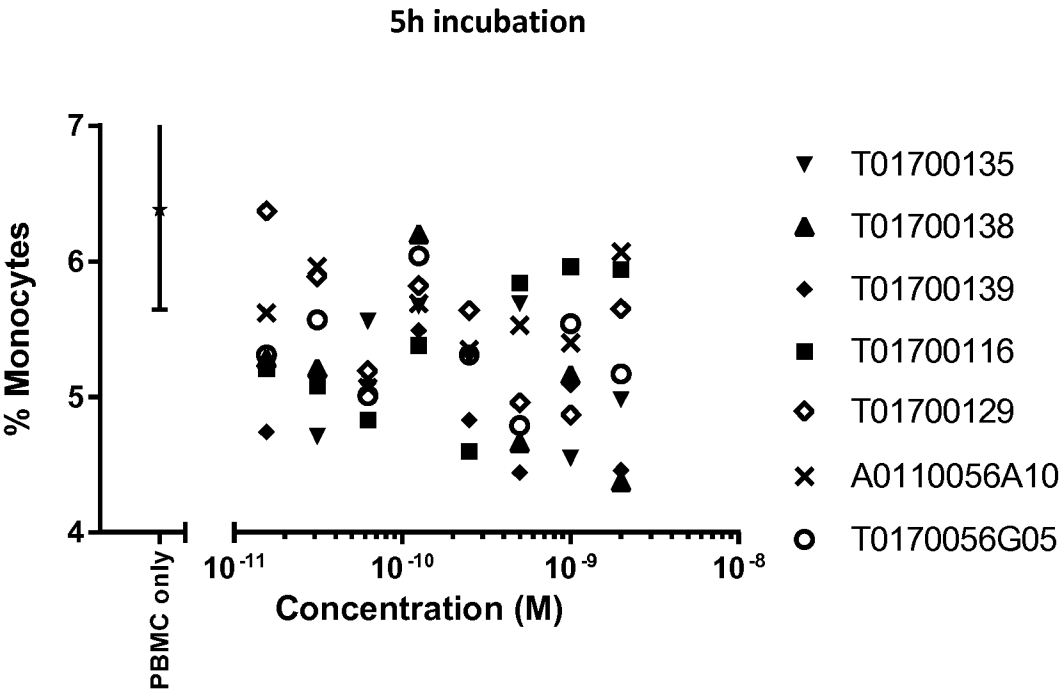
Figure 38:**A.****B.**

Figure 39:

A.



B.

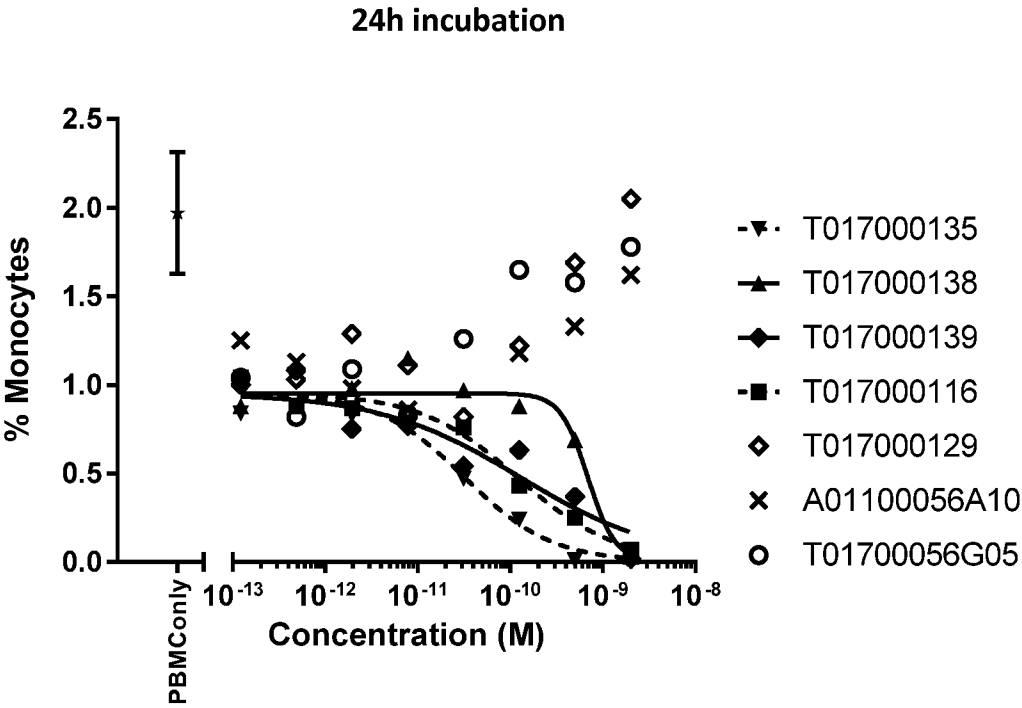


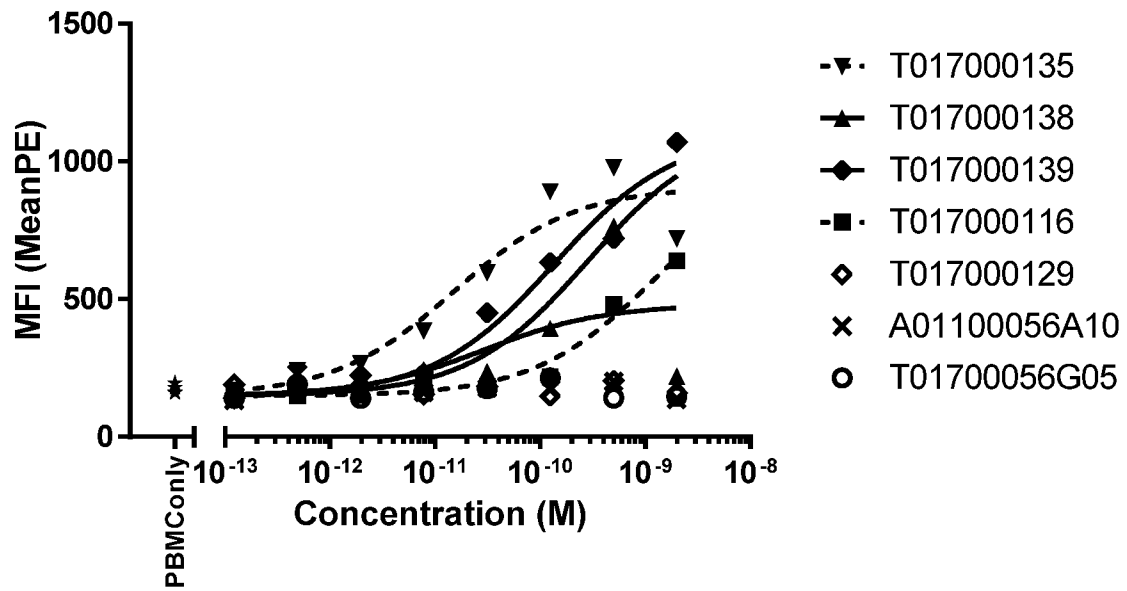
Figure 40:

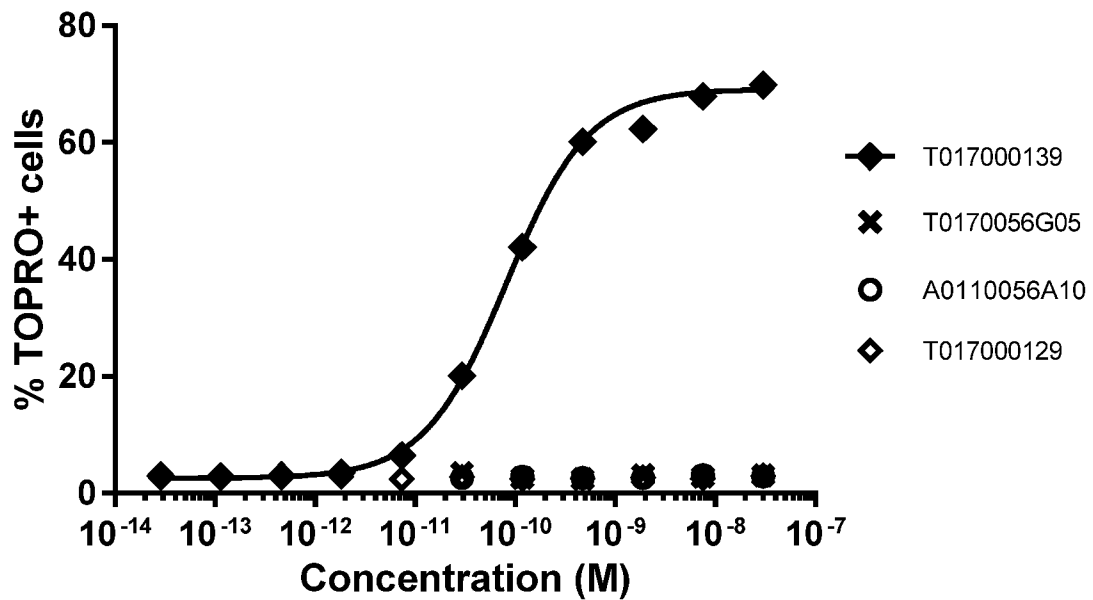
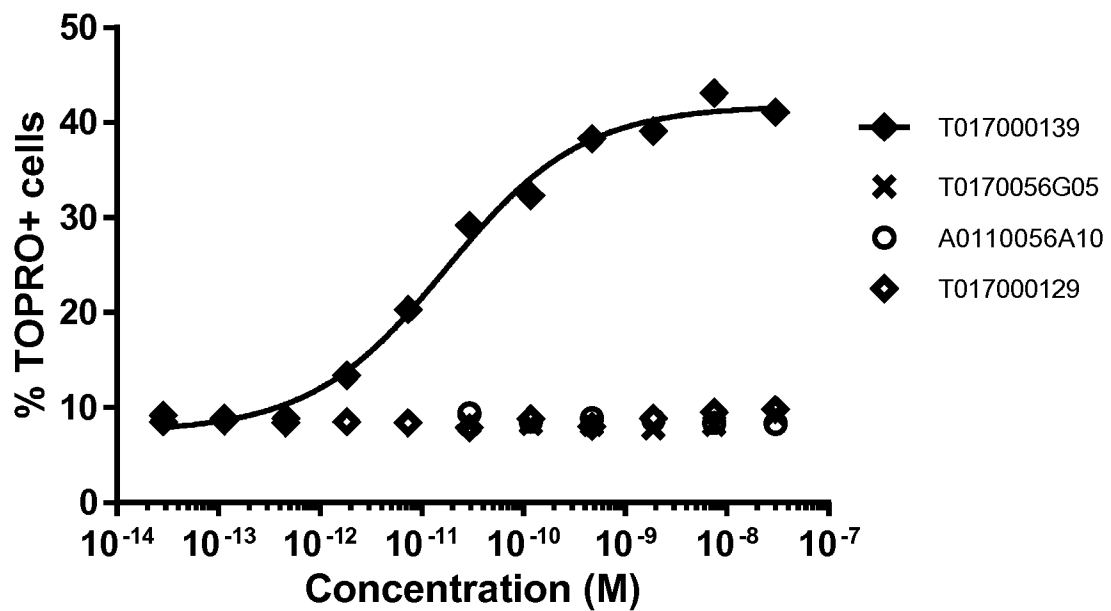
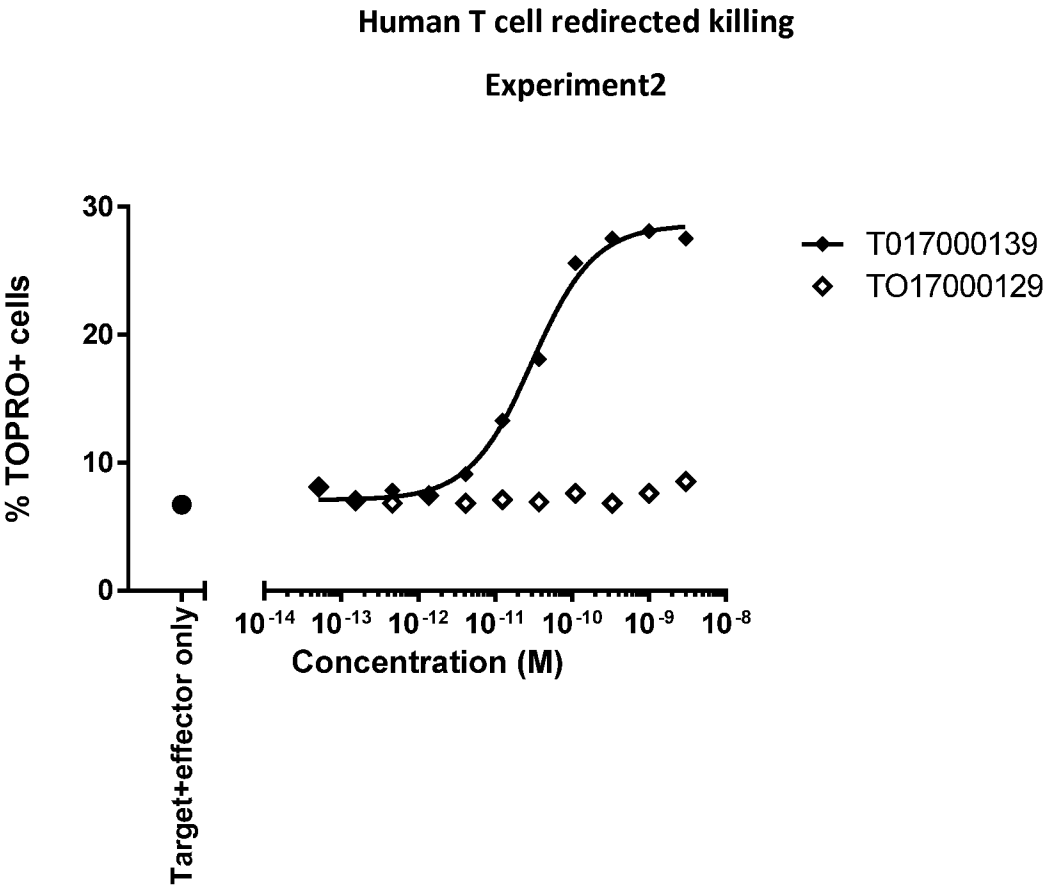
Figure 41:**A.****Human T cell redirected killing****B.****Cynomolgus T cell redirected killing**

Figure 42:

A.



