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(54) **Title:** RECOMBINANT PEPTIDE-MHC COMPLEX BINDING PROTEINS AND THEIR GENERATION AND USE

(57) **Abstract:** The present invention relates to a method of producing recombinant binding proteins with binding specificity for a peptide-MHC (pMHC) complex. The invention also relates to recombinant binding proteins comprising one, two or more designed repeat domain(s), preferably designed ankyrin repeat domain(s), with binding specificity for a pMHC complex, and to such binding proteins which further comprise a binding agent having binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell. In addition, the invention relates to nucleic acids encoding such binding proteins or repeat domains, pharmaceutical compositions comprising such binding proteins or nucleic acids, and the use of such binding proteins, nucleic acids or pharmaceutical compositions in methods for treating or diagnosing diseases, including cancer, infectious diseases and autoimmune diseases.

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RECOMBINANT PEPTIDE-MHC COMPLEX BINDING PROTEINS AND THEIR GENERATION AND USE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of priority to EP19215433.4, filed on December 11, 2019; EP19215434.2, filed on December 11, 2019; EP19215435.9, filed on December 11, 2019; EP19215436.7, filed on December 11, 2019; EP20161059.9, filed on March 4, 2020; and EP20181234.4, filed on June 19, 2020. The disclosures of these six patent applications are incorporated herein for all purposes by reference in their entirety.

FIELD OF THE DISCLOSURE

The present invention relates to a method of producing recombinant binding proteins with binding specificity for a peptide-MHC (pMHC) complex. The invention also relates to recombinant binding proteins comprising one, two or more designed repeat domain(s), preferably designed ankyrin repeat domain(s), with binding specificity for a pMHC complex, and to such binding proteins which further comprise a binding agent having binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell. In addition, the invention relates to nucleic acids encoding such binding proteins or repeat domains, pharmaceutical compositions comprising such binding proteins or nucleic acids, and the use of such binding proteins, nucleic acids or pharmaceutical compositions in methods for treating or diagnosing diseases, including cancer, infectious diseases and autoimmune diseases.

BACKGROUND

Major histocompatibility complex (MHC) class I molecules play a key role in the surveillance of aberrant or foreign proteins within cells. Peptides derived from endogenous proteins are loaded onto the peptide-binding groove of MHC class I molecules and are then displayed on the cell surface. Such MHC class I complexes are expressed in all nucleated cells, including malignant cells. Peptide-MHC (pMHC) complexes are recognized by T cell receptors (TCRs) on CD8+ cytotoxic T lymphocytes (CTLs). The presentation of peptide-MHC complexes provides a snapshot of the intracellular environment to circulating CTLs (Reeves and James, Immunology 150: 16-24 (2016)). CTLs are activated in response to detecting aberrant or foreign antigens, e.g. oncoproteins or bacterial or viral proteins, resulting in the destruction of the presenting cell. CTLs are also activated in certain autoimmune diseases upon misrecognizing a “self” antigen (Bodis et al., Rheumatol. Ther. 5: 5-20 (2018)).

MHC class I complexes presenting tumor-specific or infectious agent-specific peptides represent a unique and promising class of cell surface targets for immunotherapy of cancer and infectious diseases. Different approaches have been developed in attempts to exploit this target class, including vaccines, adoptive cell therapy and TCR-like antibodies. Among the tumor-associated antigens, cancer-testis antigens (CTAs) are considered good candidate targets for immunotherapy as they are characterized by a restricted expression in normal somatic tissues and re-expression in tumor tissues. One of the most frequently reported CTAs across various cancer types is melanoma associated antigen A3 (MAGE-A3) (J Exp Clin Cancer Res. 2019 Jul 8;38(1):294. doi: 10.1186/s13046-019-1272-2). Moreover, several CTAs have been found to induce a spontaneous immune response, NY-ESO-1 being one of the most immunogenic ones (Thomas et al., Front Immunol. 2018; 9: 947). Among the infectious agent-specific peptides, EBV nuclear antigen 1 (EBNA-1) is the only viral protein found in all EBV-related malignancies (J Gen Virol. 2009 Sep; 90(Pt 9): 2251–2259). Moreover, the sequence 18-27 of the hepatitis B virus (HBV) nucleocapsid antigen is widely recognized by CTL of HLA-A2-positive patients with acute self-limited HBV infection, and represents the main component of a peptide-based therapeutic vaccine aimed at stimulating the antiviral CTL response in patients with chronic hepatitis B (Hepatology. 1997 Oct;26(4):1027-34.c).

One obstacle to the therapeutic exploitation of virus- or tumor-specific peptide-MHC complexes has been the inherently low affinity of TCRs for peptide-MHC complexes after thymic selection. This low affinity presents a limitation, especially considering that virus- or tumor-specific peptide-MHC complexes are typically present on the surface of virus-infected or tumor cells in low density. To overcome this obstacle, affinity-enhanced TCRs have been developed and engineered T-cells expressing such affinity-enhanced TCRs have been tested in clinical studies. It has been reported that affinity-enhanced TCRs can lack specificity, and, in some instances, engineered T-cells expressing affinity-enhanced TCRs have caused serious, sometimes even deadly, medical complications due to the unexpected recognition by the TCRs of an epitope derived from an unrelated protein (see, e.g., Linette et al., Blood 122(6): 863-871 (2013)). Affinity-enhanced TCRs are also being developed as soluble TCRs, but soluble TCR expression is challenging.

A number of TCR-like antibodies with binding specificity for MHC class I complexes presenting virus- or tumor-specific peptides have been reported. Some of them were isolated using hybridoma technology. However, isolation of pMHC-specific TCR-like antibodies by hybridoma technology has been hampered by a number of factors, including the need to screen hundreds or even thousands of clones, low immunogenicity, few unique clones due to immunodominance, and poor control of fine-specificity (see, e.g., Porgador et al., Immunity 6: 715-726 (1997); Bernardeau et al., Eur. J. Immunol. 35(10): 2864-2875 (2005); Skora et al., Proc. Natl. Acad. Sci. USA 112(32): 9967-9972 (2015)). More TCR-like antibodies have been isolated using phage display. However, the affinity of TCR-like antibodies isolated from phage display libraries is generally relatively low and often not sufficient for therapeutic purposes (see, e.g., Chames et al., Proc. Natl. Acad. Sci. USA 97: 7969-7974 (2000)). In order to generate TCR-like antibodies with sufficiently high affinity, systems for affinity maturation have been developed. For example, one such system for affinity maturation of a TCR-like antibody combines mutagenesis, libraries and yeast display, and structure determinations and molecular modeling (Zhao et al., Leukemia 29(11): 2238-2247 (2015)). Only with such complex and labor-intensive affinity maturation approach were Zhao et al. able to improve the binding affinity of a TCR-like antibody by about 100-fold to obtain a pMHC-specific binding protein with sufficient affinity. Taken together, it has been challenging thus far to develop molecules that specifically bind disease-related peptide-MHC complexes with sufficient affinity, and current approaches generally involve difficult expression systems and/or time- and labor-consuming procedures such as screening of a large number of hybridoma clones or affinity maturation.

Thus, there still remains a need for new methods of producing pMHC-specific binding proteins, for new pMHC-specific binding proteins, and for therapeutic and diagnostic approaches for the treatment and characterization of diseases, including cancer, autoimmune diseases and infectious diseases, benefitting from pMHC-specific binding.

SUMMARY

The present invention provides a method of producing recombinant binding proteins with binding specificity for a peptide-MHC (pMHC) complex. The invention also provides recombinant binding proteins comprising one, two or more designed repeat domain(s), preferably designed ankyrin repeat domain(s), with binding specificity for a pMHC complex, and such binding proteins which further comprise a binding agent having binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell. In addition, the invention provides nucleic acids encoding such binding proteins or repeat domains, pharmaceutical compositions comprising such binding proteins or nucleic acids, and the use of such binding proteins, nucleic acids or pharmaceutical compositions in methods for treating or diagnosing diseases, such as, e.g., cancer, autoimmune diseases and infectious diseases, in a mammal, including a human.

The inventive method of producing recombinant binding proteins with binding specificity for a pMHC complex is surprisingly efficient and effective in generating binding proteins that bind with high affinity and/or specificity to a chosen target pMHC complex. Thus far it has been challenging to develop molecules that specifically bind disease-related pMHC complexes with sufficient affinity, and current approaches generally involve difficult expression systems and/or time- and labor-consuming procedures such as screening of a large number of hybridoma clones or affinity maturation. The inventive method disclosed herein neither involves a difficult expression system nor does it require time- and labor-consuming procedures such as those mentioned above. Furthermore, the binding interaction between binding proteins of the invention and the target peptide-MHC complex unexpectedly involves a relatively large number of amino acid residues in the target peptide. A large number of interaction residues in the target peptide is believed to reflect a highly selective or specific binding interaction with the target peptide-MHC complex within the large universe of different pMHC complexes. It appears that the pMHC-specific repeat domain(s) of the binding proteins of the invention may provide a binding surface that sterically fits very well to the composite peptide-MHC surface in a specific binding interaction. Furthermore, pMHC-specific repeat domain(s) of the binding proteins of the invention may comprise particular amino acid sequence motifs in the N-terminal capping module and/or the C-terminal capping module leading to improved pharmacokinetic properties of the designed repeat domain and of proteins comprising the designed repeat domain. The methods and binding proteins of the invention further provide the advantage that two or more of the same and/or different pMHC-specific repeat domains can be readily combined in one binding protein (generating, e.g., bivalent, biparatopic or bispecific binding proteins), thereby allowing to adapt and optimize binding avidity, binding affinity, binding specificity and/or potency of the binding proteins. Moreover, binding proteins of the invention may even further comprise a binding agent with binding specificity for a protein expressed on the surface of an immune cell, such as, e.g., a protein that is part of the T cell receptor complex expressed in cytotoxic T cells or an activating receptor expressed in natural killer (NK) cells. Binding proteins of the invention in such an immune cell engager format (e.g. a T cell engager format or a NK cell engager format) can be advantageously used in methods to activate immune cells (e.g. T cells or NK cells) and/or to engage the immune system in a localized and targeted fashion. Furthermore, the length of the linker connecting the one or more pMHC-specific repeat domains to the binding agent unexpectedly influences the potency of the binding proteins of the invention for use in an immune cell engager format, such as a T cell engager format. Furthermore, the methods and binding proteins of the invention allow to specifically target intracellular proteins, among others, thereby facilitating many new diagnostic and therapeutic opportunities, e.g. for cancers, infectious diseases and autoimmune diseases.

In one aspect, the invention provides a method of producing a peptide-MHC (pMHC)-specific binding protein, wherein said binding protein comprises a designed repeat domain with binding specificity for a target peptide-MHC complex, the method comprising the steps of:
(a) providing a collection of designed repeat domains;
(b) providing a recombinant target peptide-MHC complex; and
(c) screening said collection of designed repeat domains for specific binding to said target peptide-MHC complex to obtain at least one designed repeat domain with binding specificity for said target peptide-MHC complex. In a preferred embodiment, said designed repeat domain is a designed ankyrin repeat domain.

In another aspect, the invention provides a recombinant binding protein comprising a designed repeat domain obtainable by the above method.

In another aspect, the invention provides a recombinant binding protein comprising a first designed repeat domain, wherein said first repeat domain has binding specificity for a first target peptide-MHC complex. In a preferred embodiment said first target peptide is derived from a protein associated with a disease or disorder. As examples, in one particular preferred embodiment, said first target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder.

In one particular aspect, said first target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell, such as, e.g., NY-ESO-1 or MAGE-A3. In one preferred aspect, a target peptide which is derived from NY-ESO-1 comprises or consists of the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34, and a target peptide which is derived from MAGE-A3 comprises or consists of the amino acid sequence of SEQ ID NO: 155. In another particular aspect, said first target peptide is derived from a protein of a viral infectious agent, preferably a virus-specific protein, such as, e.g., EBNA-1 or HBV core antigen (HBcAg). In one preferred aspect, a target peptide which is derived from EBNA-1 comprises or consists of the amino acid sequence of SEQ ID NO: 92, and a target peptide which is derived from HBcAg comprises or consists of the amino acid sequence of SEQ ID NO: 255.

In one preferred aspect, the invention provides such a recombinant binding protein comprising a first designed repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first repeat domain is a designed ankyrin repeat domain.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from NY-ESO-1, and wherein said first ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from NY-ESO-1, and wherein said first ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from MAGE-A3, and wherein said first ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 175 to 217 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 175 to 217 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from MAGE-A3, and wherein said first ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 156 to 173 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 156 to 173 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 156 to 173 is optionally substituted by N.

In a further particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from EBNA-1, and wherein said first ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 111 to 154 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 111 to 154 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from EBNA-1, and wherein said first ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 93 to 110 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 93 to 110 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 93 to 110 is optionally substituted by N.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from HBcAg, and wherein said first ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 231 to 254 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 231 to 254 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is derived from HBcAg, and wherein said first ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 220 to 230, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 220 to 230 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 220 to 230 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 220 to 230 is optionally substituted by N.

In a further preferred aspect, the invention provides such a recombinant binding protein comprising a first designed repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first repeat domain comprises an N-terminal and/or a C-terminal capping module.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first ankyrin repeat domain comprises an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L, wherein said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 276 using the position numbers of SEQ ID NO: 276. SEQ ID NO: 276 is an N-terminal capping module that is identical to SEQ ID NO: 5, except that the G at position 1 and the S at position 2 of SEQ ID NO: 5 are missing. Thus, position 8 in SEQ ID NO: 276 corresponds to position 10 in SEQ ID NO: 5, and position 15 in SEQ ID NO: 276 corresponds to position 17 in SEQ ID NO: 5. In other words, said first ankyrin repeat domain comprises an N-terminal capping module having an amino acid sequence wherein the amino acid at position 10 is Q and/or the amino acid at position 17 is L, wherein said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 5 using the position numbers of SEQ ID NO: 5. Preferably, said alignment comprises no amino acid gaps. Sequence alignment generation is a procedure well known in the art.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first ankyrin repeat domain comprises a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and/or the amino acid at position 18 is Q, wherein the position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 13 using the position numbers of SEQ ID NO: 13. Preferably, said alignment comprises no amino acid gaps.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and the amino acid at position 15 is L, and/or (ii) a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and the amino acid at position 18 is Q. Preferably, said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 276 using the position numbers of SEQ ID NO: 276, and said position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 13 using the position numbers of SEQ ID NO: 13. Preferably, said alignments comprise no amino acid gaps.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first ankyrin repeat domain comprises an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 276), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 8 and position 15 are optionally exchanged by other amino acids.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first ankyrin repeat domain comprises a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 13), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid of SEQ ID NO: 13 in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

In one particular aspect, the invention provides such a recombinant binding protein comprising a first ankyrin repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 276), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 13), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid of SEQ ID NO: 13 in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

In one particular aspect, the invention provides such a recombinant binding protein further comprising a second designed repeat domain with binding specificity for a second target peptide-MHC complex. In a preferred embodiment said second target peptide is derived from a protein associated with a disease or disorder. As examples, in one particular preferred embodiment, said second target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder.

In one particular aspect, said second target peptide is derived from the same protein as said first target peptide. In one embodiment, said second target peptide has the same amino acid sequence as said first target peptide. In one embodiment, said second repeat domain has the same amino acid sequence as said first repeat domain. In one embodiment, said second repeat domain has a different amino acid sequence as compared to said first repeat domain. In one embodiment, said second target peptide has a different amino acid sequence as compared to said first target peptide.

In one particular aspect, said second target peptide is derived from a protein that is different from the protein, from which said first target peptide is derived.

In one preferred aspect, the invention provides such a recombinant binding protein further comprising a second designed repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second repeat domain is a designed ankyrin repeat domain. In one particular aspect, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from NY-ESO-1, and wherein said second ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from NY-ESO-1, and wherein said second ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N.

In another particular aspect, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from MAGE-A3, and wherein said second ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 175 to 217 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 175 to 217 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from MAGE-A3, and wherein said second ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 156 to 173 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 156 to 173 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 156 to 173 is optionally substituted by N.

In another particular aspect, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from EBNA-1, and wherein said second ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 111 to 154 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 111 to 154 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from EBNA-1, and wherein said second ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 93 to 110 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 93 to 110 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 93 to 110 is optionally substituted by N.

In another particular aspect, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from HBcAg, and wherein said second ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 231 to 254 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 231 to 254 are substituted by another amino acid. In one particular embodiment, the invention provides such a recombinant binding protein further comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second target peptide is derived from HBcAg, and wherein said second ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 220 to 230, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 220 to 230 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 220 to 230 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 220 to 230 is optionally substituted by N.

In a further preferred aspect, the invention provides such a recombinant binding protein comprising a second designed repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second repeat domain comprises an N-terminal and/or a C-terminal capping module.

In one particular aspect, the invention provides such a recombinant binding protein comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second ankyrin repeat domain comprises an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L, wherein said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 276 using the position numbers of SEQ ID NO: 276. In other words, said second ankyrin repeat domain comprises an N-terminal capping module having an amino acid sequence wherein the amino acid at position 10 is Q and/or the amino acid at position 17 is L, wherein said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 5 using the position numbers of SEQ ID NO: 5. Preferably, said alignment comprises no amino acid gaps. Sequence alignment generation is a procedure well known in the art.

In one particular aspect, the invention provides such a recombinant binding protein comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second ankyrin repeat domain comprises a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and/or the amino acid at position 18 is Q, wherein the position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 13 using the position numbers of SEQ ID NO: 13. Preferably, said alignment comprises no amino acid gaps.

In one particular aspect, the invention provides such a recombinant binding protein comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and the amino acid at position 15 is L, and/or (ii) a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and the amino acid at position 18 is Q. Preferably, said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 276 using the position numbers of SEQ ID NO: 276, and said position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 13 using the position numbers of SEQ ID NO: 13. Preferably, said alignments comprise no amino acid gaps.

In one particular aspect, the invention provides such a recombinant binding protein comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second ankyrin repeat domain comprises an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 276), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 8 and position 15 are optionally exchanged by other amino acids.

In one particular aspect, the invention provides such a recombinant binding protein comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second ankyrin repeat domain comprises a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 13), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid of SEQ ID NO: 13 in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

In one particular aspect, the invention provides such a recombinant binding protein comprising a second ankyrin repeat domain with binding specificity for a second target peptide-MHC complex, wherein said second ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 276), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 13), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid of SEQ ID NO: 13 in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

In one particular aspect, the invention provides such a recombinant binding protein, wherein the binding protein comprises a polypeptide comprising an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18.

In one preferred aspect, binding of the repeat domain to its target peptide-MHC complex comprises interaction of said repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said target peptide. In one embodiment, binding of the repeat domain to its target peptide-MHC complex alternatively or further comprises interaction of said repeat domain with at least one amino acid residue of said MHC.

In one particular aspect, the invention provides such pMHC-specific recombinant binding proteins, wherein the binding proteins further comprise a binding agent with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell or a NK cell, more preferably a CD8+ cytotoxic T-cell. In one embodiment, said protein expressed on the surface of a T-cell is a protein that is part of the T-cell receptor complex. As an example, in one particular embodiment, the invention provides such pMHC-specific recombinant binding proteins, wherein the binding proteins further comprise a binding agent with binding specificity for CD3. In another embodiment, said protein expressed on the surface of a NK cell is an activating receptor of NK cells. Examples of such activating receptors of NK cells include CD16 (also known as FcγRIIA), NKG2D, SLAM family members and the natural cytotoxicity receptors NKp30, NKp44 and NKp46.

In one preferred aspect, the invention provides such a recombinant binding protein further comprising a binding agent with binding specificity for a protein expressed on the surface of an immune cell, wherein said binding agent is a designed repeat domain, preferably a designed ankyrin repeat domain.

In another aspect, the invention provides nucleic acids encoding the designed repeat domains of the invention or encoding the pMHC-specific recombinant binding proteins of the invention, and pharmaceutical compositions comprising the pMHC-specific recombinant binding protein or nucleic acid of the invention and a pharmaceutically acceptable carrier and/or diluent.

In another aspect, the invention provides a method of tumor-localized activation of immune cells, such as T-cells or NK cells, in a mammal, preferably a human, the method comprising the step of administering to said mammal the pMHC-specific recombinant binding protein or nucleic acid of the invention, wherein said binding protein further comprises a binding agent with binding specificity for a protein expressed on the surface of an immune cell, and wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in a tumor cell.

In another aspect, the invention provides the pMHC-specific recombinant binding protein or nucleic acid according to the invention for use in a method of tumor-localized activation of immune cells, such as T-cells or NK cells, in a mammal, preferably a human, wherein said binding protein further comprises a binding agent with binding specificity for a protein expressed on the surface of an immune cell, and wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in a tumor cell.

In another aspect, the invention provides a method of infection-localized activation of immune cells, such as T-cells or NK cells, in a mammal, preferably a human, the method comprising the step of administering to said mammal the pMHC-specific recombinant binding protein or nucleic acid of the invention, wherein said binding protein further comprises a binding agent with binding specificity for a protein expressed on the surface of an immune cell, and wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein of an infectious agent.

In another aspect, the invention provides a method for treating a medical condition, the method comprising the step of administering to a patient in need thereof a therapeutically effective amount of the pMHC-specific recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention.

In another aspect, the invention provides the pMHC-specific recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention for use in a method of treating a medical condition.

In another aspect, the invention provides a method of diagnosing a medical condition in a mammal, preferably a human, the method comprising the steps of:

(i) contacting a cell or tissue sample obtained from said mammal with the pMHC-specific recombinant binding protein of the invention; and

(ii) detecting specific binding of said binding protein to said cell or tissue sample.

In one particular aspect, said medical condition is a cancer, an infectious disease, preferably a viral infectious disease, or an autoimmune disease. In one embodiment, said medical condition is a cancer. In one embodiment, said medical condition is an infectious disease, preferably a viral infectious disease. In one embodiment, said medical condition is an autoimmune disease.

In another aspect, the invention provides a method of targeting tumor cells in a patient having a tumor for destruction of the tumor cells, the method comprising the step of administering to the patient a therapeutically effective amount the pMHC-specific recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the tumor cells, preferably an intracellular protein expressed in the tumor cells. In one embodiment, said binding protein further comprises a toxic agent capable of killing a tumor cell.

In another aspect, the invention provides the pMHC-specific recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention for use in a method of targeting tumor cells in a patient having a tumor for destruction of the tumor cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the tumor cells, preferably an intracellular protein expressed in the tumor cells. In one embodiment, said binding protein further comprises a toxic agent capable of killing a tumor cell.

In another aspect, the invention provides a method of targeting infected cells in a patient having a viral infectious disease for destruction of the infected cells, the method comprising the step of administering to the patient a therapeutically effective amount the pMHC-specific recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the infected cells, preferably a virus-specific protein. In one embodiment, said binding protein further comprises a toxic agent capable of killing an infected cell.

In another aspect, the invention provides the pMHC-specific recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention for use in a method of targeting infected cells in a patient having a viral infectious disease for destruction of the infected cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the infected cells, preferably a virus-specific protein. In one embodiment, said binding protein further comprises a toxic agent capable of killing an infected cell.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A to 1C: Quality control testing of biotinylated pMHC complexes (biotin-pMHC). (A) Preparative size exclusion chromatography (SEC) for isolation of the biotinylated NYESOpMHC, NYESOAApMHC and EBNApMHC complexes. Ultraviolet (UV) absorbance at 280 nm was measured in the eluate. (B) SDS-PAGE analysis of the concentrated and refolded complexes in the absence or presence of Streptavidin. (C) Analytical size exclusion chromatography of the tripartite pMHC complexes. UV absorbance at 280 nm and 230 nm was measured in the eluate.

Figure 2: Efficient expression and purification of binding proteins comprising a repeat domain with binding specificity for a target peptide-MHC complex. Highly soluble binding proteins with binding specificity for NYESOpMHC were purified from *E. coli* culture with a high level of purity. A representative SDS-PAGE gel with elution fractions obtained after the SEC ÄKTAxpress™ run (following IMAC) for NYESOpMHC-specific DARPin® protein #21 is shown. The gel was stained with Coomassie Blue.

Figures 3A to 3C: Specific and high affinity binding of binding proteins of the invention to target peptide-MHC complex. Representative SPR traces for DARPin® protein #21 binding to NYESOpMHC (A), NYESOAApMHC (B) and EBNApMHC (C) are shown. DARPin® protein #21 bound with high affinity to NYESOpMHC, while no binding was detected to the two other pMHC complexes. Regular spikes in the binding traces are machine artifacts and may be neglected.

Figures 4A and 4B: Favorable biophysical properties and specific binding of binding proteins of the invention to target peptide-MHC complex. (A) Representative Homogeneous Time Resolved Fluorescence (HTRF) assay results for DARPin® protein #21 showed highly specific target binding to NYESOpMHC. No binding of DARPin® protein #21 to NYESOAApMHC or EBNApMHC was detected. (B) Size exclusion chromatography (SEC) of DARPin® protein #21 showed a single monodisperse peak, which eluted at a position corresponding to the expected mass of an individual DARPin® protein #21 molecule. No traces of aggregates or multimers were detected.

Figures 5A to 5F: Binding of representative binding proteins to cells. T2 cells were pulsed with NY-ESO-1-9V (157–165) peptide (NY-ESO pulsed cells) or EBNA-1 (562-570) peptide (EBNA pulsed cells) or treated with buffer not containing any peptide (non-pulsed cells). Titration binding curves of the indicated binding proteins to (A) NY-ESO pulsed cells, (B) non-pulsed cells, (C) NY-ESO pulsed cells, (D) NY-ESO pulsed cells, and (E) non-pulsed cells are shown. A titration binding curve of TCE DARPin® protein #21 to EBNA pulsed cells is also shown (F).

Figures 6A to 6C: T cell activation assay using T2 cells as target cells. (A) T2 cells pulsed with NY-ESO-1-9V (157–165) peptide (black bars) and non-pulsed T2 cells (grey bars) were incubated with effector $CD8^+$ T cells (BK112) in the presence of different TCE DARPin® proteins (1 pM): (1) TCE DARPin® protein #23, (2) TCE DARPin® protein #24, (3) TCE DARPin® protein #25, (4) TCE DARPin® protein #26, (5) TCE DARPin® protein #27, (6) TCE DARPin® protein #28, (7) TCE DARPin® protein #29, (8) TCE DARPin® protein #30, (9) TCE DARPin® protein #31, (10) TCE DARPin® protein #32, (11) TCE DARPin® protein #33, (12) TCE DARPin® protein #21, (13) TCE DARPin® protein #20, (14) TCE DARPin® protein #22, and (15) no TCE DARPin® protein. Intracellular interferon-γ (IFN-γ) in the T cells was detected by FACS. (B)-(C) In similar T cell activation assays, TCE DARPin® protein #21 (B) and TCE DARPin® protein #32 (C) were titrated over a broader concentration range (from 0.01 pM to 1 nM), as indicated.

Figure 7: T cell activation assay using tumor cells as target cells. IM9 tumor cells (round symbols) or MCF-7 tumor cells (triangular symbols) were incubated with effector $CD8^+$ T cells (BK112) in the presence of different concentrations of TCE DARPin® protein #21. As a control, effector $CD8^+$ T cells (BK112) were incubated in the presence of different concentrations of TCE DARPin® protein #21 but in the absence of tumor cells (square symbols). Intracellular IFN-γ in the T cells was detected by FACS. Ag: antigen (here, the NY-ESO-1-9V (157–165) peptide). Both tumor cell lines are $HLA\text{-}A2^+$.

Figures 8A to 8E: T cell activation assay using tumor cells as target cells. IM9 tumor cells (round symbols) or MCF-7 tumor cells (triangular symbols) were incubated with peripheral blood mononuclear cells (PBMCs) of a donor as effector cells in the presence of different concentrations of the indicated binding proteins. As a control, the PBMCs were incubated in the presence of different concentrations of the indicated binding proteins but in the absence of tumor cells (square symbols). CD25 expression in $CD8^+$ T cells was detected by FACS.

Figures 9A to 9E: T cell activation assay using tumor cells as target cells. IM9 tumor cells or MCF-7 tumor cells were incubated with PBMCs of a donor as effector cells in the presence of different concentrations of the indicated binding proteins, as for Figure 8. The levels of interferon-γ (IFN-γ) were quantified in the supernatants of the cells as an additional measure of T cell activation.

Figures 10A to 10E: T cell activation assay using tumor cells as target cells. IM9 tumor cells or MCF-7 tumor cells were incubated with PBMCs of a donor as effector cells in the presence of different concentrations of the indicated binding proteins, as for Figure 8. The levels of tumor necrosis factor-α (TNF-α) were quantified in the supernatants of the cells as an additional measure of T cell activation.

Figures 11A to 11 E: Target peptide binding analyzed by alanine scanning mutagenesis. Functional binding of the indicated binding proteins to the NY-ESO-1-9V (157–165) peptide and a series of alanine-mutated variants thereof were tested in a T cell activation assay using pulsed T2 cells as target cells and BK112 T cells as effector cells. The amino acid residues and positions in the NY-ESO-1-9V (157–165) peptide that were replaced with alanine are indicated. As a control, non-pulsed T2 cells were used. Intracellular IFN-γ was detected as a measure of T cell activation.

Figure 12: Cytotoxicity assay showing target pMHC-dependent target cell killing by effector cells mediated by a representative binding protein of the invention. Effector cells (peripheral blood mononuclear cells (PBMCs)) and target cells (either pulsed T2 cells (PT2) or non-pulsed T2 cells (NPT2)) were incubated in the presence of different concentrations (0.01 nM, 0.1 nM, 1 nM) of a NYESOpMHC-specific binding protein in TCE format (TCE DARPin® protein #21 (D)). Apoptotic levels (total green object area) at different incubation times are shown.

Figure 13: T cell activation assay using T2 cells as target cells. T2 cells pulsed with NY-ESO-1-9V (157–165) peptide (black bars) and non-pulsed T2 cells (grey bars) were incubated with effector CD8$^+$ T cells (BK112) in the presence of different bivalent or biparatopic binding proteins in TCE format (0.1 pM) or controls: (1) TCE BP DARPin® protein #20/#21, (2) TCE BP DARPin® protein #21/#22, (3) TCE BV DARPin® protein #21/#21, (4) a TCE DARPin® protein comprising an ankyrin repeat domain with binding specificity for human serum albumin instead of the ankyrin repeat domains with binding specificity for NYESOpMHC, and (5) no TCE DARPin® protein added. Intracellular interferon-γ (IFN-γ) in the T cells was detected by FACS.

Figures 14A and 14B: Binding of representative binding proteins comprising two repeat domains with binding specificity for a target pMHC complex to cells. T2 cells were pulsed with NY-ESO-1-9V (157–165) peptide (pulsed cells) or treated with buffer not containing any peptide (non-pulsed cells). Titration binding curves of the indicated binding proteins to pulsed cells (A) and non-pulsed cells (B) are shown.

Figures 15A and 15B: T cell activation assay using tumor cells as target cells. IM9 tumor cells (round symbols) or MCF-7 tumor cells (triangular symbols) were incubated with peripheral blood mononuclear cells (PBMCs) of a donor as effector cells in the presence of different concentrations of the indicated binding proteins comprising two repeat domains with binding specificity for a target pMHC complex. As a control, the PBMCs were incubated in the presence of different concentrations of the indicated binding proteins but in the absence of tumor cells (square symbols). CD25 expression in CD8$^+$ T cells was detected by FACS.

Figures 16A and 16B: T cell activation assay using tumor cells as target cells. IM9 tumor cells or MCF-7 tumor cells were incubated with PBMCs of a donor as effector cells in the presence of different concentrations of the indicated binding proteins comprising two repeat domains with binding specificity for a target pMHC complex, as for Figure 15. The levels of IFN-γ (A) and TNF-α (B) were quantified in the supernatants of the cells as an additional measure of T cell activation.

Figure 17: Target peptide binding analyzed by alanine scanning mutagenesis. Functional binding of the indicated binding protein comprising two repeat domains with binding specificity for a target pMHC complex to the NY-ESO-1-9V (157–165) peptide and a series of alanine-mutated variants thereof were tested in a T cell activation assay using pulsed T2 cells as target cells and BK112 T cells as effector cells. The amino acid residues and positions in the NY-ESO-1-9V (157–165) peptide that were replaced with alanine are indicated. As a control, non-pulsed T2 cells were used. Intracellular IFN-γ was detected as a measure of T cell activation dependent on binding of the binding proteins to the target cells.

Figures 18A to 18F: T cell activation assay using tumor cells as target cells. U266B1 tumor cells (round symbols) or Colo205 tumor cells (square symbols) ((A) and (B)), MCF-7 tumor cells transfected to express NY-ESO-1 (round symbols) or untransfected MCF-7 tumor cells (square symbols) ((C) and (D)), and IM9 tumor cells (round symbols) and MCF-7 tumor cells (square symbols) ((E) and (F)) were incubated with peripheral blood mononuclear cells (PBMCs) of a donor as effector cells in the presence of different concentrations of the indicated representative binding proteins of the invention (in TCE format). TCE DARPin® protein #21 was used in (A), (C) and (E), and TCE DARPin® protein #32 was used in (B), (D) and (F). CD69 expression in $CD8^+$ T cells was detected by FACS. Ag: NYESOpMHC.

Figures 19A and 19B: Cytotoxicity assay showing target pMHC-dependent target cell killing by effector cells mediated by a representative binding protein of the invention. Pulsed (P T2) or non-pulsed (NP T2) T2 cells (A) and HLA-$A2^+$/ NY-ESO-1^+ tumor cells (IM9, U266B1) or HLA-$A2^+$/ NY-ESO-1^- tumor cells (MCF-7) (B) were incubated, as target cells, with effector $CD8^+$ T cells in the presence or absence of a NYESOpMHC-specific binding protein in TCE format (TCE DARPin® protein #21) (1nM). Different ratios of effector cells to target cells (E:T) were used (30:1, 10:1, 5:1, and 1:1), as indicated. The percentage of specific lysis of the T2 cells (A) or tumor cells (B) obtained by the chromium release assay was plotted for different effector to target ratios. Ag: NYESOpMHC.

Figures 20A and 20B: Cytotoxicity assay showing target pMHC-dependent target cell killing by effector cells mediated by a representative binding protein of the invention. These Figures show experiments as described in Figures 19A and 19B, but using a different NYESOpMHC-specific binding protein in TCE format (TCE DARPin® protein #32).