B.

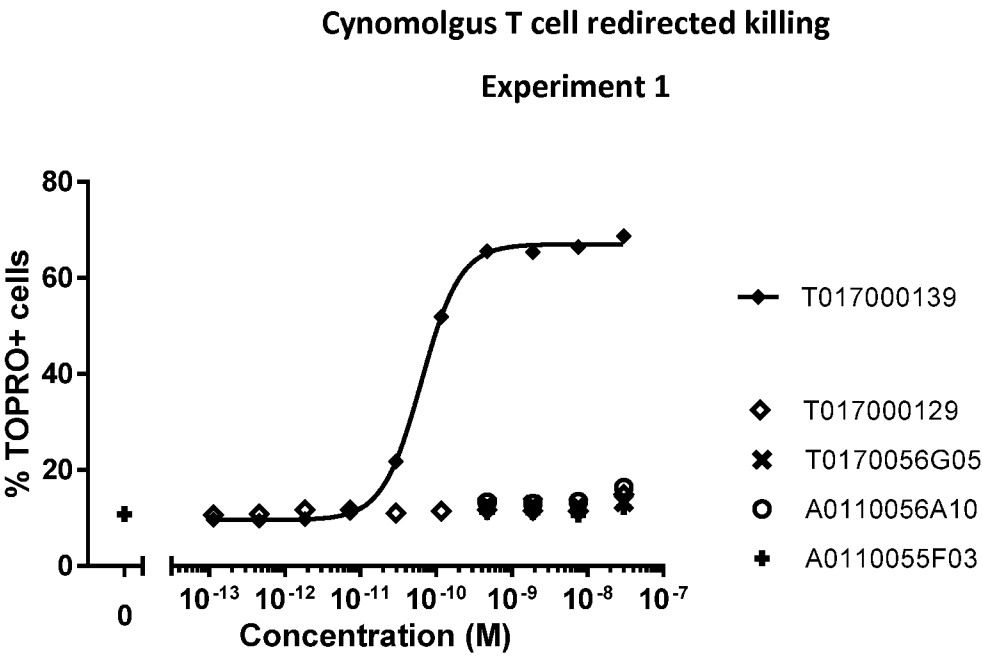
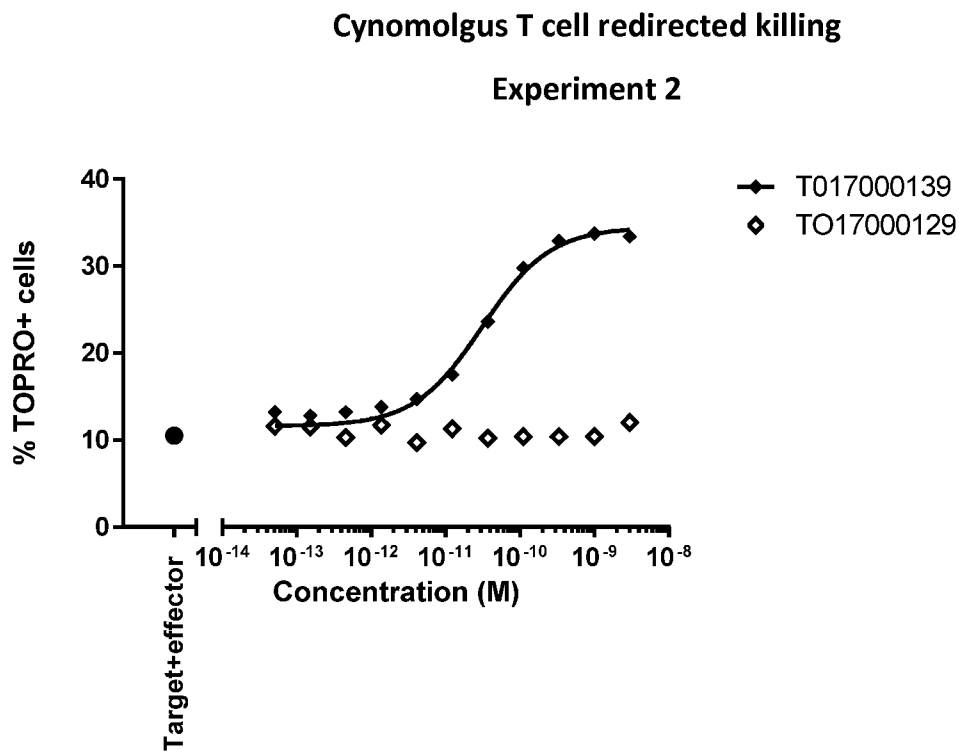


Figure 42 cont':

C.

Figure 43:

A.

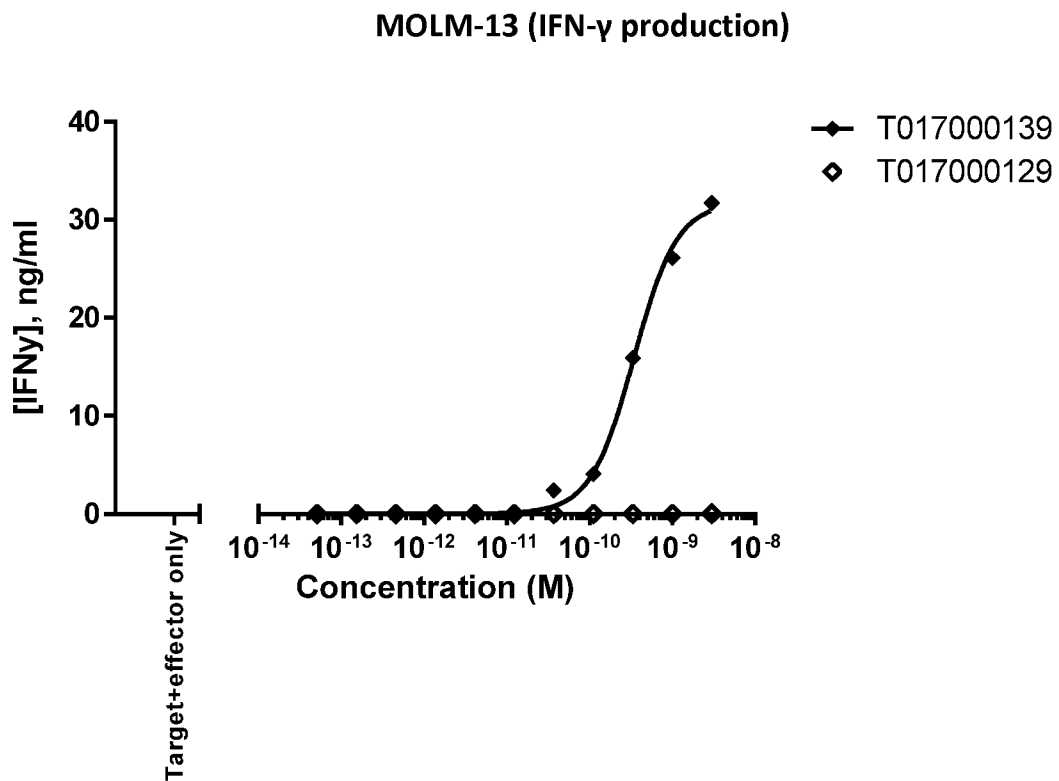


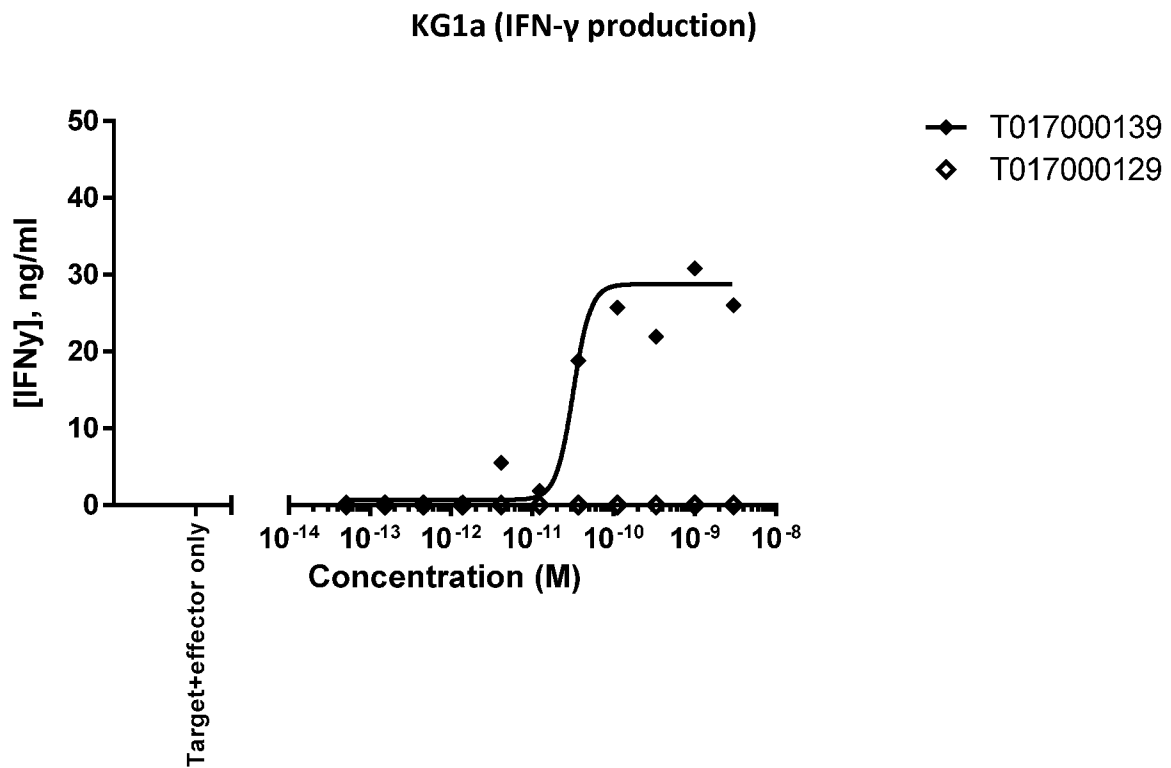
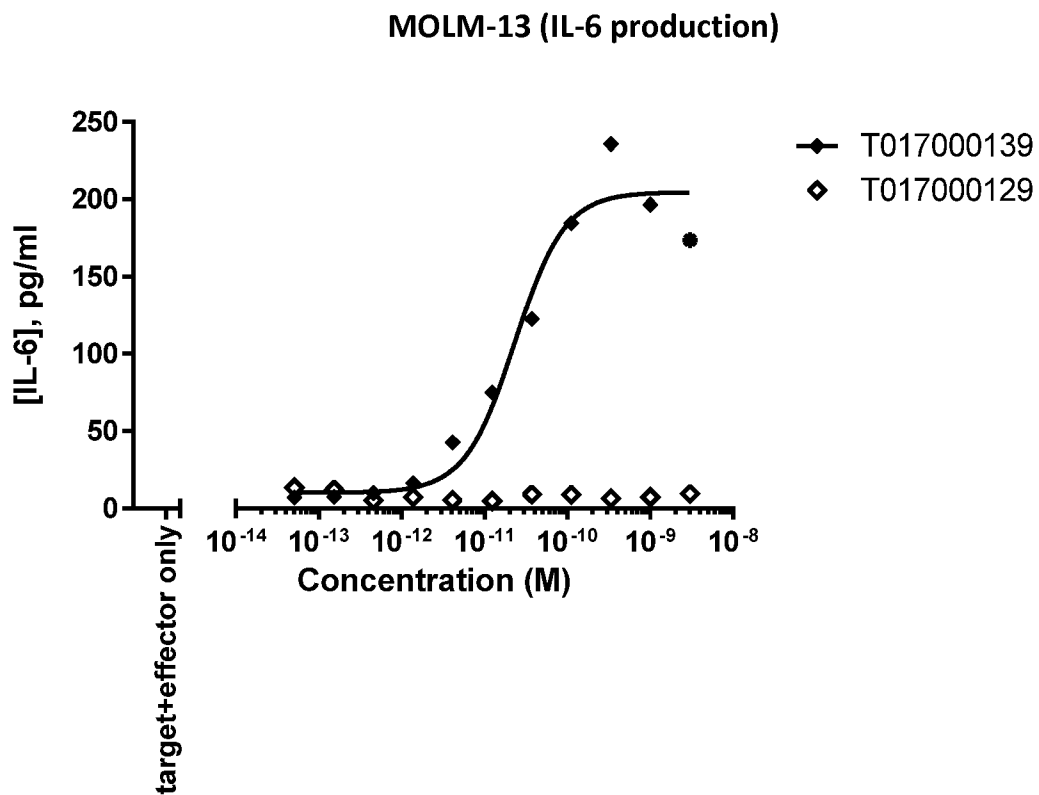
Figure 43 cont':**B.****C.**