Figures 21A and 21B: Target peptide binding analyzed by X-scanning mutagenesis. Functional binding of a NYESOpMHC-specific binding protein in TCE format (TCE DARPin® protein #21 in (A); TCE DARPin® protein #32 in (B)) to the NY-ESO-1-9V (157–165) peptide and a series of single-mutation variants thereof were tested in a T cell activation assay using pulsed T2 cells as target cells and BK112 T cells as effector cells. Each amino acid of the target peptide sequence was replaced with every one of the other 19 standard amino acids. The amino acid residues and positions in the NY-ESO-1-9V (157–165) peptide that were replaced with another amino acid are indicated on the left of the table, and the replacement amino acids are indicated on top of the table. Intracellular IFN-γ was detected as a measure of T cell activation dependent on binding of the binding proteins to the target cells. Each experiment was performed in two independent replicates. Values were averaged and normalized to 100% for the according wild-type residue (dark shaded fields) in each position. All values above 30%, indicating no loss or not a complete loss of T-cell activation, are marked in bold font and light shaded color.

Figures 22 and 23: T cell activation by pMHC-specific binding proteins in TCE format depending on length of linker connecting the pMHC-specific binding domain and the CD3-specific binding agent. HLA-$A2^+$/NY-ESO-1^+ tumor cells (IM9) (continuous lines) or HLA-$A2^+$/NY-ESO-1^- tumor cells (MCF-7) (dashed lines) were incubated with PBMCs for 48 hours in the presence or absence of TCE DARPin® protein #21 with different linker lengths (Figure 22) or of TCE DARPin® protein #32 with different linker lengths (Figure 23). After 48 hours, CD25 expression was measured on $CD8^+$ T cells. Results obtained in the presence of TCE

DARPin® protein are shown. No T cell activation was observed in the absence of TCE DARPin® protein. Ag: antigen/NY-ESO-1. Linker lengths: standard, XXS, XS, S and L (see Example 11).

Figures 24 and 25: T cell activation by pMHC-specific binding proteins in TCE format depending on length of linker connecting the pMHC-specific binding domain and the CD3-specific binding agent. HLA-A2$^+$/NY-ESO-1$^+$ tumor cells (U266B1) (continuous lines) or HLA-A2$^+$/NY-ESO-1$^-$ tumor cells (Colo205) (dashed lines) were incubated with PBMCs for 48 hours in the presence or absence of TCE DARPin® protein #21 with different linker lengths (Figure 24) or of TCE DARPin® protein #32 with different linker lengths (Figure 25). After 48 hours, CD25 expression was measured on CD8$^+$ T cells. Results obtained in the presence of TCE DARPin® protein are shown. No T cell activation was observed in the absence of TCE DARPin® protein. Ag: antigen/NY-ESO-1. Linker lengths: standard, XXS, XS, S and L (see Example 11).

Figure 26. Pharmacokinetic profiles in mouse of variants of designed ankyrin repeat domains (each genetically linked to an identical designed ankyrin repeat domain with binding specificity for serum albumin via an identical polypeptide linker). (a) Pharmacokinetic profile in mouse of Protein #281, and variant Proteins #282 and #283. (b) Pharmacokinetic profile in mouse of Protein #284, and variant Proteins #285, #286, and #287. (c) Pharmacokinetic profile in mouse of Protein #288, and variant Proteins #289, #290, and #291. (d) Pharmacokinetic profile in mouse of Protein #292, and variant Proteins #293, #294, and #295. The experiment was performed as described in Example 17 using Balb/c mice and 1 mg/kg intravenous dosing. Proteins #281 to #295 (comprising SEQ ID NOs: 281 to 295, respectively, with each having a His-tag (SEQ ID NO: 326) at the N-terminus; symbol indicated in the figure) were produced and purified as described in Example 17. C: concentration in [nM]; t: time in [hours].

DETAILED DESCRIPTION OF THE INVENTION

As disclosed and exemplified herein, the disclosure provides designed repeat proteins, preferably designed ankyrin repeat proteins, that specifically target a peptide-MHC complex. Designed repeat protein libraries, including designed ankyrin repeat protein libraries (WO2002/020565; Binz et al., Nat. Biotechnol. *22*, 575-582, 2004; Stumpp et al., Drug Discov. Today *13*, 695-701, 2008), can be used for the selection of target-specific designed repeat domains that bind to their target with high affinity. Such target-specific designed repeat domains in turn can be used as valuable components of recombinant binding proteins for the treatment of diseases. Whether designed repeat protein libraries can be used to identify proteins that specifically and with high affinity bind to a composite epitope, such as the ones presented by peptide-MHC complexes, had not been shown. Generating molecules that specifically bind disease-related peptide-MHC complexes with sufficient affinity has been notoriously difficult.

Designed ankyrin repeat proteins are a class of binding molecules which have the potential to overcome limitations of monoclonal antibodies, hence allowing novel therapeutic approaches. Such ankyrin repeat proteins may comprise a single designed ankyrin repeat domain, or may comprise a combination of two, three, four, five or more designed ankyrin repeat domains with the same or different target specificities (Stumpp et al., Drug Discov. Today *13*, 695-701, 2008; U.S. Patent No. 9,458,211). Ankyrin repeat proteins comprising only a single designed ankyrin repeat domain are small proteins (14 kDa) which can be selected to bind a given target protein with high affinity and specificity. These characteristics, and the possibility of combining two, three, four, five or more designed ankyrin repeat domains in one protein, make designed ankyrin repeat proteins ideal agonistic, antagonistic and/or inhibitory drug candidates. Furthermore, such ankyrin repeat proteins can be engineered to carry various effector functions, e.g. cytotoxic agents or half-life extending agents, enabling completely new drug formats. Taken together, designed ankyrin repeat proteins are an example of the next generation of protein therapeutics with the potential to surpass existing antibody drugs.

DARPin® is a trademark owned by Molecular Partners AG, Switzerland.

In one aspect, the invention provides a method of producing a peptide-MHC (pMHC)-specific binding protein, wherein said binding protein comprises a designed repeat domain with binding specificity for a target peptide-MHC complex, the method comprising the steps of:

(a) providing a collection of designed repeat domains;
(b) providing a recombinant target peptide-MHC complex; and
(c) screening said collection of designed repeat domains for specific binding to said target peptide-MHC complex to obtain at least one designed repeat domain with binding specificity for said target peptide-MHC complex.

In one embodiment, said designed repeat domain is a designed ankyrin repeat domain and said collection of designed repeat domains is a collection of designed ankyrin repeat domains.

In one embodiment, said collection comprises designed repeat domains that comprise fixed positions and randomized positions, and wherein designed repeat domains of said collection differ from each other in at least one of the randomized positions.

In one embodiment, said collection of designed repeat proteins is provided by ribosome display.

In one embodiment, the method further comprises the steps of (i) providing a second recombinant peptide-MHC complex, wherein said peptide of said second peptide-MHC complex comprises an amino acid sequence that differs from said target peptide by at least one amino acid residue; and (ii) removing from said collection by negative selection designed repeat domains with binding specificity for said second recombinant peptide-MHC complex.

In one embodiment, said repeat domain with binding specificity for a target peptide-MHC complex binds to said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5×10^{-8}M, or below 3×10^{-8}M, or below 2×10^{-8}M, or below 10^{-8}M, or below 5×10^{-9}M, or below 3×10^{-9}M, or below 2×10^{-9}M, or below 10^{-9}M, or below 5×10^{-10}M, or below 3×10^{-10}M, or below 2×10^{-10}M, or below 10^{-10}M. In one embodiment, said repeat domain with binding specificity for a target peptide-MHC complex binds to said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M.

In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said target peptide. Thus, in one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least one amino acid residue of said target peptide. In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least two amino acid residues of said target peptide. In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least three amino acid residues of said target peptide. In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least four amino acid residues of said target peptide. In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least five amino acid residues of said target peptide. In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least six amino acid residues of said target peptide. In one embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least seven amino acid residues of said target peptide. In a further or alternative embodiment, binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least one amino acid residue of said MHC.

Methods to determine the amino acid residues involved in the binding interaction between proteins or between a protein and a peptide, such as, e.g., alanine scanning mutagenesis, are well known to the person skilled in the art.

In the context of the present invention, a typical and preferred determination of the amino acid residues of a target peptide that are involved in the binding interaction between a binding protein or a designed repeat domain with binding specificity for a target peptide-MHC complex and the target peptide-MHC complex is performed by alanine scanning mutagenesis as described in Example 7. Thus, in one embodiment said amino acid residues of a target peptide that are involved in the binding interaction between a binding protein or a designed repeat domain of the invention with binding specificity for a target peptide-MHC complex and the target peptide-MHC complex are determined by alanine scanning mutagenesis. In one embodiment said amino acid residues of a target peptide that are involved in the binding interaction between a binding protein or a designed repeat domain of the invention with binding specificity for a target peptide-MHC complex and the target peptide-MHC complex are determined by alanine scanning mutagenesis as described in Example 7.

In the context of the present invention, the phrase “binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said target peptide”, or similar phrases, refers to any amino acid residues of the target peptide, the mutation of which to alanine results in a reduction of T cell activation in an assay as described in Example 7 (or a similar assay) by at least 50% compared to the wild-type peptide.

The inventors of the present invention have discovered that a surprisingly large number of peptide residues are important for the specific interaction between the binding proteins of the invention comprising an ankyrin repeat domain with binding specificity for a target peptide-MHC complex and the target peptide. For instance, in the appended examples, several peptide residues that are important for the specific interaction between the binding proteins of the invention comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and the NY-ESO-1 target peptide have been identified (see Figure 11). The inventors theorize that this finding may reflect a structural difference between the binding surface formed by the designed ankyrin repeat domains of the present invention and that formed by other binding proteins, such as antibodies and T cell receptors (TCRs). Without being bound by any theory, it is believed that the higher the number of amino acid residues involved in the binding of a repeat domain and its target peptide, the higher is the binding specificity. In one embodiment, said target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, such as a bacterial infectious agent or a viral infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder. In one embodiment, said target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell. In one embodiment, said target peptide is derived from NY-ESO-1. In one embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34. In one embodiment, said target peptide is derived from MAGE-A3. In one embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 155. In one embodiment, said target peptide is derived from a protein of an infectious agent, such as, e.g., a viral infectious agent, preferably a virus-specific protein. In one embodiment, said target peptide is derived from EBNA-1. In one embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 92. In one embodiment, said target peptide is derived from HBcAg. In one embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 255.

In one embodiment, said MHC is MHC class I. In one embodiment, said MHC class I is HLA-A*02. In one embodiment, said HLA-A*02 is HLA-A*0201. In one embodiment, said HLA-A*0201 has the amino acid sequence of SEQ ID NO: 73. Alternatively, in another embodiment, said MHC is not HLA-A*02. In one embodiment, said MHC class I is HLA-A*01. In one embodiment, said HLA-A*01 is HLA-A*0101. In one embodiment, said HLA-A*0101 has the amino acid sequence of SEQ ID NO: 218. Alternatively, in another embodiment, said MHC is not HLA-A*01.

In another aspect, the invention relates to a recombinant binding protein comprising a designed repeat domain obtainable by one of the methods of the invention as described herein.

In another aspect, the invention relates to a recombinant binding protein comprising a first designed repeat domain, wherein said first repeat domain has binding specificity for a first target peptide-MHC complex.

In a preferred embodiment, the binding protein of the invention comprises a first designed repeat domain with binding specificity for a first target peptide-MHC complex, wherein said first target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, such as a bacterial infectious agent or a viral infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder.

In one embodiment, said first target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell. In one embodiment, said first target peptide is derived from a tumor-specific intracellular protein. In one embodiment, said first target peptide is derived from NY-ESO-1. In one embodiment, said first target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34. In one embodiment, said first target peptide is derived from MAGE-A3. In one embodiment, said first target peptide has the amino acid sequence of SEQ ID NO: 155. In one embodiment, said target peptide is derived from a protein of an infectious agent, such as, e.g., a viral infectious agent, preferably a virus-specific protein. In one embodiment, said target peptide is derived from EBNA-1. In one embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 92. In one embodiment, said target peptide is derived from HBcAg. In one embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 255. In a preferred embodiment, the first MHC is MHC class I. In one embodiment, said first MHC class I is HLA-A*02. In one embodiment, said HLA-A*02 is HLA-A*0201. In one embodiment, said HLA-A*0201 has the amino acid sequence of SEQ ID NO: 73. Alternatively, in another embodiment, said first MHC is not HLA-A*02. In one embodiment, said first MHC class I is HLA-A*01 In one embodiment, said HLA-A*01 is HLA-A*0101. In one embodiment, said HLA-A*0101 has the amino acid sequence of SEQ ID NO: 218. Alternatively, in another embodiment, said first MHC is not HLA-A*01.

In one embodiment, said first repeat domain binds to said first target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M.

In one embodiment, said binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said first target peptide. Thus, in one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least one amino acid residue of said first target peptide. In one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least two amino acid residues of said first target peptide. In one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least three amino acid residues of said first target peptide. In one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least four amino acid residues of said first target peptide. In one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least five amino acid residues of said first target peptide. In one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least six amino acid residues of said first target peptide. In one embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least seven amino acid residues of said first target peptide. In a further or alternative embodiment, binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least one amino acid residue of said first MHC.

In a preferred embodiment, said first designed repeat domain is a designed ankyrin repeat domain. In a particularly preferred embodiment, said first designed ankyrin repeat domain is a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects or embodiments herein.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. Thus, in one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. Thus, in one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 3 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 2 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 1 amino acid in any of SEQ ID NOs: 37 to 72 is substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 37 to 72. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 are substituted by another amino acid. Thus, in one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 and (2) sequences in which up to 3 amino acids in any of SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 are substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 and (2) sequences in which up to 2 amino acids in any of SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 are substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 and (2) sequences in which up to 1 amino acid in any of SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 is substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42 and 67 to 69 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 37 to 42 and 67 to 69 are substituted by another amino acid. Thus, in one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42 and 67 to 69 and (2) sequences in which up to 3 amino acids in any of SEQ ID NOs: 37 to 42 and 67 to 69 are substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42 and 67 to 69 and (2) sequences in which up to 2 amino acids in any of SEQ ID NOs: 37 to 42 and 67 to 69 are substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42 and 67 to 69 and (2) sequences in which up to 1 amino acid in any of SEQ ID NOs: 37 to 42 and 67 to 69 is substituted by another amino acid. In one embodiment, said ankyrin repeat module comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 37 to 42 and 67 to 69. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In another particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 111 to 154 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 111 to 154 are substituted by another amino acid. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 92.

In a further particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 175 to 217 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 175 to 217 are substituted by another amino acid. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 155.

In a further particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 231 to 254 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 231 to 254 are substituted by another amino acid. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 255.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module and a third ankyrin repeat module. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first, a second and optionally a third ankyrin repeat module, wherein each of said first, said second and, if present, said third ankyrin repeat module independently comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid. In one embodiment, each of said first, said second and, if present, said third ankyrin repeat module independently comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 37 to 42, 54, 55 and 67 to 72 are substituted by another amino acid. In one embodiment, each of said first, said second and, if present, said third ankyrin repeat module independently comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 42 and 67 to 69 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 37 to 42 and 67 to 69 are substituted by another amino acid. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in SEQ ID NO: 54 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 55 are substituted by another amino acid. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which up to 6 amino acids in SEQ ID NO: 54 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 55 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which up to 5 amino acids in SEQ ID NO: 54 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 55 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which up to 4 amino acids in SEQ ID NO: 54 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 55 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which up to 3 amino acids in SEQ ID NO: 54 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 55 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which up to 2 amino acids in SEQ ID NO: 54 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 55 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 54 and (2) sequences in which 1 amino acid in SEQ ID NO: 54 is substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 55 and (2) sequences in which 1 amino acid of SEQ ID NO: 55 is substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 54, and said second ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 55. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module and a third ankyrin repeat module, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in SEQ ID NO: 37 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 38 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 39 are substituted by another amino acid. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which up to 6 amino acids in SEQ ID NO: 37 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 38 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 39 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which up to 5 amino acids in SEQ ID NO: 37 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 38 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 39 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which up to 4 amino acids in SEQ ID NO: 37 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 38 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 39 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which up to 3 amino acids in SEQ ID NO: 37 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 38 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 39 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which up to 2 amino acids in SEQ ID NO: 37 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 38 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 39 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 37 and (2) sequences in which 1 amino acid in SEQ ID NO: 37 is substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 38 and (2) sequences in which 1 amino acid of SEQ ID NO: 38 is substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 39 and (2) sequences in which 1 amino acid of SEQ ID NO: 39 is substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 37, and said second ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 38, and said third ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 39. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module and a third ankyrin repeat module, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in SEQ ID NO: 40 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 41 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 41 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 42 are substituted by another amino acid. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which up to 6 amino acids in SEQ ID NO: 40 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 41 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 41 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 42 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which up to 5 amino acids in SEQ ID NO: 40 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 41 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 41 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 42 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which up to 4 amino acids in SEQ ID NO: 40 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 41 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 41 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 42 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which up to 3 amino acids in SEQ ID NO: 40 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 41 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 41 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 42 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which up to 2 amino acids in SEQ ID NO: 40 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 41 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 41 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 42 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 40 and (2) sequences in which 1 amino acid in SEQ ID NO: 40 is substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ

ID NO: 41 and (2) sequences in which 1 amino acid of SEQ ID NO: 41 is substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 42 and (2) sequences in which 1 amino acid of SEQ ID NO: 42 is substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 40, and said second ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 41, and said third ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 42. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module and a third ankyrin repeat module, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in SEQ ID NO: 67 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 68 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 69 are substituted by another amino acid. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which up to 6 amino acids in SEQ ID NO: 67 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 68 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 69 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which up to 5 amino acids in SEQ ID NO: 67 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 68 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 69 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which up to 4 amino acids in SEQ ID NO: 67 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 68 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 69 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which up to 3 amino acids in SEQ ID NO: 67 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 68 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 69 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which up to 2 amino acids in SEQ ID NO: 67 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 68 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 69 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 67 and (2) sequences in which 1 amino acid in SEQ ID NO: 67 is substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 68 and (2) sequences in which 1 amino acid of SEQ ID NO: 68 is substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 69 and (2) sequences in which 1 amino acid of SEQ ID NO: 69 is substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 67, and said second ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 68, and said third ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 69. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first ankyrin repeat module and a second ankyrin repeat module and a third ankyrin repeat module, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in SEQ ID NO: 70 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 71 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids of SEQ ID NO: 72 are substituted by another amino acid. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which up to 6 amino acids in SEQ ID NO: 70 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 71 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which up to 6 amino acids of SEQ ID NO: 72 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which up to 5 amino acids in SEQ ID NO: 70 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 71 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which up to 5 amino acids of SEQ ID NO: 72 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which up to 4 amino acids in SEQ ID NO: 70 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 71 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which up to 4 amino acids of SEQ ID NO: 72 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which up to 3 amino acids in SEQ ID NO:

70 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 71 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which up to 3 amino acids of SEQ ID NO: 72 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which up to 2 amino acids in SEQ ID NO: 70 are substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 71 are substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which up to 2 amino acids of SEQ ID NO: 72 are substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 70 and (2) sequences in which 1 amino acid in SEQ ID NO: 70 is substituted by another amino acid, and said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 71 and (2) sequences in which 1 amino acid of SEQ ID NO: 71 is substituted by another amino acid, and said third ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 72 and (2) sequences in which 1 amino acid of SEQ ID NO: 72 is substituted by another amino acid. In one embodiment, in such an ankyrin repeat domain said first ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 70, and said second ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 71, and said third ankyrin repeat module comprises the amino acid sequence of SEQ ID NO: 72. In one embodiment, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain, and said second ankyrin repeat module is located N-terminally of said third ankyrin repeat module within said ankyrin repeat domain. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In another particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first, a second and optionally a third ankyrin repeat module, wherein each of said first, said second and, if present, said third ankyrin repeat module independently comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 111 to 154 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 111 to 154 are substituted by another amino acid. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 92.

In a further particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a first, a second and optionally a third ankyrin repeat module, wherein each of said first, said second and, if present, said third ankyrin repeat module independently comprises an amino acid sequence selected from the group consisting of (1) SEQ

ID NOs: 175 to 217 and (2) sequences in which up to 9, or up to 8, or up to 7, or up to 6, or up to 5, or up to 4, or up to 3, or up to 2, or up to 1 amino acids in any of SEQ ID NOs: 175 to 217 are substituted by another amino acid. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 155.

In one preferred embodiment, all of said amino acid substitutions of said ankyrin repeat module(s) as described and referred to herein occur in framework positions of said ankyrin repeat module(s), wherein typically the overall structure of the module(s) is not affected by the substitutions.

In one preferred embodiment, all of said amino acid substitutions of said ankyrin repeat module(s) as described and referred to herein occur in positions other than the randomized positions 3, 4, 6, 14 and 15 of said ankyrin repeat module(s) of SEQ ID NOs: 37 to 60, 62 to 66 and 68 to 72 or the randomized positions 3, 4, 6, 13 and 14 of said ankyrin repeat module(s) of SEQ ID NO: 61 and 67. In another preferred embodiment, all of said amino acid substitutions of said ankyrin repeat module(s) as described and referred to herein occur in positions other than the randomized positions 3, 4, 6, 14 and 15 of said ankyrin repeat module(s) of SEQ ID NOs: 111 to 122 and 124 to 154 or the randomized positions 3, 4, 6, 15 and 16 of said ankyrin repeat module(s) of SEQ ID NO: 123. In another preferred embodiment, all of said amino acid substitutions of said ankyrin repeat module(s) as described and referred to herein occur in positions other than the randomized positions 3, 4, 6, 14 and 15 of said ankyrin repeat module(s) of SEQ ID NOs: 175 to 217. In another preferred embodiment, all of said amino acid substitutions of said ankyrin repeat module(s) as described and referred to herein occur in positions other than the randomized positions 3, 4, 6, 14 and 15 of said ankyrin repeat module(s) of SEQ ID NOs: 231 to 254.

In a further preferred embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention further comprises an N-terminal and/or a C-terminal capping module.

In accordance with the present invention, the N-terminal capping module of the ankyrin repeat domain of the invention with binding specificity for a target peptide-MHC complex has an amino acid sequence which may or may not comprise an amino acid G at position 1 and/or an amino acid S at position 2. An example of such an N-terminal capping module having an amino acid sequence comprising the amino acid G at position 1 and the amino acid S at position 2 is provided in SEQ ID NO: 5. An example of such an N-terminal capping module having an amino acid sequence not comprising the amino acid G at position 1 and the amino acid S at position 2 is provided in SEQ ID NO: 276. Thus, SEQ ID NO: 5 and SEQ ID NO: 276 are identical except that G at position 1 and S at position 2 of SEQ ID NO: 5 are missing in SEQ ID NO: 276. A skilled person therefore readily understands that the position numbering provided above with respect to a N-terminal capping module comprising the surface design of the invention may vary depending on whether these amino acid residues are present or not. Accordingly, an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L, wherein said position numbers are defined by reference to SEQ ID NO: 276, corresponds to an N-terminal capping module having an amino acid sequence wherein the amino acid at position 10 is Q and/or the amino acid at position 17 is L, wherein said position numbers are defined by reference to SEQ ID NO:

5, which comprises a “GS” sequence at its N-terminus, In the following, SEQ ID NO: 276 will generally be used as the reference sequence.

In one embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L, wherein said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 276, using the position numbers of SEQ ID NO: 276. Preferably, said alignment comprises no amino acid gaps. Sequence alignment generation is a procedure well known in the art.

In one embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and/or the amino acid at position 18 is Q, wherein the position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 13 using the position numbers of SEQ ID NO: 13. Preferably, said alignment comprises no amino acid gaps.

In one embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises (i) an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L, and/or (ii) a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and/or the amino acid at position 18 is Q. In one embodiment, the designed ankyrin repeat domain of the invention comprises (i) an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and/or the amino acid at position 15 is L, and (ii) a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and/or the amino acid at position 18 is Q. In one embodiment, the designed ankyrin repeat domain of the invention comprises (i) an N-terminal capping module having an amino acid sequence wherein the amino acid at position 8 is Q and the amino acid at position 15 is L, and (ii) a C-terminal capping module having an amino acid sequence wherein the amino acid at position 14 is R and the amino acid at position 18 is Q. Preferably, said position numbers of positions of the N-terminal capping module are determined by alignment to SEQ ID NO: 276 using the position numbers of SEQ ID NO: 276, and said position numbers of positions of the C-terminal capping module are determined by alignment to SEQ ID NO: 13 using the position numbers of SEQ ID NO: 13. Preferably, said alignments comprise no amino acid gaps.

In a further embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 276), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 8 and position 15 are optionally exchanged by other amino acids.

In one embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 13), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid of SEQ ID NO: 13 in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

In one embodiment, the designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA (SEQ ID NO: 276), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA (SEQ ID NO: 13), wherein up to 10 amino acids, up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid of SEQ ID NO: 13 in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 9 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 8 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 7 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 6 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 5 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 4 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 3 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to 2 amino acids in positions other than position 8 and position 15 are optionally exchanged by other amino acids, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids. In one embodiment, said designed ankyrin repeat domain comprises (i) an N-terminal capping module having an amino acid sequence DLGKKLLQAARAGQLDEVRELLKAGADVNA, wherein up to one amino acid in a position other than position 8 and position 15 is optionally exchanged by another amino acid, and (ii) a C-terminal capping module having an amino acid sequence QDKSGKTPADLAARAGHQDIAEVLQKAA, wherein up to 9 amino acids, up to 8 amino acids, up to 7 amino acids, up to 6 amino acids, up to 5 amino acids, up to 4 amino acids, up to 3 amino acids, up to 2 amino acids, or up to one amino acid in positions other than position 14 and position 18 are optionally exchanged by other amino acids.

Applicant has surprisingly discovered that ankyrin binding domains comprising one or more of the above-mentioned amino acids at the above-mentioned positions in the N-terminal capping module (i.e. positions 8 and 15) and/or the C-terminal capping module (i.e. positions 14 and 18) of the designed ankyrin repeat domain results in improved pharmacokinetic properties, including a prolonged terminal half-life, of the designed ankyrin repeat domain and of proteins comprising the designed ankyrin repeat domain (Example 17 and Figure 26).

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence selected from SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 20, 21, 27, 32 and 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20, 21, 27, 32 and 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20, 21, 27, 32 and 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20, 21, 27, 32 and 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20, 21, 27, 32 and 33, wherein G at position 1 and/or

S at position 2 of SEQ ID NOs: 20, 21, 27, 32 and 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20, 21, 27, 32 and 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20, 21, 27, 32 and 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 20, 21, 27, 32 and 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 20, 21, 27, 32 and 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 20, 21, 27, 32 and 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 20, 21, 27, 32 and 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 20, 21, 27, 32 and 33. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence selected from SEQ ID NOs: 20, 21, 27, 32 and 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20, 21, 27, 32 and 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20, 21, 27, 32 and 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20, 21, 27, 32 and 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 20, 21 and 32, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20, 21 and 32 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20, 21 and 32 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20, 21 and 32 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20, 21 and 32, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20, 21 and 32 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20, 21 and 32 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20, 21 and 32 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 20, 21 and 32. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 20, 21 and 32; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 20, 21 and 32. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 20, 21 and 32; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 20, 21 and 32. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence selected from SEQ ID NOs: 20, 21 and 32, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20, 21 and 32 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20, 21 and 32 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20, 21 and

32 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 20, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 20 are optionally missing, and wherein A at the second last position of SEQ ID NO: 20 is optionally substituted by L and/or A at the last position of SEQ ID NO: 20 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 20, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 20 are optionally missing, and wherein A at the second last position of SEQ ID NO: 20 is optionally substituted by L and/or A at the last position of SEQ ID NO: 20 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 20. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 20; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 20. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 20; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 20. Thus, in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 20, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 20 are optionally missing, and wherein A at the second last position of SEQ ID NO: 20 is optionally substituted by L and/or A at the last position of SEQ ID NO: 20 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 21, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 21 are optionally missing, and wherein A at the second last position of SEQ ID NO: 21 is optionally substituted by L and/or A at the last position of SEQ ID NO: 21 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 21, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 21 are optionally missing, and wherein A at the second last position of SEQ ID NO: 21 is optionally substituted by L and/or A at the last position of SEQ ID NO: 21 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 21. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 21; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 21. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 21; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 21. Thus, in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 21, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 21 are optionally missing, and wherein A at the second last position of SEQ ID NO: 21 is optionally substituted by L and/or A at the last position of SEQ ID NO: 21 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 27, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 27 are optionally missing, and wherein A at the second last position of SEQ ID NO: 27 is optionally substituted by L and/or A at the last position of SEQ ID NO: 27 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 27, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 27 are optionally missing, and wherein A at the second last position of SEQ ID NO: 27 is optionally substituted by L and/or A at the last position of SEQ ID NO: 27 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 27. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 27; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 27. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 27; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 27. Thus, in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 27, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 27 are optionally missing, and wherein A at the second last position of SEQ ID NO: 27 is optionally substituted by L and/or A at the last position of SEQ ID NO: 27 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 32, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 32 are optionally missing, and wherein A at the second last position of SEQ ID NO: 32 is optionally substituted by L and/or A at the last position of SEQ ID NO: 32 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 32, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 32 are optionally missing, and wherein A at the second last position of SEQ ID NO: 32 is optionally substituted by L and/or A at the last position of SEQ ID NO: 32 is optionally substituted by N.

Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 32. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 32; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 32. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 32; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 32. Thus, in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 32, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 32 are optionally missing, and wherein A at the second last position of SEQ ID NO: 32 is optionally substituted by L and/or A at the last position of SEQ ID NO: 32 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 33, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 33 are optionally missing, and wherein A at the second last position of SEQ ID NO: 33 is optionally substituted by L and/or A at the last position of SEQ ID NO: 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 33, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 33 are optionally missing, and wherein A at the second last position of SEQ ID NO: 33 is optionally substituted by L and/or A at the last position of SEQ ID NO: 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 33. Thus, in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 33, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 33 are optionally missing, and wherein A at the second last position of SEQ ID NO: 33 is optionally substituted by L and/or A at the last position of SEQ ID NO: 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 93 to 110 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 93 to 110 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 93 to 110 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 93 to 110 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 93 to 110 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 93 to 110 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 93 to 110; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 93 to 110. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence selected from SEQ ID NOs: 93 to 110, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 93 to 110 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 93 to 110 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 93 to 110 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 92.

In one particular embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 156 to 173 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 156 to 173 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 156 to 173 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 156 to 173 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 156 to 173 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 156 to 173 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 156 to 173; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 156 to 173. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence selected from SEQ ID NOs: 156 to 173, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 156 to 173 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 156 to 173 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 156 to 173 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 155.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, or below 3 x 10^{-10}M, or below 2 x 10^{-10}M, or below 10^{-10}M. Thus, in one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 5 x 10^{-8}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 3 x 10^{-8}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 2 x 10^{-8}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-8}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 5 x 10^{-9}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 3 x 10^{-9}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 2 x 10^{-9}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-9}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 5 x 10^{-10}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 3 x 10^{-10}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 2 x 10^{-10}M. In one embodiment, said ankyrin repeat domain binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-10}M.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, and comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of any one of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 5×10^{-8}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 5×10^{-8}M, and comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of any one of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said first target peptide-MHC complex in PBS with a dissociation constant (K_D) below 2×10^{-8}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 2×10^{-8}M, and comprises the amino acid sequence of any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of any one of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5×10^{-8}M, or below 3×10^{-8}M, or below 2×10^{-8}M, or below 10^{-8}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 20, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 20 are optionally missing, and wherein A at the second last position of SEQ ID NO: 20 is optionally substituted by L and/or A at the last position of SEQ ID NO: 20 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 20. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 20; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 20. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 20; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 20. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5×10^{-8}M, or below 3×10^{-8}M, or below 2×10^{-8}M, or below 10^{-8}M, and comprises the amino acid sequence of SEQ ID NO: 20, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 20 are optionally missing, and wherein A at the second last position of SEQ ID NO: 20 is optionally substituted by L and/or A at the last position of SEQ ID NO: 20 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 21, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 21 are optionally missing, and wherein A at the second last position of SEQ ID NO: 21 is optionally substituted by L and/or A at the last position of SEQ ID NO: 21 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 21. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 21; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 21. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 21; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 21. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, and comprises the amino acid sequence of SEQ ID NO: 21, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 21 are optionally missing, and wherein A at the second last position of SEQ ID NO: 21 is optionally substituted by L and/or A at the last position of SEQ ID NO: 21 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said first target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 27, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 27 are optionally missing, and wherein A at the second last position of SEQ ID NO: 27 is optionally substituted by L and/or A at the last position of SEQ ID NO: 27 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 27. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 27; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 27. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 27; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 27. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, and comprises the amino acid sequence of SEQ ID NO: 27, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 27 are optionally missing, and wherein A at the second last position of SEQ ID NO: 27 is optionally substituted by L and/or A at the last position of SEQ ID NO: 27 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 32, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 32 are optionally missing, and wherein A at the second last position of SEQ ID NO: 32 is optionally substituted by L and/or A at the last position of SEQ ID NO: 32 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 32. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 32; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 32. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 32; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 32. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, and comprises the amino acid sequence of SEQ ID NO: 32, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 32 are optionally missing, and wherein A at the second last position of SEQ ID NO: 32 is optionally substituted by L and/or A at the last position of SEQ ID NO: 32 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, or below 3 x 10^{-10}M, or below 2 x 10^{-10}M, and comprises an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with SEQ ID NO: 33, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 33 are optionally missing, and wherein A at the second last position of SEQ ID NO: 33 is optionally substituted by L and/or A at the last position of SEQ ID NO: 33 is optionally substituted by N. Thus, in one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 90% amino acid sequence identity with SEQ ID NO: 33. In another embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 93% amino acid sequence identity with SEQ ID NO: 33; and in a further embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 95% amino acid sequence identity with SEQ ID NO: 33. In one embodiment, said ankyrin repeat domain comprises an amino acid sequence with at least 98% amino acid sequence identity with SEQ ID NO: 33; and in one embodiment, said ankyrin repeat domain comprises the amino acid sequence of SEQ ID NO: 33. Thus, in one embodiment, a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex according to the present invention binds said first target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, or below 3 x 10^{-10}M, or below 2 x 10^{-10}M, and comprises the amino acid sequence of SEQ ID NO: 33, wherein G at position 1 and/or S at position 2 of SEQ ID NO: 33 are optionally missing, and wherein A at the second last position of SEQ ID NO: 33 is optionally substituted by L and/or A at the last position of SEQ ID NO: 33 is optionally substituted by N. In one preferred embodiment, said target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In the context of the present invention, a typical and preferred determination of dissociation constants (K_D) of the recombinant binding proteins or designed repeat domains of the invention with binding specificity for a target peptide-MHC complex by Surface Plasmon Resonance (SPR) analysis is described in Example 2. Thus, in one embodiment said binding specificity for a target peptide-MHC complex of the recombinant binding proteins or designed repeat domains of the invention is determined in PBS by Surface Plasmon Resonance (SPR). In one embodiment said binding specificity for a target peptide-MHC complex of the recombinant binding proteins or designed repeat domains of the invention is determined in PBS by Surface Plasmon Resonance (SPR) as described in Example 2.

In one aspect of the invention, the recombinant binding protein of the invention further comprises a second designed repeat domain with binding specificity for a second target peptide-MHC complex.

In a preferred embodiment, said second target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, such as a bacterial infectious agent or a viral infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder.

In one embodiment, said second target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell. In one embodiment, said second target peptide is derived from an intracellular protein expressed in a tumor cell.

In one embodiment, said second target peptide is derived from a protein of an infectious agent, preferably a viral infectious agent. In one embodiment, said second target peptide is derived from a virus-specific protein.

In one embodiment, said second repeat domain with binding specificity for a second target peptide-MHC complex binds to said second target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10^{-9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, or below 3 x 10^{-10}M, or below 2 x 10^{-10}M, or below 10^{-10}M. In one embodiment, said second repeat domain with binding specificity for a second target peptide-MHC complex binds to said second target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M.

In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said second target peptide. Thus, in one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least one amino acid residue of said second target peptide. In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least two amino acid residues of said second target peptide. In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least three amino acid residues of said second target peptide. In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least four amino acid residues of said second target peptide. In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least five amino acid residues of said second target peptide. In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least six amino acid residues of said second target peptide. In one embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least seven amino acid residues of said second target peptide. In a further or alternative embodiment, said binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least one amino acid residue of said second MHC.

In one embodiment, said second MHC is MHC class I. In one embodiment, said second MHC is HLA-A*02. In one embodiment, said HLA-A*02 is HLA-A*0201. In one embodiment, said HLA-A*0201 has the amino acid sequence of SEQ ID NO: 73. Alternatively, in another embodiment, said second MHC is not HLA-A*02.

In one embodiment, said second MHC is MHC class I. In one embodiment, said second MHC is HLA-A*01. In one embodiment, said HLA-A*01 is HLA-A*0101. In one embodiment, said HLA-A*0101 has the amino acid sequence of SEQ ID NO: 218. Alternatively, in another embodiment, said second MHC is not HLA-A*01.

In one embodiment, said second target peptide is derived from the same protein as said first target peptide. In one embodiment, said second target peptide has the same amino acid sequence as said first target peptide. In a further embodiment, said second repeat domain has the same amino acid sequence as said first repeat domain. Alternatively, in another further embodiment, said second repeat domain has a different amino acid sequence as compared to said first repeat domain. In one embodiment, said second target peptide has a different amino acid sequence as compared to said first target peptide.

In another embodiment, said second target peptide is derived from a protein that is different from the protein, from which said first target peptide is derived.

In a preferred embodiment, said second designed repeat domain is a designed ankyrin repeat domain. In a particularly preferred embodiment, said second designed ankyrin repeat domain is a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects and embodiments herein.

In one aspect of the invention, the recombinant binding protein of the invention further comprises a third designed repeat domain with binding specificity for a third target peptide-MHC complex. In a preferred embodiment, said third designed repeat domain is a designed ankyrin repeat domain. In a particularly preferred embodiment, said third designed ankyrin repeat domain is a designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects and embodiments herein.

In the context of the present invention, when the recombinant binding protein of the invention comprises a first and a second designed repeat domain, and when said first and said second repeat domains have binding specificity for the same target peptide-MHC complex, i.e. the second target peptide has the same amino acid sequence as the first target peptide, and when said first and said second repeat domains are identical in sequence, said recombinant binding protein is bivalent. In the context of the present invention, when the recombinant binding protein of the invention comprises a first and a second designed repeat domain, and when said first and said second repeat domains have binding specificity for the same target peptide-MHC complex, i.e. the second target peptide has the same amino acid sequence as the first target peptide, and when said first and said second repeat domains are different in sequence, said recombinant binding protein is biparatopic. In the context of the present invention, when the recombinant binding protein of the invention comprises a first and a second designed repeat domain, and when said first and said second repeat domains have binding specificity for different target peptide-MHC complexes, i.e. the second target peptide has a different amino acid sequence as compared to the first target peptide, said recombinant binding protein is bispecific. The different target peptides may be derived from the same protein or from different proteins. In the context of the present invention, the same definitions apply analogously to a recombinant binding protein of the invention which comprises a first and a second and a third designed repeat domain, wherein each of the first, second and third designed repeat domains has binding specificity for a target peptide-MHC complex.

In one embodiment, the recombinant binding protein of the invention is monovalent, bivalent, trivalent, multivalent, monoparatopic, biparatopic, triparatopic, multiparatopic, monospecific, bispecific, trispecific, or multispecific. In one particular embodiment, the recombinant binding protein of the invention is monovalent. In one particular embodiment, the recombinant binding protein of the invention is bivalent. In one particular embodiment, the recombinant binding protein of the invention is trivalent. In one particular embodiment, the recombinant binding protein of the invention is multivalent. In one particular embodiment, the recombinant binding protein of the invention is monoparatopic. In one particular embodiment, the recombinant binding protein of the invention is biparatopic, In one particular embodiment, the recombinant binding protein of the invention is triparatopic, In one particular embodiment, the recombinant binding protein of the invention is multiparatopic. In one particular embodiment, the recombinant binding protein of the invention is monospecific. In one particular embodiment, the recombinant binding protein of the invention is bispecific. In one particular embodiment, the recombinant binding protein of the invention is trispecific. In one particular embodiment, the recombinant binding protein of the invention is multispecific. It should be understood that the above embodiments are combinable with each other. As an example, a recombinant binding protein of the invention comprising two identical repeat domains with specificity for a first pMHC complex and further comprising a third repeat domain with specificity for a second, different pMHC complex would be bivalent and bispecific at the same time. Accordingly, in one particular embodiment, the recombinant binding protein of the invention is any combination of monovalent, bivalent, trivalent or multivalent, and monoparatopic, biparatopic, triparatopic or multiparatopic, and/or monospecific, bispecific, trispecific, or multispecific.

As a non-limiting example, a bivalent binding protein according to the invention may comprise at least two occurrences of a repeat domain having the amino acid sequence of SEQ ID NO: 21. An example of such a bivalent protein is provided by SEQ ID NO: 16. As a further non-limiting example, a biparatopic binding protein according to the invention may comprise at least one repeat domain having the amino acid sequence of SEQ ID NO: 20 and at least one repeat domain having the amino acid sequence of SEQ ID NO: 21, or it may comprise at least one repeat domain having the amino acid sequence of SEQ ID NO: 21 and at least one repeat domain having the amino acid sequence of SEQ ID NO: 22. Examples of such biparatopic proteins are provided by SEQ ID NO: 17 and SEQ ID NO: 18, respectively.

In one embodiment, when the recombinant binding protein of the invention comprises two or more ankyrin repeat domains, for example when the recombinant binding protein of the invention comprises two or three ankyrin repeat domains, said two or more ankyrin repeat domains, for example said two or three ankyrin repeat domains, may be linked with a peptide linker. In one embodiment, said peptide linker is a proline-threonine rich peptide linker. In one embodiment, said peptide linker is the proline-threonine rich peptide linker of SEQ ID NO: 1 or 2. In one embodiment, said two or more ankyrin repeat domains, for example said two or three ankyrin repeat domains, are linked with the proline-threonine rich peptide linker of SEQ ID NO: 1 or 2. In another embodiment, said peptide linker is a glycine-serine rich peptide linker. In one embodiment, said peptide linker is the glycine-serine rich peptide linker of SEQ ID NO: 3. In one embodiment, said two or more ankyrin repeat domains, for example said two or three ankyrin repeat domains, are linked with the glycine-serine rich peptide linker of SEQ ID NO: 3. In one embodiment, when the recombinant binding protein of the invention comprises three or more ankyrin repeat domains, said three or more ankyrin repeat domains may be linked with different peptide linkers, for example, proline-threonine rich peptide linkers and serine-glycine rich peptides linkers, such as, for example, the peptide linkers of SEQ ID NOs: 1 to 3.

In one embodiment, the recombinant binding protein of the invention comprises two or three ankyrin repeat domains, wherein each of said two or three ankyrin repeat domains independently comprises an ankyrin repeat module as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein comprises two ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said two ankyrin repeat domains independently comprises an ankyrin repeat module as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises two ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said two ankyrin repeat domains comprises a first ankyrin repeat module and a second ankyrin repeat module as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises two ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said two ankyrin repeat domains comprises a first ankyrin repeat module, a second ankyrin repeat module and a third ankyrin repeat module as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises two or three ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said two or three ankyrin repeat domains independently comprises an amino acid sequence as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises exactly two ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said two ankyrin repeat domains independently comprises an amino acid sequence as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises three ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said three ankyrin repeat domains independently comprises an amino acid sequence as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises exactly three ankyrin repeat domains with binding specificity for a target peptide-MHC complex, wherein each of said three ankyrin repeat domains independently comprises an amino acid sequence as described more specifically in any of the aspects and embodiments herein.

In one embodiment, the recombinant binding protein of the invention comprises a polypeptide consisting of a first and a second ankyrin repeat domain with binding specificity for a first and a second target peptide-MHC complex, respectively, linked with a peptide linker, wherein said polypeptide comprises or consists of an amino acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 16 to 18 are optionally missing, and wherein A at the second last position of each of said ankyrin repeat domains of SEQ ID NOs: 16 to 18 is optionally substituted by L and/or A at the last position of each of said ankyrin repeat domains of SEQ ID NOs: 16 to 18 is optionally substituted by N. Thus, in one embodiment, said polypeptide comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18. In one embodiment, said polypeptide comprises an amino acid sequence with at least 90% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18. In one embodiment, said polypeptide comprises an amino acid sequence with at least 93% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18. In one embodiment, said polypeptide comprises an amino acid sequence with at least 95% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18. In one embodiment, said polypeptide comprises an amino acid sequence with at least 98% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18. In one embodiment, said polypeptide comprises the amino acid sequence of any one of SEQ ID NOs: 16 to 18. In one embodiment, said polypeptide consists of the amino acid sequence of any one of SEQ ID NOs: 16 to 18. In one embodiment, said recombinant binding protein comprising a polypeptide consisting of two pMHC-specific ankyrin repeat domains binds the target peptide-MHC complex(es) in PBS with a dissociation constant (K_D) below 10^{-7}M, or below 5 x 10^{-8}M, or below 3 x 10^{-8}M, or below 2 x 10^{-8}M, or below 10^{-8}M, or below 5 x 10^{-9}M, or below 3 x 10-^{9}M, or below 2 x 10^{-9}M, or below 10^{-9}M, or below 5 x 10^{-10}M, or below 3 x 10^{-10}M, or below 2 x 10^{-10}M, or below 10^{-10}M. In one preferred embodiment, said first and said second target peptides independently have the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

In one embodiment, the recombinant binding protein of the invention comprises a single designed ankyrin repeat domain with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects and embodiments herein or comprises a combination of two, three, four, five or more designed ankyrin repeat domains with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects and embodiments herein.

In one aspect of the invention, the recombinant binding protein of the invention further comprises a binding agent.

In one particular embodiment, said binding agent has binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In a further embodiment, said protein expressed on the surface of a T-cell is a protein that is part of the T-cell receptor complex. As an example, in one particular embodiment, a recombinant binding protein of the invention further comprises a binding agent with binding specificity for cluster of differentiation 3 (CD3).

In the context of the present invention, a binding agent may be an antibody, an antibody mimetic, including a scaffold protein or a repeat protein, a designed repeat domain, preferably a designed ankyrin repeat domain, or any other suitable binding molecules known in the art. In one embodiment, said binding agent is an antibody. In another embodiment, said binding agent is a designed repeat domain, preferably a designed ankyrin repeat domain.

In one particular embodiment, said binding agent comprises or consists of an antibody with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In a further embodiment, said protein expressed on the surface of a T-cell is a protein that is part of the T-cell receptor complex. As an example, in one particular embodiment, a recombinant binding protein of the invention further comprises an antibody with binding specificity for CD3.

In one particular embodiment, said binding agent comprises or consists of a designed repeat domain, preferably a designed ankyrin repeat domain, with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In a further embodiment, said protein expressed on the surface of a T-cell is a protein that is part of the T-cell receptor complex. As an example, in one particular embodiment, a recombinant binding protein of the invention further comprises a designed ankyrin repeat domain with binding specificity for CD3.

In one embodiment, said binding agent is linked, conjugated, fused or otherwise physically attached to said pMHC-specific ankyrin repeat domain or said two, three or more pMHC-specific ankyrin repeat domains. In one embodiment, said binding agent is covalently linked to said pMHC-specific ankyrin repeat domain or said two, three or more pMHC-specific ankyrin repeat domains. In one embodiment, said binding agent is covalently linked to said pMHC-specific ankyrin repeat domain or said two, three or more pMHC-specific ankyrin repeat domains with a peptide linker. In one embodiment, said peptide linker is a proline-threonine rich peptide linker. In one embodiment, said peptide linker is the proline-threonine rich peptide linker of SEQ ID NO: 1 or 2. In one embodiment, said binding agent is covalently linked to said pMHC-specific ankyrin repeat domain or said two, three or more pMHC-specific ankyrin repeat domains with the proline-threonine rich peptide linker of SEQ ID NO: 1 or 2. In another embodiment, said peptide linker is a glycine-serine rich peptide linker. In one embodiment, said peptide linker is the glycine-serine rich peptide linker of SEQ ID NO: 3. In one embodiment, said binding agent is covalently linked to said pMHC-specific ankyrin repeat domain or said two, three or more pMHC-specific ankyrin repeat domains with the glycine-serine rich peptide linker of SEQ ID NO: 3.

It was surprisingly and unexpectedly found by the inventors of the present invention that the shorter the linker between said binding agent and said pMHC-specific ankyrin repeat domain (or said two, three or more pMHC-specific ankyrin repeat domains) the higher the potency of the construct in a T cell engager format (see Example 11). Thus, in a further preferred embodiment, the amino acid sequence of the peptide linker described herein has a length of less than 38 amino acids, preferably of at most 37, more preferably at most 24, even more preferably at most 18, even more preferably at most 11, most preferably at most 6 amino acids. In another preferred embodiment, the amino acid sequence of said peptide linker has a length from 1 to 37, from 1 to 24, from 1 to 23, from 1 to 18, from 1 to 17, from 1 to 11, from 1 to 10, from 1 to 6, from 6 to 37, from 6 to 24, from 6 to 23, from 6 to 18, from 6 to 17, or from 6 to 11 amino acid residues. Preferably, said peptide linker is a proline-threonine-rich peptide linker. In a further preferred embodiment, the amino acid sequence of said peptide linker has a length of less than 18 amino acids and of at least 1 amino acid, i.e. the peptide linker has a length from 1 to 17 amino acids. In one embodiment, said linker has the amino acid sequence as provided in any one of SEQ ID NOs: 1 and 277 to 280. In a preferred embodiment said peptide linker has the amino acid sequence as provided in SEQ ID NO: 278. In a more preferred embodiment said peptide linker has the amino acid sequence as provided in SEQ ID NO: 279. In a most preferred embodiment said peptide linker has the amino acid sequence as provided in SEQ ID NO: 280.

In one aspect of the invention, the recombinant binding protein of the invention, comprises one, two, three or more pMHC-specific ankyrin repeat domains as described more specifically in any of the aspects and embodiments herein, and further comprises a CD3-specific binding agent, wherein the linker between said binding agent and said pMHC-specific ankyrin repeat domain (or said two, three or more pMHC-specific ankyrin repeat domains) is a peptide linker as described in any of the above embodiments. Preferably, said peptide linker is a proline-threonine rich peptide linker having a length from 1 to 17 amino acids (such as, e.g., a peptide linker having the amino acid sequence as provided in SEQ ID NO: 279 or SEQ ID NO: 280).

In one aspect of the invention, the recombinant binding protein of the invention, wherein said binding protein comprises one, two, three or more pMHC-specific ankyrin repeat domains as described more specifically in any of the aspects and embodiments herein, and wherein said binding protein further comprises a CD3-specific binding agent, is capable of facilitating infection- or tumor-localized activation of T cells. In such instances, the recombinant binding protein of the invention may be used in a T-cell engager format to locally activate T-cells against target peptide-MHC complex presenting tumor cells and/or target peptide-MHC complex presenting infectious cells.

In one aspect of the invention, the recombinant binding protein of the invention is capable of inhibiting recognition of said first target peptide-MHC complex and/or, if said binding protein comprises said second repeat domain, of said second target peptide-MHC complex by T-cell receptors. In other words, the recombinant binding protein of the invention is capable of inhibiting the ability of the immune system to recognize the target peptide-MHC complex(es). In such instances, the recombinant binding protein of the invention may be used for the treatment of an autoimmune disease. Measuring the recognition of a target peptide-MHC complex by T-cell receptors can be performed by any suitable methods known to the skilled person in the art or by suitable T-cell activation assays, such as the assays described in the Examples of the present application or variations thereof.

In one embodiment, the recombinant binding protein of the invention further comprises a polypeptide tag. In the context of the present invention, a polypeptide tag is an amino acid sequence attached to a polypeptide/protein, wherein said amino acid sequence is useful for the purification, detection, or targeting of said polypeptide/protein, or wherein said amino acid sequence improves the physicochemical behavior of the polypeptide/protein, or wherein said amino acid sequence possesses an effector function. The individual polypeptide tags of a binding protein may be connected to other parts of the binding protein directly or via peptide linkers. Polypeptide tags are all well known in the art and are fully available to the person skilled in the art. Examples of polypeptide tags are small polypeptide sequences, for example, His, HA, myc, FLAG, or Strep-tags, or polypeptides such as enzymes (for example alkaline phosphatase), which allow the detection of said polypeptide/protein, or polypeptides which can be used for targeting (such as immunoglobulins or fragments thereof) and/or as effector molecules.

In one embodiment, the recombinant binding protein of the invention further comprises a peptide linker. In the context of the present invention, a peptide linker is an amino acid sequence, which is able to link, for example, two protein domains, a polypeptide tag and a protein domain, a protein domain and a non-proteinaceous compound or polymer such as polyethylene glycol, a protein domain and a biologically active molecule, a protein domain and a T-cell-specific ankyrin repeat domain, or two sequence tags. Peptide linkers are known to the person skilled in the art. A list of examples is provided in the description of patent application WO2002/020565. Particular examples of such linkers are glycine-serine-linkers and proline-threonine-linkers of variable lengths. Examples of a glycine-serine-linker are the amino acid sequence GS and the amino acid sequence of SEQ ID NO: 3, and examples of a proline-threonine-linker are the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NOs: 277 to 280.

In another aspect, the invention relates to a nucleic acid encoding the amino acid sequence of the designed repeat domain of the present invention or of the pMHC-specific recombinant binding protein of the present invention. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of the pMHC-specific recombinant binding protein of the present invention. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of the designed repeat domain of the present invention. In one embodiment, the invention relates to a nucleic acid encoding an amino acid sequence selected from the group consisting of SEQ ID NO: 20 to 33. In one embodiment, the invention relates to a nucleic acid encoding an amino acid sequence selected from the group consisting of SEQ ID NO: 20, 21, 25, 32 and 33. In one embodiment, the invention relates to a nucleic acid encoding an amino acid sequence selected from the group consisting of SEQ ID NO: 20, 21 and 33. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of SEQ ID NO: 20. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of SEQ ID NO: 21. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of SEQ ID NO: 27. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of SEQ ID NO: 32. In one embodiment, the invention relates to a nucleic acid encoding the amino acid sequence of SEQ ID NO: 33. In one embodiment, the invention relates to the nucleic acid sequence of SEQ ID NO: 78, which encodes the amino acid sequence of SEQ ID NO: 20. In one embodiment, the invention relates to the nucleic acid sequence of SEQ ID NO: 79, which encodes the amino acid sequence of SEQ ID NO: 21. In one embodiment, the invention relates to the nucleic acid sequence of SEQ ID NO: 80, which encodes the amino acid sequence of SEQ ID NO: 32. Furthermore, the invention relates to vectors comprising any nucleic acid of the invention. Nucleic acids are well known to the skilled person in the art. In the examples, nucleic acids were used to produce designed ankyrin repeat domains or recombinant binding proteins of the invention in *E. coli*.

In another aspect, the invention relates to a pharmaceutical composition comprising the pMHC-specific recombinant binding protein of the present invention and/or the nucleic acid of the present invention, and optionally a pharmaceutically acceptable carrier and/or diluent.

Pharmaceutically acceptable carriers and/or diluents are known to the person skilled in the art and are explained in more detail below. Even further, a diagnostic composition is provided comprising one or more of the above-mentioned recombinant binding proteins and/or nucleic acids, in particular recombinant binding proteins of the present invention.

A pharmaceutical composition comprises a recombinant binding protein and/or a nucleic acid, preferably a recombinant binding protein and/or a nucleic acid as described more specifically in any of the aspects or embodiments herein, and a pharmaceutically acceptable carrier, excipient or stabilizer, for example as described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed., 1980.

Suitable carriers, excipients or stabilizers known to one of skill in the art include, for example, saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. A pharmaceutical composition may also be a combination formulation, comprising an additional active agent, such as an anti-cancer agent or an anti-angiogenic agent, or an additional bioactive compound.

The formulations to be used for *in vivo* administration must be aseptic or sterile. This is readily accomplished by filtration through sterile filtration membranes.

In one embodiment, a pharmaceutical composition comprises at least one recombinant binding protein as described herein and a detergent, such as, e.g., a nonionic detergent, a buffer, such as, e.g., phosphate buffer, and a sugar, such as, e.g., sucrose. In one embodiment, such a composition comprises recombinant binding proteins as described above and PBS.

In another aspect, the invention provides a method of tumor-localized activation of immune cells, preferably T-cells, in a mammal, preferably a human, the method comprising the step of administering to said mammal the pMHC-specific recombinant binding protein of the invention comprising a first repeat domain with binding specificity for a first target peptide-MHC complex and optionally comprising a second repeat domain with binding specificity for a second target peptide-MHC complex and further comprising a binding agent with binding specificity for a protein expressed on the surface of immune cells, preferably T-cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in a tumor cell. In one embodiment, said first and/or, if present, said second target peptide is derived from an intracellular protein expressed in a tumor cell. In one embodiment, said first and/or, if present, said second target peptide is derived from a tumor-specific protein. In one embodiment, said first and/or, if present, said second target peptide is derived from an intracellular tumor-specific protein. In one embodiment, said first and/or, if present, said second target peptide is derived from NY-ESO-1. In one embodiment, said first and/or, if present, said second target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34. In one embodiment, said first and/or, if present, said second target peptide is derived from MAGE-A3. In one embodiment, said first and/or, if present, said second target peptide has the amino acid sequence of SEQ ID NO: 155. In one embodiment, said binding agent has binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In one embodiment, said binding agent is an antibody with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In one embodiment, said binding agent is a designed repeat domain with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In one embodiment, said binding agent is a designed ankyrin repeat domain with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In a preferred embodiment, said protein expressed on the surface of an immune cell is a protein that is part of the T-cell receptor complex, such as, e.g., CD3. In one embodiment, said first ankyrin repeat domain and/or, if present, said second ankyrin repeat are independently designed ankyrin repeat domains with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects and embodiments herein.

Further provided is the pMHC-specific recombinant binding protein of the present invention, the nucleic acid of the invention, or the pharmaceutical composition of the invention for use in a method of tumor-localized activation of immune cells as described herein.

In another aspect, the invention provides a method of infection-localized activation of immune cells, preferably T-cells, in a mammal, preferably a human, the method comprising the step of administering to said mammal the pMHC-specific recombinant binding protein of the invention comprising a first repeat domain with binding specificity for a first target peptide-MHC complex and optionally comprising a second repeat domain with binding specificity for a second target peptide-MHC complex and further comprising a binding agent with binding specificity for a protein expressed on the surface of immune cells, preferably T-cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein of an infectious agent. In one embodiment, said infection is a viral infection. In one embodiment, said first and/or, if present, said second target peptide is derived from a protein of a viral infectious agent. In one embodiment, said first and/or, if present, said second target peptide is derived from a virus-specific protein. In one embodiment, said first and/or, if present, said second target peptide is derived from EBNA-1. In one embodiment, said first and/or, if present, said second target peptide has the amino acid sequence of SEQ ID NO: 92. In one embodiment, said first and/or, if present, said second target peptide is derived from HBcAg. In one embodiment, said first and/or, if present, said second target peptide has the amino acid sequence of SEQ ID NO: 255. In one embodiment, said binding agent has binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In one embodiment, said binding agent is an antibody with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In one embodiment, said binding agent is a designed repeat domain with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In one embodiment, said binding agent is a designed ankyrin repeat domain with binding specificity for a protein expressed on the surface of an immune cell, preferably a T-cell, even more preferably a CD8+ cytotoxic T-cell. In a preferred embodiment, said protein expressed on the surface of an immune cell is a protein that is part of the T-cell receptor complex, such as, e.g., CD3. In one embodiment, said first ankyrin repeat domain and/or, if present, said second ankyrin repeat are independently designed ankyrin repeat domains with binding specificity for a target peptide-MHC complex as described more specifically in any of the aspects and embodiments herein.

Further provided is the pMHC-specific recombinant binding protein of the present invention, the nucleic acid of the invention, or the pharmaceutical composition of the invention for use in a method of infection-localized activation of immune cells as described herein.

In another aspect, the invention provides a method of treating a medical condition, the method comprising the step of administering to a patient in need thereof a therapeutically effective amount of the pMHC-specific recombinant binding protein of the invention, the nucleic acid of the invention or the pharmaceutical composition of the invention.

Further provided is the pMHC-specific recombinant binding protein, the nucleic acid, or the pharmaceutical composition of the invention for use in a method of treating a medical condition as described herein.

In another aspect, the invention provides a method of diagnosing a medical condition in a mammal, preferably a human, the method comprising the step of:

(i) contacting a cell or tissue sample obtained from said mammal with the recombinant binding protein of the invention; and

(ii) detecting specific binding of said binding protein to said cell or tissue sample. In one embodiment, said tissue is tumor tissue.

In the context of the invention, the terms “medical condition”, “disease” and “disorder” are used interchangeably and include but are not limited to cancer, infectious disease, and autoimmune disease. In one preferred embodiment, said medical condition is a cancer, an infectious disease, preferably a viral infectious disease, or an autoimmune disease. In one preferred embodiment, said medical condition is a cancer. In one embodiment, such cancer is selected from the group consisting of epithelial malignancies (primary and metastatic), including but not limited to lung, colorectal, gastric, bladder, ovarian and breast carcinomas, blood cell malignancies, including but not limited to leukemia, lymphoma, and myeloma, sarcomas, including but not limited to bone and soft tissue sarcomas, and melanoma. In one preferred embodiment, such cancer is selected from the group consisting of liposarcoma, neuroblastoma, synovial sarcoma, melanoma and ovarian cancer. In another preferred embodiment, such cancer is selected from the group consisting of melanoma, lung cancer, liver cancer, stomach cancer, skin cancer, neuroblastoma, soft tissue sarcoma, bladder cancer, testicular cancer and ovarian cancer. In one preferred embodiment, said medical condition is an infectious disease, preferably a viral infectious disease. In one embodiment, such infectious disease is a viral infection caused by hepatitis B virus (HBV). In another embodiment such infectious disease is a viral infection caused by Epstein–Barr virus (EBV). In one preferred embodiment, said medical condition is an autoimmune disease. In one embodiment, such autoimmune disease is selected from the group consisting of systemic lupus erythematosus, rheumatoid arthritis and type I diabetes.

In one embodiment, the invention relates to the use of the pharmaceutical composition, nucleic acid or recombinant binding protein according to the present invention for the treatment of a disease. For that purpose, the pharmaceutical composition, the nucleic acid or the recombinant binding protein according to the present invention is administered, to a patient in need thereof, in a therapeutically effective amount. Administration may include topical administration, oral administration, and parenteral administration. The typical route of administration is parenteral administration. In parental administration, the pharmaceutical composition of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. The dosage and mode of administration will depend on the individual to be treated and the particular disease.

Further, any of the above-mentioned pharmaceutical compositions or recombinant binding proteins is considered for the treatment of a disorder.

In one embodiment, said recombinant binding protein or pharmaceutical composition of the invention as described herein is applied intravenously. For parenteral application, said recombinant binding protein or pharmaceutical composition can be injected as bolus injection or by slow infusion at a therapeutically effective amount.

In one embodiment, the invention relates to a method of treatment of a medical condition, the method comprising the step of administering, to a patient in need of such a treatment, a therapeutically effective amount of the recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention. In one embodiment, the invention relates to the use of the recombinant binding protein, nucleic acid, or pharmaceutical composition of the present invention for the treatment of a medical condition. In one embodiment, the invention relates to the recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention for use in the treatment of a medical condition. In one embodiment, the invention relates to the use of the pharmaceutical composition, recombinant binding protein, or nucleic acid of the invention, as a medicament for the treatment of a medical condition. In one embodiment, the invention relates to the use of the pharmaceutical composition, recombinant binding protein, or nucleic acid of the invention, for manufacturing of a medicament. In one embodiment, the invention relates to the use of the pharmaceutical composition, recombinant binding protein, or nucleic acid of the invention, for manufacturing of a medicament for the treatment of a medical condition. In one embodiment, the invention relates to a process for the manufacturing of a medicament for the treatment of a medical condition, wherein the pharmaceutical composition, recombinant binding protein, or nucleic acid of the invention is an active ingredient of the medicament. In one embodiment, the invention relates to a process of treatment of a medical condition using the pharmaceutical composition, recombinant binding protein, or nucleic acid of the invention.

In a further embodiment, the invention relates to the use of the recombinant binding protein, nucleic acid or pharmaceutical composition of the invention for the manufacture of a medicament that is used for the treatment of a medical condition, preferably a neoplastic disease, more preferably cancer.

In one embodiment, the invention relates to a recombinant binding protein comprising any of the above mentioned designed ankyrin repeat domains for therapeutic purposes.

In one aspect, the invention relates to a kit comprising the recombinant binding protein of the invention. In one embodiment, the invention relates to a kit comprising a nucleic acid encoding the recombinant binding protein of the invention. In one embodiment, the invention relates to a kit comprising the pharmaceutical composition of the invention. In one embodiment, the invention relates to a kit comprising the recombinant binding protein of the invention, and/or a nucleic acid encoding the recombinant binding protein of the invention, and/or the pharmaceutical composition of the invention. In one embodiment, the invention relates to a kit comprising a recombinant binding protein comprising one or more peptide-MHC-specific ankyrin repeat domains of the invention, for example one or more peptide-MHC-specific ankyrin repeat domains independently having the amino acid sequence of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 27, SEQ ID NO: 32 or SEQ ID NO: 33, and/or a nucleic acid encoding a recombinant binding protein comprising one or more peptide-MHC-specific ankyrin repeat domains of the invention, for example one or more peptide-MHC-specific ankyrin repeat domains independently having the amino acid sequence of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 27, SEQ ID NO: 32 or SEQ ID NO: 33, and/or a pharmaceutical composition comprising a recombinant binding protein comprising one or more peptide-MHC-specific ankyrin repeat domains of the invention, for example one or more peptide-MHC-specific ankyrin repeat domains independently having the amino acid sequence of SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 27, SEQ ID NO: 32 or SEQ ID NO: 33.

In another aspect, the invention provides a method of targeting tumor cells in a patient having a tumor for destruction of the tumor cells, the method comprising the step of administering to the patient a therapeutically effective amount of the binding protein, nucleic acid or pharmaceutical composition of the invention, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the tumor cells, preferably an intracellular protein expressed in the tumor cells. In one embodiment, said binding protein further comprises a toxic agent capable of killing a tumor cell.

Further provided is the recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention for use in a method of targeting tumor cells in a patient having a tumor for destruction of the tumor cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the tumor cells, preferably an intracellular protein expressed in the tumor cells. In one embodiment, said binding protein further comprises a toxic agent capable of killing a tumor cell.

In another aspect, the invention provides a method of targeting infected cells in a patient having a viral infectious disease for destruction of the infected cells, the method comprising the step of administering to the patient a therapeutically effective amount of the binding protein, nucleic acid or pharmaceutical composition of the invention, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the infected cells, preferably a virus-specific protein. In one embodiment, said binding protein further comprises a toxic agent capable of killing an infected cell.

Further provided is the recombinant binding protein, nucleic acid, or pharmaceutical composition of the invention for use in a method of targeting infected cells in a patient having a viral infectious disease for destruction of the infected cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the infected cells, preferably a virus-specific protein. In one embodiment, said binding protein further comprises a toxic agent capable of killing an infected cell.

In a preferred embodiment, the medical condition to be treated by the method of treatment of the invention or by the binding protein, the nucleic acid or the pharmaceutical composition of the invention is a cancer or tumor selected from the group consisting of liposarcoma, neuroblastoma, synovial sarcoma, melanoma and ovarian cancer.In another preferred embodiment, the recombinant binding proteins of the invention have binding specificity for a peptide-MHC (pMHC) complex, wherein the peptide is derived from NY-ESO-1.

The invention is not restricted to the particular embodiments described in the Examples.

This specification refers to a number of amino acid sequences, nucleic acid sequences and SEQ ID NOs that are disclosed in the appended Sequence Listing, which is herewith incorporated by reference in its entirety.

DEFINITIONS

Unless defined otherwise herein, all technical and scientific terms used herein shall have the meanings that are commonly understood by those of ordinary skill in the art to which the present invention belongs.

In the context of the present invention, the term "collection" refers to a population comprising at least two different entities or members. Preferably, such a collection comprises at least 10^5, more preferably more than 10^7, and most preferably more than 10^9 different members. A "collection" may as well be referred to as a "library" or a "plurality".

The term "nucleic acid molecule" refers to a polynucleotide molecule, which may be a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule, either single stranded or double stranded, and includes modified and artificial forms of DNA or RNA. A nucleic acid molecule may either be present in isolated form, or be comprised in recombinant nucleic acid molecules or vectors.

In the context of the present invention the term "protein" refers to a molecule comprising a polypeptide, wherein at least part of the polypeptide has, or is able to acquire, a defined three-dimensional arrangement by forming secondary, tertiary, and/or quaternary structures within a single polypeptide chain and/or between multiple polypeptide chains. If a protein comprises two or more polypeptide chains, the individual polypeptide chains may be linked non-covalently or covalently, e.g. by a disulfide bond between two polypeptides. A part of a protein, which individually has, or is able to acquire, a defined three-dimensional arrangement by forming secondary and/or tertiary structure, is termed "protein domain". Such protein domains are well known to the practitioner skilled in the art.