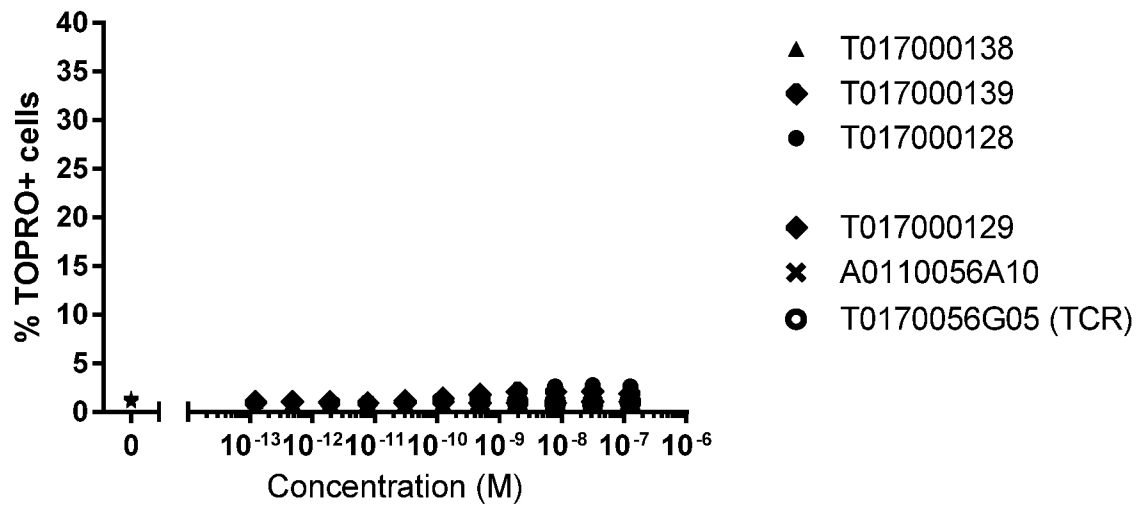
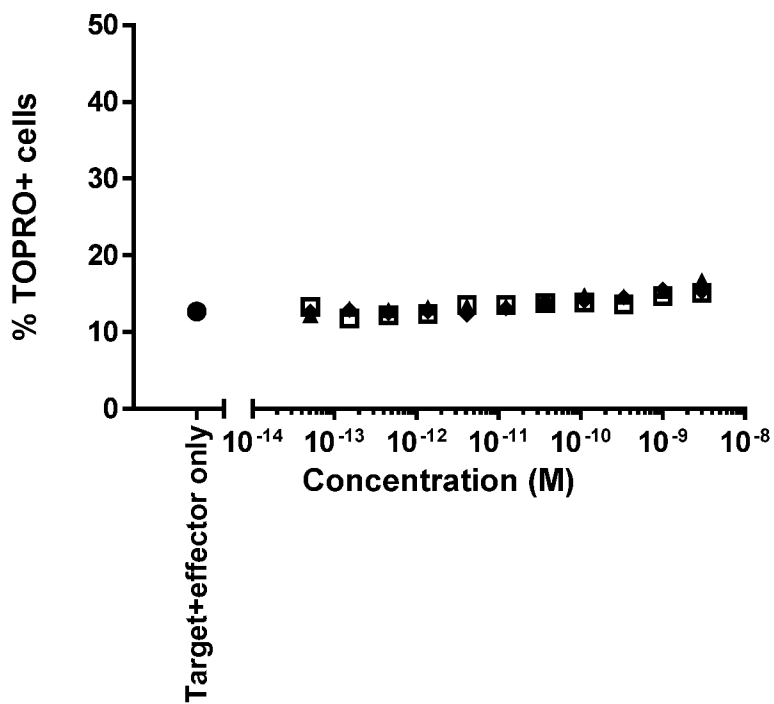
Figure 44:**A.****B.**

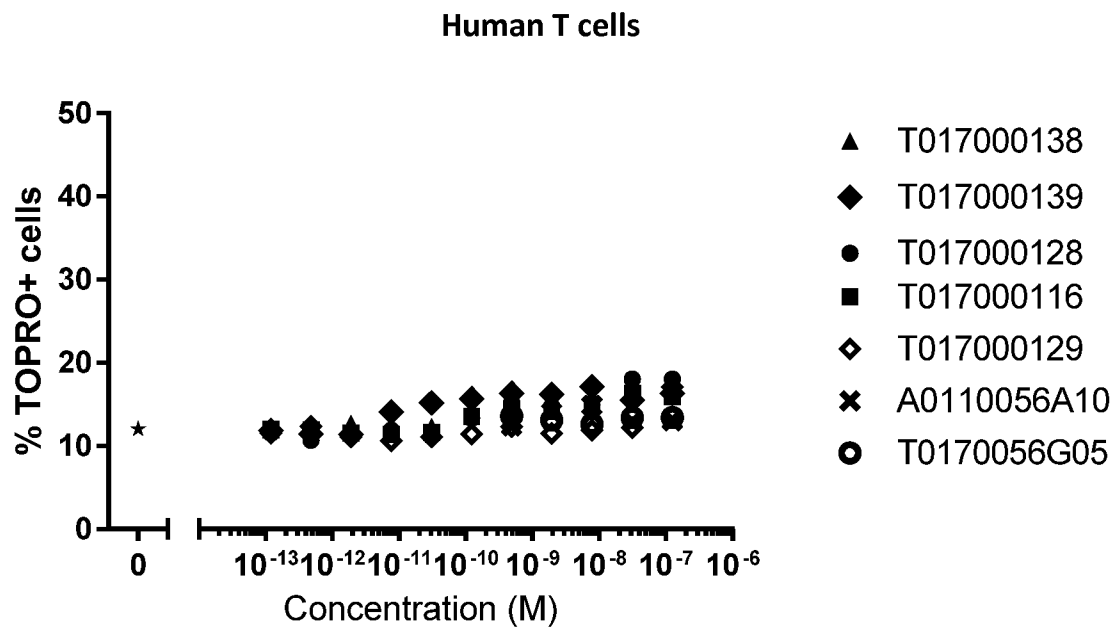
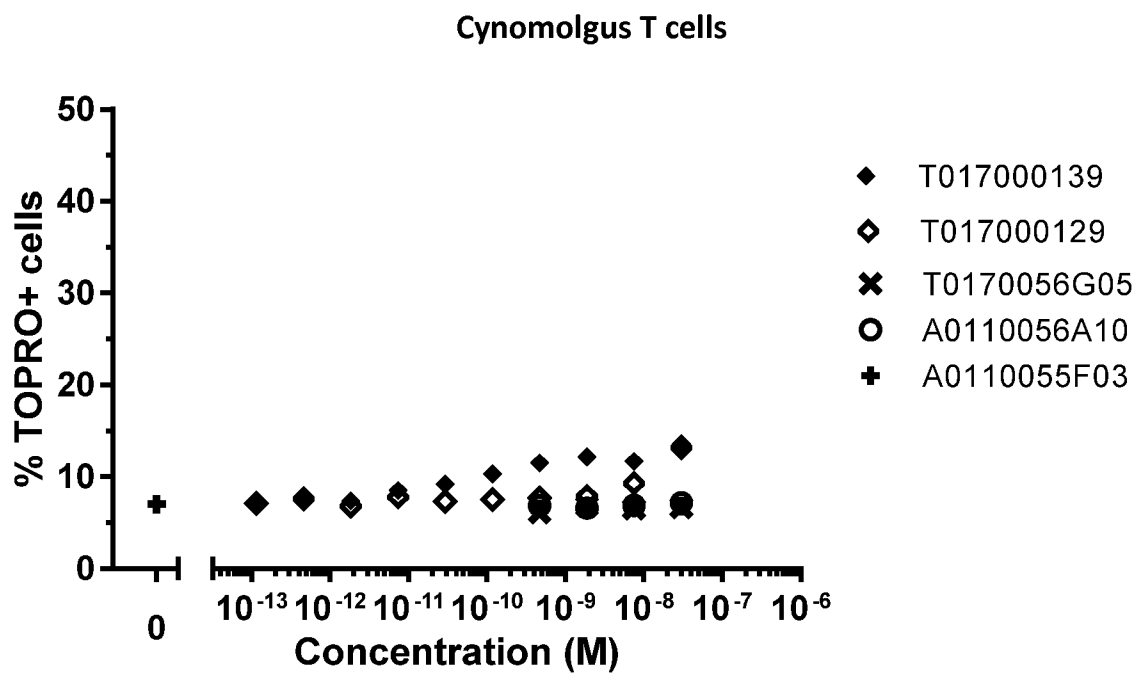
Figure 45:**A.****B.**

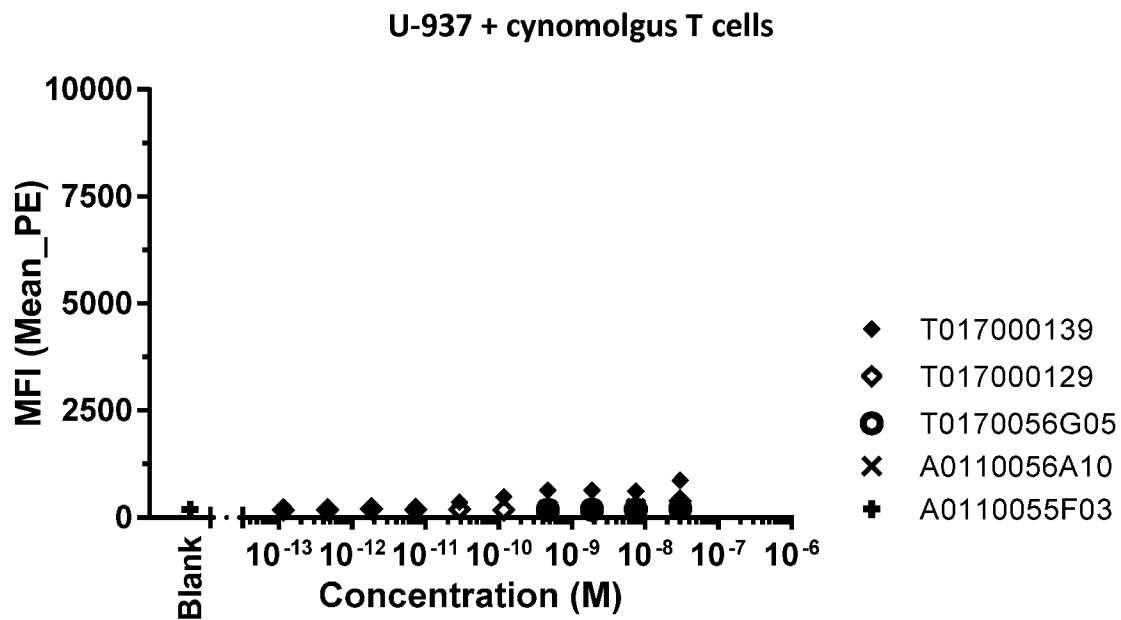
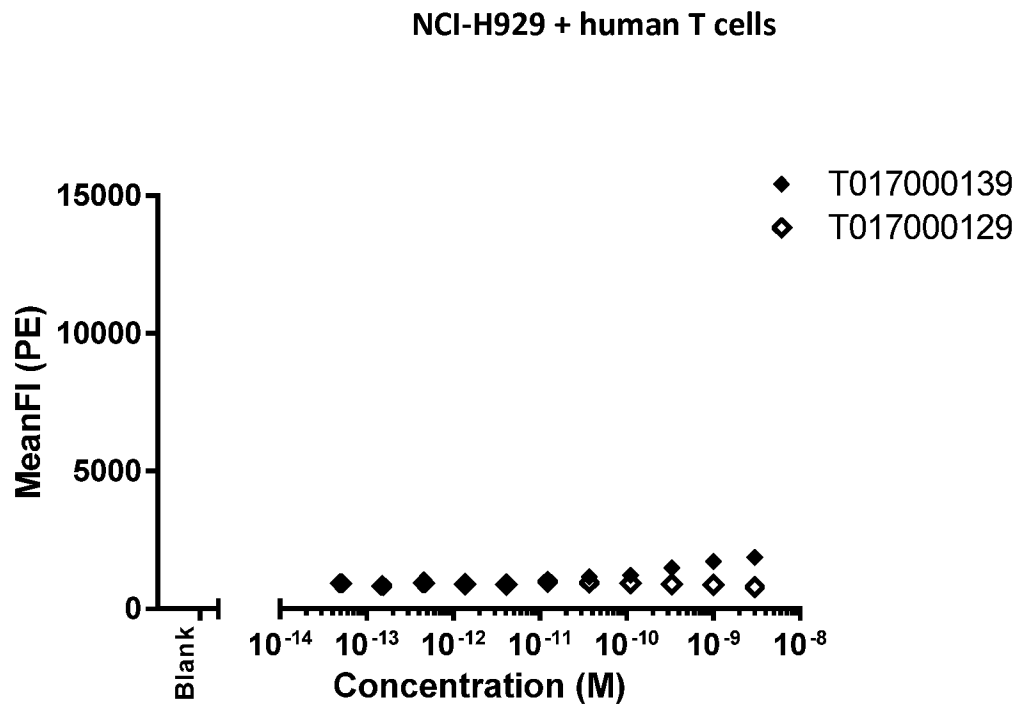
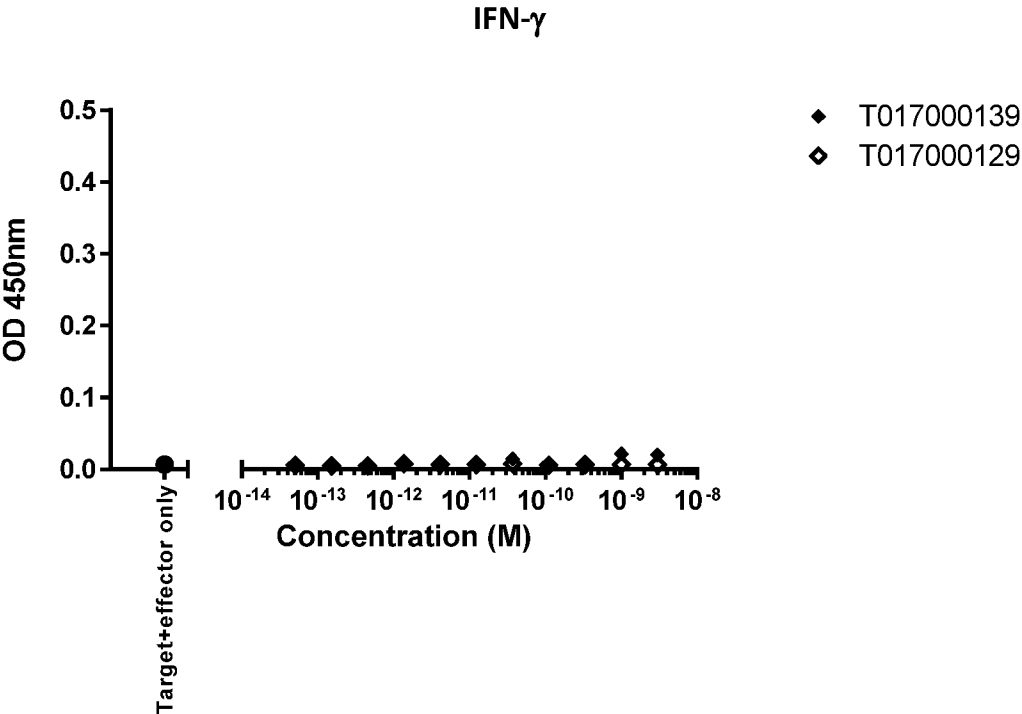
Figure 46:**A.****B.**

Figure 47:

A.