The term “recombinant” as used in recombinant protein, recombinant polypeptide and the like, means that said protein or polypeptide is produced by the use of recombinant DNA technologies well known to the practitioner skilled in the art. For example, a recombinant DNA molecule (e.g. produced by gene synthesis) encoding a polypeptide can be cloned into a bacterial expression plasmid (e.g. pQE30, QIAgen), yeast expression plasmid, mammalian expression plasmid, or plant expression plasmid, or a DNA enabling in vitro expression. If, for example, such a recombinant bacterial expression plasmid is inserted into appropriate bacteria (e.g. *Escherichia coli*), these bacteria can produce the polypeptide(s) encoded by this recombinant DNA. The correspondingly produced polypeptide or protein is called a recombinant polypeptide or recombinant protein.

In the context of the present invention, the term "binding protein" refers to a protein comprising a binding domain. A binding protein may also comprise two, three, four, five or more binding domains. Preferably, said binding protein is a recombinant binding protein. More preferably, the binding proteins of the instant invention comprise an ankyrin repeat domain with binding specificity for a pMHC complex.

Furthermore, any such binding protein may comprise additional polypeptides (such as e.g. polypeptide tags, peptide linkers, fusion to other proteinaceous domains with binding specificity, cytokines, hormones, or antagonists), or chemical modifications (such as coupling to polyethylene-glycol, toxins, small molecules, antibiotics and alike) well known to the person skilled in the art. In some embodiments, the binding protein further comprises a binding agent with binding specificity for a protein expressed on the surface of an immune cell. In some embodiments, the binding protein further comprises a toxic agent capable of killing a tumor cell and/or an infected cell. Examples of toxic agents include, but are not limited to, vinblastine, doxorubicin, topoisomerase I inhibitors, calicheamicins, duocarmycin-hydroxybenzamide-azaindole (DUBA), pyrrolobenzodiazepine dimers (PBD), and derivatives of the microtubule inhibitor family, such as auristatin and maytansine, e.g. monomethyl auristatin E (MMAE), monomethyl auristatin F (MMAF), drug maytansinoid 1 (DM1) and drug maytansinoid 4 (DM4).

The term “binding domain” means a protein domain exhibiting binding specificity for a target. Preferably, said binding domain is a recombinant binding domain.

The term "target" refers to an individual molecule such as a nucleic acid molecule, a peptide, polypeptide or protein, a carbohydrate, or any other naturally occurring molecule, including any part of such individual molecule, or to complexes of two or more of such molecules, or to a whole cell or a tissue sample, or to any non-natural compound. Preferably, a target is a naturally occurring or non-natural polypeptide or protein, or a polypeptide or protein containing chemical modifications, for example, naturally occurring or non-natural phosphorylation, acetylation, or methylation. In the context of the present invention, peptide-MHC complexes and peptide-MHC presenting cells and tissues are targets of pMHC-specific binding proteins. Furthermore, CD3 and CD3-expressing cells, such as, e.g., T-cells, are targets of pMHC-specific binding proteins of the invention that further comprise a binding agent with binding specificity for a protein expressed on the surface of an immune cell, such as CD3.

In the context of the present invention, the term "polypeptide" relates to a molecule consisting of a chain of multiple, i.e. two or more, amino acids linked via peptide bonds. Preferably, a polypeptide consists of more than eight amino acids linked via peptide bonds. The term “polypeptide” also includes multiple chains of amino acids, linked together by S-S bridges of cysteines. Polypeptides are well-known to the person skilled in the art.

Patent application WO2002/020565 and Forrer et al., 2003 (Forrer, P., Stumpp, M.T., Binz, H.K., Plückthun, A., 2003. FEBS Letters 539, 2-6), contain a general description of repeat protein features and repeat domain features, techniques and applications. The term "repeat protein" refers to a protein comprising one or more repeat domains. Preferably, a repeat protein comprises one, two, three, four, five or six repeat domains. Furthermore, said repeat protein may comprise additional non-repeat protein domains, polypeptide tags and/or peptide linkers. The repeat domains can be binding domains.

The term "repeat domain" refers to a protein domain comprising two or more consecutive repeat modules as structural units, wherein said repeat modules have structural and sequence homology. Preferably, a repeat domain also comprises an N-terminal and/or a C-terminal capping module. For clarity, a capping module can be a repeat module. Such repeat domains, repeat modules, and capping modules, sequence motives, as well as structural homology and sequence homology are well known to the practitioner in the art from examples of ankyrin repeat domains (Binz et al., J. Mol. Biol. *332*, 489–503, 2003; Binz et al., 2004, loc. cit.; WO2002/020565; WO2012/069655), leucine-rich repeat domains (WO2002/020565), tetratricopeptide repeat domains (Main, E.R., Xiong, Y., Cocco, M.J., D'Andrea, L., Regan, L., Structure 11(5), 497-508, 2003), and armadillo repeat domains (WO2009/040338). It is further well known to the practitioner in the art, that such repeat domains are different from proteins comprising repeated amino acid sequences, where every repeated amino acid sequence is able to form an individual domain (for example FN3 domains of Fibronectin).

The term "ankyrin repeat domain" refers to a repeat domain comprising two or more consecutive ankyrin repeat modules as structural units, wherein said ankyrin repeat modules have structural and sequence homology.

The term "designed" as used in designed repeat protein, designed repeat domain and the like refers to the property that such repeat proteins and repeat domains, respectively, are man-made and do not occur in nature. The binding proteins of the instant invention are designed repeat proteins and they comprise at least one designed repeat domain. Preferably, the designed repeat domain is a designed ankyrin repeat domain.

The term "target interaction residues" refers to amino acid residues of a repeat module, which contribute to the direct interaction with a target.

The terms "framework residues" or “framework positions” refer to amino acid residues of a repeat module, which contribute to the folding topology, i.e. which contribute to the fold of said repeat module or which contribute to the interaction with a neighboring module. Such contribution may be the interaction with other residues in the repeat module, or the influence on the polypeptide backbone conformation as found in α-helices or β-sheets, or the participation in amino acid stretches forming linear polypeptides or loops.

Such framework and target interaction residues may be identified by analysis of the structural data obtained by physicochemical methods, such as X-ray crystallography, NMR and/or CD spectroscopy, or by comparison with known and related structural information well known to practitioners in structural biology and/or bioinformatics.

The term "repeat modules" refers to the repeated amino acid sequence and structural units of the designed repeat domains, which are originally derived from the repeat units of naturally occurring repeat proteins. Each repeat module comprised in a repeat domain is derived from one or more repeat units of a family or subfamily of naturally occurring repeat proteins, preferably the family of ankyrin repeat proteins. Furthermore, each repeat module comprised in a repeat domain may comprise a “repeat sequence motif” deduced from homologous repeat modules obtained from repeat domains selected on a target, e.g. as described in Example 1, and having the same target specificity.

Accordingly, the term "ankyrin repeat module" refers to a repeat module, which is originally derived from the repeat units of naturally occurring ankyrin repeat proteins. Ankyrin repeat proteins are well known to the person skilled in the art.

Repeat modules may comprise positions with amino acid residues which have not been randomized in a library for the purpose of selecting target-specific repeat domains ("non-randomized positions" or “fixed positions” used interchangeably herein) and positions with amino acid residues which have been randomized in the library for the purpose of selecting target-specific repeat domains ("randomized positions"). The non-randomized positions comprise framework residues. The randomized positions comprise target interaction residues. “Have been randomized” means that two or more amino acids were allowed at an amino acid position of a repeat module, for example, wherein any of the usual twenty naturally occurring amino acids were allowed, or wherein most of the twenty naturally occurring amino acids were allowed, such as amino acids other than cysteine, or amino acids other than glycine, cysteine and proline. For the purpose of this patent application, amino acid residues 3, 4, 6, 14 and 15 of SEQ ID NOs: 37 to 60, 62 to 66, 68 to 72, 111 to 122, 124 to 154 175 to 217, and 231 to 254, amino acid residues 3, 4, 6, 13 and 14 of SEQ ID NOs: 61 and 67 and amino acid residues 3, 4, 6, 15 and 16 of SEQ ID NO: 123 are randomized positions of the ankyrin repeat modules of the instant invention.

The term "repeat sequence motif" refers to an amino acid sequence, which is deduced from one or more repeat modules. Preferably, said repeat modules are from repeat domains having binding specificity for the same target. Such repeat sequence motifs comprise framework residue positions and target interaction residue positions. Said framework residue positions correspond to the positions of framework residues of the repeat modules. Likewise, said target interaction residue positions correspond to the positions of target interaction residues of the repeat modules. Repeat sequence motifs comprise non-randomized positions and randomized positions.

The term “repeat unit” refers to amino acid sequences comprising sequence motifs of one or more naturally occurring proteins, wherein said "repeat units" are found in multiple copies, and exhibit a defined folding topology common to all said motifs determining the fold of the protein. Examples of such repeat units include leucine-rich repeat units, ankyrin repeat units, armadillo repeat units, tetratricopeptide repeat units, HEAT repeat units, and leucine-rich variant repeat units.

The term “binding specificity”, “has binding specificity for a target”, “specifically binding to a target”, “binding to a target with high specificity”, “specific for a target” or “target specificity” and the like means that a binding protein or binding domain binds in PBS to a target with a lower dissociation constant (i.e. it binds with higher affinity) than it binds to an unrelated protein such as the *E. coli* maltose binding protein (MBP). Preferably, the dissociation constant (“K_D”) in PBS for the target is at least 10^2; more preferably, at least 10^3; more preferably, at least 10^4; or more preferably, at least 10^5 times lower than the corresponding dissociation constant for MBP. Methods to determine dissociation constants of protein-protein interactions, such as surface plasmon resonance (SPR) based technologies (e.g. SPR equilibrium analysis) or isothermal titration calorimetry (ITC) are well known to the person skilled in the art. The measured K_D values of a particular protein-protein interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of K_D values are preferably made with standardized solutions of protein and a standardized buffer, such as PBS. A typical and preferred determination of dissociation constants (K_D) of the inventive recombinant binding proteins with binding specificity for pMHC by Surface Plasmon Resonance (SPR) analysis is described in Example 2.

The term “about” means the mentioned value +/- 20%; for example “about 50” shall mean 40 to 60.

The term “PBS” means a phosphate buffered water solution containing 137 mM NaCl, 10 mM phosphate and 2.7 mM KCl and having a pH of 7.4.

The major histocompatibility complex (MHC) is a group of genes that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances. The proteins encoded by the major histocompatibility complex, or complexes of such proteins, are called “MHC proteins” or “MHC molecules”. MHC proteins are found in all higher vertebrates. Most notable are the MHC class I and class II glycoproteins that present peptides to an immune cell receptor, such as the T-cell receptor. In humans, the major histocompatibility complex is also called the human leukocyte antigen (HLA) system.

The term “MHC” as used herein refers to the major histocompatibility complex, preferably the major histocompatibility complex of a mammal, even more preferably the major histocompatibility complex of a human. The term “HLA” as used herein refers to the human leukocyte antigen system. An MHC molecule displays a peptide and presents it to the immune system of the vertebrate. The terms "peptide antigen" and "MHC peptide antigen" are used interchangeably herein and refer to an MHC ligand that can bind in the peptide binding groove of an MHC molecule. A peptide antigen typically has between 8 and 25 amino acids that are linked via peptide bonds. The MHC peptide antigen can be either a self or a non-self peptide. The peptide antigen can typically be presented to the immune system by the MHC molecule. MHC-class I molecules typically present the peptide antigen to CD8 positive T-cells whereas MHC-class II molecules typically present the peptide antigen to CD4 positive T-cells. In the context of the present invention, a peptide antigen that is specifically bound by a binding protein of the invention, when the peptide antigen is bound to a MHC molecule, is also called a “target peptide”. Accordingly, the terms "peptide-MHC complex", “pMHC complex”, "peptide-MHC", and "pMHC" are used interchangeably in the present application, and refer to a complex formed by the binding of a peptide antigen to an MHC molecule. Preferably, the MHC molecule is an MHC-class I molecule. Also preferably, the peptide is a peptide derived from a protein expressed in a tumor cell, a peptide derived from a protein of an infectious agent, e.g., a viral infectious agent, or a peptide derived from a protein associated with an autoimmune disorder. In one preferred embodiment, said protein expressed in a tumor cell is a tumor-specific protein.

The term “tumor-specific protein” means a protein that is expressed in tumor cells and that is not expressed or is expressed only at a lower level in many or all non-tumorigenic tissues or that is expressed only in a limited number of non-tumorigenic tissues in addition to the tumor tissue. Preferably, a tumor-specific protein has the capacity to elicit an immune response that is cancer-specific. Examples of tumor-specific proteins are known to the skilled person in the art and include but are not limited to MAGE-A1, MAGE-A3, MAGE-A4, NY-ESO-1, PRAME, CT83, SSX2 and the like.

The term “virus-specific protein” means a protein that is expressed from a viral genome in a virus-infected cell and that is not expressed in non-infected host cells. Examples of virus-specific proteins are known to the skilled person in the art and include, but are not limited to, EBNA-1, EBNA-2, EBNA-3, LMP-1, LMP-2, NSP1, NSP2, NSP4, NSP5, NSP6, E1, E2, HBx, HBsAg, HBcAg, and the like.

Examples of proteins associated with an autoimmune disorder are known to the skilled person in the art and include but are not limited to Carboxypeptidase H, Chromogranin A, Insulin, β-arrestin, Aquaporin-4, Citrullinated protein and the like.

A “target peptide-MHC complex” refers to a peptide-MHC complex, in which the peptide antigen is a target peptide.

More preferably, the peptide antigen is derived from an intracellular protein, even more preferably an intracellular protein expressed in a tumor cell or a tumor-specific protein.

Most preferably, the peptide antigen is derived from NY-ESO-1. NY-ESO-1 or New York esophageal squamous cell carcinoma 1 is a well-known cancer-testis antigen (CTAs) with re-expression in numerous cancer types (WO 98/14464; Chen Y T et al., Proc Natl Acad Sci USA 1997, 94: 1914-18; Scanlan et al., 2004, Cancer Immunity, 4, 1). Its ability to elicit spontaneous humoral and cellular immune responses, together with its restricted expression pattern, have rendered it a good candidate target for cancer immunotherapy (Thomas R et al., Front Immunol. 2018; 9: 947). In particular, NY-ESO-1 is expressed at a very high frequency for example in liposarcoma, neuroblastoma, synovial sarcoma, melanoma and ovarian cancer. The protein and polynucleotide sequence for NY-ESO-1 is provided in Genbank ACCESSION No. U87459, Version U87459.1. Preferably, the peptide antigen derived from NY-ESO-1 has the amino acid sequence SLLMWITQV (SEQ ID NO: 19) or SLLMWITQC (SEQ ID NO: 34).

Alternatively, the peptide antigen may be derived from MAGE-A3. MAGE-A3 or Melanoma-associated antigen 3 is a protein that in humans is encoded by the MAGEA3 gene. Cancer/testis antigen MAGE-A3 is a member of Melanoma Antigen Gene (MAGE) family, which has restricted expression to the testis and is aberrantly expressed in cancer cells. MAGE-A3 has been found to be broadly expressed in a variety of malignancies, including melanoma, breast cancer, head and neck cancer, lung cancer, gastric cancer, skin squamous cell carcinoma, colorectal cancer, etc. The relatively restricted expression of MAGE-A3 and its immunogenicity has made it an ideal target for immunotherapies (Int J Med Sci. 2018; 15(14): 1702–1712). The protein and polynucleotide sequence for MAGE-A3 is provided in GenPept ACCESSION NP_005353, Version NP_005353.1. Preferably, the peptide antigen derived from MAGE-A3 has the amino acid sequence EVDPIGHLY (SEQ ID NO: 155).

The peptide antigen may also be derived from a protein of an infectious agent, e.g., a protein of a viral infectious agent, preferably a virus-specific protein. The viral infectious agent may be Epstein-Barr virus (EBV), wherein said protein of EBV is preferably a virus-specific protein. The viral infectious agent may be Hepatitis B virus (HBV), wherein said protein of HBV is preferably a virus-specific protein.

The peptide antigen may be derived from EBNA-1. EBNA-1 or Epstein-Barr nuclear antigen 1 was the first Epstein-Barr virus (EBV) protein detected and is the most widely studied. EBNA1 is expressed in both latent and lytic modes of EBV infection, although it has mainly been studied in latency, where it plays multiple important roles. The importance of EBNA1 in EBV latency is reflected in the fact that EBNA1 is the only viral protein expressed in all forms of latency in proliferating cells and in all EBV-associated tumours (Scientifica (Cairo). 2012; 2012: 438204). Preferably, the peptide antigen derived from EBNA-1 has the amino acid sequence FMVFLQTHI (SEQ ID NO: 92).

Alternatively, the peptide antigen may be derived from the hepatitis B virus core antigen (HBcAg). Preferably, the peptide antigen derived from HBcAg is the sequence 18-27 of the HBV core antigen, abbreviated as “HBVc18” or “c18 peptide” herein, and has the amino acid sequence FLPSDFFPSV (SEQ ID NO: 255).

The term “peptide derived from” as used in “peptide derived from a tumor-specific protein”, “peptide derived from an intracellular protein”, “peptide derived from a protein of an infectious agent”, “peptide derived from a protein associated with an autoimmune disorder" and the like refers to a molecular fraction or a peptide fragment of the respective protein. Such a peptide fragment may result from the degradation of normal or pathogenic proteins in order to be presented, i.e. displayed, in association with an MHC molecule on the surface of a cell, for recognition by certain lymphocytes such as T cells. The presented peptide can be either *self* or *non-self*, and an organism’s immune system can normally distinguish between self and non-self, thus preventing an organism's immune system from targeting its own cells. Alternatively, such a peptide fragment may be produced by methods known in the art. Preferably, the peptide is derived from an intracellular protein.

The term “binding agent” refers to any molecule capable of specifically binding a target molecule and includes, for example, antibodies, antibody fragments, aptamers, peptides (e.g., Williams et al., J Biol Chem 266:5182-5190 (1991)), antibody mimics, repeat proteins, e.g. designed anykrin repeat proteins, receptor proteins and any other naturally occurring interaction partners of the target molecule, and can comprise natural proteins and proteins modified or genetically engineered, e.g., to include non-natural residues and/or to lack natural residues.

The term “negative selection” or “de-selection” of a binding domain means the selective removal of a binding domain, which is not or less suitable for the purpose of the invention, from a collection of binding domains. In the context of the present invention, negative selection or de-selection preferably means the selective removal, from a collection of designed repeat domains, of unwanted designed repeat domain(s) with binding specificity for a peptide-MHC complex other than the target peptide-MHC complex or with binding specificity for the common HLA-A scaffold of different pMHC complexes. Methods for negatively selecting or de-selecting an unwanted member of a collection, such as, e.g., an unwanted binding domain of a collection of binding domains, are known to the skilled person of the art, such as, e.g., the method used in Example 1 of the present application.

The term “peptide-MHC presenting cells” as used herein refers to any cells capable of expressing or displaying a peptide-MHC on the cell surface. This includes, but is not limited to, tumor cells, infected cells,

cells associated with autoimmune disorders, classical antigen-presenting cells such as dendritic cells, macrophages, and B cells, as well as cells that have been made to display MHC molecules bound to exogenously administered peptides, such as peptide-pulsed T2 cells.

The term “CD3-expressing cells” as used herein refers to any cells expressing CD3 (cluster of differentiation 3) on the cell surface, including, but not limited, to T cells such as cytotoxic T cells (CD8+ T cells) and T helper cells (CD4+ T cells).

The term “tumor-localized activation of T cells” means that T cells are activated preferentially in tumor tissue as compared to a non-tumor tissue.

The term “infection-localized activation of T cells” means that T cells are activated preferentially in an infected tissue as compared to a non-infected tissue.

The term “medical condition“ (or “disorder” or “disease”) includes but is not limited to autoimmune disorders, inflammatory disorders, retinopathies (particularly proliferative retinopathies), neurodegenerative disorders, infectious diseases, metabolic diseases, and neoplastic diseases. Any of the recombinant binding proteins described herein may be used for the preparation of a medicament for the treatment of such a disorder, particularly a disorder selected from the group comprising: an autoimmune disorder, an inflammatory disorder, an immune disorder, an infectious disease (e.g. a viral or bacterial infectious disease) and a neoplastic disease. A “medical condition” may be one that is characterized by inappropriate cell proliferation. A medical condition may be a hyperproliferative condition. The invention particularly relates to a method of treating a medical condition, the method comprising the step of administering, to a patient in need of such treatment, a therapeutically effective amount of a recombinant binding protein or pharmaceutical composition of the invention. In a preferred embodiment, said medical condition is a neoplastic disease. The term "neoplastic disease", as used herein, refers to an abnormal state or condition of cells or tissue characterized by rapidly proliferating cell growth or neoplasm. In one embodiment, said medical condition is a malignant neoplastic disease. In one embodiment, said medical condition is a cancer. In another preferred embodiment, said medical condition is an infectious disease. In another preferred embodiment, said medical condition is an autoimmune disease. The term “therapeutically effective amount” means an amount that is sufficient to produce a desired effect on a patient.

The term "antibody" means not only intact antibody molecules, but also any fragments and variants of antibody molecules that retain immunogen-binding ability. Such fragments and variants are also well known in the art and are regularly employed both in vitro and in vivo. Accordingly, the term "antibody" encompasses intact immunoglobulin molecules, antibody fragments such as, e.g., Fab, Fab', $F(ab')_2$, and single chain V region fragments (scFv), bispecific antibodies, chimeric antibodies, humanized antibodies, antibody fusion polypeptides, and unconventional antibodies.

The terms "cancer" and "cancerous" are used herein to refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Cancer encompasses solid tumors and liquid tumors, as well as primary tumors and metastases. A "tumor" comprises one or more cancerous cells. Solid tumors typically also comprise tumor stroma. Examples of cancer include, but are not limited to, primary and metastatic carcinoma, lymphoma, blastoma, sarcoma, myeloma, melanoma and leukemia, and any other epithelial and blood cell malignancies. More particular examples of such cancers include brain cancer, bladder cancer, breast cancer, ovarian cancer, kidney cancer, colorectal cancer, gastric cancer, head and neck cancer, lung cancer, pancreatic cancer, prostate cancer, malignant melanoma, osteosarcoma, soft tissue sarcoma, carcinoma, squameous cell carcinoma, clear cell kidney cancer, head/neck squamous cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma, small-cell lung cancer (SCLC), triple negative breast cancer, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), diffuse large B- cell lymphoma (DLBCL), follicular lymphoma, Hodgkin's lymphoma (HL), mantle cell lymphoma (MCL), multiple myeloma (MM), myelodysplastic syndrome (MDS), non-Hodgkin's lymphoma (NHL), Squamous Cell Carcinoma of the Head and Neck (SCCHN), chronic myelogenous leukemia (CML), small lymphocytic lymphoma (SLL), malignant mesothelioma, liposarcoma, neuroblastoma, or synovial sarcoma.

The term “therapeutically effective amount” refers to the amount sufficient to induce a desired biological, pharmacological, or therapeutic outcome in a subject. A therapeutically effective amount in the context of the invention means a sufficient amount of the binding protein to treat or prevent a disease or disorder at a reasonable benefit/risk ratio applicable to any medical treatment.

The term “treatment” or “treating” refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those who have already the disorder as well as those in which the disorder is to be prevented.

The term “mammal” for purposes of treatment refers to any animal classified as a mammal, including human, domestic and farm animals, nonhuman primates, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. The terms "autoimmune disease" and "autoimmune disorder" are used herein to refer to or describe disorders wherein the immune system of a mammal mounts a humoral or cellular immune response to the mammal's own tissue or to antigens that are not intrinsically harmful to the mammal, thereby producing tissue injury in such a mammal. Examples of autoimmune disorders are numerous and include, but are not limited to, systemic lupus erythematosus, rheumatoid arthritis and type I diabetes. Autoimmune diseases also include acute glomerulonephritis, Addison's disease, adult onset idiopathic hypoparathyroidism (AOIH), alopecia totalis, amyotrophic lateral sclerosis, ankylosing spondylitis, autoimmune aplastic anemia, autoimmune hemolytic anemia, Behcet's disease, Celiac disease, chronic active hepatitis, CREST syndrome, Crohn's disease, dermatomyositis, dilated cardiomyopathy, eosinophilia-myalgia syndrome, epidernolisis bullosa acquisita (EBA), giant cell arteritis, Goodpasture's syndrome, Graves' disease, Guillain-Barre syndrome, hemochromatosis, Henoch-Schonlein purpura, idiopathic IgA nephropathy, insulin-dependent diabetes mellitus (IDDM), juvenile rheumatoid arthritis, Lambert-Eaton syndrome, linear IgA dermatosis, lupus erythematosus, multiple sclerosis, myasthenia gravis, myocarditis, narcolepsy, necrotizing vasculitis, neonatal lupus syndrome (NLE), nephrotic syndrome, pemphigoid, phemphigus, polymyositis, primary sclerosing cholangitis, psoriasis, rapidly progressive glomerulonephritis (RPGN), Reiter's syndrome, rheumatoid arthritis, scleroderma, Sjogren's syndrome, stiff-man syndrome, thyroiditis, and ulcerative colitis.

The terms "infectious disease" and "infection" are used herein to refer to or describe the invasion and multiplication of microorganisms in body tissues, especially causing pathological symptoms. Examples of infectious diseases include without limitation, viral diseases and bacterial diseases, such as, e.g., HIV infection, West Nile virus infection, hepatitis A, B, and C, small pox, tuberculosis, Vesicular Stomatitis Virus (VSV) infection, Respiratory Syncytial Virus (RSV) infection, human papilloma virus (HPV) infection, SARS, influenza, Ebola, viral meningitis, herpes, anthrax, lyme disease, and E. Coli infections, among others.

EXAMPLES

Starting materials and reagents disclosed below are known to those skilled in the art, are commercially available and/or can be prepared using well-known techniques.

Materials

Chemicals were purchased from Sigma-Aldrich (USA). Oligonucleotides were from Microsynth (Switzerland). Unless stated otherwise, DNA polymerases, restriction enzymes and buffers were from New England Biolabs (USA) or Fermentas/Thermo Fisher Scientific (USA). Inducible *E. coli* expression strains were used for cloning and protein production, e.g. *E. coli* XL1-blue (Stratagene, USA) or BL21 (Novagen, USA).

Molecular Biology

Unless stated otherwise, methods are performed according to known protocols (see, e.g., Sambrook J., Fritsch E.F. and Maniatis T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1989, New York).

Designed ankyrin repeat protein libraries

Methods to generate designed ankyrin repeat protein libraries have been described, e.g. in U.S. Patent No. 7,417,130; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.. By such methods designed ankyrin repeat protein libraries having randomized ankyrin repeat modules and/or randomized capping modules can be constructed. For example, such libraries could accordingly be assembled based on a fixed N-terminal capping module (e.g. the N-terminal capping module of SEQ ID NO: 5, 6 or 7) or a randomized N-terminal capping module according to SEQ ID NO: 8, one or more randomized repeat modules according to the sequence motif of SEQ ID NO: 9, 10 or 11, and a fixed C-terminal capping module (e.g. the C-terminal capping module of SEQ ID NO: 12, 13 or 14) or a randomized C-terminal capping module according to SEQ ID NO: 15. Preferably, such libraries are assembled to not have any of the amino acids C, G, M, N (in front of a G residue) and P at randomized positions of repeat or capping modules. In addition, randomized repeat modules according to the sequence motif of SEQ ID NO: 9, 10 or 11 could be further randomized at position 10 and/or position 17; the randomized N-terminal capping module according to the sequence motif of SEQ ID NO: 8 could be further randomized at position 7 and/or position 9; and the randomized C-terminal capping modules according to the sequence motif of SEQ ID NO: 15 could be further randomized at positions 10, 11 and/or 17.

Furthermore, such randomized modules in such libraries may comprise additional polypeptide loop insertions with randomized amino acid positions. Examples of such polypeptide loop insertions are complement determining region (CDR) loop libraries of antibodies or de novo generated peptide libraries. For example, such a loop insertion could be designed using the structure of the N-terminal ankyrin repeat domain of human ribonuclease L (Tanaka, N., Nakanishi, M, Kusakabe, Y, Goto, Y., Kitade, Y, Nakamura, K.T., EMBO J. *23(30)*, 3929-3938, 2004) as guidance. In analogy to this ankyrin repeat domain where ten amino acids are inserted in the beta-turn present close to the border of two ankyrin repeats, ankyrin repeat protein libraries may contain randomized loops (with fixed and randomized positions) of variable length (e.g. 1 to 20 amino acids) inserted in one or more beta-turns of an ankyrin repeat domain.

Any such N-terminal capping module of an ankyrin repeat protein library preferably possesses the RILLAA, RILLKA or RELLKA motif (e.g. present from position 21 to 26 in SEQ ID NO: 20) and any such C-terminal capping module of an ankyrin repeat protein library preferably possesses the KLN, KLA or KAA motif (e.g. present at the last three amino acids in SEQ ID NO: 20).

The design of such an ankyrin repeat protein library may be guided by known structures of an ankyrin repeat domain interacting with a target. Examples of such structures, identified by their Protein Data Bank (PDB) unique accession or identification codes (PDB-IDs), are 1WDY, 3V31, 3V30, 3V2X, 3V2O, 3UXG, 3TWQ-3TWX, 1N11, 1S70 and 2ZGD.

Examples of designed ankyrin repeat protein libraries, such as N2C and N3C designed ankyrin repeat protein libraries, have been described (U.S. Patent No. 7,417,130; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.). The digit in N2C and N3C describes the number of randomized repeat modules present between the N-terminal and C-terminal capping modules.

The nomenclature used to define the positions inside the repeat units and modules is based on Binz et al. 2004, loc. cit. with the modification that borders of the ankyrin repeat modules and ankyrin repeat units are shifted by one amino acid position. For example, position 1 of an ankyrin repeat module of Binz et al. 2004 (loc. cit.) corresponds to position 2 of an ankyrin repeat module of the current disclosure and consequently position 33 of an ankyrin repeat module of Binz et al. 2004, loc. cit. corresponds to position 1 of a following ankyrin repeat module of the current disclosure.

All the DNA sequences were confirmed by Sanger sequencing.

<u>Example 1</u>: Selection of binding proteins comprising an ankyrin repeat domain with binding specificity for NY-ESO-1 peptide-MHC complex (NYESOpMHC)

Summary

Using ribosome display (Hanes, J. and Plückthun, A., PNAS *94*, 4937-42, 1997), multiple ankyrin repeat domains with binding specificity for NY-ESO-1 peptide-MHC complex (NYESOpMHC) were selected from DARPin® libraries in a way similar to the one described by Binz et al. 2004 (loc. cit.), with specific conditions and additional de-selection steps as described below. The binding and specificity of the selected clones towards recombinant NYESOpMHC and NYESOAApMHC target were assessed by *E. coli* crude extract Homogeneous Time Resolved Fluorescence (HTRF), indicating that multiple NYESOpMHC-specific binding proteins were successfully selected. For example, the ankyrin repeat domains of SEQ ID NO: 20 to 33 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC. Individual ankyrin repeat modules from such ankyrin repeat domains with binding specificity to NYESOpMHC are provided, e.g., in SEQ ID NO: 37 to 72. Examples of nucleic acids encoding such ankyrin repeat domains with binding specificity for NYESOpMHC are provided in SEQ ID NO: 78 to 80.

Production of biotinylated pMHC complexes as target and selection material

Tripartite complexes of HLA-A*0201 (SEQ ID NO: 73), human beta-2-microglobulin (hβ2m; SEQ ID NO: 74) and one of the peptides NY-ESO-1-9V (aa 157-165; SLLMWITQV; SEQ ID NO: 19), NY-ESO-1-9VAA (aa 157-165; SLLAAITQV; SEQ ID NO: 35) and EBNA-1 (aa 562-570; FMVFLQTHI; SEQ ID NO: 36) were produced according to established protocols (Garboczi et al, 1992; Celie et al, 2009). Codon-optimized HLA-A*0201 comprising a linker (GSGGSGGSAGG; SEQ ID NO: 75) and the Avi-tag (GLNDIFEAQKIEWHE; SEQ ID NO: 76; Fairhead & Howarth, 2015) for biotinylation (HLA-A*0201avi; SEQ ID NO: 77) and wild-type human beta-2-microglobulin (hβ2m) were expressed in *E. coli* BL21 (DE3) at 37°C as inclusion bodies (IB) and dissolved in 50 mM MES, 5 mM EDTA, 5 mM DTT, 8M urea, pH 6.5 after IB purification. HLA-A*0201avi and hβ2m molecules were refolded in the presence of the respective peptides at final concentrations of 25, 30 and 15 mg, respectively, per 500 mL volume in 50 mM Tris pH 8.3, 230 mM L-Arginine, 3 mM EDTA, 255 µM GSSG; 2.5 mM GSH; 250 µM PMSF. The three resulting pMHC complexes were (1) NYESOpMHC comprising the NY-ESO-1-9V (157–165) peptide, (2) NYESOAApMHC comprising the NY-ESO-1-9VAA (157–165) peptide, and (3) EBNApMHC comprising the EBNA-1 (562-570) peptide (see **Table 1**).

For biotinylation, samples were concentrated to a volume of 7.5 mL, and the buffer was exchanged to 100 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5 using PD10 columns. HLA-A*0201avi was biotinylated by adding 5 mM ATP, 400 µM Biotin, 200 µM PMSF and 20 µg BirA enzyme. BirA was produced in-house following the procedure described in Shen et al, 2009. The refolded tripartite, biotinylated complexes were isolated using size exclusion chromatography (Superdex 200 HiLoad 16/600) in PBS supplemented with 150 mM NaCl, 1mM EDTA, 10% glycerol. Samples were concentrated to about 1 mg/mL and flash-frozen as 25 and 50 µL aliquots by liquid nitrogen.

Table 1: pMHC complexes for selection and screening of designed ankyrin repeat proteins

pMHC complexes (HLA:peptide)	Peptide sequence, format, description
NYESOpMHC (HLA-A*0201:NYESO1-9V)	Peptide sequence: SLLMWITQV Biotinylated via Avi-tag Valine-modified antigenic peptide of NY-ESO-1, a frequently expressed tumor antigen of the cancer-testis family; Cys to Val mutation improves pMHC stability with structural mimicry of the wild-type peptide (Webb et al, J. Biol. Chem. *279 (22)*, 23438–23446, 2004)
NYESOAApMHC	Peptide sequence: SLLAAITQV Biotinylated via Avi-tag

(HLA-A*0201:NYESO1-9VAA)	Alanine mutated NY-ESO-1 peptide used to test specificity of designed ankyrin repeat proteins in the screening phase
EBNApMHC (HLA-A*0201:EBNA-1)	Peptide sequence: FMVFLQTHI Biotinylated via Avi-tag Epstein Barr virus (EBV) nuclear antigen-derived antigenic peptide (Sim et al, Sci. Rep. *3*, 3232, 2013)

For quality control purposes, 25 µg of biotinylated pMHC complexes (biotin-pMHC) were incubated with 50 µg Streptavidin (IBA Lifesciences), either with or without adding 100 mM DTT and incubation at 95°C for 5 min. 50 µg samples were run over analytical size exclusion chromatography (GE Superdex 200 10/300 GL). The biotinylated NYESOpMHC, NYESOAApMHC and EBNApMHC complexes all eluted at a peak maximum of about 82 mL from the Superdex 200 HiLoad 16/600 column (**Figure 1A**). The final amounts of obtained NYESOpMHC, NYESOAApMHC and EBNApMHC complexes after size exclusion were about 3.5, 3.5 and 5 mg, respectively. SDS-PAGE analysis of the concentrated and thawed flash-frozen refolded complexes indicated efficient biotinylation, since HLA-A*0201avi was almost completely bound to Streptavidin (**Figure 1B**). Analytical size exclusion chromatography revealed single peaks at retention volumes corresponding to apparent molecular weights of 45 kDa, close to the theoretical MW of the tripartite pMHC complexes of about 48 kDa (**Figure 1C**).

Selection of NYESOpMHC-specific ankyrin repeat proteins by ribosome display

The selection of pMHC-specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using the NYESOpMHC complex as a target, libraries of ankyrin repeat proteins as described above, and established protocols (See, e.g., Zahnd, C., Amstutz, P. and Plückthun, A., Nat. Methods *4*, 69-79, 2007). The number of reverse transcription (RT)-PCR cycles after each selection round was continuously reduced, adjusting to the yield due to enrichment of binders. The first four rounds of selection employed standard ribosome display selection, using decreasing target concentrations and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. 2004, loc. cit.), but incorporated an unusual de-selection step.

During the ribosome display rounds, a de-selection (or negative selection) step was incorporated, wherein the ternary complexes were pre-incubated with the corresponding isotype HLA molecule containing another peptide, and only then transferred to the target NYESOpMHC complex, in order to direct binding of ankyrin repeat proteins towards the peptide-embedded epitope and away from the common HLA-A scaffold. In other words, de-selection (or negative selection) was performed in order to de-select ankyrin repeat proteins that bind predominantly to the common HLA-A scaffold of pMHC complexes rather than to the specific epitope provided by the embedded peptide. Furthermore, ankyrin repeat proteins that cross-react with the epitope provided by the embedded peptide used for de-selection were also de-selected. Here, the EBNApMHC complex comprising the EBNA-1 (562-570) peptide was used for de-selection.

In detail, for the de-selection step, Nunc MaxiSorp plates were coated with 100 µl solution of 66 nM neutravidin in PBS and incubated at 4°C overnight. The following day, the MaxiSorp 96-well microplates were washed three times with 300 µl PBST per well and blocked with 300 µl PBST-BSA for 1h at 4°C, rotating at 700 rpm, prior to the de-selection step. After emptying the wells, 100 µl of a 50 nM biotinylated pMHC de-selection target solution in PBST-BSA was added to each well, rotating with 700 rpm at 4°C for 1h. During this incubation step, mRNA in-vitro translations according to the ribosome display protocol were performed separately. Shortly after in vitro translations and the generation of ternary complexes (i.e. mRNA, ribosome, and translated ankyrin repeat protein), the pMHC-PBST-BSA solutions were discarded and Nunc MaxiSorp microplate wells were washed three times with 300 µl PBST and finally incubated with Tris-wash buffer containing BSA (WBT-BSA). Just prior to the actual de-selection step, the WBT-BSA solution was discarded and aliquots of 150 µl (for the first selection round) or 100 µl (for selection rounds 2 to 4) of the in vitro translation ternary complexes were transferred consecutively three times to a prepared Nunc MaxiSorp well containing the immobilized de-selection pMHC complex and incubated in each of the three wells for 20 min at 4°C. At the end of the de-selection process, all the 100µl ternary complex aliquots of each selection pool were combined and the according volumes were taken forward into the selection on the actual target pMHC complex as described above.

Selected clones bind specifically to NYESOpMHC complex as shown by crude extract HTRF

Individually selected ankyrin repeat proteins specifically binding NYESOpMHC complex in solution were identified by a Homogeneous Time Resolved Fluorescence (HTRF) assay using crude extracts of ankyrin repeat protein-expressing *Escherichia coli* cells using standard protocols. Ankyrin repeat protein clones selected by ribosome display were cloned into a derivative of the pQE30 (Qiagen) expression vector, transformed into *E. coli* XL1-Blue (Stratagene), plated on LB-agar (containing 1% glucose and 50 µg/ml ampicillin) and then incubated overnight at 37°C. Single colonies were picked into a 96 well plate (each clone in a single well) containing 165 µl growth medium (LB containing 1% glucose and 50 µg/ml ampicillin) and incubated overnight at 37°C, shaking at 800 rpm. 150 µl of fresh LB medium containing 50 µg/ml ampicillin was inoculated with 8.5 µl of the overnight culture in a fresh 96-deep-well plate. After incubation for 120 minutes at 37°C and 850 rpm, expression was induced with IPTG (0.5 mM final concentration) and continued for 6 hours. Cells were harvested by centrifugation of the plates, supernatant was discarded and the pellets were frozen at -20°C overnight before resuspension in 8.5 µl µl B-PERII (Thermo Scientific) and incubation for one hour at room temperature with shaking (600 rpm). Then, 160 µl PBS was added and cell debris was removed by centrifugation (3220 g for 15 min).

The extract of each lysed clone was applied as a 1:200 dilution (final concentration) in PBSTB (PBS supplemented with 0.1% Tween 20® and 0.2% (w/v) BSA, pH 7.4) together with 20 nM (final concentration) biotinylated pMHC complex, 1:400 (final concentration) of anti-6His-D2 HTRF antibody – FRET acceptor conjugate (Cisbio) and 1:400 (final concentration) of anti-strep-Tb antibody FRET donor conjugate (Cisbio, France) to a well of a 384-well plate and incubated for 120 minutes at 4°C. The HTRF was read-out on a Tecan M1000 using a 340 nm excitation wavelength and a 620 ±10 nm emission filter for background fluorescence detection and a 665 ±10 nm emission filter to detect the fluorescence signal for specific binding.

The extract of each lysed clone was tested for binding to each of the three biotinylated pMHC complexes, i.e. NYESOpMHC, NYESOAApMHC and EBNApMHC, in order assess binding and specificity to the target

NYESOpMHC complex. NYESOAApMHC and EBNApMHC served as pMHC complexes distinct from NYESOpMHC to allow selection of ankyrin repeat proteins with high binding specificity for NYESOpMHC.

In order to calculate the specificity of each ankyrin repeat protein for NYESOpMHC, the ratio of the HTRF signal for the target NYESOpMHC to the HTRF signal for the distinct NYESOAApMHC was determined. All binders which generated at least 25-times higher HTRF signals on the target NYESOpMHC than on NYESOAApMHC were regarded as specific hits and taken forward for sequencing. Surprisingly, screening of several hundred clones by such a crude cell extract HTRF analysis revealed many different ankyrin repeat domains with specificity for NYESOpMHC. Specific binding of ankyrin repeat proteins to a composite epitope comprising an HLA scaffold and a short peptide, wherein only the peptide differs from other composite epitopes which are not specifically bound, has never been shown before and developing specific binders to such composite epitopes using antibody or TCR technology has been challenging.

A total of 95 hits were sequenced using the sequencing service at Microsynth (Balgach; Switzerland). Examples of amino acid sequences of selected ankyrin repeat domains that specifically bind to NYESOpMHC are provided in SEQ ID NO: 20 to 33.

These ankyrin repeat domains with binding specificity for NYESOpMHC were cloned into a pQE (QIAgen, Germany) based expression vector providing an N-terminal His-tag (SEQ ID NO: 4) to facilitate simple protein purification as described below. For example, expression vectors encoding the following ankyrin repeat proteins were constructed:

DARPin® protein #20 (SEQ ID NO:20 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #21 (SEQ ID NO:21 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #22 (SEQ ID NO:22 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #23 (SEQ ID NO:23 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #24 (SEQ ID NO:24 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #25 (SEQ ID NO:25 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #26 (SEQ ID NO:26 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #27 (SEQ ID NO:27 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #28 (SEQ ID NO:28 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #29 (SEQ ID NO:29 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #30 (SEQ ID NO:30 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #31 (SEQ ID NO:31 with a His-tag (SEQ ID NO:4) fused to its N terminus);
DARPin® protein #32 (SEQ ID NO:32 with a His-tag (SEQ ID NO:4) fused to its N terminus); and
DARPin® protein #33 (SEQ ID NO:33 with a His-tag (SEQ ID NO:4) fused to its N terminus).

High level and soluble expression of NYESOpMHC-specific ankyrin repeat proteins

For further analysis, the selected clones showing specific NYESOpMHC binding in the crude cell extract HTRF as described above were expressed in *E. coli* cells and purified using their His-tag according to standard protocols. 25 ml of stationary overnight cultures (TB, 1% glucose, 50 mg/l of ampicillin; 37°C) were used to inoculate 500 ml cultures (TB, 50 mg/l ampicillin, 37°C). At an absorbance of 1.0 to 1.5 at 600 nm, the cultures were induced with 0.5 mM IPTG and incubated at 37°C for 4-5 h while shaking. The cultures were centrifuged and the resulting pellets were re-suspended in 25 ml of TBS_{500} (50 mM Tris-HCl, 500 mM NaCl, pH 8) and lysed (sonication or French press). Following the lysis, the samples were mixed with 50 KU DNase/ml and incubated for 15 minutes prior to a heat-treatment step for 30 minutes at 62.5 °C, centrifuged and the supernatant was collected and filtrated. Triton X100 (1% (v/v) final concentration) and imidazole (20 mM final concentration) were added to the homogenate. Proteins were purified over a Ni-nitrilotriacetic acid (Ni-NTA) column followed by a size exclusion chromatography on an ÄKTAxpress™ system according to standard protocols and resins known to the person skilled in the art. Alternatively, selected ankyrin repeat domains devoid of a His-tag are produced by high cell density fermentation in *E. coli* and purified by a series of chromatography and ultra/diafiltration steps according to standard resins and protocols known to the person skilled in the art. Highly soluble ankyrin repeat proteins with binding specificity for NYESOpMHC were purified from *E. coli* culture (up to 200 mg ankyrin repeat protein per liter of culture) with a purity > 95% as estimated from 4-12% SDS-PAGE. A representative example of such SDS-PAGE analysis is shown in **Figure 2** for DARPin® protein #21.

<u>Example 2</u>: Determination of dissociation constants (K_D) of ankyrin repeat proteins with binding specificity for NYESOpMHC by Surface Plasmon Resonance (SPR) analysis

The binding affinities of the purified ankyrin repeat proteins on the NYESOpMHC target were analyzed using a ProteOn Surface Plasmon Resonance (SPR) instrument (BioRad) and the measurement was performed according standard procedures known to the person skilled in the art. Similarly, the binding affinities of the purified ankyrin repeat proteins on the NYESOAApMHC and EBNApMHC complexes were also analyzed to compare binding and confirm the binding specificity of the ankyrin repeat proteins for the NYESOpMHC target.

Briefly, biotinylated NYESOpMHC, NYESOAApMHC and EBNApMHC were diluted in PBST (PBS, pH 7.4 containing 0.005% Tween 20®) and coated on a NLC chip (BioRad) to a level of around 500 resonance units (RU) (respectively 1000 RU for multi-trace SPR measurements). The interaction of ankyrin repeat protein and pMHC complex was then measured by injecting 150 µl running buffer (PBS, pH 7.4 containing 0.005% Tween 20®) containing either single concentrations or serial dilutions of ankyrin repeat proteins covering a concentration range between 3.7 nM and 300nM for multi-trace SPR measurements or 250 nM for single trace measurements (on-rate measurement), followed by a running buffer flow for at least 60 minutes at a constant flow rate of 100 µl/min (off-rate measurement). No target regeneration was performed. The signals (i.e. resonance unit (RU) values) of the interspots were subtracted from the RU traces obtained after injection of ankyrin repeat protein. Based on the SPR traces obtained from the on-rate and off-rate measurements, the on- and off-rates of the corresponding ankyrin repeat proteins towards the pMHC complexes were determined.

As representative examples, **Figures 3A-3C** show SPR traces obtained for binding of DARPin® protein #21 to NYESOpMHC (Fig. 3A), NYESOAApMHC (Fig. 3B) and EBNApMHC (Fig. 3C). Dissociation constants (K_D) were calculated from the estimated on- and off-rates using standard procedures known to the person skilled in the art. K_D values of the binding interactions of selected ankyrin repeat proteins with NYESOpMHC were determined to be in the low nanomolar range or below. None of these selected ankyrin repeat proteins displayed measurable binding interaction with NYESOAApMHC or EBNApMHC. **Table 2** provides the K_D values of some selected ankyrin repeat proteins as examples.

Table 2. K_D values of ankyrin repeat protein - NYESOpMHC interactions

DARPin® protein #	K_D [M]
#20	57.9×10^{-10}
#21	8.61×10^{-10}
#22	0.685×10^{-10}
#23	0.939×10^{-10}
#24	9.39×10^{-10}
#25	1.72×10^{-10}
#26	12.9×10^{-10}
#27	4.94×10^{-10}
#28	25.7×10^{-10}
#29	6.35×10^{-10}
#30	23.3×10^{-10}
#31	13.2×10^{-10}
#32	16.3×10^{-10}
#33	1.08×10^{-10}

Figures 4A and 4B show further characterization of DARPin® protein **#21** as a representative example of the NYESOpMHC-specific ankyrin repeat domains of the invention. Binding of DARPin® protein **#21** to NYESOpMHC, NYESOAApMHC and EBNApMHC was investigated using a HTRF assay, demonstrating highly specific target binding to NYESOpMHC (**Figure 4A**). No binding of DARPin® protein **#21** to NYESOAApMHC or EBNApMHC was observed.
The biophysical properties of DARPin® protein **#21** were also investigated by size exclusion chromatography (SEC) using a Superdex 200 column. DARPin® protein **#21** was injected at 50 μM concentration. The SEC elution profile showed a single monodisperse peak, which eluted at a position corresponding to the expected mass of an individual DARPin® protein **#21** molecule (**Figure 4B**). No traces of aggregates or multimers were detected.

These experimental data and K_D values demonstrate that designed ankyrin repeat proteins with high binding affinity and specificity for a specific pMHC complex, e.g. the NYESOpMHC complex, as well as with beneficial biophysical properties, can be generated, using screening and selection procedures, e.g., as described in Example 1.

<u>Example 3</u>: Binding of ankyrin repeat proteins with binding specificity for NYESOpMHC to cells

After biophysical characterization of the ankyrin repeat domains with binding specificity for NYESOpMHC, they were also tested for cell binding. For this purpose, T2 cells (ATCC® CRL-1992™) were used, which are hybrids of T- and B-lymphoblasts that lack the transporter associated with antigen processing (TAP) protein complex and are therefore a model system for studying antigen processing and T cell recognition. T2 cells are HLA-A2 positive and NY-ESO-1 negative. These cells can be loaded with peptides that bind to HLA-A2, such as, e.g. the NY-ESO-1-9V (157–165) peptide, thereby generating corresponding pMHC complexes on their surface.

A titration of peptide showed that the addition of peptide in the µM range to T2 cells generates sufficient pMHC complexes to allow studying the binding of ankyrin repeat domains to the pMHC complexes formed between HLA-A2 and the added peptide (data not shown). T2 cells were pulsed overnight at 37°C with 20 µM of NY-ESO-1-9V (157–165) peptide or the EBNA-1 (562-570) peptide. In parallel, T2 cells were treated with buffer not containing any peptide (non-pulsed cells). Ankyrin repeat proteins with binding specificity for NYESOpMHC were added at different concentrations to the cells and, after 30 minutes incubation at 4°C, binding of the ankyrin repeat proteins to the cells was determined by flow cytometry using an anti-His tag antibody (Penta·His Alexa Fluor 488 Conjugate, Qiagen).

As representative examples, Figures 5A and 5B show the obtained binding curves for DARPin® protein #20, DARPin® protein #21, and DARPin® protein #22, using T2 cells pulsed with NY-ESO-1-9V (157–165) peptide (Figure 5A) and non-pulsed T2 cells (Figure 5B). EC_{50} values for binding to T2 cells pulsed with NY-ESO-1-9V (157–165) peptide were determined using standard procedures known to the person skilled in the art. EC_{50} values of selected ankyrin repeat proteins were determined to be in the low nanomolar range, demonstrating efficient binding to the cells. **Table 3** provides the EC_{50} values of selected ankyrin repeat proteins as examples.

Table 3. EC_{50} values of binding to T2 cells pulsed with NY-ESO-1-9V (157–165) peptide

DARPin® protein #	EC_{50} [nM]
#20	20.6
#21	4.5
#22	3.7

In order to test the ability of the ankyrin repeat domains with binding specificity for NYESOpMHC to function in an immune cell engager format, selected ankyrin repeat domains were genetically linked, using conventional cloning methods, to a binding agent with binding specificity for a protein expressed on the surface of an immune cell. By this procedure, binding proteins were generated comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and further comprising a binding agent with binding specificity for CD3.

The ability of such binding proteins in T cell engager (TCE) format to bind to pulsed T2 cells was tested as described above. As shown in **Figures 5C and 5D**, specific binding of DARPin® protein #20, DARPin® protein #21, and DARPin® protein #22 to T2 cells pulsed with NY-ESO-1-9V (157–165) peptide was conserved in the T cell engager format (TCE DARPin® protein #20, TCE DARPin® protein #21 and TCE DARPin® protein #22). TCE DARPin® protein #20, TCE DARPin® protein #21 and TCE DARPin® protein

#22 did not bind to non-pulsed T2 cells (**Figure 5E**), or to T2 cells pulsed with the EBNA-1 (562-570) peptide (except minor binding at high concentrations), as shown exemplary for TCE DARPin® protein **#21** (**Figure 5F**). No significant difference in binding was observed between a binding protein comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and the same binding protein in a TCE format (Figure 5D). The EC_{50} values of the ankyrin repeat proteins in T cell engager format were determined to be in the low nanomolar range, demonstrating efficient binding to the cells. **Table 4** provides the EC_{50} values of selected ankyrin repeat proteins in T cell engager format as examples.

Table 4. EC_{50} values of binding to T2 cells pulsed with NY-ESO-1-9V (157–165) peptide

TCE DARPin® protein #	EC_{50} [nM]
#20	13.6
#21	6.7
#22	3.4

In conclusion, the binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC were able to specifically bind to NYESOpMHC on the surface of cells, with an EC50 in the low nanomolar range. Binding to NYESOpMHC on the surface of cells was specific, since no binding was observed to non-pulsed T2 cells or to T2 cells pulsed with an unrelated peptide. The specific binding properties were conserved when the NYESOpMHC-specific ankyrin repeat domains were linked to a binding agent with binding specificity for a protein expressed on the surface of an immune cells, such as CD3 expressed on the surface of T cells (T cell engager (TCE) format).

Example 4: T cell activation by binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC, using pulsed T2 cells

The ability of the NYESOpMHC-specific ankyrin repeat domains in TCE format to activate T cells upon binding to NYESOpMHC-displaying target cells was analyzed in T cell activation assays.

T2 cells pulsed with NY-ESO-1-9V (157–165) peptide were used as NYESOpMHC-displaying target cells. The effector cells were BK112 T cells, which are monoclonal $CD8^+$ T cells expanded from healthy donors (Levitsky et al., *J. Immunol.* 161:594-601 (1998)). For the T cell activation assay, T2 cells were pulsed overnight at 37°C with 10 µM of peptide. Effector BK112 T cells were added at an effector to target cell ratio of 1:5 in the presence of TCE DARPin® proteins at different concentrations (e.g. 1pM) and incubated for 4-5 hours at 37°C. Intracellular IFN-γ in the CD8+ T cells was then determined by fluorescence-activated cell sorting (FACS), as a measure of T cell activation. The reagents used for FACS included APC mouse anti-human IFN-γ and Pacific Blue™ mouse anti-human CD8 from BD Biosciences.

Figure 6 shows the result of testing a panel of different NYESOpMHC-specific ankyrin repeat domains in TCE format (1 pM) in this T cell activation assay. The NYESOpMHC-specific ankyrin repeat domains in TCE format were (corresponding to the numbers at the x-axis in Figure 6) (1) TCE DARPin® protein **#23**, (2) TCE DARPin® protein **#24**, (3) TCE DARPin® protein **#25**, (4) TCE DARPin® protein **#26**, (5) TCE

DARPin® protein #27, (6) TCE DARPin® protein #28, (7) TCE DARPin® protein #29, (8) TCE DARPin® protein #30, (9) TCE DARPin® protein #31, (10) TCE DARPin® protein #32, (11) TCE DARPin® protein #33, (12) TCE DARPin® protein #21, (13) TCE DARPin® protein #20, and (14) TCE DARPin® protein #22. As a control, the experiment was also performed with no TCE DARPin® protein added (15). After 4 hours incubation, intracellular IFN-γ in the T cells was determined by FACS.

All 14 TCE DARPin® proteins specifically mediated T cell activation in the presence of target peptide-MHC complex in pulsed T2 cells, but not (or much less) in the absence of target peptide-MHC complex in non-pulsed T2 cells. For those TCE DARPin® proteins, for which a signal was detected also with non-pulsed T2 cells at 1 pM of TCE DARPin® protein, a larger window between pulsed and non-pulsed T2 cells was obtained at lower concentrations of TCE DARPin® protein (data not shown). Thus, all of these NYESOpMHC-specific ankyrin repeat domains in TCE format were able to specifically activate effector T cells dependent on the presence of NYESOpMHC-displaying target cells.

The dependency of specific activation of effector T cells on the presence of NYESOpMHC-displaying target cells was further shown in similar T cell activation assays, in which the TCE DARPin® proteins were titrated over a broader concentration range. **Figures 6B and 6C** show the results of such experiments for TCE DARPin® protein #21 (B) and TCE DARPin® protein #32 (C). Specific activation of the effector T cells was achieved by both TCE DARPin® proteins starting at very low concentrations (sub-picomolar) and over the entire concentration range tested (up to nanomolar) in the presence of pulsed T2 cells. In contrast, no T cell activation was observed over the entire concentration range in the presence of non-pulsed T2 cells. This confirmed the ability of the NYESOpMHC-specific ankyrin repeat domains in TCE format to specifically activate effector T cells dependent on the presence of NYESOpMHC-displaying target cells.

<u>Example 5</u>: T cell activation by binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC, using tumor cells

The ability of the NYESOpMHC-specific ankyrin repeat domains in TCE format to mediate T cell activation upon binding to NYESOpMHC-displaying target cells was also analyzed in T cell activation assays using tumor cell lines as target cells. T cell activation was compared using a tumor cell line (IM9; ATCC® CCL-159™)) that endogenously expresses HLA-A2 and NY-ESO-1 (hence, HLA-A2$^+$ NY-ESO-1$^+$ cells) and displays NYESOpMHC on its surface, and a tumor cell line (MCF-7; ATCC® HTB-22™) that expresses HLA-A2 but not NY-ESO-1 (hence, HLA-A2$^+$ NY-ESO-1$^-$ cells) and does not display NYESOpMHC on its surface. BK112 T cells were used as effector cells and the experiments were performed essentially as described in Example 4, but with different target cells. As a control, effector BK112 T cells were treated identically, except that no target cells were added.

As shown exemplary for TCE DARPin® protein #21, the NYESOpMHC-specific ankyrin repeat domains in TCE format can effectively activate T cells in the presence of NYESOpMHC-expressing tumor cells (IM9) (**Figure 7**, round symbols). The EC_{50} value for activation of IM9 tumor cells by TCE DARPin® protein #21 was determined, using standard procedures known to the person skilled in the art, to be 18.7 nM, demonstrating potent activity of the TCE DARPin® protein. In contrast, no activation of the T cells was observed in the presence of tumor cells that do not express NYESOpMHC on their cell surface (MCF-7)

(**Figure 7**, triangular symbols) or if no tumor cells were added to the T cells (i.e. BK112 T cells only) (**Figure 7**, square symbols).

These data indicate that pMHC-specific ankyrin repeat domains in TCE format can potently activate T cells upon binding to target peptide-MHC complex on the surface of tumor cells.

Example 6: Immune cell activation by binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC, using tumor cells

The ability of the NYESOpMHC-specific ankyrin repeat domains in TCE format to mediate T cell activation upon binding to NYESOpMHC-displaying target cells was also analyzed in T cell activation assays using tumor cell lines as target cells and peripheral blood mononuclear cells (PBMCs) isolated from a donor as effector cells. Immune cell activation was compared using a tumor cell line (IM9) that endogenously expresses HLA-A2 and NY-ESO-1 and displays NYESOpMHC on its surface, and a tumor cell line (MCF-7) that expresses HLA-A2 but not NY-ESO-1 and does not display NYESOpMHC on its surface (see also Example 5). PBMCs isolated from a donor were used as effector cells.

For the T cell activation assay, target tumor cells and effector PBMCs were combined at an effector to target cell ratio of 5:1, NYESOpMHC-specific ankyrin repeat domains in TCE format were added at different concentrations, and the mixtures were incubated for 48 hours at 37°C. Supernatant was then stored at -80°C for further analysis (e.g. IFN-γ and TNF-α quantification) and the levels of activation markers (CD25 and CD69) on $CD8^+$ T cells were determined by FACS (using CD25 Monoclonal Antibody (BC96), PerCP-Cyanine5.5, eBioscience™; and Alexa Fluor® 488 mouse anti-human CD8 and PE-Cy™7 mouse anti-human CD69 from BD Biosciences). As a control, effector PBMCs were treated identically, except that no target cells were added. The CD25 levels obtained upon incubation with TCE DARPin® protein #20, TCE DARPin® protein #21, TCE DARPin® protein #27, TCE DARPin® protein #32 and TCE DARPin® protein #33 are shown in **Figures 8A to 8E**, as indicated. Similar results were obtained for CD69 (data not shown).

The data demonstrate that the NYESOpMHC-specific ankyrin repeat domains in TCE format can potently activate natural $CD8^+$ T cells derived from a donor in the presence of NYESOpMHC-expressing tumor cells (IM9) (**Figures 8A to 8E**, round symbols). The EC_{50} values for activation of the T cells in the presence of IM9 tumor cells by TCE DARPin® proteins were determined, using standard procedures known to the person skilled in the art. The EC_{50} values were in the low nM range or below (**Table 5**), demonstrating potent activity of the TCE DARPin® proteins.

Table 5. EC_{50} values of activating T cells in the presence of IM9 tumor cells

TCE DARPin® protein #	EC_{50} [nM]
#20	1.2
#21	1.9
#27	2.2
#32	1.9

#33	0.03

In contrast to IM9 cells, no activation of the T cells (or smaller activation only at much higher TCE DARPin® protein concentrations) was observed in the presence of tumor cells that do not express NYESOpMHC on their cell surface (MCF-7) (**Figures 8A to 8E**, triangular symbols) or if no tumor cells were added to the T cells (i.e. PBMCs only) (**Figures 8A to 8E**, square symbols).

As additional measures of immune cell activation, the levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were quantified in the supernatants of the cells after incubation (see above). The protocol provided by the manufacturer was followed (ProcartalPlexTM Multiplex Immunoassay EPX01A-10228-901 and -10223-90, Thermo Fischer Scientific). Briefly, supernatants were added to a mixture of color-coded beads pre-coated with cytokine specific capture antibodies. Secondary biotinylated detection antibodies specific for the cytokines of interest were added and formed an antibody-cytokine sandwich. PE-conjugated streptavidin was then added and bound to the biotinylated detection antibodies and the addition of reading buffer allowed the acquisition of data with the MAG/Pix Luminex instrument. Cytokine levels could be calculated using the standards provided.

The levels of IFN-γ (**Figures 9A to 9E**) and TNF-α (**Figures 10A to 10E**) in the supernatants strongly increased in the presence of IM9 tumor cells in a TCE DARPin® protein concentration-dependent manner. These results confirm that the NYESOpMHC-specific ankyrin repeat domains in TCE format can potently mediate T cell activation in the presence of NYESOpMHC-expressing tumor cells (IM9). Furthermore, no increase of IFN-γ (**Figures 9A to 9E**) and TNF-α (**Figures 10A to 10E**) (or a smaller increase only at much higher TCE DARPin® protein concentrations) was observed in the presence of tumor cells that do not express NYESOpMHC on their cell surface (MCF-7). In all of the assays described in this Example, TCE DARPin® protein #33 was potent already at lower concentrations than the other tested TCE DARPin® proteins, but less specific at higher concentrations.

Similar T cell activation assays as described above in this Example were also performed using another tumor cell line (U266B1) that endogenously expresses HLA-A2 and NY-ESO-1 and displays NYESOpMHC on its surface, and another tumor cell line (Colo205) that expresses HLA-A2 but not NY-ESO-1 and does not display NYESOpMHC on its surface. Furthermore, similar assays were also performed comparing MCF-7 tumor cells (which do not express NYESOpMHC on their cell surface) and MCF-7 tumor cells that were transfected to express NY-ESO-1 and thus express NYESOpMHC on their cell surface. The CD69 levels obtained upon incubation with TCE DARPin® protein #21 or TCE DARPin® protein #32 are shown in **Figures 18A to 18F**, as indicated, as a measure of T cell activation. With each of the tested binding proteins of the invention, significant T cell activation was only observed in the presence of tumor cells that express NYESOpMHC on their cell surface (U266B1, transfected MCF-7, IM9), but not in the presence of tumor cells that do not express NYESOpMHC on their cell surface (Colo205, MCF-7).

In conclusion, these data show that a recombinant binding protein comprising both an ankyrin repeat domain with binding specificity for a target peptide-MHC complex and a binding agent with binding specificity for a protein expressed on the surface of T cells can potently activate T cells upon binding to target peptide-MHC complex on the surface of tumor cells.

Example 7: Specificity of target peptide binding by binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC, analyzed by alanine scanning mutagenesis

In order to analyze the specific interaction between the ankyrin repeat domains with binding specificity for NYESOpMHC and the NYESOpMHC target, alanine-scanning mutagenesis was performed. For this purpose, the NY-ESO-1-9V (157–165) peptide and a series of variants thereof, in each of which one amino acid of the NY-ESO-1-9V (157–165) peptide was replaced with alanine, were tested in a T cell activation assay. The assay used pulsed T2 cells as target cells and BK112 T cells as effector cells, essentially as described in Example 4. If an amino acid residue in the NY-ESO-1-9V (157–165) peptide is important for the binding interaction between the ankyrin repeat domain with binding specificity for NYESOpMHC and the NYESOpMHC target, then a reduced or no T cell activation will be observed if this amino acid residue is replaced with alanine.

The alanine-mutated peptides were obtained from Genscript and had the following sequences: ALLMWITQV (SEQ ID NO: 81; S to A at position 1), SLAMWITQV (SEQ ID NO: 82; L to A at position 3), SLLAWITQV (SEQ ID NO: 83; M to A at position 4), SLLMAITQV (SEQ ID NO: 84; W to A at position 5), SLLMWATQV (SEQ ID NO: 85; I to A at position 6), SLLMWIAQV (SEQ ID NO: 86; T to A at position 7), SLLMWITAV (SEQ ID NO: 87; Q to A at position 8), and SALMWITQA (SEQ ID NO: 88; L to A at position 2 and V to A at position 9). In the peptide with SEQ ID NO: 88, the anchor positions 2 and 9 were both substituted with alanine. The anchor residues at positions 2 and 9 are critical for binding of the peptide to the HLA molecule. T2 cells were pulsed with the NY-ESO-1-9V (157–165) peptide and each of the mutated peptides (10 µM). Pulsed T2 cells were incubated with effector BK112 T cells at an effector to target cell ratio of 1:5 in the presence of TCE DARPin® protein.

The alanine scanning mutagenesis analysis showed which peptide residues were important for the binding interaction between the TCE DARPin® proteins and the NYESOpMHC target (**Figures 11A to 11E**). These data demonstrate that in each case at least 3 peptide residues were important for this binding interaction, causing a decrease in T cell activation by at least 50% as compared to the NY-ESO-1-9V (157–165) peptide when mutated to alanine. Surprisingly, in some cases, interactions with at least 5 peptide residues located across almost the entire peptide sequence were important for binding of the ankyrin repeat domain to the target peptide-MHC complex. As an example, for DARPin® protein #20, at least positions 3, 4, 5, 6, and 7 of the NY-ESO-1-9V (157–165) target peptide were important for the functional binding interaction leading to T cell activation (**Figure 11A**). As another example, for DARPin® protein #21, at least positions 4, 5, 6, 7 and 8 of the NY-ESO-1-9V (157–165) target peptide were important for the functional binding interaction leading to T cell activation (**Figure 11B**). As another example, for DARPin® protein #32, at least positions 1, 3, 4, 5, 6, and 7 of the NY-ESO-1-9V (157–165) target peptide were important for the functional binding interaction leading to T cell activation (**Figure 11D**). As expected, no functional binding interaction leading to T cell activation was observed when the two anchoring residues at positions 2 and 9 were mutated or when non-pulsed T2 cells were used.

In conclusion, these data show that a surprisingly large number of peptide residues are important for the specific interaction between the binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and the NY-ESO-1-9V (157–165) target peptide. This may reflect a structural difference between the binding surface formed by the designed ankyrin repeat domains and that formed by other binding proteins, such as antibodies and T cell receptors (TCRs).

Example 8: pMHC-dependent target cell killing by effector cells mediated by a binding protein comprising an ankyrin repeat domain with binding specificity for NYESOpMHC

The ability of binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC in TCE format to mediate pMHC-dependent killing of target cells by effector cells was tested in Incucyte® assays (Essen Biosciences) following the protocol provided by the manufacturer.

Briefly, effector cells (peripheral blood mononuclear cells (PBMCs)) and target cells (either pulsed T2 cells (PT2) or non-pulsed T2 cells (NPT2) cells) were incubated in the presence of different concentrations (0.01 nM, 0.1 nM, 1 nM) of an NYESOpMHC-specific ankyrin repeat protein in TCE format (TCE DARPin® protein #21 (D)). The ratio of effector to target cells was 20:1. The Incucyte® Caspase 3/7 reagent, a substrate that is cleaved during cell apoptosis to release green-fluorescent DNA dye and thus stain nuclear DNA, was added. Cells were scanned every two hours and levels of tumor cell death could be followed over time.