B.

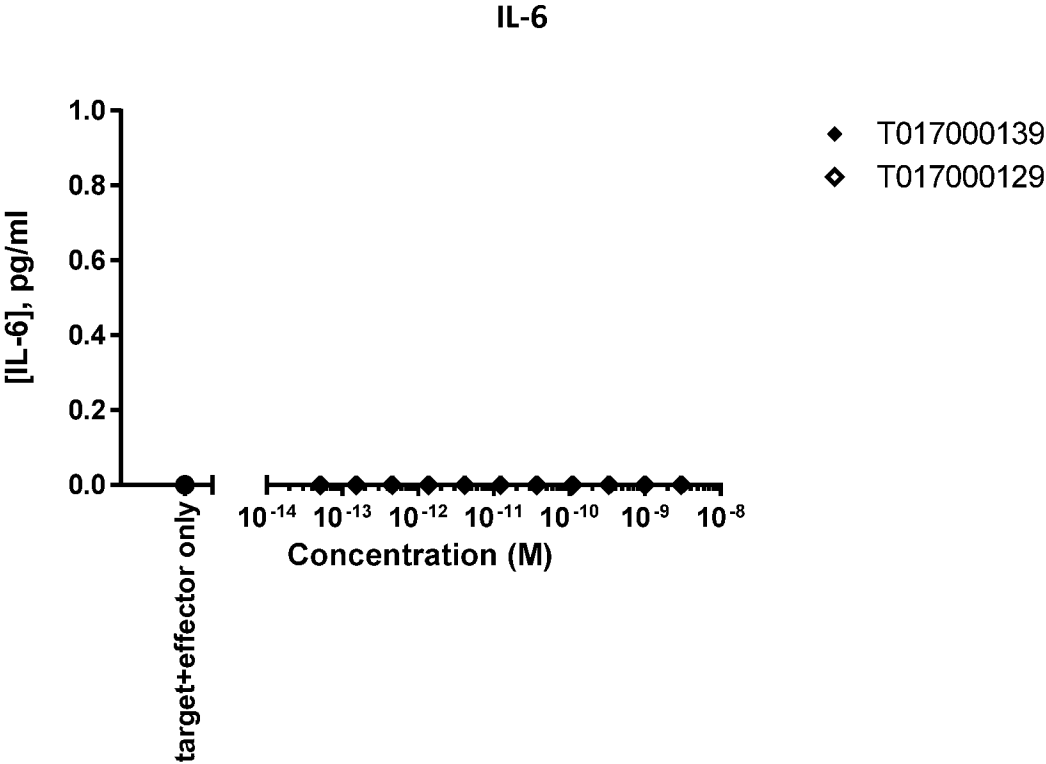


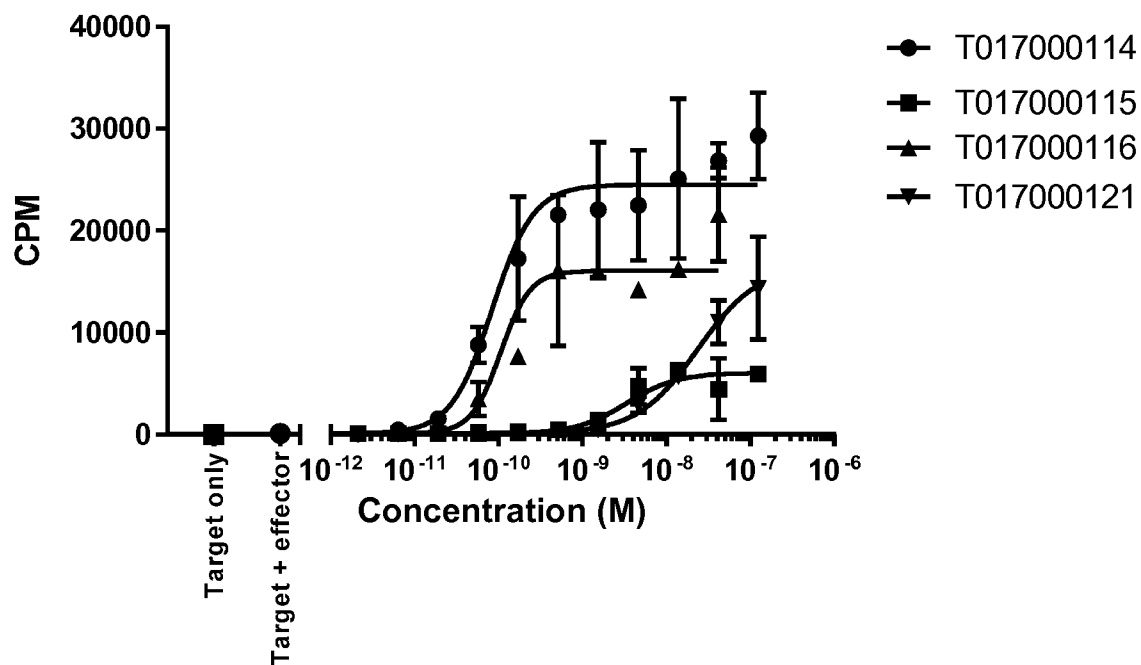
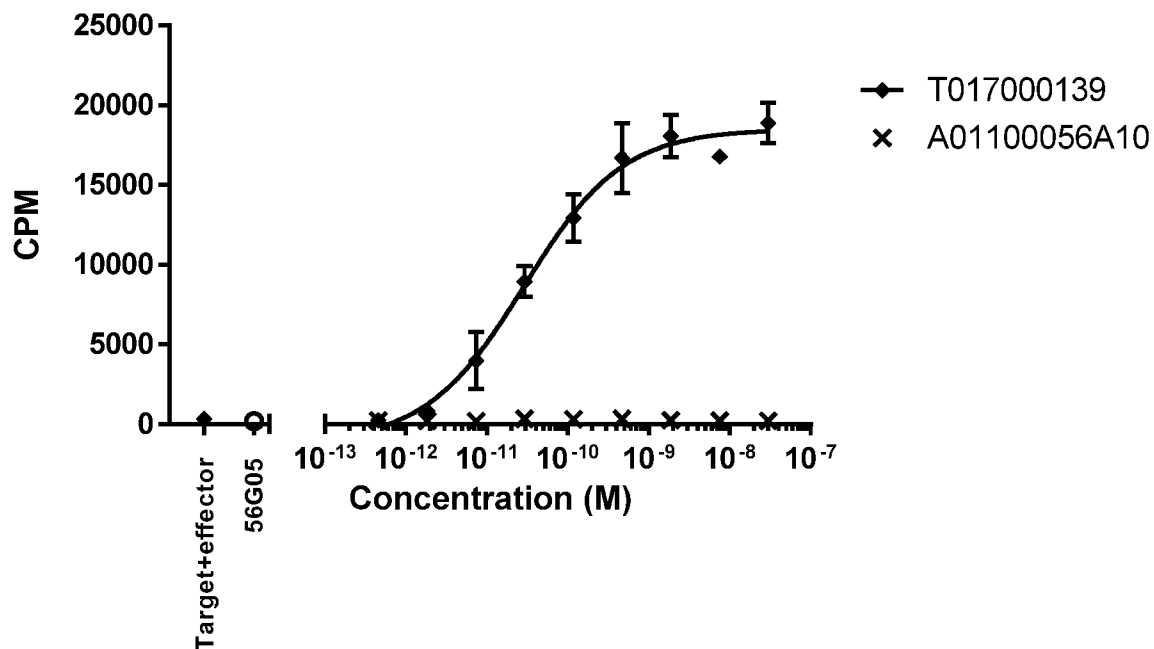
Figure 48:**A.****B.**

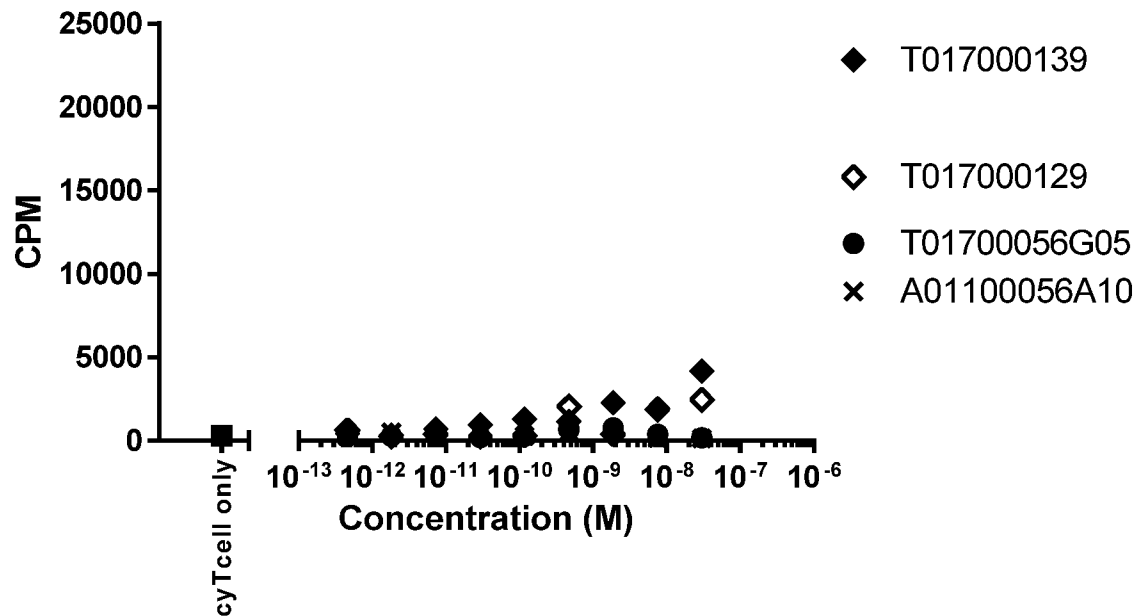
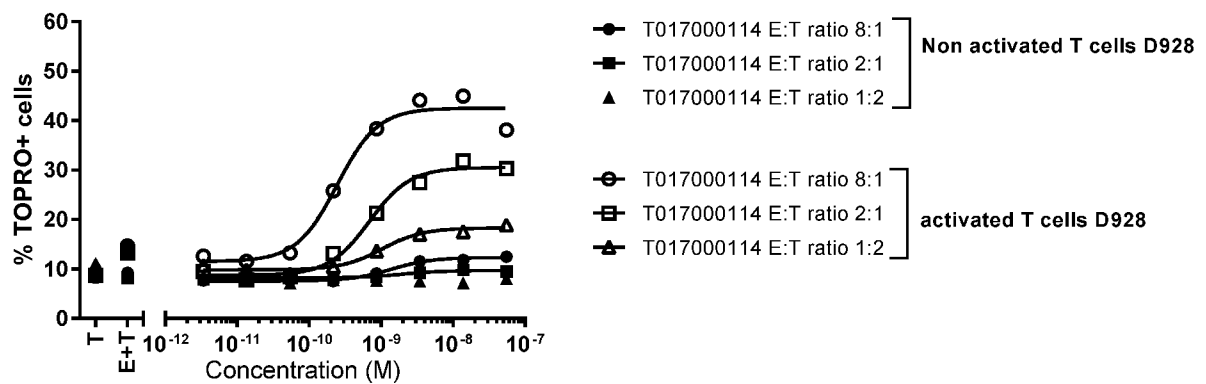
Figure 49:**Figure 50**