The results are represented in **Figure 12**, which shows the levels of cell apoptosis (total green object area). The binding protein comprising an ankyrin repeat domain with binding specificity for NYESOpMHC in TCE format was able to effectively mediate the killing of target cells displaying NYESOpMHC on their surface (PT2 cells) in a concentration-dependent manner. In contrast, no significant effect was observed on target cells that do not display NYESOpMHC on their surface (NPT2 cells).

The ability of binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC in TCE format to mediate pMHC-dependent killing of target cells by effector cells was also tested in a different type of assay. Here, not only pulsed or non-pulsed T2 cells were used as target cells, but also tumor cells that express (IM9; U266B1) or do not express (MCF-7) NYESOpMHC on their cell surface.

Briefly, radioactively-labelled (Cr51) T2 cells (pulsed with with NY-ESO-1-9V (157–165) peptide or non-pulsed) or radioactively labeled (Cr51) HLA-A2$^+$/NY-ESO-1$^+$ tumor cells lines (IM9, U266B1) or HLA-A2$^+$/ NY-ESO-1$^-$ tumor cell lines (MCF-7) were incubated, as target cells, with pre-activated effector CD8$^+$ T cells for 4 hours in the presence or absence of 1nM of TCE DARPin® protein #21 or TCE DARPin® protein #32. After the incubation, the amount of radioactivity release from the lysed target cells was determined in the supernatant with a LumaPlate TopCount NXT microplate scintillation counter. The percentage of specific lysis of the T2 cells or tumor cell lines obtained by the chromium release assay was plotted for different effector cell to target cell ratios (30:1; 10:1; 5:1; 1:1).

The results are represented in **Figures 19A and 19B** for TCE DARPin® protein #21 and in **Figures 20A and 20B** for TCE DARPin® protein #32. The results show that the binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC in TCE format were able to effectively mediate the killing of target cells displaying NYESOpMHC on their surface (pulsed T2 cells or IM9 or U266B1 tumor cells) in a manner dependent on the ratio of effector cells to target cells. An even higher level of target cell killing was achieved with higher concentrations of the TCE DARPin® proteins (data not shown). In contrast, no significant effect was observed on target cells that do not display NYESOpMHC on their surface (non-pulsed T2 cells or MCF-7 tumor cells).

Together, these data demonstrate that a binding protein comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and further comprising a binding agent with binding specificity for a protein expressed on the surface of a T cell can effectively mediate target peptide-MHC-dependent killing of target cells (including tumor cells) by effector cells (T cells). Thus, the ankyrin repeat domains of the invention can be used in a T cell engager format to mediate NYESOpMHC-specific target cell (such as tumor cell) killing by effector cells (T cells).

<u>Example 9</u>: Bivalent and biparatopic binding proteins comprising two ankyrin repeat domains with binding specificity for NYESOpMHC

Various bivalent and biparatopic binding proteins comprising two ankyrin repeat domains with binding specificity for NYESOpMHC were constructed to test if such constructs are functional in specific binding to cells displaying NYESOpMHC on their surface. Furthermore, bivalent and biparatopic binding proteins comprising two ankyrin repeat domains with binding specificity for NYESOpMHC were constructed that further comprise a binding agent with binding specificity for a protein expressed on the surface of an immune cell, such as CD3 expressed on the surface of T cells. These bivalent or biparatopic binding proteins in T cell engager format were tested for their ability to mediate T cell activation upon binding to NYESOpMHC-displaying target cells. All the constructs were generated by conventional cloning methods.

An example of a bivalent binding protein comprising two ankyrin repeat domains with binding specificity for NYESOpMHC is a binding protein, which comprises two ankyrin repeat domains of SEQ ID NO: 21 connected by a peptide linker (SEQ ID NO: 16 BV DARPin® protein #21/#21). Linking BV DARPin® protein #21/#21 to a binding agent with binding specificity for CD3 resulted in a bivalent binding protein in T cell engager format (TCE BV DARPin® protein #21/#21).

Examples of biparatopic binding proteins comprising two ankyrin repeat domains with binding specificity for NYESOpMHC are binding proteins which comprise one ankyrin repeat domain of SEQ ID NO: 20 and one ankyrin repeat domain of SEQ ID NO: 21 connected by a peptide linker (SEQ ID NO: 17; BP DARPin® protein #20/#21) or one ankyrin repeat domain of SEQ ID NO: 21 and one ankyrin repeat domain of SEQ ID NO: 22 connected by a peptide linker (SEQ ID NO: 18; BP DARPin® protein #21/#22). Linking BP DARPin® protein #20/#21 or BP DARPin® protein #21/#22 to a binding agent with binding specificity for CD3 resulted in biparatopic binding proteins in T cell engager format (TCE BP DARPin® protein #20/#21 and TCE BP DARPin® protein #21/#22).

T cell activation assay using pulsed T2 cells as target cells and BK112 T cells as effector cells

The ability of bivalent and biparatopic binding proteins in TCE format to activate T cells upon binding to NYESOpMHC-displaying target cells was analyzed using the T cell activation assay described in Example 4. Briefly, T2 cells pulsed with NY-ESO-1-9V (157–165) peptide were used as NYESOpMHC-displaying target cells and BK112 T cells were used as effector cells. The T cell activation assay was performed essentially as described in Example 4.

Figure 13 shows the result of testing different bivalent and biparatopic NYESOpMHC-specific ankyrin repeat proteins in TCE format (0.1 pM) in this T cell activation assay. The NYESOpMHC-specific ankyrin repeat proteins in TCE format were (corresponding to the numbers at the x-axis in Figure 13) (1) TCE BP DARPin® protein #20/#21, (2) TCE BP DARPin® protein #21/#22, and (3) TCE BV DARPin® protein #21/#21. As controls, the experiment was also performed with a TCE DARPin® protein comprising an ankyrin repeat domain with binding specificity for human serum albumin instead of the ankyrin repeat domains with binding specificity for NYESOpMHC (4) and with no TCE DARPin® protein added (5).

All three bivalent or biparatopic TCE DARPin® proteins specifically mediated T cell activation in the presence of pulsed T2 cells, but not (or much less) in the presence of non-pulsed T2 cells. Thus, the bivalent and biparatopic NYESOpMHC-specific ankyrin repeat proteins in TCE format were able to specifically activate effector T cells dependent on the presence of NYESOpMHC-displaying target cells.

Specific binding to NYESOpMHC-displaying cells

The ability of bivalent or biparatopic pMHC-specific binding proteins to specifically bind to target peptide-MHC complex-displaying cells was investigated using the T2 cell binding assay described in Example 3. The binding proteins were also tested in a TCE format. The experiments were performed essentially as described in Example 3.

As an example, **Figures 14A and 14B** show the obtained binding curves for BP DARPin® protein #21/#22 and TCE BP DARPin® protein #21/#22, using T2 cells pulsed with NY-ESO-1-9V (157–165) peptide (Figure 14A) and non-pulsed T2 cells (Figure 14B). EC_{50} values for binding to T2 cells pulsed with NY-ESO-1-9V (157–165) peptide were determined using standard procedures known to the person skilled in the art. EC_{50} values of these biparatopic NYESOpMHC-specific ankyrin repeat proteins were determined to be in the low nanomolar range (**Table 6**), demonstrating efficient binding to the cells. There was no significant difference in binding to pulsed or non-pulsed T2 cells between the biparatopic binding protein (BP DARPin® protein #21/#22) and the same biparatopic binding protein in TCE format (TCE BP DARPin® protein #21/#22).

Table 6. EC_{50} values of binding to T2 cells pulsed with NY-ESO-1-9V (157–165) peptide

Binding protein	EC_{50} [nM]
BP DARPin® protein #21/#22	1.4
TCE BP DARPin® protein #21/#22	2.5

In conclusion, the binding proteins comprising two ankyrin repeat domains with binding specificity for NYESOpMHC were able to specifically and efficiently bind to NYESOpMHC on the surface of cells, with an EC50 in the low nanomolar range. Binding to NYESOpMHC on the surface of cells was specific, since no binding was observed to non-pulsed T2 cells. The specific and efficient binding properties were conserved when the two NYESOpMHC-specific ankyrin repeat domains were linked to a binding agent with binding specificity for a protein expressed on the surface of immune cells, such as CD3 expressed on the surface of T cells (T cell engager (TCE) format).

Immune cell activation in the presence of NYESOpMHC-expressing tumor cells

The ability of bivalent and biparatopic NYESOpMHC-specific ankyrin repeat proteins in TCE format to activate T cells upon binding to NYESOpMHC-displaying target cells was also analyzed in T cell activation assays using tumor cell lines as target cells and peripheral blood mononuclear cells (PBMCs) isolated from a donor as effector cells, in an assay performed essentially as described in Example 6.

The levels of the T cell activation marker CD25 obtained upon incubation with the bivalent binding protein TCE BV DARPin® protein #21/#21 or the biparatopic binding protein TCE BP DARPin® protein #20/#21 are shown in **Figures 15A and 15B**, as indicated. The data demonstrate that bivalent or biparatopic NYESOpMHC-specific ankyrin repeat proteins in TCE format can potently mediate activation of $CD8^+$ T cells derived from a donor in the presence of NYESOpMHC-expressing tumor cells (IM9) (**Figures 15A and 15B**, round symbols). The EC_{50} values for activation of the T cells in the presence of IM9 tumor cells by bivalent or biparatopic TCE DARPin® proteins were determined, using standard procedures known to the person skilled in the art. The EC_{50} values were in the low nM range or below (**Table 7**), demonstrating potent activity of the bivalent and biparatopic TCE DARPin® proteins.

Table 7. EC_{50} values of activating T cells in the presence of IM9 tumor cells

Binding protein	EC_{50} [nM]
TCE BV DARPin® protein #21/#21	0.9
TCE BP DARPin® protein #20/#21	2.0

In contrast to IM9 cells, no activation of the T cells (or smaller activation only at much higher TCE DARPin® protein concentrations) was observed in the presence of tumor cells that do not express NYESOpMHC on their cell surface (MCF-7) (**Figures 15A and 15B**, triangular symbols) or if no tumor cells were added to the T cells (i.e. PBMCs only) (**Figures 15A and 15B**, square symbols).

As additional measures of immune cell activation, the levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) were quantified in the supernatants of the cells after incubation with the biparatopic binding protein (TCE BP DARPin® protein #20/#21), essentially as described in Example 6. The levels of IFN-γ (**Figure 16A**) and TNF-α (**Figure 16B**) in the supernatants strongly increased in the presence of IM9 tumor cells in a TCE DARPin® protein concentration-dependent manner. These results confirm that the biparatopic NYESOpMHC-specific ankyrin repeat protein in TCE format can potently mediate activation of

T cells in the presence of NYESOpMHC-expressing tumor cells (IM9). No significant increase of IFN-γ (**Figure 16A**) and TNF-α (**Figure 16B**) was observed in the presence of tumor cells that do not express NYESOpMHC on their cell surface (MCF-7).

In conclusion, these data show that a recombinant binding protein comprising two ankyrin repeat domains with binding specificity for a target peptide-MHC complex and a binding agent with binding specificity for a protein expressed on the surface of T cells can potently activate T cells upon binding to target peptide-MHC complex on the surface of tumor cells.

Specificity of target peptide binding analyzed by alanine scanning mutagenesis

In order to analyze the specific interaction between binding proteins comprising two ankyrin repeat domains with binding specificity for NYESOpMHC and the NYESOpMHC target, alanine-scanning mutagenesis was performed essentially as described in Example 7.

The alanine scanning mutagenesis analysis for the biparatopic binding protein TCE BP DARPin® protein #20/#21 (1 pM) is shown in **Figure 17**. These data demonstrate that at least 3 peptide residues, i.e. the residues at positions 4, 5, and 6, were important for the binding interaction between this biparatopic binding protein and the target peptide-MHC complex on the surface of the target cells. As expected, no binding was observed when the two anchoring residues at positions 2 and 9 were mutated or when non-pulsed T2 cells were used.

<u>Example 10</u>: Specificity of target peptide binding by binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC, analyzed by X-scanning mutagenesis

In order to analyze further the specific interaction between the ankyrin repeat domains with binding specificity for NYESOpMHC and the NYESOpMHC target, X-scanning mutagenesis was performed. For this purpose, the NY-ESO-1-9V (157–165) peptide and a series of single-mutation variants thereof were tested in a T cell activation assay. For the variant peptides, each amino acid of the NY-ESO-1-9V (157–165) peptide was replaced with every one of the other 19 standard amino acids found in proteins. These mutated peptides were obtained from Genscript. The assay used pulsed T2 cells as target cells and BK112 T cells as effector cells, essentially as described in Example 4. In brief, T2 cells were pulsed with each of the mutated peptides (10 μM) and incubated with effector $CD8^+$ T cells (BK112 T cells) for 4 hours in the presence of TCE DARPin® protein concentrations allowing EC90 levels for the wildtype peptide (TCE DARPin® protein #21: 1 pM; TCE DARPin® protein #32: 10 pM). After the incubation, intracellular IFN-γ was detected by FACS. Each of the experiments were performed in two independent replicates.

The X-scanning mutagenesis analysis expands on the alanine-scanning mutagenesis described in Example 7. Testing each of the other 19 amino acids at each position of the NY-ESO-1-9V (157–165) peptide allows to characterize the specificity of the binding interaction between a binding protein of the invention comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and the NYESOpMHC complex displayed on the surface of cells. Such an analysis shows which amino acid residue(s) at a given position of the peptide allow or do not allow an efficient binding interaction. Furthermore, such analysis allows to identify potentially cross-reactive peptides.

For further analysis, the intracellular IFN-γ values obtained in the independent replicates of the T cell activation assays were averaged and normalized to 100% for the according wild-type residue (dark shaded fields) in each position. The averaged and normalized results of the analysis are shown in **Figure 21A** for TCE DARPin® protein #21 and in **Figure 21B** for TCE DARPin® protein #32. All values above 30%, indicating no loss or not a complete loss of T-cell activation, are marked in bold font and light shaded color.

The X-scan analysis confirmed that binding proteins of the invention comprising an ankyrin repeat domain with binding specificity for NYESOpMHC interact with multiple residues of the NY-ESO-1-9V (157–165) peptide presented in the peptide binding groove of the MHC class I molecule. For the tested TCE DARPin® proteins, i.e. TCE DARPin® protein #21 and TCE DARPin® protein #32, interactions with at least 5 peptide residues were important for binding of the ankyrin repeat domain to the target peptide-MHC complex. For DARPin® protein #21, at least positions 4, 5, 6, 7 and 8 of the NY-ESO-1-9V (157–165) target peptide were important for the functional binding interaction leading to T cell activation (**Figure 21A**). For DARPin® protein #32, at least positions 3, 4, 5, 6, and 7 of the NY-ESO-1-9V (157–165) target peptide were important for the functional binding interaction leading to T cell activation (**Figure 21B**). Positions 2 and 9 were not considered in these conclusions, since the residues at these positions are the anchor residues, which are critical for binding of the peptide to the HLA molecule.

Search for potential cross-reactive peptides using the Expasy Prosite database (https://prosite.expasy.org/scanprosite/) identified 43 unique human peptide sequences for TCE DARPin® protein #21 and 68 unique human peptide sequences for TCE DARPin® protein #32. These numbers are comparable to values previously reported for natural T-cell receptors.

In conclusion, these data show again that a surprisingly large number of peptide residues are important for the specific interaction between the binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC and the NY-ESO-1-9V (157–165) target peptide. This may reflect a structural difference between the binding surface formed by the designed ankyrin repeat domains and that formed by other binding proteins, such as antibodies and T cell receptors (TCRs).

Furthermore, the results demonstrate that the method of producing a peptide-MHC (pMHC)-specific binding protein described herein allows efficient identification and characterization of binding proteins which target pMHC complexes, such as, e.g., tumor-specific or tumor-associated pMHC complexes, and which have advantageous properties. The method allows to efficiently produce pMHC-specific binding proteins with high affinity to the target pMHC complex and with a level of specificity comparable to that of natural or affinity-matured T-cell receptors, as assessed by the low number of potentially cross-reactive peptides identified by X-scan and in vitro cellular assays.

Example 11: Influence of linker length on immune cell activation by binding proteins comprising an ankyrin repeat domain with binding specificity for NYESOpMHC

In order to test the influence of the length of the linker between the pMHC-specific binding domain and the CD3-specific binding agent in a binding protein of the invention in T cell engager (TCE) format on the potency of such binding proteins in TCE format, different constructs comprising a NYESOpMHC binding domain connected to a CD3 binding domain with linkers of varying length were tested in a T cell activation assay using different tumor cell lines.

Linker length design & abbreviations

The following binding proteins in TCE format were generated comprising DARPin® protein #21 or DARPin® protein #32 as the NYESOpMHC-specific binding domain:

Table 8: TCE binding proteins and the linkers used

Binding protein in TCE format	PT linker
TCE DARPin® protein #21 (standard)	standard
TCE DARPin® protein #21 (XXS)	XXS
TCE DARPin® protein #21 (XS)	XS
TCE DARPin® protein #21 (S)	S
TCE DARPin® protein #21 (L)	L
TCE DARPin® protein #32 (standard)	standard
TCE DARPin® protein #32 (XXS)	XXS
TCE DARPin® protein #32 (XS)	XS
TCE DARPin® protein #32 (S)	S
TCE DARPin® protein #32 (L)	L

Table 9: Overview of the linkers tested in the binding proteins of Table 8

PT linker abbreviation	SEQ ID NO	Length (a.a.)	Amino Acid Sequence
standard	1	24	GSPTPTPTTPTPTPTTPTPTPTGS
XXS	280	6	GSPTGS
XS	279	11	GSPTPTPTTGS
S	278	18	GSPTPTPTTPTPTPTTGS
L	277	38	GSPTPTPTTPTPTPTTPTPTPTTPTPTPTTPTPTPTGS

Potency and specificity of binding proteins in TCE format in T cell activation assays

The potency and specificity of binding proteins in TCE format generated with different linkers was tested using different tumor cell lines in a T cell activation assay. Some of the tumor cell lines (IM9 and U266B1) endogenously express NY-ESO-1 and present the NY-ESO-1 target peptide in a pMHC complex on their cell surface. Other tumor cell lines (MCF7 and Colo205) do not express NY-ESO-1 and hence do no present the NY-ESO-1 target peptide in a pMHC complex on their cell surface.

T cell activation assays were essentially performed as described above in Example 6. Accordingly, target tumor cells and effector PBMCs were combined at an effector-to-target cell ratio of 5:1, NYESOpMHC-specific ankyrin repeat domains in TCE format (see constructs in **Table 8**) were added at different concentrations, and the mixtures were incubated for 48 hours at 37°C. The levels of activation marker CD25 on CD8+ T cells were determined by FACS (using CD25 Monoclonal Antibody (BC96), PerCP-Cyanine5.5, eBioscience™; and Alexa Fluor® 488 mouse anti-human CD8 from BD Biosciences). As a control, effector PBMCs were treated identically, except that no target cells were added. The same sets of tumor cells were used as in Example 6.

The levels of T cell activation obtained in the presence of the binding proteins are shown in **Figures 22 to 25**. Enhanced potency was observed with decreasing linker length. The highest potency was observed for the constructs designed with the shortest linker (XXS), with the potency of the constructs designed with the second shortest linker (XS) also strongly enhanced compared to the constructs designed with longer linkers. No increase in the levels of non-specific activation was observed for any of the cell lines tested.

The EC50 values obtained from the data shown in Figure 22 are listed in Table 10 below.

Table 10 : EC50 values obtained from the data shown in Figure 22

Binding protein in TCE format	**EC50 [pM] IM9 (Ag+)**
TCE DARPin® protein #21 (L)	1245
TCE DARPin® protein #21 (standard)	365.5
TCE DARPin® protein #21 (S)	422.1
TCE DARPin® protein #21 (XS)	304.6
TCE DARPin® protein #21 (XXS)	143.4

The EC50 values obtained from the data shown in Figure 23 are listed in Table 11 below.

Table 11 : EC50 values obtained from the data shown in Figure 23

Binding protein in TCE format	**EC50 [pM] IM9 (Ag+)**
TCE DARPin® protein #32 (L)	2949
TCE DARPin® protein #32 (standard)	1236
TCE DARPin® protein #32 (S)	1075
TCE DARPin® protein #32 (XS)	437.3
TCE DARPin® protein #32 (XXS)	272.8

The EC50 values obtained from the data shown in Figure 24 are listed in Table 12 below.

Table 12 : EC50 values obtained from the data shown in Figure 24

Binding protein in TCE format	EC50 [pM] U266B1 (Ag+)
TCE DARPin® protein #21 (L)	~67000
TCE DARPin® protein #21 (standard)	10120
TCE DARPin® protein #21 (S)	3361
TCE DARPin® protein #21 (XS)	558.9
TCE DARPin® protein #21 (XXS)	491.8

The EC50 values obtained from the data shown in Figure 25 are listed in Table 13 below.

Table 13 : EC50 values obtained from the data shown in Figure 25

Binding protein in TCE format	EC50 [pM] U266B1 (Ag+)
TCE DARPin® protein #32 (L)	37480
TCE DARPin® protein #32 (standard)	5655
TCE DARPin® protein #32 (S)	5776
TCE DARPin® protein #32 (XS)	2188
TCE DARPin® protein #32 (XXS)	555.2

From the data, it can be concluded that, for the pMHC-specific binding domains in TCE format, preferably a linker is used that is at least as short as the L linker, more preferably at least as short as the standard linker, more preferably at least as short as the S linker, more preferably at least as short as the XS linker, more preferably at least as short as short as the XXS linker and most preferably as short or about as short as the XXS linker. The specific linkers provided in this Example are examples of such linkers with the preferred lengths.

<u>Example 12</u>: Selection of binding proteins comprising an ankyrin repeat domain with binding specificity for EBNA-1 peptide-MHC complex (EBNA1pMHC)

Summary

Using ribosome display (Hanes, J. and Plückthun, A., PNAS *94*, 4937-42, 1997), multiple ankyrin repeat domains with binding specificity for EBNA-1 peptide-MHC complex (EBNA1pMHC) were selected from DARPin® libraries in a way similar to the one described by Binz et al. 2004 (loc. cit.), with specific conditions and additional de-selection steps as described below. The binding and specificity of the selected clones towards recombinant EBNA1pMHC and other pMHC complexes were assessed by *E. coli* crude extract Homogeneous Time Resolved Fluorescence (HTRF), indicating that multiple EBNA1pMHC-specific binding proteins were successfully selected. For example, the ankyrin repeat domains of SEQ ID NOs: 93 to 110 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for EBNA1pMHC. Individual ankyrin repeat modules from such ankyrin repeat domains with binding specificity to EBNA1pMHC are provided, e.g., in SEQ ID NOs: 111 to 154.

Production of biotinylated pMHC complexes as target and selection material

Tripartite complexes of HLA-A*0201 (SEQ ID NO: 73), human beta-2-microglobulin (hβ2m; SEQ ID NO: 74) and one of the peptides of EBNA-1 (FMVFLQTHI; SEQ ID NO: 92) or the NY-ESO-1 related peptides of SEQ ID NO: 19 or SEQ ID NO: 35 were produced according to established protocols (Garboczi et al, 1992; Celie et al, 2009). Codon-optimized HLA-A*0201 comprising a linker (GSGGSGGSAGG; SEQ ID NO: 75 and the Avi-tag (GLNDIFEAQKIEWHE; SEQ ID NO: 76; Fairhead & Howarth, 2015) for biotinylation (HLA-A*0201avi; SEQ ID NO: 77) and wild-type human beta-2-microglobulin (hβ2m) were expressed in *E. coli* BL21 (DE3) at 37°C as inclusion bodies (IB) and dissolved in 50 mM MES, 5 mM EDTA, 5 mM DTT, 8M urea, pH 6.5 after IB purification. HLA-A*0201avi and hβ2m molecules were refolded in the presence of the respective peptides at final concentrations of 25, 30 and 15 mg, respectively, per 500 mL volume in 50 mM Tris pH 8.3, 230 mM L-Arginine, 3 mM EDTA, 255 μM GSSG; 2.5 mM GSH; 250 μM PMSF. The resulting pMHC complexes were (1) EBNA1pMHC comprising the EBNA-1 peptide of SEQ ID NO: 92, (2) NYESOpMHC comprising the peptide of SEQ ID NO: 19, and (3) NYESOAApMHC comprising the peptide of SEQ ID NO: 35.

For biotinylation, samples were concentrated to a volume of 7.5 mL, and the buffer was exchanged to 100 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5 using PD10 columns. HLA-A*0201avi was biotinylated by adding 5 mM ATP, 400 μM Biotin, 200 μM PMSF and 20 μg BirA enzyme. BirA was produced in-house following the procedure described in Shen et al, 2009. The refolded tripartite, biotinylated complexes were isolated using size exclusion chromatography (Superdex 200 HiLoad 16/600) in PBS supplemented with 150 mM NaCl, 1mM EDTA, 10% glycerol. Samples were concentrated to about 1 mg/mL and flash-frozen as 25 and 50 μL aliquots by liquid nitrogen.

For quality control purposes, 25 μg of biotinylated pMHC complexes (biotin-pMHC) were incubated with 50 μg Streptavidin (IBA Lifesciences), either with or without adding 100 mM DTT and incubation at 95°C for 5 min. 50 μg samples were run over analytical size exclusion chromatography (GE Superdex 200 10/300 GL). The biotinylated EBNA1pMHC, NYESOpMHC and NYESOAApMHC complexes all eluted at a peak maximum of about 82 mL from the Superdex 200 HiLoad 16/600 column. SDS-PAGE analysis of the concentrated and thawed flash-frozen refolded complexes indicated efficient biotinylation, since HLA-A*0201avi was almost completely bound to Streptavidin. Analytical size exclusion chromatography revealed single peaks at retention volumes corresponding to apparent molecular weights close to the theoretical MW of the tripartite pMHC complexes (45 kD).

Selection of EBNA1pMHC-specific ankyrin repeat proteins by ribosome display

The selection of pMHC-specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using the EBNA1pMHC complex as a target, libraries of ankyrin repeat proteins as described above, and established protocols (See, e.g., Zahnd, C., Amstutz, P. and Plückthun, A., Nat. Methods *4*, 69-79, 2007). The number of reverse transcription (RT)-PCR cycles after each selection round was continuously reduced, adjusting to the yield due to enrichment of binders. The first four rounds of selection employed standard ribosome display selection, using decreasing target concentrations and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. 2004, loc. cit.), but incorporated an unusual de-selection step.

During the ribosome display rounds, a de-selection (or negative selection) step was incorporated, wherein the ternary complexes were pre-incubated with the corresponding isotype HLA molecule containing another peptide, and only then transferred to the target EBNA1pMHC complex, in order to direct binding of ankyrin repeat proteins towards the peptide-embedded epitope and away from the common HLA-A scaffold. In other words, de-selection (or negative selection) was performed in order to de-select ankyrin repeat proteins that bind predominantly to the common HLA-A scaffold of pMHC complexes rather than to the specific epitope provided by the embedded peptide. Furthermore, ankyrin repeat proteins that cross-react with the epitope provided by the embedded peptide used for de-selection were also de-selected. Here, the NYESOpMHC complex was used for de-selection.

In detail, for the de-selection step, Nunc MaxiSorp plates were coated with 100 µl solution of 66 nM neutravidin in PBS and incubated at 4°C overnight. The following day, the MaxiSorp 96-well microplates were washed three times with 300 µl PBST per well and blocked with 300 µl PBST-BSA for 1h at 4°C, rotating at 700 rpm, prior to the de-selection step. After emptying the wells, 100 µl of a 50 nM biotinylated pMHC de-selection target solution in PBST-BSA was added to each well, rotating with 700 rpm at 4°C for 1h. During this incubation step, mRNA in-vitro translations according to the ribosome display protocol were performed separately. Shortly after in vitro translations and the generation of ternary complexes (i.e. mRNA, ribosome, and translated ankyrin repeat protein), the pMHC-PBST-BSA solutions were discarded and Nunc MaxiSorp microplate wells were washed three times with 300 µl PBST and finally incubated with Tris-wash buffer containing BSA (WBT-BSA). Just prior to the actual de-selection step, the WBT-BSA solution was discarded and aliquots of 150 µl (for the first selection round) or 100 µl (for selection rounds 2 to 4) of the in vitro translation ternary complexes were transferred consecutively three times to a prepared Nunc MaxiSorp well containing the immobilized de-selection pMHC complex and incubated in each of the three wells for 20 min at 4°C. At the end of the de-selection process, all the 100µl ternary complex aliquots of each selection pool were combined and the according volumes were taken forward into the selection on the actual target pMHC complex as described above.

Selected clones bind specifically to EBNA1pMHC complex as shown by crude extract HTRF

Individually selected ankyrin repeat proteins specifically binding EBNA1pMHC complex in solution were identified by a Homogeneous Time Resolved Fluorescence (HTRF) assay using crude extracts of ankyrin repeat protein-expressing *Escherichia coli* cells using standard protocols. Ankyrin repeat protein clones selected by ribosome display were cloned into a derivative of the pQE30 (Qiagen) expression vector, transformed into *E. coli* XL1-Blue (Stratagene), plated on LB-agar (containing 1% glucose and 50 µg/ml ampicillin) and then incubated overnight at 37°C. Single colonies were picked into a 96 well plate (each clone in a single well) containing 165 µl growth medium (LB containing 1% glucose and 50 µg/ml ampicillin) and incubated overnight at 37°C, shaking at 800 rpm. 150 µl of fresh LB medium containing 50 µg/ml ampicillin was inoculated with 8.5 µl of the overnight culture in a fresh 96-deep-well plate. After incubation for 120 minutes at 37°C and 850 rpm, expression was induced with IPTG (0.5 mM final concentration) and continued for 6 hours. Cells were harvested by centrifugation of the plates, supernatant was discarded and the pellets were frozen at -20°C overnight before resuspension in 8.5 µl µl B-PERII (Thermo Scientific) and incubation for one hour at room temperature with shaking (600 rpm). Then, 160 µl PBS was added and cell debris was removed by centrifugation (3220 g for 15 min).

The extract of each lysed clone was applied as a 1:200 dilution (final concentration) in PBSTB (PBS supplemented with 0.1% Tween 20® and 0.2% (w/v) BSA, pH 7.4) together with 20 nM (final concentration) biotinylated pMHC complex, 1:400 (final concentration) of anti-6His-D2 HTRF antibody – FRET acceptor conjugate (Cisbio) and 1:400 (final concentration) of anti-strep-Tb antibody FRET donor conjugate (Cisbio, France) to a well of a 384-well plate and incubated for 120 minutes at 4°C. The HTRF was read-out on a Tecan M1000 using a 340 nm excitation wavelength and a 620 ±10 nm emission filter for background fluorescence detection and a 665 ±10 nm emission filter to detect the fluorescence signal for specific binding.

The extract of each lysed clone was tested for binding to each of the two biotinylated pMHC complexes, i.e. EBNA1pMHC and NYESOpMHC, in order to assess binding and specificity to the target EBNA1pMHC complex. NYESOpMHC served as pMHC complex distinct from EBNA1pMHC to allow selection of ankyrin repeat proteins with high binding specificity for EBNA1pMHC.

In order to calculate the specificity of each ankyrin repeat protein for EBNA1pMHC, the ratio of the HTRF signal for the target EBNA1pMHC to the HTRF signal for the distinct NYESOpMHC was determined. All binders which generated at least 25-times higher HTRF signals on the target EBNA1pMHC than on NYESOpMHC were regarded as specific hits and taken forward for sequencing. Surprisingly, screening of several hundred clones by such a crude cell extract HTRF analysis revealed many different ankyrin repeat domains with specificity for EBNA1pMHC. Specific binding of ankyrin repeat proteins to a composite epitope comprising an HLA scaffold and a short peptide, wherein only the peptide differs from other composite epitopes which are not specifically bound, has never been shown before and developing specific binders to such composite epitopes using antibody or TCR technology has been challenging.

<u>Example 13</u>: Selection of binding proteins comprising an ankyrin repeat domain with binding specificity for MAGE-A3 peptide-MHC complex (MAGEA3pMHC)

Summary

Using ribosome display (Hanes, J. and Plückthun, A., PNAS *94*, 4937-42, 1997), multiple ankyrin repeat domains with binding specificity for MAGE-A3 peptide-MHC complex (MAGEA3pMHC) were selected from DARPin® libraries in a way similar to the one described by Binz et al. 2004 (loc. cit.), with specific conditions and additional de-selection steps as described below. The binding and specificity of the selected clones towards recombinant MAGEA3pMHC and other pMHC complexes were assessed by *E. coli* crude extract Homogeneous Time Resolved Fluorescence (HTRF), indicating that multiple MAGEA3pMHC-specific binding proteins were successfully selected. For example, the ankyrin repeat domains of SEQ ID NOs: 156 to 173 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for MAGEA3pMHC. Individual ankyrin repeat modules from such ankyrin repeat domains with binding specificity to MAGEA3pMHC are provided, e.g., in SEQ ID NOs: 175 to 217.

Production of biotinylated pMHC complexes as target and selection material

Tripartite complexes of HLA-A*0101 (SEQ ID NO: 218), human beta-2-microglobulin (hβ2m; SEQ ID NO: 74) and one of the peptides of MAGE-A3 (EVDPIGHLY; SEQ ID NO: 155) or Titin (ESDPIVAQY, SEQ ID NO: 174) were produced according to established protocols (Garboczi et al, 1992; Celie et al, 2009). Codon-optimized HLA-A*0101 comprising a linker (GSGGSGGSAGG; SEQ ID NO: 75 and the Avi-tag (GLNDIFEAQKIEWHE; SEQ ID NO: 76; Fairhead & Howarth, 2015) for biotinylation (HLA-A*0101avi; SEQ ID NO: 219) and wild-type human beta-2-microglobulin (hβ2m) were expressed in *E. coli* BL21 (DE3) at 37°C as inclusion bodies (IB) and dissolved in 50 mM MES, 5 mM EDTA, 5 mM DTT, 8M urea, pH 6.5 after IB purification. HLA-A*0101avi and hβ2m molecules were refolded in the presence of the respective peptides at final concentrations of 25, 30 and 15 mg, respectively, per 500 mL volume in 50 mM Tris pH 8.3, 230 mM L-Arginine, 3 mM EDTA, 255 µM GSSG; 2.5 mM GSH; 250 µM PMSF. The resulting pMHC complexes were (1) MAGEA3pMHC comprising the MAGE-A3 peptide of SEQ ID NO: 155, and (2) TITINpMHC comprising the Titin peptide of SEQ ID NO: 174.

For biotinylation, samples were concentrated to a volume of 7.5 mL, and the buffer was exchanged to 100 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5 using PD10 columns. HLA-A*0101avi was biotinylated by adding 5 mM ATP, 400 µM Biotin, 200 µM PMSF and 20 µg BirA enzyme. BirA was produced in-house following the procedure described in Shen et al, 2009. The refolded tripartite, biotinylated complexes were isolated using size exclusion chromatography (Superdex 200 HiLoad 16/600) in PBS supplemented with 150 mM NaCl, 1mM EDTA, 10% glycerol. Samples were concentrated to about 1 mg/mL and flash-frozen as 25 and 50 µL aliquots by liquid nitrogen.

For quality control purposes, 25 µg of biotinylated pMHC complexes (biotin-pMHC) were incubated with 50 µg Streptavidin (IBA Lifesciences), either with or without adding 100 mM DTT and incubation at 95°C for 5 min. 50 µg samples were run over analytical size exclusion chromatography (GE Superdex 200 10/300 GL). The biotinylated MAGEA3pMHC and TITINpMHC complexes both eluted at a peak maximum of about 82 mL from the Superdex 200 HiLoad 16/600 column. SDS-PAGE analysis of the concentrated and thawed flash-frozen refolded complexes indicated efficient biotinylation, since HLA-A*0101avi was almost completely bound to Streptavidin. Analytical size exclusion chromatography revealed single peaks at retention volumes corresponding to apparent molecular weights close to the theoretical MW of the tripartite pMHC complexes.

Selection of MAGEA3pMHC-specific ankyrin repeat proteins by ribosome display

The selection of pMHC-specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using the MAGEA3pMHC complex as a target, libraries of ankyrin repeat proteins as described above, and established protocols (See, e.g., Zahnd, C., Amstutz, P. and Plückthun, A., Nat. Methods *4*, 69-79, 2007). The number of reverse transcription (RT)-PCR cycles after each selection round was continuously reduced, adjusting to the yield due to enrichment of binders. The first four rounds of selection employed standard ribosome display selection, using decreasing target concentrations and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. 2004, loc. cit.), but incorporated an unusual de-selection step.

During the ribosome display rounds, a de-selection (or negative selection) step was incorporated, wherein the ternary complexes were pre-incubated with the corresponding isotype HLA molecule containing another peptide, and only then transferred to the target MAGEA3pMHC complex, in order to direct binding of ankyrin repeat proteins towards the peptide-embedded epitope and away from the common HLA-A scaffold. In other words, de-selection (or negative selection) was performed in order to de-select ankyrin repeat proteins that bind predominantly to the common HLA-A scaffold of pMHC complexes rather than to the specific epitope provided by the embedded peptide. Furthermore, ankyrin repeat proteins that cross-react with the epitope provided by the embedded peptide used for de-selection were also de-selected. Here, the TITINpMHC complex was used for de-selection.

In detail, for the de-selection step, Nunc MaxiSorp plates were coated with 100 µl solution of 66 nM neutravidin in PBS and incubated at 4°C overnight. The following day, the MaxiSorp 96-well microplates were washed three times with 300 µl PBST per well and blocked with 300 µl PBST-BSA for 1h at 4°C, rotating at 700 rpm, prior to the de-selection step. After emptying the wells, 100 µl of a 50 nM biotinylated pMHC de-selection target solution in PBST-BSA was added to each well, rotating with 700 rpm at 4°C for 1h. During this incubation step, mRNA in-vitro translations according to the ribosome display protocol were performed separately. Shortly after in vitro translations and the generation of ternary complexes (i.e. mRNA, ribosome, and translated ankyrin repeat protein), the pMHC-PBST-BSA solutions were discarded and Nunc MaxiSorp microplate wells were washed three times with 300 µl PBST and finally incubated with Tris-wash buffer containing BSA (WBT-BSA). Just prior to the actual de-selection step, the WBT-BSA solution was discarded and aliquots of 150 µl (for the first selection round) or 100 µl (for selection rounds 2 to 4) of the in vitro translation ternary complexes were transferred consecutively three times to a prepared Nunc MaxiSorp well containing the immobilized de-selection pMHC complex and incubated in each of the three wells for 20 min at 4°C. At the end of the de-selection process, all the 100µl ternary complex aliquots of each selection pool were combined and the according volumes were taken forward into the selection on the actual target pMHC complex as described above.

Selected clones bind specifically to MAGEA3pMHC complex as shown by crude extract HTRF

Individually selected ankyrin repeat proteins specifically binding MAGEA3pMHC complex in solution were identified by a Homogeneous Time Resolved Fluorescence (HTRF) assay using crude extracts of ankyrin repeat protein-expressing *Escherichia coli* cells using standard protocols. Ankyrin repeat protein clones selected by ribosome display were cloned into a derivative of the pQE30 (Qiagen) expression vector, transformed into *E. coli* XL1-Blue (Stratagene), plated on LB-agar (containing 1% glucose and 50 µg/ml ampicillin) and then incubated overnight at 37°C. Single colonies were picked into a 96 well plate (each clone in a single well) containing 165 µl growth medium (LB containing 1% glucose and 50 µg/ml ampicillin) and incubated overnight at 37°C, shaking at 800 rpm. 150 µl of fresh LB medium containing 50 µg/ml ampicillin was inoculated with 8.5 µl of the overnight culture in a fresh 96-deep-well plate. After incubation for 120 minutes at 37°C and 850 rpm, expression was induced with IPTG (0.5 mM final concentration) and continued for 6 hours. Cells were harvested by centrifugation of the plates, supernatant was discarded and the pellets were frozen at -20°C overnight before resuspension in 8.5 µl µl B-PERII (Thermo Scientific) and incubation for one hour at room temperature with shaking (600 rpm). Then, 160 µl PBS was added and cell debris was removed by centrifugation (3220 g for 15 min).

The extract of each lysed clone was applied as a 1:200 dilution (final concentration) in PBSTB (PBS supplemented with 0.1% Tween 20® and 0.2% (w/v) BSA, pH 7.4) together with 20 nM (final concentration) biotinylated pMHC complex, 1:400 (final concentration) of anti-6His-D2 HTRF antibody – FRET acceptor conjugate (Cisbio) and 1:400 (final concentration) of anti-strep-Tb antibody FRET donor conjugate (Cisbio, France) to a well of a 384-well plate and incubated for 120 minutes at 4°C. The HTRF was read-out on a Tecan M1000 using a 340 nm excitation wavelength and a 620 ±10 nm emission filter for background fluorescence detection and a 665 ±10 nm emission filter to detect the fluorescence signal for specific binding.

The extract of each lysed clone was tested for binding to each of the two biotinylated pMHC complexes, i.e. MAGEA3pMHC and TITINpMHC, in order to assess binding and specificity to the target MAGEA3pMHC complex. TITINpMHC served as pMHC complex distinct from MAGEA3pMHC to allow selection of ankyrin repeat proteins with high binding specificity for MAGEA3pMHC.

In order to calculate the specificity of each ankyrin repeat protein for MAGEA3pMHC, the ratio of the HTRF signal for the target MAGEA3pMHC to the HTRF signal for the distinct TITINpMHC was determined. All binders which generated at least 25-times higher HTRF signals on the target MAGEA3pMHC than on TITINpMHC were regarded as specific hits and taken forward for sequencing. Surprisingly, screening of several hundred clones by such a crude cell extract HTRF analysis revealed many different ankyrin repeat domains with specificity for MAGEA3pMHC. Specific binding of ankyrin repeat proteins to a composite epitope comprising an HLA scaffold and a short peptide, wherein only the peptide differs from other composite epitopes which are not specifically bound, has never been shown before and developing specific binders to such composite epitopes using antibody or TCR technology has been challenging.

Example 14: Determination of dissociation constants (K_D) of ankyrin repeat proteins with binding specificity for MAGEA3pMHC by Surface Plasmon Resonance (SPR) analysis

In order to test the ability of the ankyrin repeat domains with binding specificity for MAGEA3pMHC to function in an immune cell engager format and to bind to MAGEA3pMHC in that format, selected ankyrin repeat domains were genetically linked, using conventional cloning methods, to a binding agent with binding specificity for a protein expressed on the surface of an immune cell. By this procedure, binding proteins were generated comprising an ankyrin repeat domain with binding specificity for MAGEA3pMHC and further comprising a binding agent with binding specificity for CD3. These proteins were purified with conventional chromatography methods.

The binding affinities of the purified ankyrin repeat proteins on the MAGEA3pMHC target were analyzed using a ProteOn Surface Plasmon Resonance (SPR) instrument (BioRad) and the measurement was performed according standard procedures known to the person skilled in the art.

Dissociation constants (K_D) were calculated from the estimated on- and off-rates using standard procedures known to the person skilled in the art. K_D values of the binding interactions of selected ankyrin repeat proteins with MAGEA3pMHC were determined to be mostly in the low nanomolar range or below. None of these selected ankyrin repeat proteins displayed measurable binding interaction with control peptides. As a control, binding to the Titin peptide-MHC was tested in parallel. No significant binding to Titin peptide-MHC was detected, demonstrating the specificity of binding. **Table 14** provides the K_D values of some selected ankyrin repeat proteins as examples.

Table 14. K_D values of ankyrin repeat protein – MAGEA3pMHC interactions

DARPin® protein comprising SEQ ID NO	K_D [M]
#156	1.76×10^{-8}
#158	3.91×10^{-9}
#159	1.20×10^{-9}
#161	3.39×10^{-10}
#162	2.52×10^{-10}
#163	1.08×10^{-9}
#164	1.07×10^{-7}
#166	1.60×10^{-9}
#168	8.60×10^{-9}
#170	4.09×10^{-10}
#171	7.55×10^{-9}
#173	7.91×10^{-10}

<u>Example 15</u>: Selection of binding proteins comprising an ankyrin repeat domain with binding specificity for HBVc18 peptide-MHC complex (HBVc18pMHC)

Summary

Using ribosome display (Hanes, J. and Plückthun, A., PNAS *94*, 4937-42, 1997), multiple ankyrin repeat domains with binding specificity for HBVc18 peptide-MHC complex (HBVc18pMHC) were selected from DARPin® libraries in a way similar to the one described by Binz et al. 2004 (loc. cit.), with specific conditions and additional de-selection steps as described below. The binding and specificity of the selected clones towards recombinant HBVc18pMHC and other pMHC complexes were assessed by *E. coli* crude extract

Homogeneous Time Resolved Fluorescence (HTRF), indicating that multiple HBVc18pMHC-specific binding proteins were successfully selected. For example, the ankyrin repeat domains of SEQ ID NOs: 220 to 230 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for HBVc18pMHC. Individual ankyrin repeat modules from such ankyrin repeat domains with binding specificity to HBVc18pMHC are provided, e.g., in SEQ ID NOs: 231 to 254.

Production of biotinylated pMHC complexes as target and selection material

Biotinylated tripartite complexes of HLA-A*0201 (SEQ ID NO: 73), human beta-2-microglobulin (hβ2m; SEQ ID NO: 74) and one of the peptides listed in **Table 15** were produced according to established protocols (Garboczi et al, 1992; Celie et al, 2009).

Table 15: Peptides used for generation of pMHC complexes for selection and screening of designed ankyrin repeat proteins

Peptide description	Peptide Sequence
HLA-A*0201:HBVc18	FLPSDFFPSV
HLA-A*0201:HBe183	FLLTRILTI
HLA-A*0201:HBe183mut	FLLRILTI
HLA-A*0201:NY-ESO-1	SLLMWITQV
HLA-A*0201:EBNA-1	FMVFLQTHI
HLA-A*0201:human off-target 1	ALPTLIPSV
HLA-A*0201:human off-target 2	FLPDANSSV
HLA-A*0201:human off-target 3	FLPQGFPDSV
HLA-A*0201:human off-target 4	RLPPDFFGV
HLA-A*0201:human off-target 5	YLDLFGDPSV
HLA-A*0201:alanine scan 1	ALPSDFFPSV
HLA-A*0201:alanine scan 2	FLASDFFPSV
HLA-A*0201:alanine scan 3	FLPADFFPSV
HLA-A*0201:alanine scan 4	FLPSAFFPSV
HLA-A*0201:alanine scan 5	FLPSDAFPSV
HLA-A*0201:alanine scan 6	FLPSDFAPSV
HLA-A*0201:alanine scan 7	FLPSDFFASV
HLA-A*0201:alanine scan 8	FLPSDFFPAV

Amino acids with homology to the HBVc18 peptide are underlined.

All pMHC complexes used in experiments underwent prior quality control testing.

Selection of HBVc18pMHC-specific ankyrin repeat proteins by ribosome display

The selection of pMHC-specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using the HBVc18pMHC complex as a target, libraries of ankyrin repeat proteins as described above, and established protocols (See, e.g., Zahnd, C., Amstutz, P. and Plückthun, A., Nat. Methods *4*, 69-79, 2007). The number of reverse transcription (RT)-PCR cycles after each selection round was continuously reduced, adjusting to the yield due to enrichment of binders. The first four or five rounds of selection employed standard ribosome display selection, using decreasing target concentrations and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. 2004, loc. cit.). Unusual de-selection steps were incorporated into the selection strategy, as described below.

During the ribosome display rounds, de-selection (or negative selection) steps were incorporated, wherein the ternary complexes were pre-incubated with the corresponding isotype HLA molecule containing another peptide, and only then transferred to the target HBVc18pMHC complex, in order to direct binding of ankyrin repeat proteins towards the peptide-embedded epitope and away from the common HLA-A scaffold. In other words, de-selection (or negative selection) was performed in order to de-select ankyrin repeat proteins that bind predominantly to the common HLA-A scaffold of pMHC complexes rather than to the specific epitope provided by the embedded peptide. Furthermore, ankyrin repeat proteins that cross-react with the epitope provided by the embedded peptide used for de-selection were also de-selected. Here, the peptides HBe183 (FLLTRILTI), HBe183mut (FLLRILTI), EBNA-1 (FMVFLQTHI) and NY-ESO-1 (SLLMWITQV) were used for de-selection.
In detail, Nunc MaxiSorp plates were coated with 100 µl solution of 66 nM neutravidin in PBS and incubated at 4°C over night. The following day, the MaxiSorp 96-well microplate were washed three times with 300 µl PBST per well and blocked with 300 µl PBST-BSA for 1 h at 4°C, rotating at 700 rpm, prior to the de-selection step. After emptying the wells, 100 µl of a 50 nM biotinylated pMHC de-selection target solutions in PBST-BSA was added to each well, rotating with 700 rpm at 4°C, 1h. During this incubation step, mRNA in-vitro translations were performed. Shortly after translations and generation of the ternary complex mixes the pMHC-PBST-BSA solutions were discarded and Nunc MaxiSorp microplate wells were washed three times with 300 µl PBST and finally incubated with WBT-BSA. For the actual de-selection step, the WBT-BSA solutions were discarded and aliquots of 100 µl (for selection rounds 2-5, respectively 150 µl for the first selection round) of the translated ternary complexes were transferred subsequently three times to a prepared Nunc MaxiSorp well containing the immobilized de-selection pMHC target and incubated in each of the three wells for 20 min at 4°C. At the end of the deselection process, the 100µl of the ternary complex aliquots of each selection pool were merged again and the according volumes were taken forward into the selection of the actual target.

After the 4th and 5th ribosome display selection rounds, the pools were cloned with BamHI and PstI into a bacterial expression vector and transformed into E.coli XL-1 Blue and plated on LB/Glu/Amp Agar plates.

Selected clones bind specifically to HBVc18pMHC complex as determined by crude extract HTRF (data not shown).

Furthermore, the proteins were purified with conventional chromatography methods.

<u>Example 16</u>: Determination of dissociation constants (K_D) of ankyrin repeat proteins with binding specificity for HBVc18pMHC by Surface Plasmon Resonance (SPR) analysis

In order to test the ability of the ankyrin repeat domains with binding specificity for HBVc18pMHC to function in an immune cell engager format and to bind to HBVc18pMHC in that format, selected ankyrin repeat domains were genetically linked, using conventional cloning methods, to a binding agent with binding specificity for a protein expressed on the surface of an immune cell. By this procedure, binding proteins were generated comprising an ankyrin repeat domain with binding specificity for HBVc18pMHC and further comprising a binding agent with binding specificity for CD3. These proteins were purified with conventional chromatography methods.

The binding affinities of the purified ankyrin repeat proteins on the HBVc18pMHC target were analyzed using a ProteOn Surface Plasmon Resonance (SPR) instrument (BioRad) and the measurement was performed according standard procedures known to the person skilled in the art.

Dissociation constants (K_D) were calculated from the estimated on- and off-rates using standard procedures known to the person skilled in the art. K_D values of the binding interactions of selected ankyrin repeat proteins with HBVc18pMHC were determined to be in the low nanomolar range or below. None of these selected ankyrin repeat proteins displayed measurable binding interaction with control peptides. **Table 16** provides the K_D values of some selected ankyrin repeat proteins as examples.

Table 16. K_D values of ankyrin repeat protein - HBVc18pMHC interactions

DARPin® protein comprising SEQ ID NO	K_D [M]
220	4.65×10^{-9}
221	3.69×10^{-10}
222	7.15×10^{-9}
224	7.36×10^{-10}
225	9.23×10^{-9}
226	1.38×10^{-8}
227	7.10×10^{-9}
228	4.72×10^{-9}
229	1.03×10^{-9}
230	9.33×10^{-9}

Example 17: Mouse pharmacokinetic profiles of protein variants

The present example provides amino acid sequences for designed ankyrin repeat domains that lead to improved pharmacokinetic profiles. It was surprisingly found that the pharmacokinetic properties can be modulated by applying certain amino acid mutations in the designed ankyrin repeat domains.

Expression and purification of proteins

The DNA encoding each of the designed ankyrin repeat domains consisting of SEQ ID NOs: 281 to 325 was cloned into a pQE (QIAgen, Germany) based expression vector providing an N-terminal His-tag to facilitate simple protein purification as described below. Proteins consisting of SEQ ID NOs: 281 to 325, additionally having a His-tag (SEQ ID NO: 326) fused to their N-termini, were produced in *E. coli*, purified to homogeneity, and stored in PBS buffer. Methods for the production and purification of proteins are well known to the practitioner in the art. Proteins #281 to #325 consist of two designed ankyrin repeat domains, of which one is a designed ankyrin repeat domain with binding specificity for serum albumin.

Alternatively, proteins consisting of SEQ ID NOs: 281 to 325, additionally having the amino acids GS at the N terminus, are produced in *E. coli*, purified to homogeneity, and stored in PBS buffer. In case the amino acids GS are at the N terminus, the Met residue additionally encoded by the expression vector is efficiently cleaved off in the cytoplasm of *E. coli* from the expressed polypeptide since the start Met is followed by a small Gly residue. The proteins consisting of SEQ ID NOs: 281 to 325, additionally having the amino acids GS at the N terminus, exhibit equivalent results in the pharmacokinetic profiling experiment described below as the proteins consisting of SEQ ID NOs: 281 to 325, additionally having a His-tag (SEQ ID NO: 326) fused to the N terminus.

Mouse pharmacokinetic profile measurements

Pharmacokinetic analyses were performed in female Balb/c mice using Proteins #281 to #295, produced as described above. Proteins were applied at 1 mg/kg by intravenous injection into the tail vein. Six mice, divided in two groups of 3 mice each, were used for each protein. For every protein, blood was collected from the mice of one group 5 min, 24 h, 72 h, and 168 h post injection, and from the mice of the other group 6 h, 48 h, 96 h, and 168 h post injection. The blood samples were allowed to stand at room temperature and were centrifuged to generate serum using procedures well-known to the person skilled in the art, followed by storage at -80°C pending analyses. Serum concentrations of Proteins #281 to #295 were determined by sandwich ELISA using a rabbit monoclonal anti-DARPin antibody as capture reagent and an anti-RGS-His antibody-HRP conjugate as detection reagent, and using a standard curve. The monoclonal anti-DARPin antibody was generated using conventional rabbit immunization and hybridoma generation techniques well known to the person skilled in the art, and the binding of the monoclonal antibody to Proteins #281 to #295 was verified prior to concentration determination experiments. Briefly, 100 μl of goat-anti-rabbit antibody (10 nM) (Thermo Scientific) in PBS per well were immobilized in a Maxisorp plate (Nunc, Denmark) overnight at 4°C. After washing 5 times with 300 μl PBST (PBS supplemented with 0.1% Tween 20), the wells were blocked with 300 μl PBST-C (PBST supplemented with 0.25% casein) for 1 h at room temperature with shaking at 450 rpm on a Titramax 1000 shaker (Heidolph, Germany). After washing 5 times as described above, 100 μl/well rabbit-anti-DARPin antibody (5 nM) in PBST-C were added for 1 h at room temperature with shaking at 450 rpm. After washing 5 times as described above, different dilutions of serum samples or standard references, diluted in PBST-C, were added for 2 hours at room temperature with shaking at 450 rpm. After washing 5 times as described above, 50 μl mouse anti-RGS-His antibody-HRP conjugate (QIAgen) (100 ng/ml) in PBST-C was added for 30 min at room temperature with shaking at 450 rpm. After washing 5 times as described above, the ELISA was developed using 50 μl TMB substrate. The reaction was stopped after 5 min using 100 μl 1 M H_2SO_4. The OD (OD 450 nm - OD 620 nm) was then recorded. Pharmacokinetic parameters were determined using standard software such as Phoenix WinNonLin (Certara, Princeton, USA) or GraphPadPrism (GraphPad Software, La Jolla, USA) and standard analyses such as non-compartmental analyses, all well-known to the person skilled in the art. The resulting pharmacokinetic profiles are shown in **Figure 26**. The pharmacokinetic parameters area under the curve, clearance, volume of distribution, and half-life, derived from the measurements, are listed in **Table 177**, **Table 188**, **Table 1919**, and **Table 2020**, respectively.

Table 17. Mouse pharmacokinetic parameters of Proteins #281 to #283

Parameter Protein	AUCINF_D_pred h*(nmol/L)	Cl_pred L/(h*kg)	Vss_pred L/kg	HL_Lambda_z h
#281*	4398	0.0071	0.031	5.1
#282*	12221	0.0025	0.06	18.8
#283*	27102	0.0011	0.057	37.3

* Proteins #281 to #283 in this table represent proteins consisting of the corresponding amino acid sequence of SEQ ID NO: 281 to 283, and additionally an N-terminal His-tag (SEQ ID NO: 326).

Table 18. Mouse pharmacokinetic parameters of Proteins #284 to #287

Parameter Protein	AUCINF_D_pred h*(nmol/L)	Cl_pred L/(h*kg)	Vss_pred L/kg	HL_Lambda_z h
#284*	11332	0.003	0.046	13.1
#285*	21910	0.0016	0.075	36.7
#286*	30314	0.0011	0.051	33.1
#287*	22127	0.0016	0.052	25.4

* Proteins #284 to #287 in this table represent proteins consisting of the corresponding amino acid sequence of SEQ ID NO: 284 to 287, and additionally an N-terminal His-tag (SEQ ID NO: 326).

Table 19. Mouse pharmacokinetic parameters of Proteins #288 to #291

Parameter Protein	AUCINF_D_pred h*(nmol/L)	Cl_pred L/(h*kg)	Vss_pred L/kg	HL_Lambda_z h
#288*	11619	0.003	0.046	13.9
#289*	21758	0.0016	0.057	27.9

#290*	42071	0.0008	0.036	34.5
#291*	23398	0.0015	0.043	25.8

* Proteins #288 to #291 in this table represent proteins consisting of the corresponding amino acid sequence of SEQ ID NO: 288 to 291, and additionally an N-terminal His-tag (SEQ ID NO: 326).

Table 20. Mouse pharmacokinetic parameters of Proteins #292 to #295

Parameter	AUCINF_D_pred	Cl_pred	Vss_pred	HL_Lambda_z
Protein	h*(nmol/L)	L/(h*kg)	L/kg	h
#292*	4222	0.0082	0.036	4.4
#293*	28590	0.0012	0.057	36.6
#294*	23517	0.0015	0.083	42.7
#295*	30107	0.0011	0.04	27.4

* Proteins #292 to #295 in this table represent proteins consisting of the corresponding amino acid sequence of SEQ ID NO: 292 to 295, and additionally an N-terminal His-tag (SEQ ID NO: 326).

These findings indicate that the sequence modifications described here lead to improved pharmacokinetic properties. In particular, Proteins #282 and #283 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Proteins #281. Also, Proteins #285, #286 and #287 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #284. Similarly, Proteins #289, #290 and #291 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #288. And Proteins #293, #294 and #295 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #292.

Similar results are obtained when comparing the mouse pharmacokinetic parameters of Proteins #296 to #310. In particular, Proteins #297 and #298 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #296. Also, Proteins #300, #301 and #302 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #299. Similarly, Proteins #304, #305 and #306 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #303. And Proteins #308, #309 and #310 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #307. Likewise, similar results are obtained when comparing the mouse pharmacokinetic parameters of Proteins #311 to #325. In particular, Proteins #312 and #313 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #311. Also, Proteins #315, #316 and #317 exhibit slower clearance, larger area under the curve, and longer terminal half-life than

Protein #314. Similarly, Proteins #319, #320 and #321 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #318. And Proteins #323, #324 and #325 exhibit slower clearance, larger area under the curve, and longer terminal half-life than Protein #322. The effect of the sequence modifications on pharmacokinetic properties of the proteins in mouse is thus observed when using different designed ankyrin repeat domains with binding specificity for serum albumin as means for half-life extension. The effect of the sequence modifications on pharmacokinetic properties of the proteins in mouse is thus also observed when using different linker sequences (e.g. Pro-Thr-rich linker instead of Gly-Ser-rich linker).

CLAIMS

1. A method of producing a peptide-MHC (pMHC)-specific binding protein, wherein said binding protein comprises a designed repeat domain with binding specificity for a target peptide-MHC complex, the method comprising the steps of:
   (a) providing a collection of designed repeat domains;
   (b) providing a recombinant target peptide-MHC complex; and
   (c) screening said collection of designed repeat domains for specific binding to said target peptide-MHC complex to obtain at least one designed repeat domain with binding specificity for said target peptide-MHC complex.

2. The method of claim 1, wherein said designed repeat domain is a designed ankyrin repeat domain.

3. The method of claim 1 or 2, wherein said collection comprises designed repeat domains that comprise fixed positions and randomized positions, and wherein designed repeat domains of said collection differ from each other in at least one of the randomized positions.

4. The method of any one of the preceding claims, wherein said collection of designed repeat proteins is provided by ribosome display.

5. The method of any one of the preceding claims, wherein the method further comprises the steps of (i) providing a second recombinant peptide-MHC complex, wherein said peptide of said second peptide-MHC complex comprises an amino acid sequence that differs from said target peptide by at least one amino acid residue; and (ii) removing from said collection by negative selection designed repeat domains with binding specificity for said second recombinant peptide-MHC complex.

6. The method of any one of the preceding claims, wherein said repeat domain with binding specificity for a target peptide-MHC complex binds to said target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M.

7. The method of any one of the preceding claims, wherein binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said target peptide.

8. The method of any one of the preceding claims, wherein binding of said repeat domain with binding specificity for a target peptide-MHC complex to said target peptide-MHC complex comprises interaction of said repeat domain with at least one amino acid residue of said MHC.

9. The method of any one of the preceding claims, wherein said target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell.

10. The method of any one of claims 1 to 9, wherein said target peptide is derived from NY-ESO-1, preferably wherein said target peptide comprises or consists of the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

11. The method of any one of claims 1 to 9, wherein said target peptide is derived from EBNA-1, preferably wherein said target peptide comprises or consists of the amino acid sequence of SEQ ID NO: 92.

12. The method of any one of claims 1 to 9, wherein said target peptide is derived from MAGE-A3, preferably wherein said target peptide comprises or consists of the amino acid sequence of SEQ ID NO: 155.

13. The method of any one of claims 1 to 9, wherein said target peptide is derived from HBcAg, preferably wherein said target peptide comprises or consists of the amino acid sequence of SEQ ID NO: 255.

14. The method of any one of the preceding claims, wherein said MHC is MHC class I.

15. A recombinant binding protein comprising a designed repeat domain obtainable by the method of any one of the preceding claims.

16. A recombinant binding protein comprising a first designed repeat domain, wherein said first repeat domain has binding specificity for a first target peptide-MHC complex, wherein preferably said first target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder.

17. The binding protein of claim 16, wherein said first target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell.

18. The binding protein of claim 16 or 17, wherein said first repeat domain binds to said first target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M.

19. The binding protein of any one of claims 16 to 18, wherein binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said first target peptide.

20. The binding protein of any one of claims 16 to 19, wherein binding of said first repeat domain to said first target peptide-MHC complex comprises interaction of said first repeat domain with at least one amino acid residue of said first MHC.

21. The binding protein of any one of claims 16 to 20, wherein said first MHC is MHC class I.

22. The binding protein of claim 21, wherein said MHC class I is HLA-A*02.

23. The binding protein of any one of claims 16 to 22, wherein said first target peptide is derived from NY-ESO-1.

24. The binding protein of any one of claims 16 to 23, wherein said first target peptide has the amino acid sequence of SEQ ID NO: 19 or SEQ ID NO: 34.

25. The binding protein of any one of claims 16 to 24, wherein said first repeat domain is a designed ankyrin repeat domain.

26. The binding protein of claim 25, wherein said first ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid.

27. The binding protein of claim 25 or 26, wherein said first ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N.

28. The binding protein of any one of claims 15 to 27, wherein said binding protein further comprises a second designed repeat domain, wherein said second repeat domain has binding specificity for a second target peptide-MHC complex, wherein preferably said second target peptide is selected from the group consisting of (i) a peptide derived from a protein expressed in a tumor cell, (ii) a peptide derived from a protein of an infectious agent, preferably a viral infectious agent, and (iii) a peptide derived from a protein associated with an autoimmune disorder.

29. The binding protein of claim 28, wherein said second target peptide is derived from an intracellular protein, preferably an intracellular protein expressed in a tumor cell.

30. The binding protein of claim 28 or 29, wherein said second repeat domain with binding specificity for a second target peptide-MHC complex binds to said second target peptide-MHC complex in PBS with a dissociation constant (K_D) below 10^{-7}M.

31. The binding protein of any one of claims 28 to 30, wherein binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least one, at least two, at least three, at least four, at least five, at least six, or at least seven amino acid residues of said second target peptide.

32. The binding protein of any one of claims 28 to 31, wherein binding of said second repeat domain to said second target peptide-MHC complex comprises interaction of said second repeat domain with at least one amino acid residue of said second MHC.

33. The binding protein of any one of claims 28 to 32, wherein said second MHC is MHC class I.

34. The binding protein of any one of claims 28 to 33, wherein said second target peptide is derived from the same protein as said first target peptide.

35. The binding protein of any one of claims 28 to 34, wherein said second target peptide has the same amino acid sequence as said first target peptide.

36. The binding protein of any one of claims 28 to 35, wherein said second repeat domain has the same amino acid sequence as said first repeat domain.

37. The binding protein of any one of claims 28 to 35, wherein said second repeat domain has a different amino acid sequence as compared to said first repeat domain.

38. The binding protein of any one of claims 28 to 34, wherein said second target peptide has a different amino acid sequence as compared to said first target peptide.

39. The binding protein of any one of claims 28 to 33, wherein said second target peptide is derived from a protein that is different from the protein, from which said first target peptide is derived.

40. The binding protein of any one of claims 28 to 39, wherein said second MHC is HLA-A*02.

41. The binding protein of any one of claims 28 to 39, wherein said second MHC is not HLA-A*02.

42. The binding protein of any one of claims 28 to 41, wherein said second repeat domain is a designed ankyrin repeat domain.

43. The binding protein of claim 42, wherein said second ankyrin repeat domain comprises an ankyrin repeat module comprising an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 37 to 72 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 37 to 72 are substituted by another amino acid.

44. The binding protein of claim 42 or 43, wherein said second ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 20 to 33, wherein G at position 1 and/or S at position 2 of SEQ ID NOs: 20 to 33 are optionally missing, and wherein A at the second last position of SEQ ID NOs: 20 to 33 is optionally substituted by L and/or A at the last position of SEQ ID NOs: 20 to 33 is optionally substituted by N.

45. The binding protein of any one of claims 28 to 34, 40, and 42 to 44, wherein said binding protein comprises a polypeptide comprising an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 16 to 18.

46. The binding protein of any one of claims 15 to 45, wherein said binding protein further comprises a binding agent with binding specificity for a protein expressed on the surface of an immune cell.

47. The binding protein of claim 46, wherein said immune cell is a T-cell.

48. The binding protein of claim 47, wherein said T-cell is a CD8+ cytotoxic T-cell.

49. The binding protein of any one of claims 46 to 47, wherein said protein expressed on the surface of an immune cell is a protein that is part of the T-cell receptor complex.

50. The binding protein of claim 49, wherein said protein that is part of the T-cell receptor complex is cluster of differentiation 3 (CD3).

51. The binding protein of any one of claims 46 to 50, wherein said binding agent is an antibody, an antibody mimetic, a scaffold protein, a repeat protein, or a designed repeat domain.

52. The binding protein of any one of claims 46 to 51, wherein said binding agent is a designed ankyrin repeat domain.

53. The binding protein of any one of claims 46 to 52, wherein said binding agent is covalently linked to said first repeat domain and/or, if present, said second repeat domain with a peptipe linker.

54. The binding protein of claim 53, wherein said peptide linker is a proline-threonine-rich peptipe linker.

55. The binding protein of any one of claims 53 to 54, wherein the amino acid sequence of said peptide linker has a length from 1 to 17 amino acids.

56. The binding protein of any one of claims 15 to 55, wherein said binding protein is capable of inhibiting recognition of said first target peptide-MHC complex and/or, if said binding protein comprises said second repeat domain, of said second target peptide-MHC complex by T-cell receptors.

57. A nucleic acid encoding the designed repeat domain with binding specificity for a first or a second target peptide-MHC complex as defined in any one of claims 15 to 56 or encoding the binding protein of any one of claims 15 to 56.

58. A pharmaceutical composition comprising the binding protein of any one of claims 15 to 56 or the nucleic acid of claim 57, and a pharmaceutically acceptable carrier and/or diluent.

59. A method of tumor-localized activation of T-cells in a mammal, preferably a human, the method comprising the step of administering to said mammal the binding protein of any one of claims 15 to 55 or the nucleic acid of claim 57, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in a tumor cell.

60. The binding protein of any one of claims 15 to 55 or the nucleic acid of claim 57 for use in a method of tumor-localized activation of T-cells in a mammal, preferably a human, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in a tumor cell.

61. A method of infection-localized activation of T-cells in a mammal, preferably a human, the method comprising the step of administering to said mammal the binding protein of any one of claims 15 to 55 or the nucleic acid of claim 57, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein of an infectious agent.