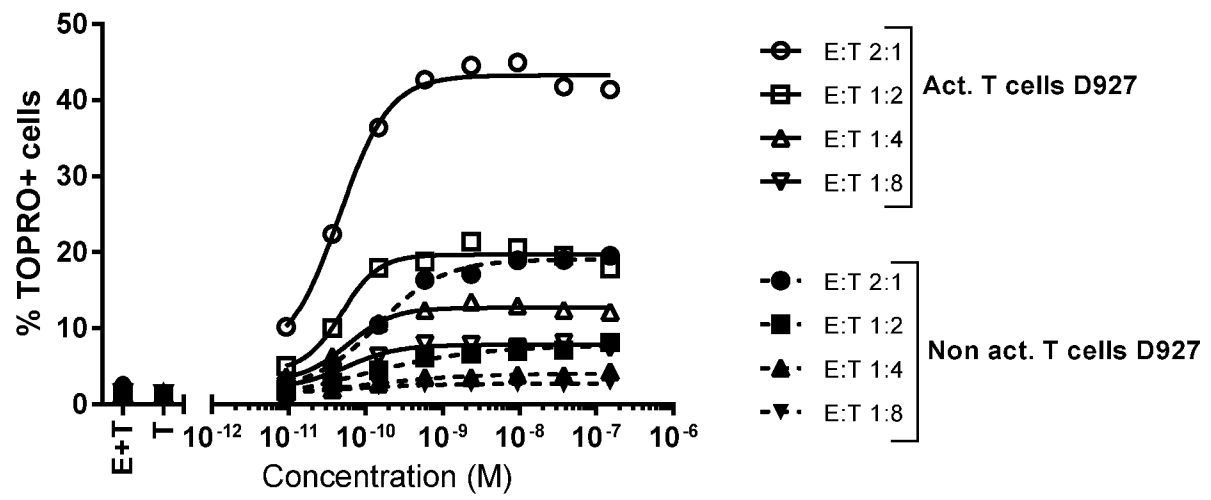
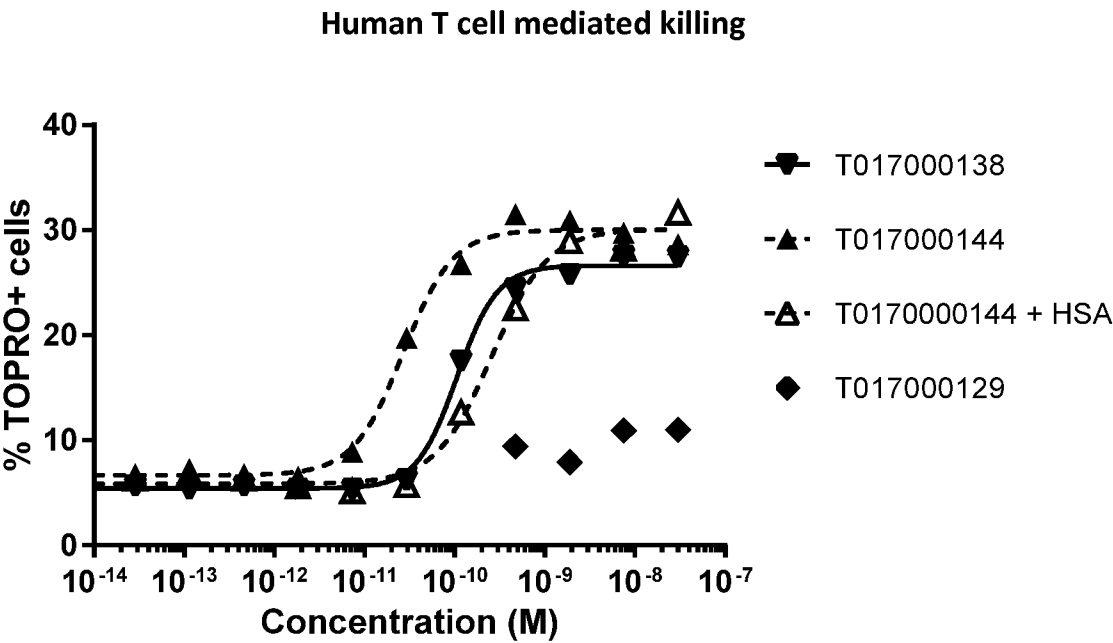
Figure 51:

Figure 52:

A.



B.

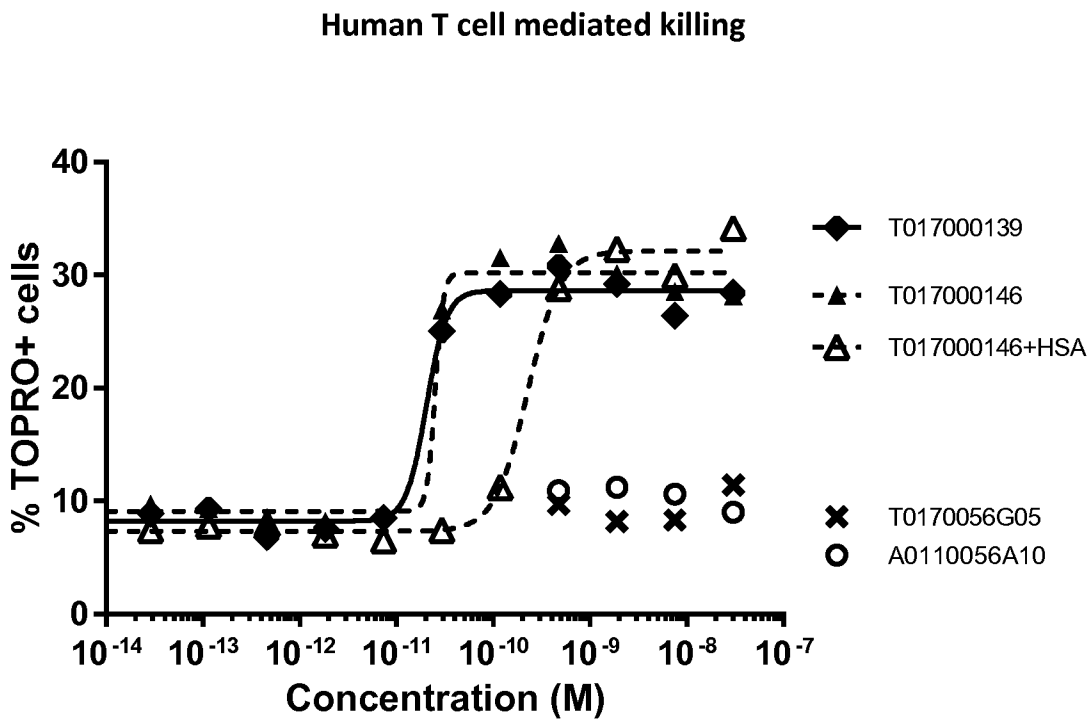
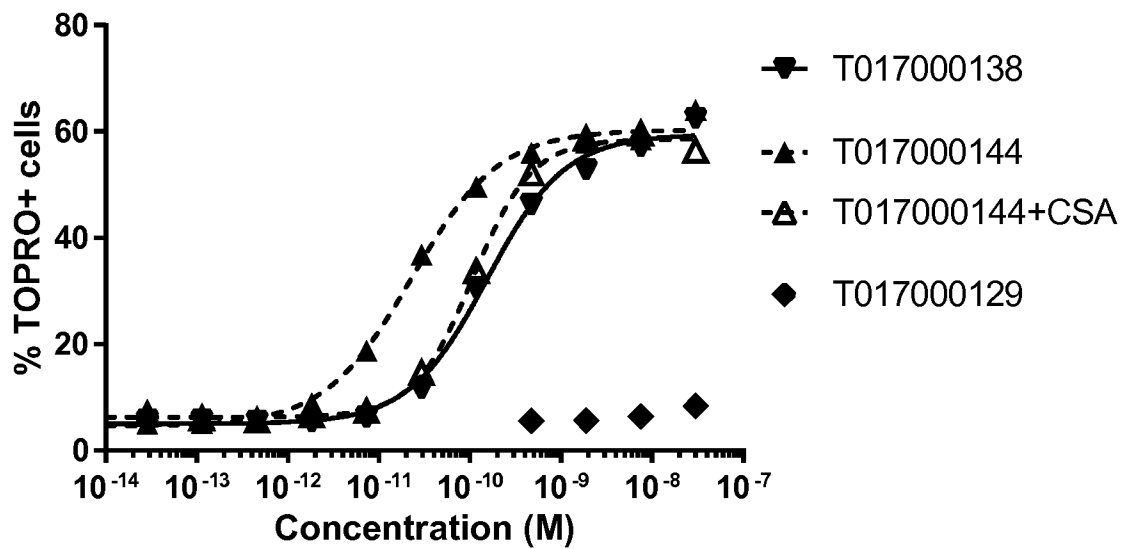


Figure 52 cont':

C.

Cynomolgus mediated killing



D.

Cynomolgus mediated killing

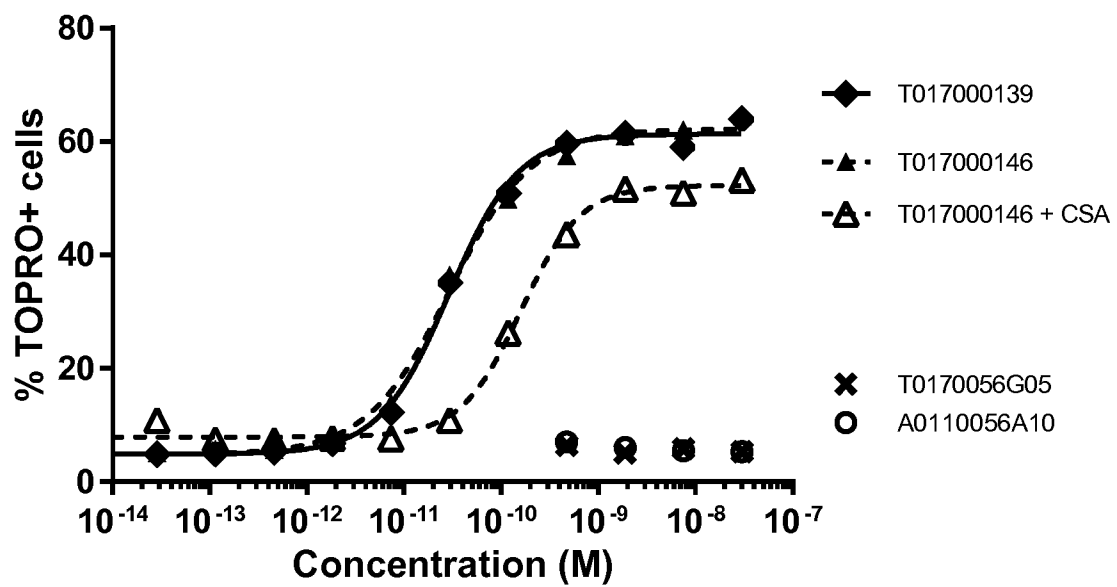


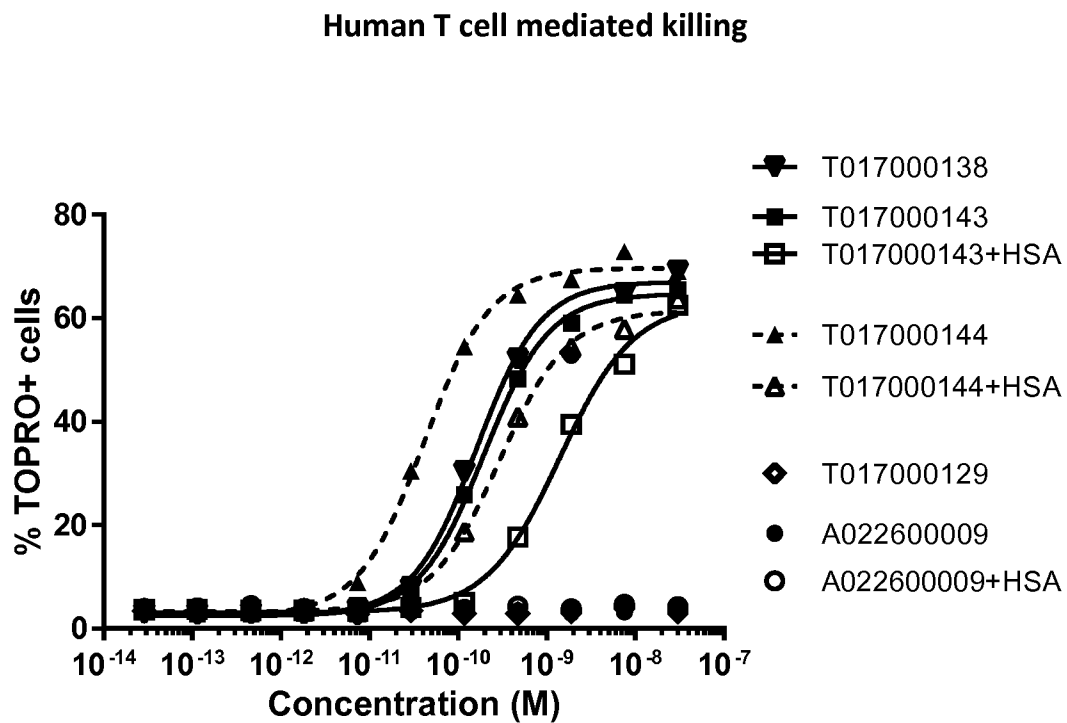
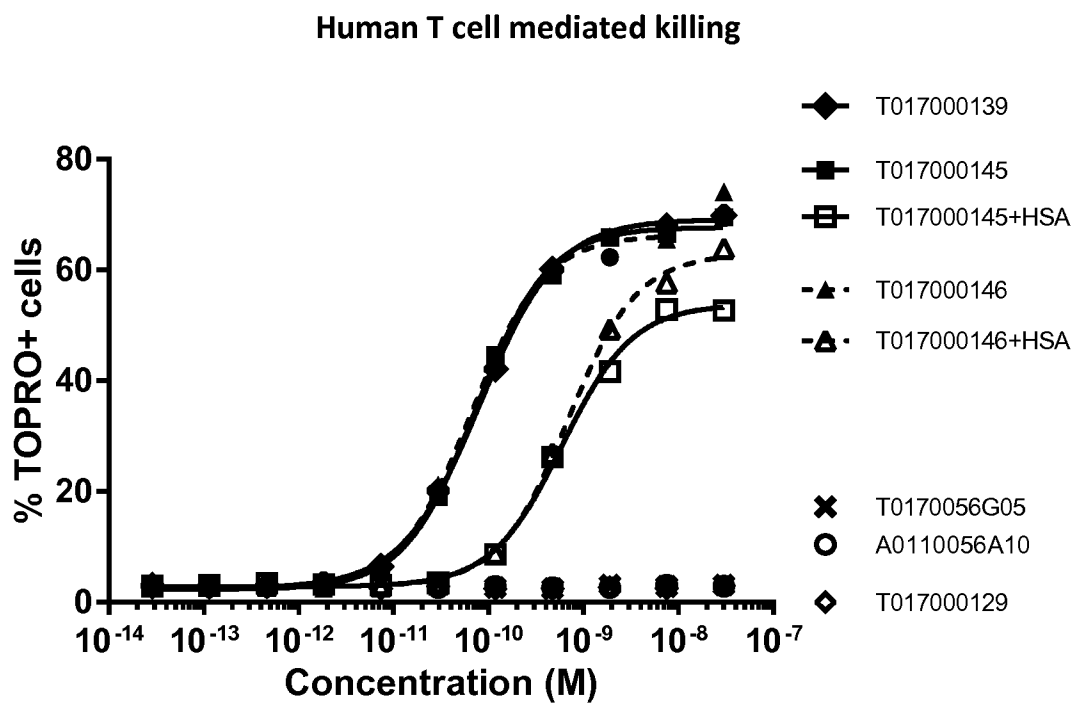
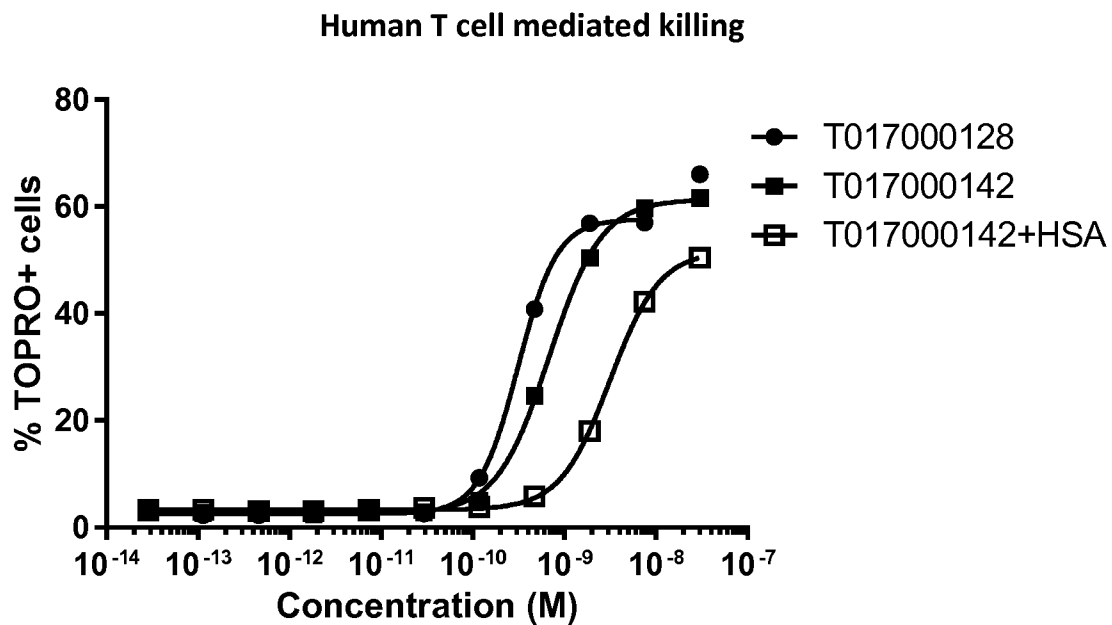
Figure 53:**A.****B.**

Figure 53 cont':

C.



D.

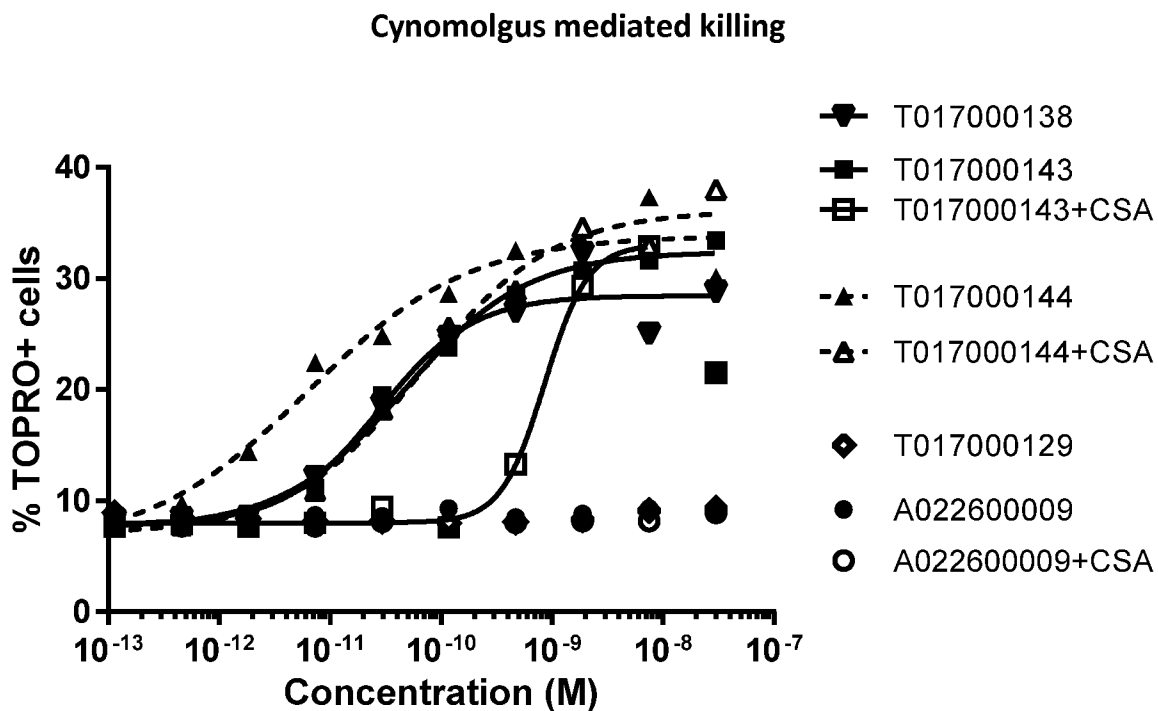
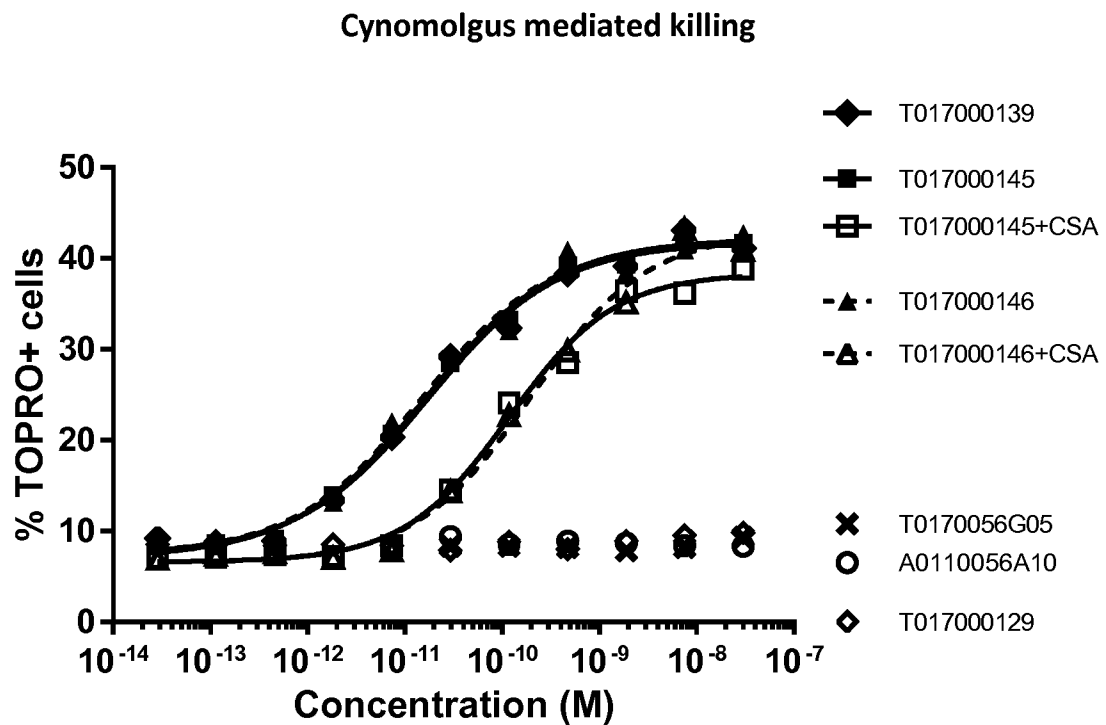


Figure 53 cont':

E.



F.

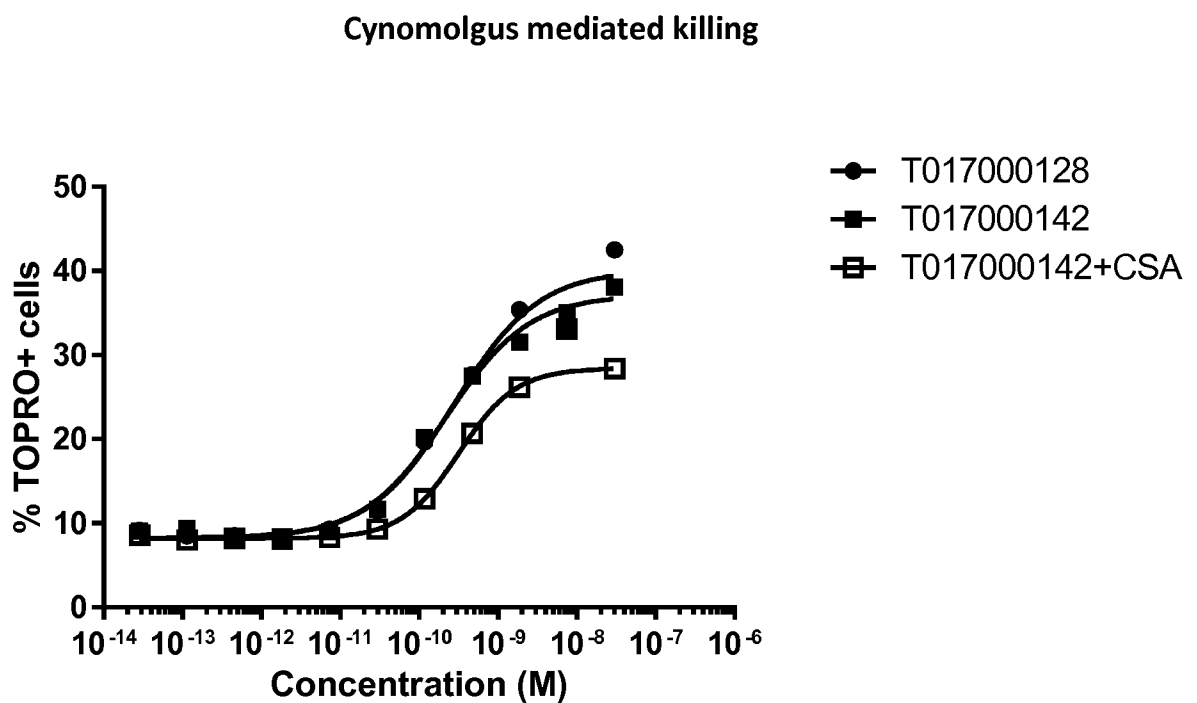
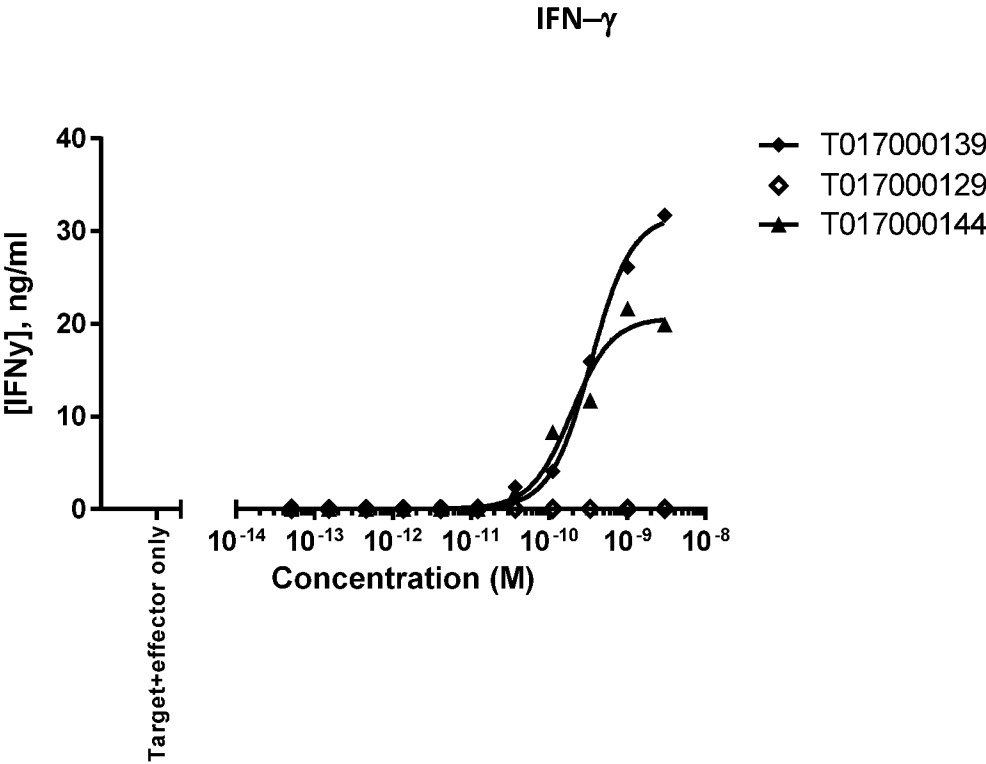


Figure 54:

A.



B.

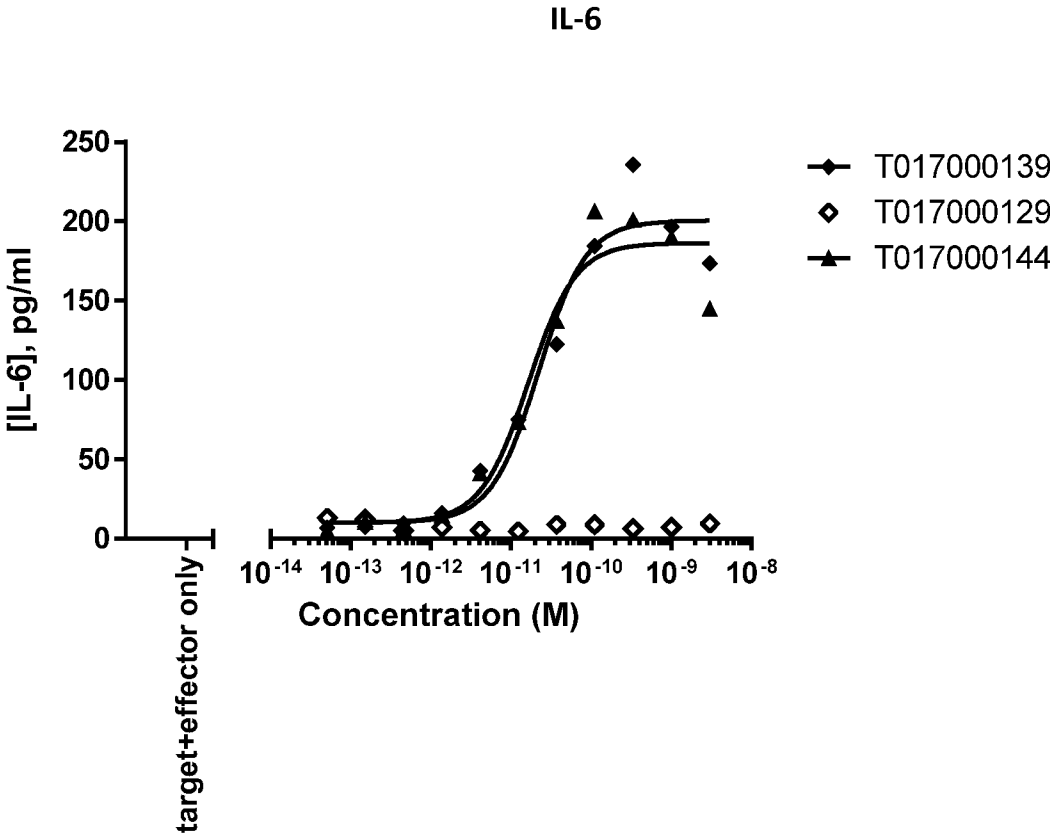


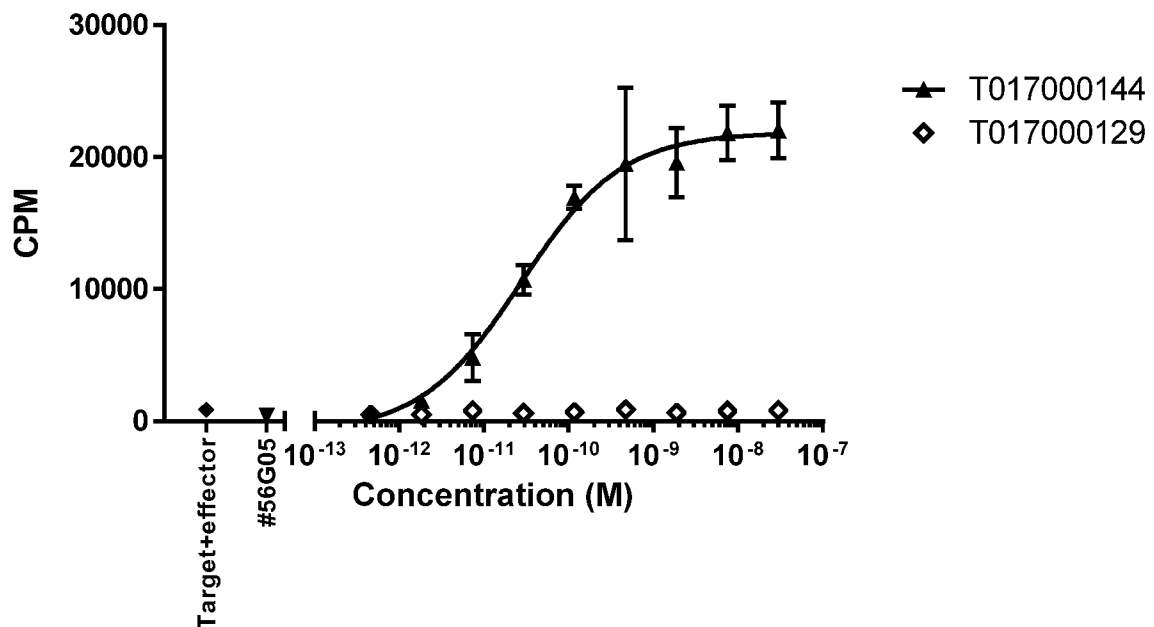
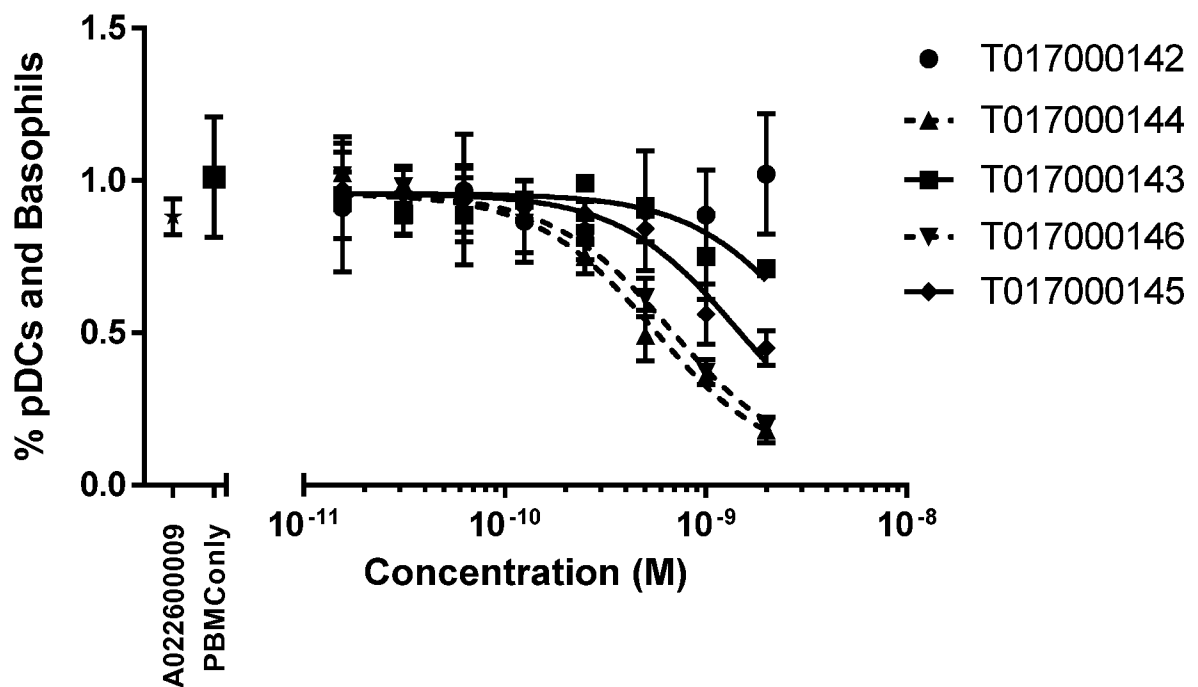
Figure 55:**Figure 56:**

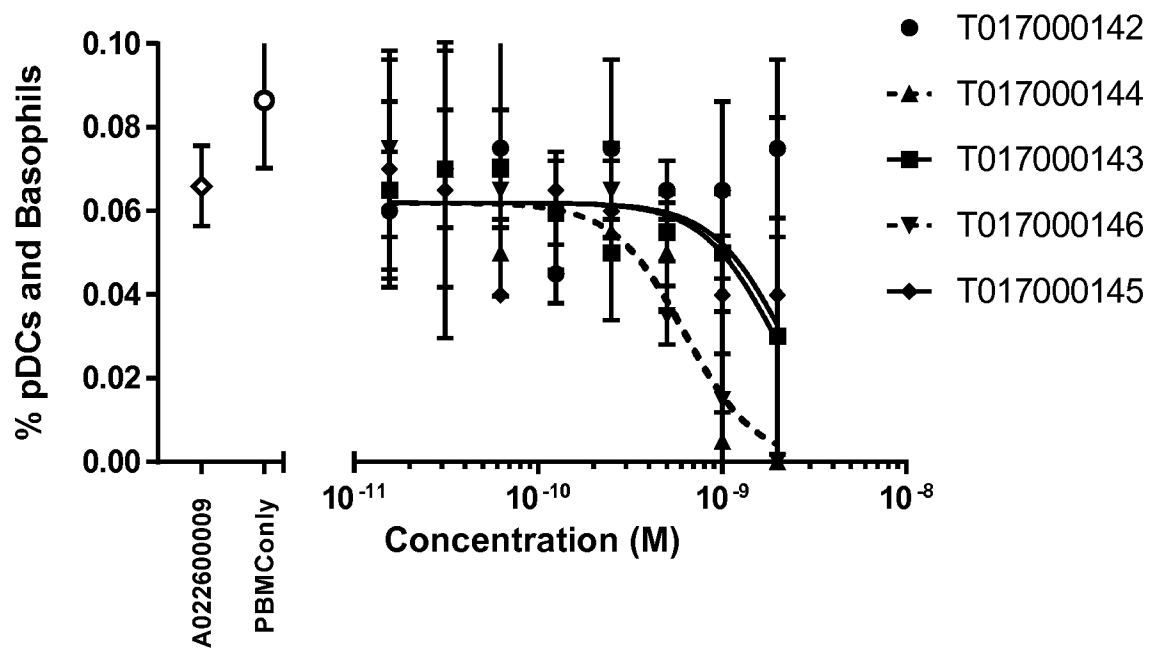
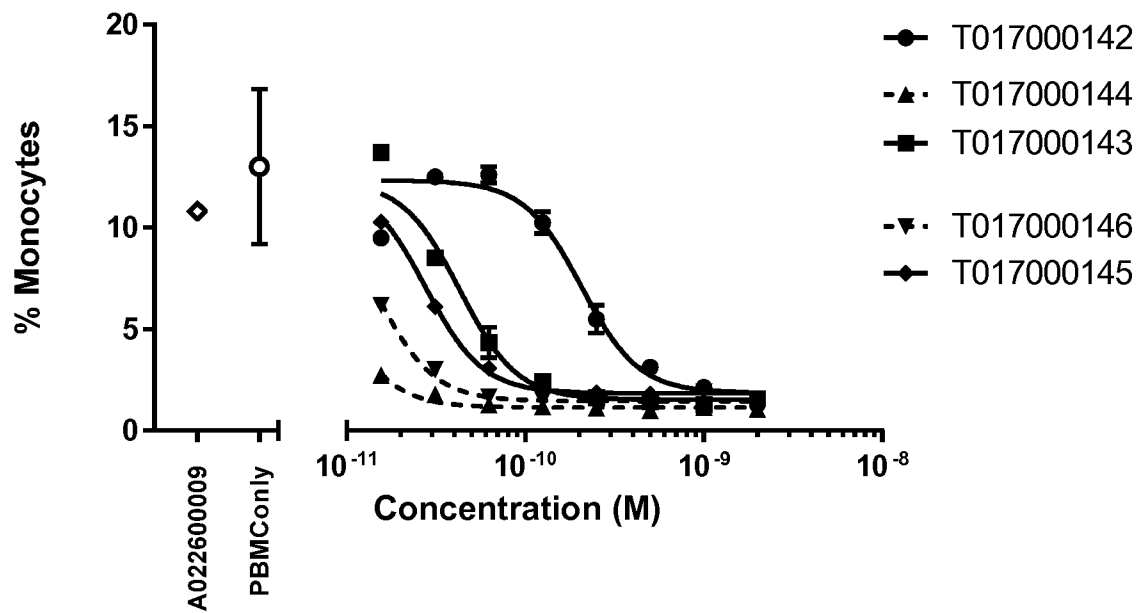
Figure 57:**Figure 58:**

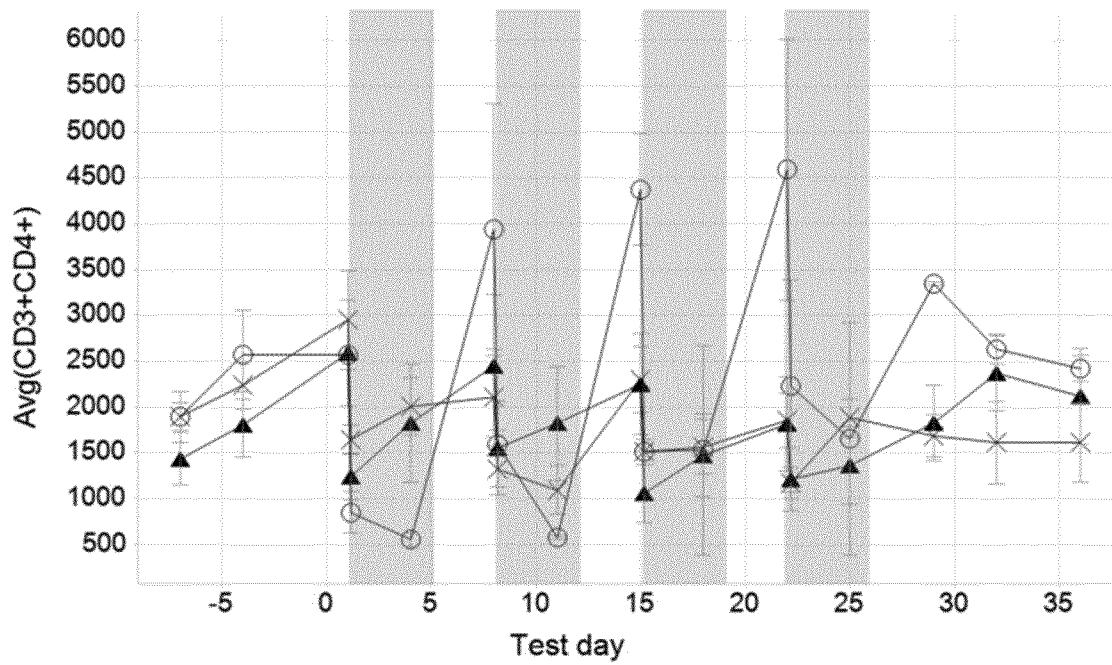
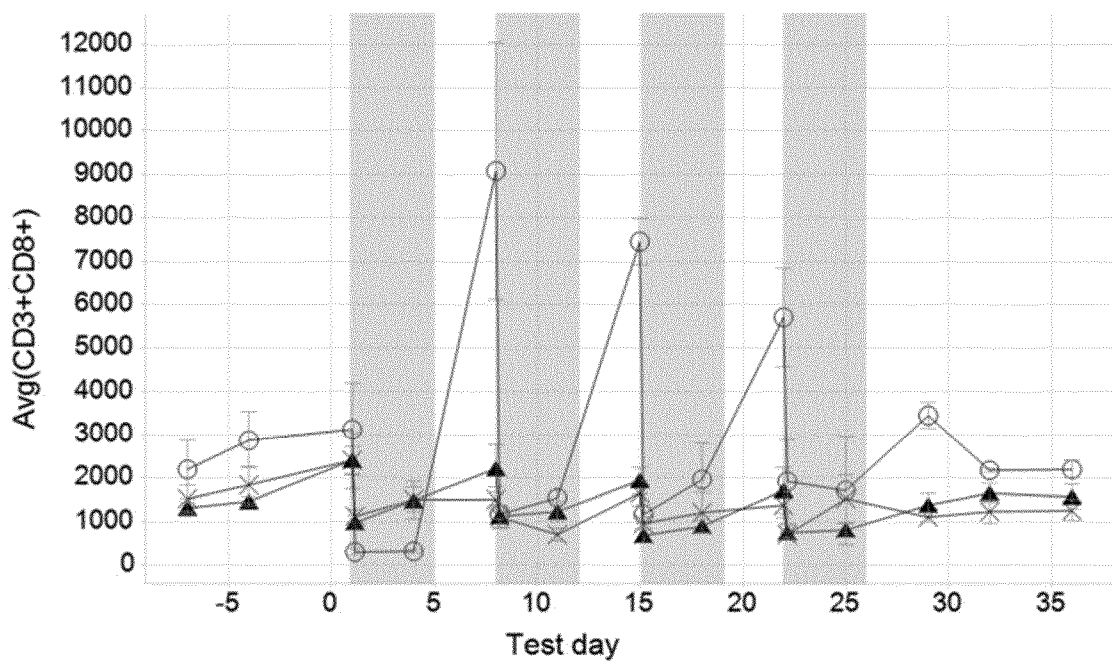
Figure 59:**A.****B.**

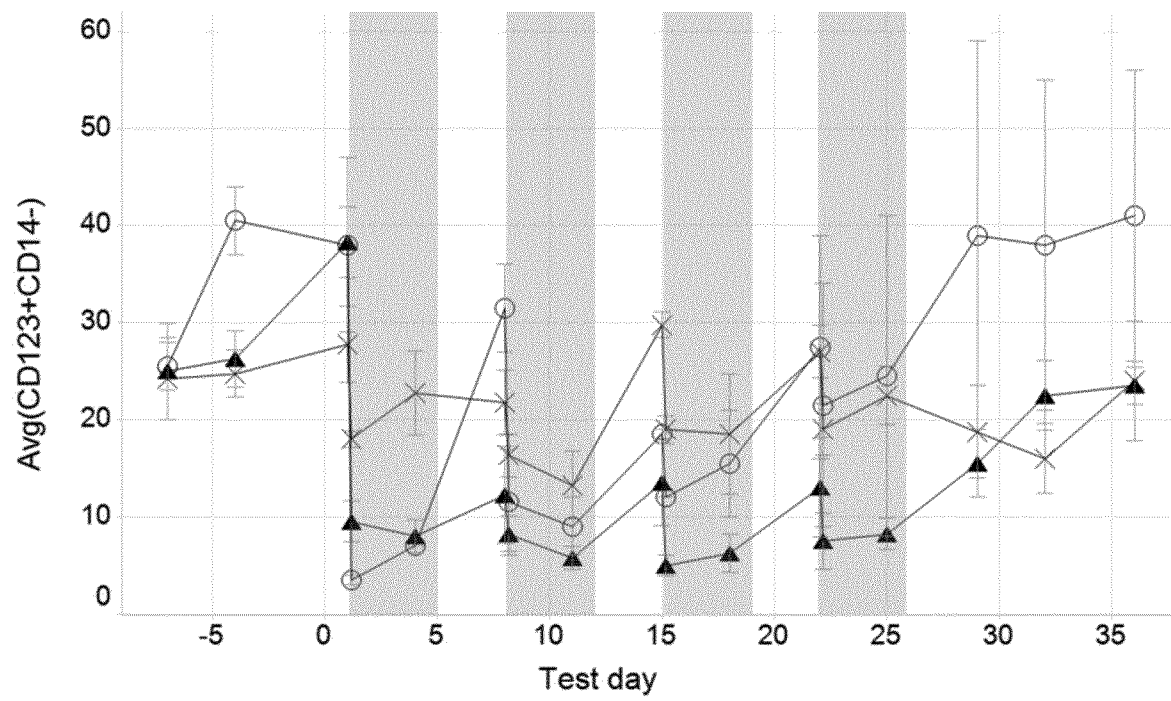
Figure 60:

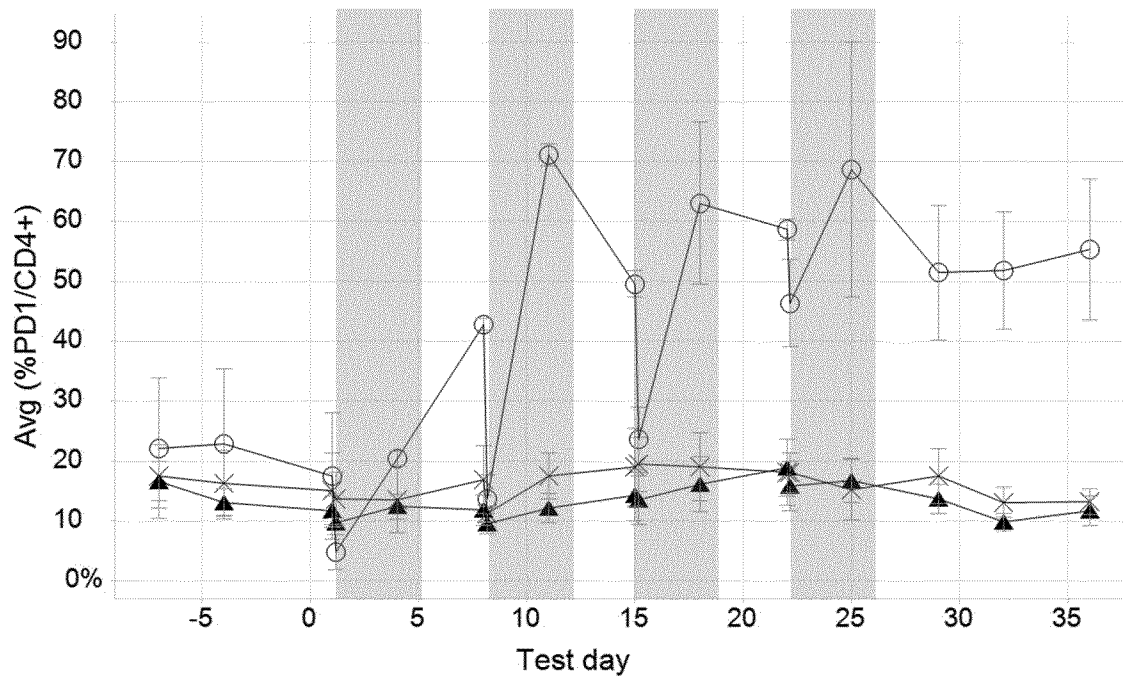
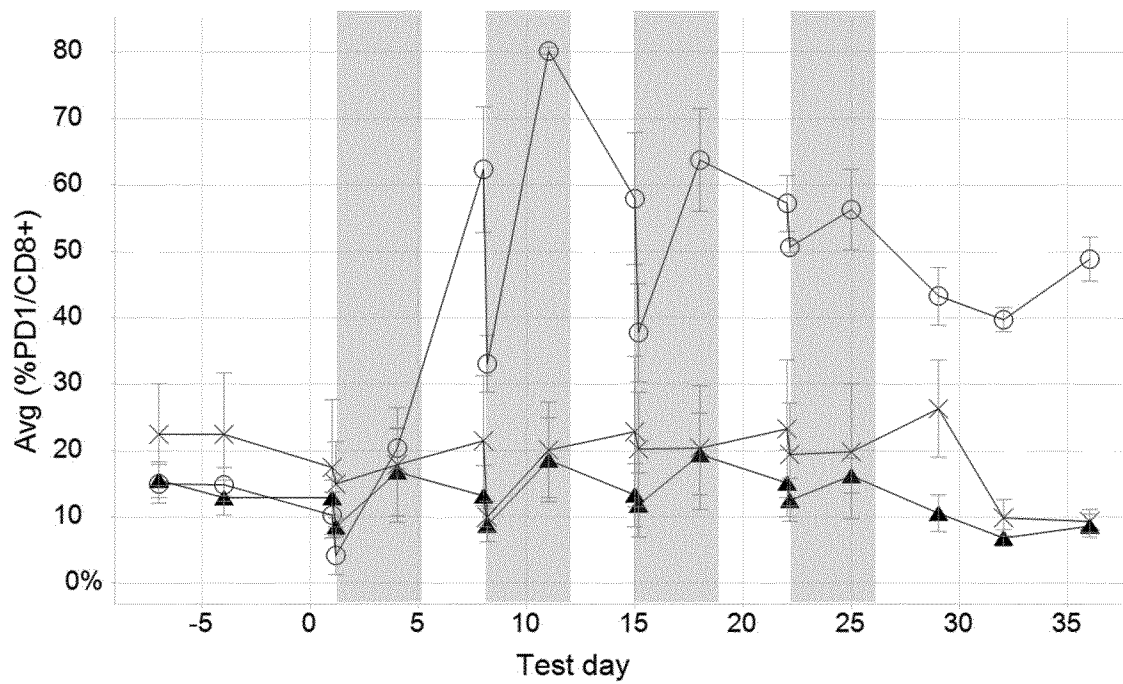
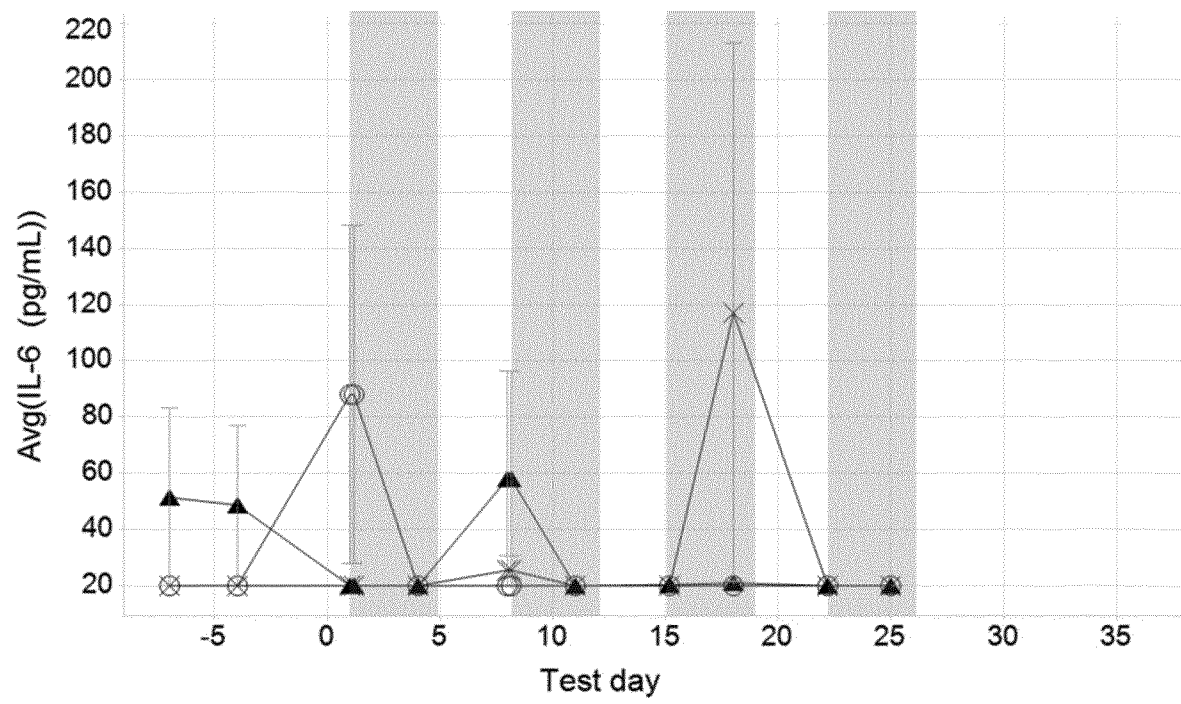
Figure 61:**A.****B.**

Figure 62:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/079507

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/30 A61K39/00 C07K16/28 C07K16/46
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/116626 A1 (SANOFI SA [FR]) 28 July 2016 (2016-07-28) claims 1-31	1-98
A	WO 2012/066058 A1 (BOEHRINGER INGELHEIM INT [DE]; MICROMET GMBH [DE]; BORGES ERIC [AT]; H) 24 May 2012 (2012-05-24) page 4, paragraph 3 - paragraph 4 ----- -/-	1-98



Further documents are listed in the continuation of Box C.



See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

13 March 2018

Date of mailing of the international search report

22/03/2018

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Klee, Barbara

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/079507

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHU-RU KUO ET AL: "Engineering a CD123xCD3 bispecific scFvimmunofusion for the treatment of leukemia and elimination of leukemia stem cells", PROTEIN ENGINEERING, DESIGN AND SELECTION, OXFORD JOURNAL, LONDON, GB, vol. 25, no. 10, 1 October 2012 (2012-10-01), pages 561-569, XP002721301, ISSN: 1741-0126, DOI: 10.1093/PROTEIN/GZS040 [retrieved on 2012-06-27] abstract; figures 1-8 page 567, column 2, paragraph 2 - page 568, column 2, paragraph 1 -----	1-98
X	WO 2013/173820 A2 (SCOTT & WHITE HEALTHCARE [US]) 21 November 2013 (2013-11-21) page 30, paragraph 109; claims 1-19 -----	1-98
X,P	WO 2016/180969 A1 (ABLYNX NV [BE]) 17 November 2016 (2016-11-17) claims 1, 11, 29, 34-36, 48; figures 1-42; tables 1-31 -----	1-98

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Information on patent family members

International application No

PCT/EP2017/079507

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