62. The binding protein of any one of claims 15 to 55 or the nucleic acid of claim 57 for use in a method of infection-localized activation of T-cells in a mammal, preferably a human, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein of an infectious agent.

63. A method of treating a medical condition, the method comprising the step of administering to a patient in need thereof a therapeutically effective amount of the binding protein of any one of claims 15 to 56, the nucleic acid of claim 57, or the pharmaceutical composition of claim 58.

64. The binding protein of any one of claims 15 to 56, the nucleic acid of claim 57, or the pharmaceutical composition of claim 58 for use in a method of treating a medical condition.

65. A method of diagnosing a medical condition in a mammal, preferably a human, the method comprising the steps of:
    (i) contacting a cell or tissue sample obtained from said mammal with the binding protein of any one of claims 15 to 56; and
    (ii) detecting specific binding of said binding protein to said cell or tissue sample.

66. The method of claim 63 or 65 or the binding protein for use according to claim 64, wherein said medical condition is a cancer, an infectious disease, preferably a viral infectious disease, or an autoimmune disease.

67. The method of claim 63 or 65 or the binding protein for use according to claim 64, wherein said medical condition is a cancer.

68. The method of claim 63 or 65 or the binding protein for use according to claim 64, wherein said medical condition is an infectious disease, preferably a viral infectious disease.

69. The method of claim 63 or 65 or the binding protein for use according to claim 64, wherein said medical condition is an autoimmune disease.

70. A method of targeting tumor cells in a patient having a tumor for destruction of the tumor cells, the method comprising the step of administering to the patient a therapeutically effective amount of the binding protein of any one of claims 15 to 56, the nucleic acid of claim 57, or the pharmaceutical composition of claim 58, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the tumor cells, preferably an intracellular protein expressed in the tumor cells.

71. The method of targeting tumor cells of claim 70, wherein said binding protein further comprises a toxic agent capable of killing a tumor cell.

72. The binding protein of any one of claims 15 to 56, the nucleic acid of claim 57, or the pharmaceutical composition of claim 58 for use in a method of targeting tumor cells in a patient having a tumor for destruction of the tumor cells, wherein said first target peptide and/or, if said binding protein

comprises said second repeat domain, said second target peptide is derived from a protein expressed in the tumor cells, preferably an intracellular protein expressed in the tumor cells.

73. The binding protein, nucleic acid or pharmaceutical composition for use according to claim 72, wherein the binding protein further comprises a toxic agent capable of killing a tumor cell.

74. A method of targeting infected cells in a patient having a viral infectious disease for destruction of the infected cells, the method comprising the step of administering to the patient a therapeutically effective amount of the binding protein of any one of claims 15 to 56, the nucleic acid of claim 57, or the pharmaceutical composition of claim 58, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the infected cells, preferably a virus-specific protein expressed in the infected cells.

75. The method of targeting tumor cells of claim 74, wherein said binding protein further comprises a toxic agent capable of killing an infected cell.

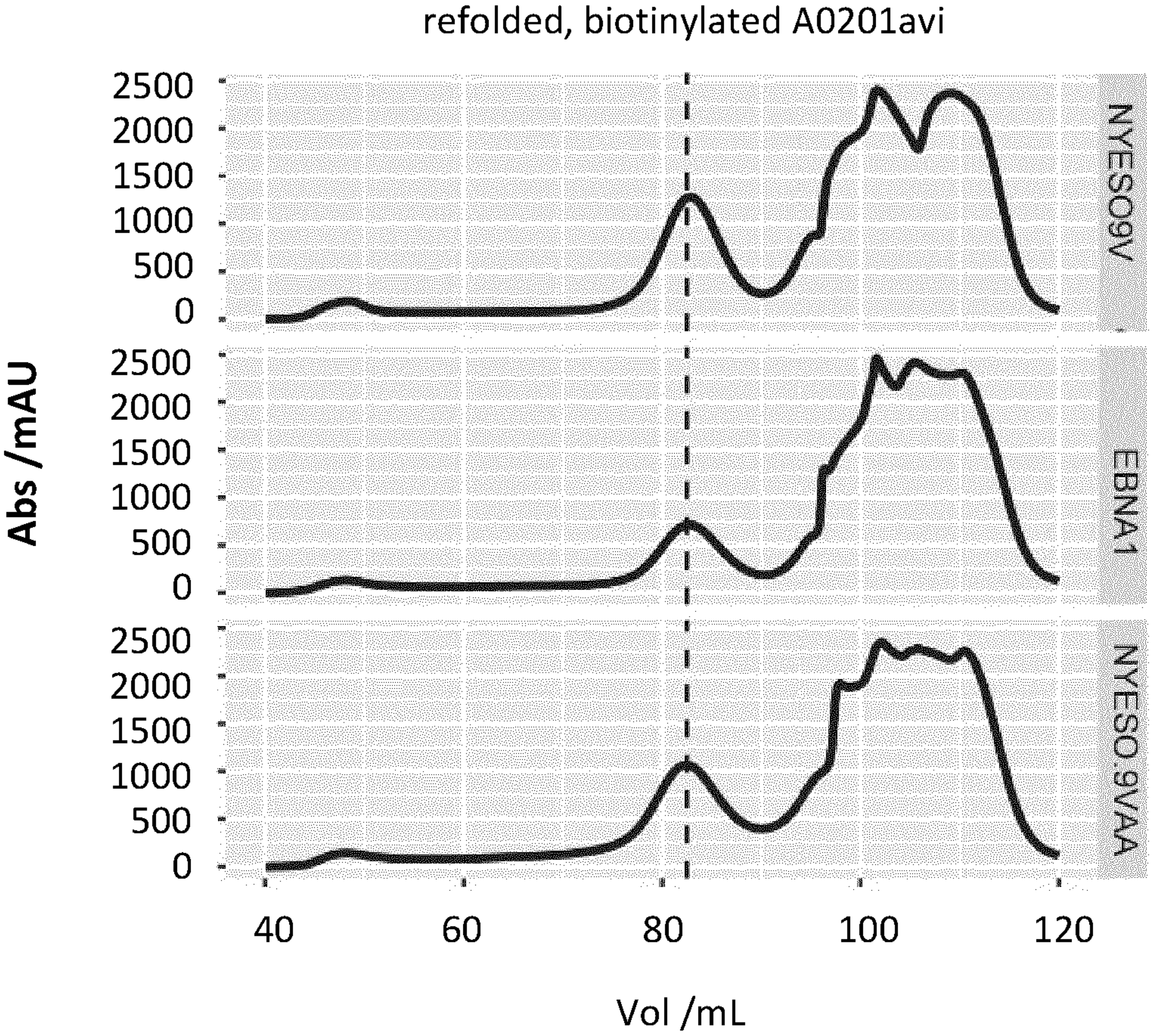
76. The binding protein of any one of claims 15 to 56, the nucleic acid of claim 57, or the pharmaceutical composition of claim 58 for use in a method of targeting infected cells in a patient having a viral infectious disease for destruction of the infected cells, wherein said first target peptide and/or, if said binding protein comprises said second repeat domain, said second target peptide is derived from a protein expressed in the infected cells, preferably a virus-specific protein expressed in the infected cells.

77. The binding protein, nucleic acid or pharmaceutical composition for use according to claim 76, wherein the binding protein further comprises a toxic agent capable of killing an infected cell.

78. The method of any one of claims 63, 66 to 67, 69 and 70 or the binding protein, nucleic acid or pharmaceutical composition of any of claims 64, 72 and 73, wherein said medical condition or tumor is selected from the group consisting of liposarcoma, neuroblastoma, synovial sarcoma, melanoma and ovarian cancer.

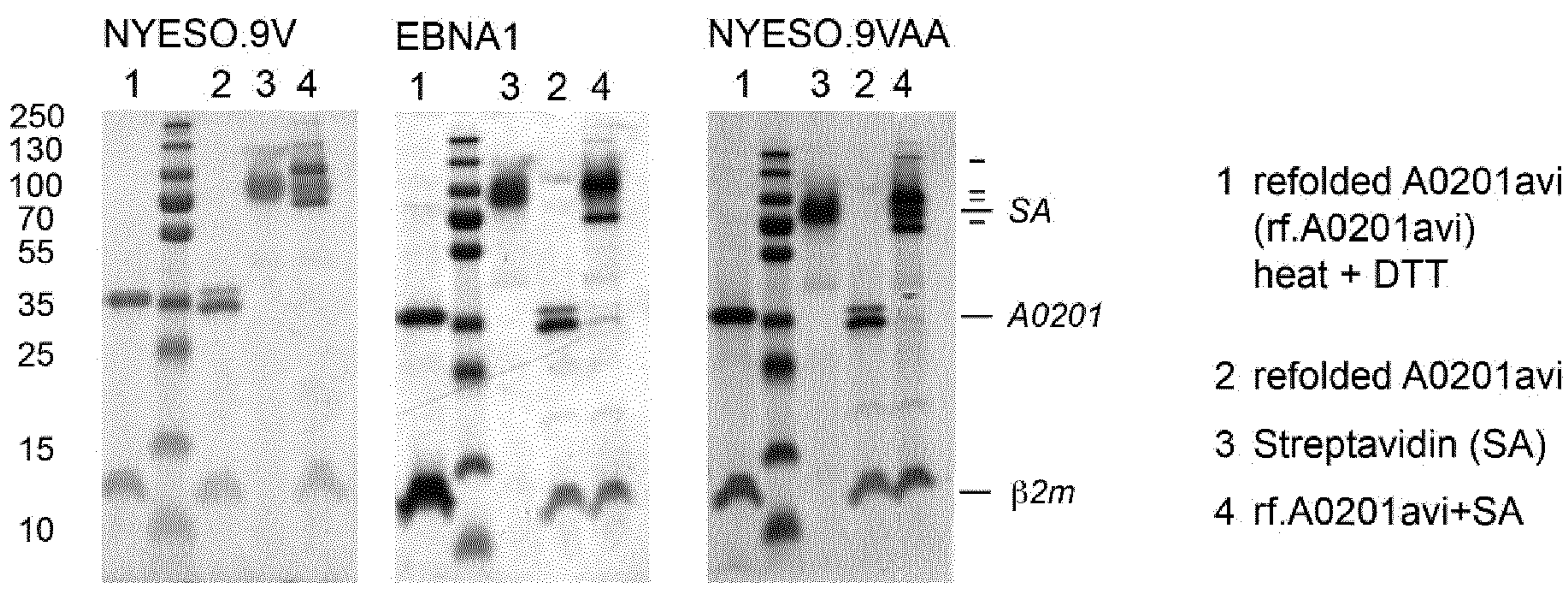
FIGURE 1

A



refolded, biotinylated A0201avi
Abs /mAU
2500
2000
1500
1000
500
0
NYESO9V
EBNA1
NYESO.9VAA
40
60
80
100
120
Vol /mL

B



NYESO.9V
EBNA1
NYESO.9VAA
1 2 3 4
1 3 2 4
1 3 2 4
250
130
100
70
55
35
25
15
10
SA
A0201
β2m
1 refolded A0201avi (rf.A0201avi) heat + DTT
2 refolded A0201avi
3 Streptavidin (SA)
4 rf.A0201avi+SA

FIGURE 1 (cont.)

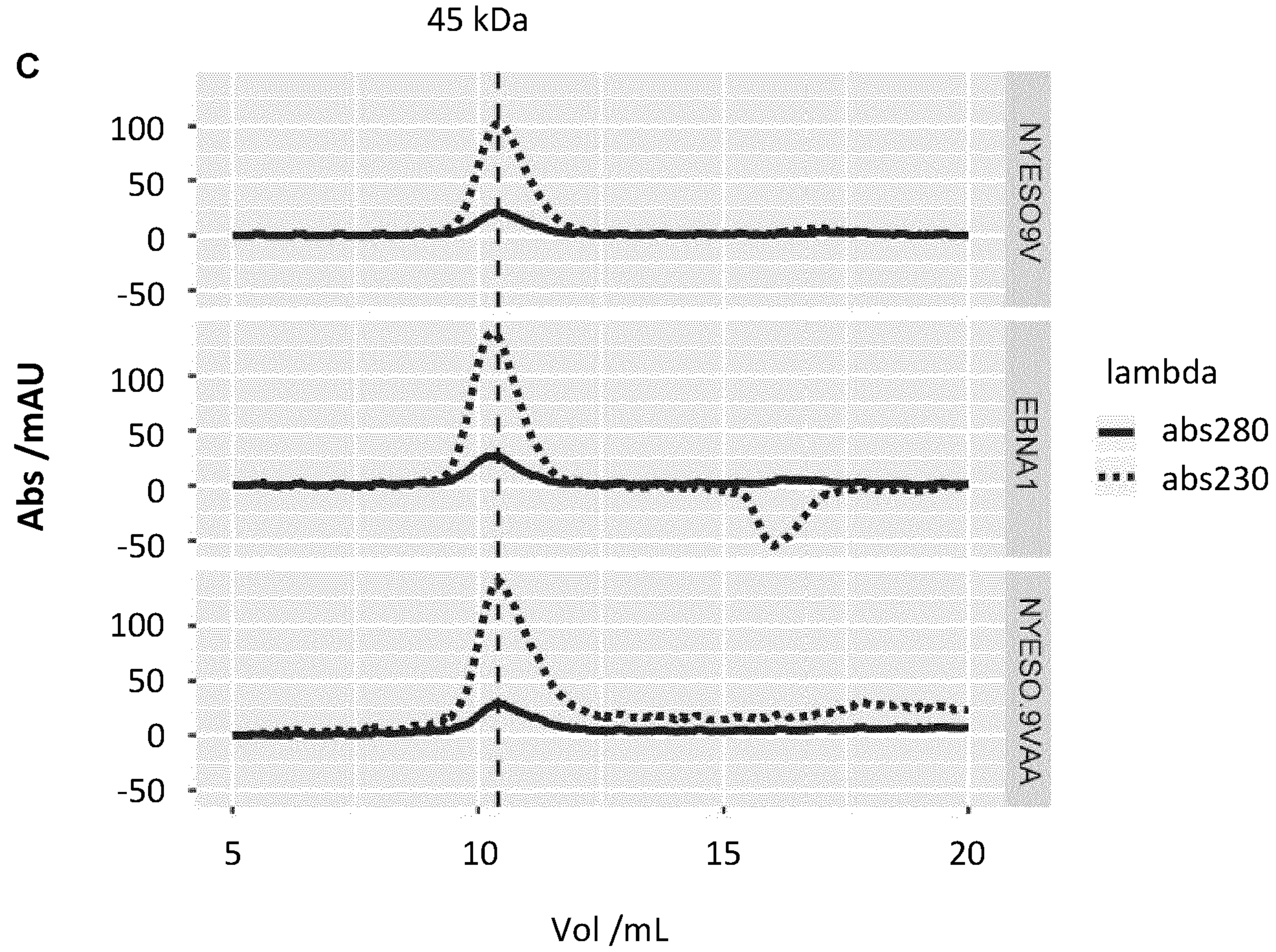


FIGURE 2

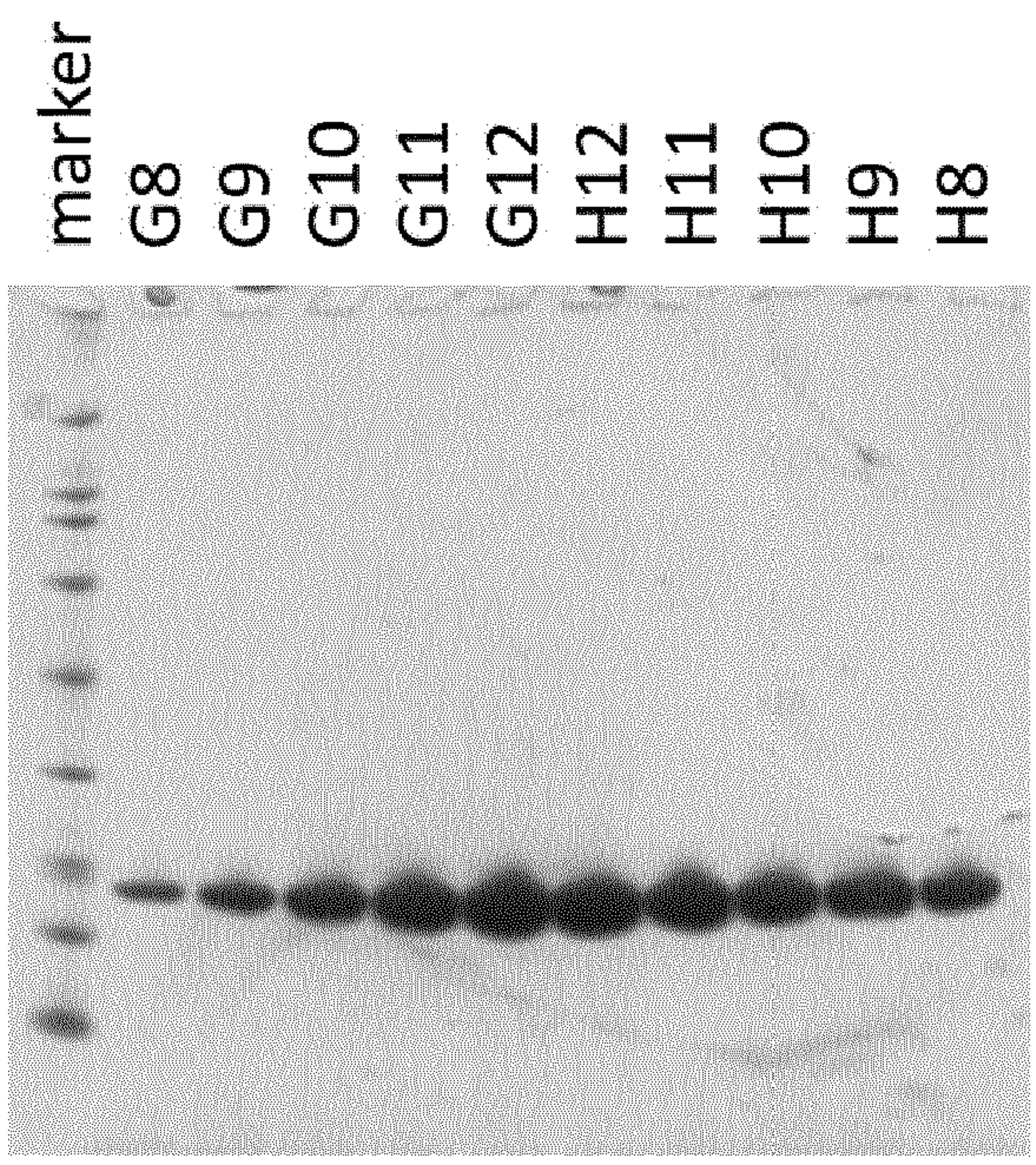


FIGURE 3

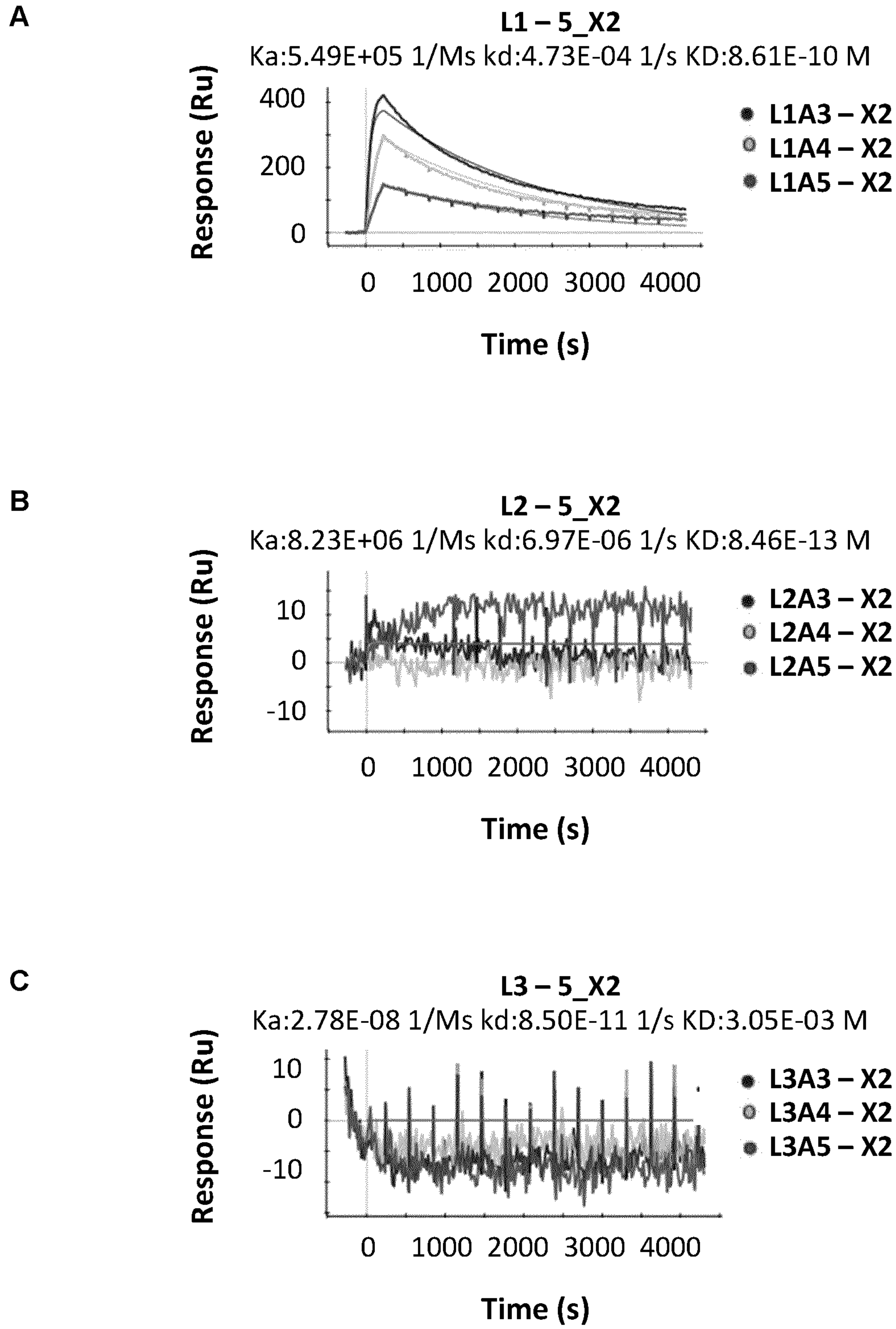
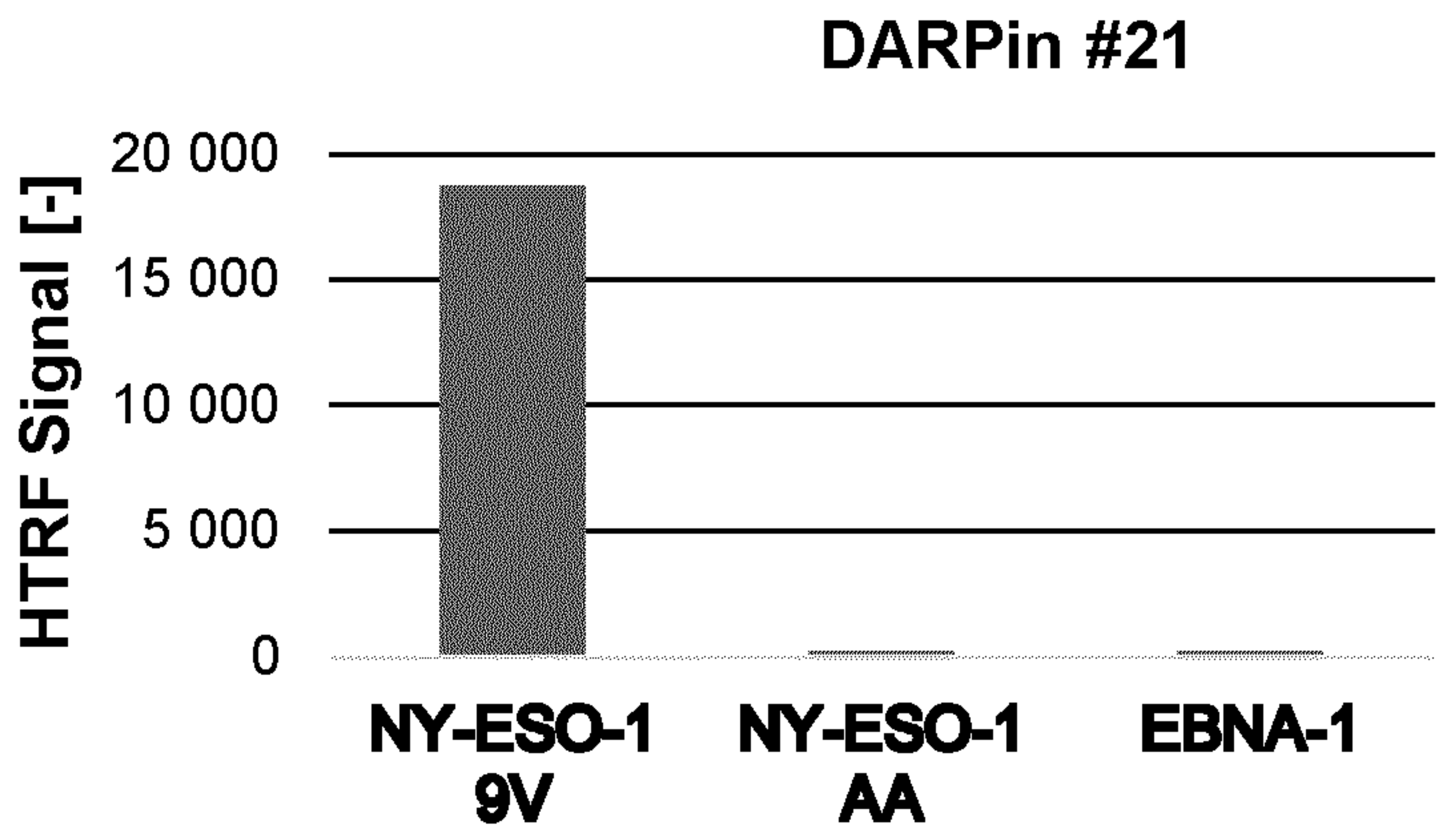

A
L1 – 5_X2
Ka:5.49E+05 1/Ms kd:4.73E-04 1/s KD:8.61E-10 M
Response (Ru)
400
200
0
0 1000 2000 3000 4000
Time (s)
L1A3 – X2
L1A4 – X2
L1A5 – X2
B
L2 – 5_X2
Ka:8.23E+06 1/Ms kd:6.97E-06 1/s KD:8.46E-13 M
Response (Ru)
10
0
-10
0 1000 2000 3000 4000
Time (s)
L2A3 – X2
L2A4 – X2
L2A5 – X2
C
L3 – 5_X2
Ka:2.78E-08 1/Ms kd:8.50E-11 1/s KD:3.05E-03 M
Response (Ru)
10
0
-10
0 1000 2000 3000 4000
Time (s)
L3A3 – X2
L3A4 – X2
L3A5 – X2

FIGURE 4

A


DARPin #21
HTRF Signal [-]
20 000
15 000
10 000
5 000
0
NY-ESO-1 9V
NY-ESO-1 AA
EBNA-1

B

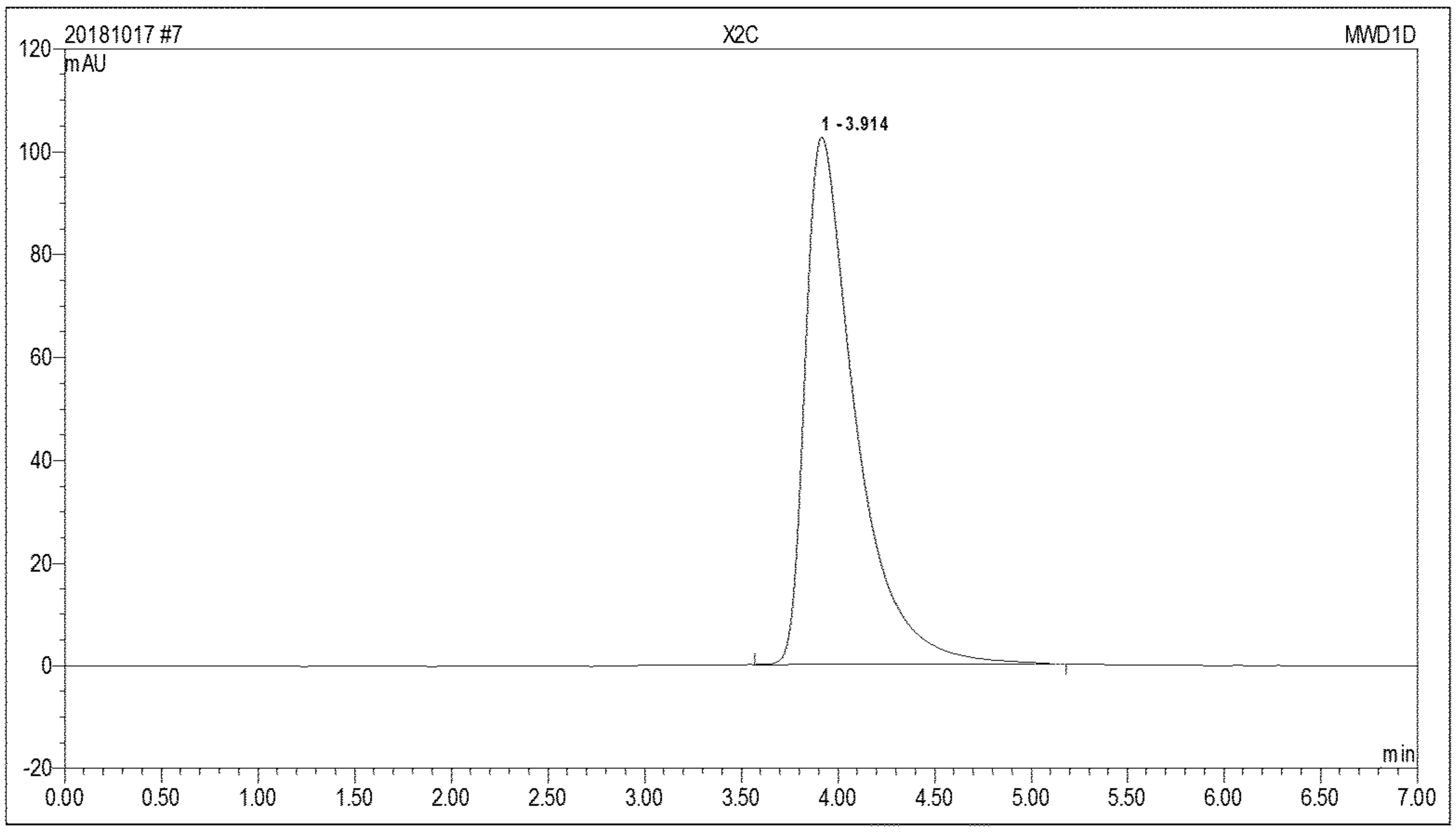
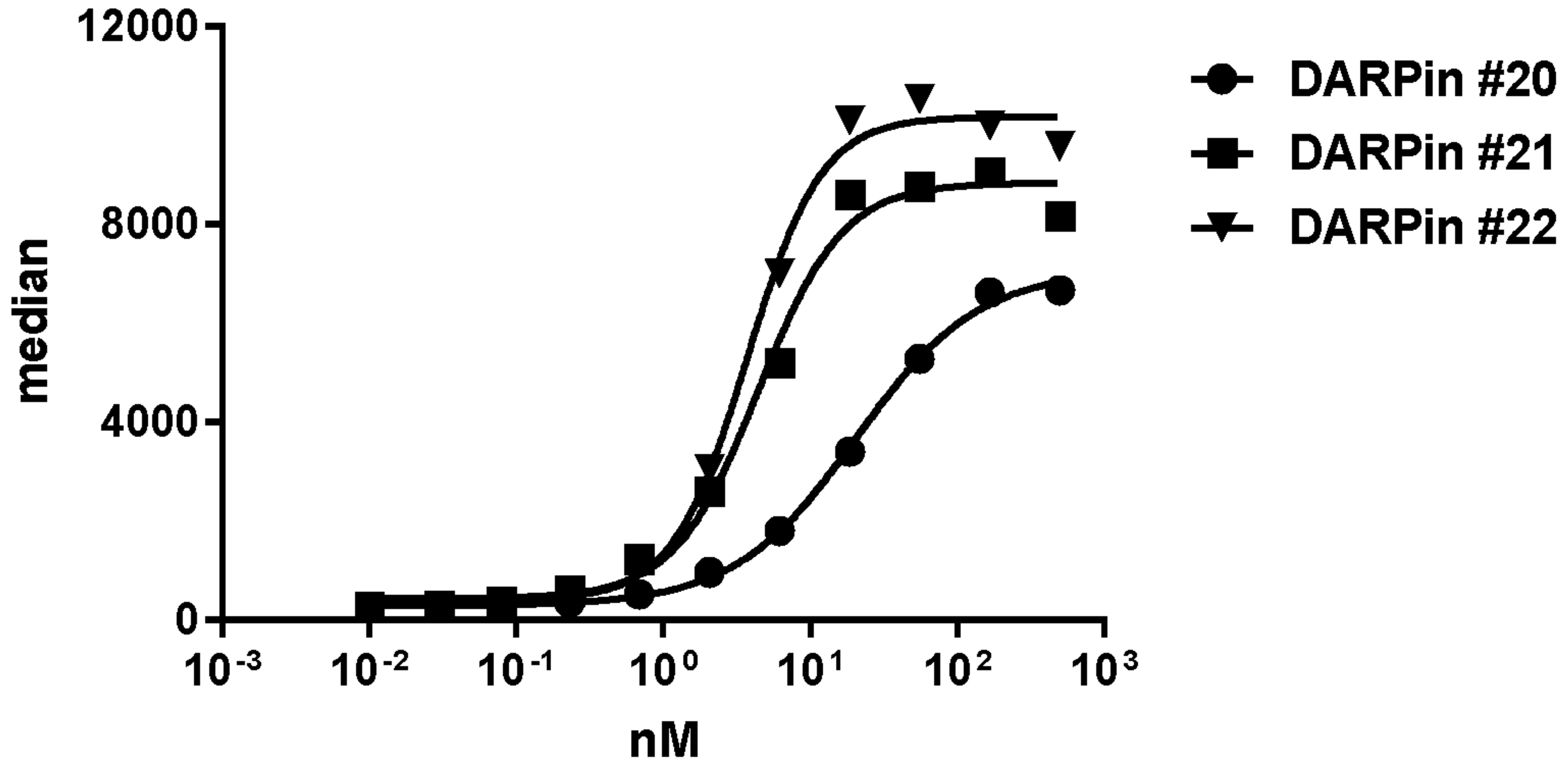
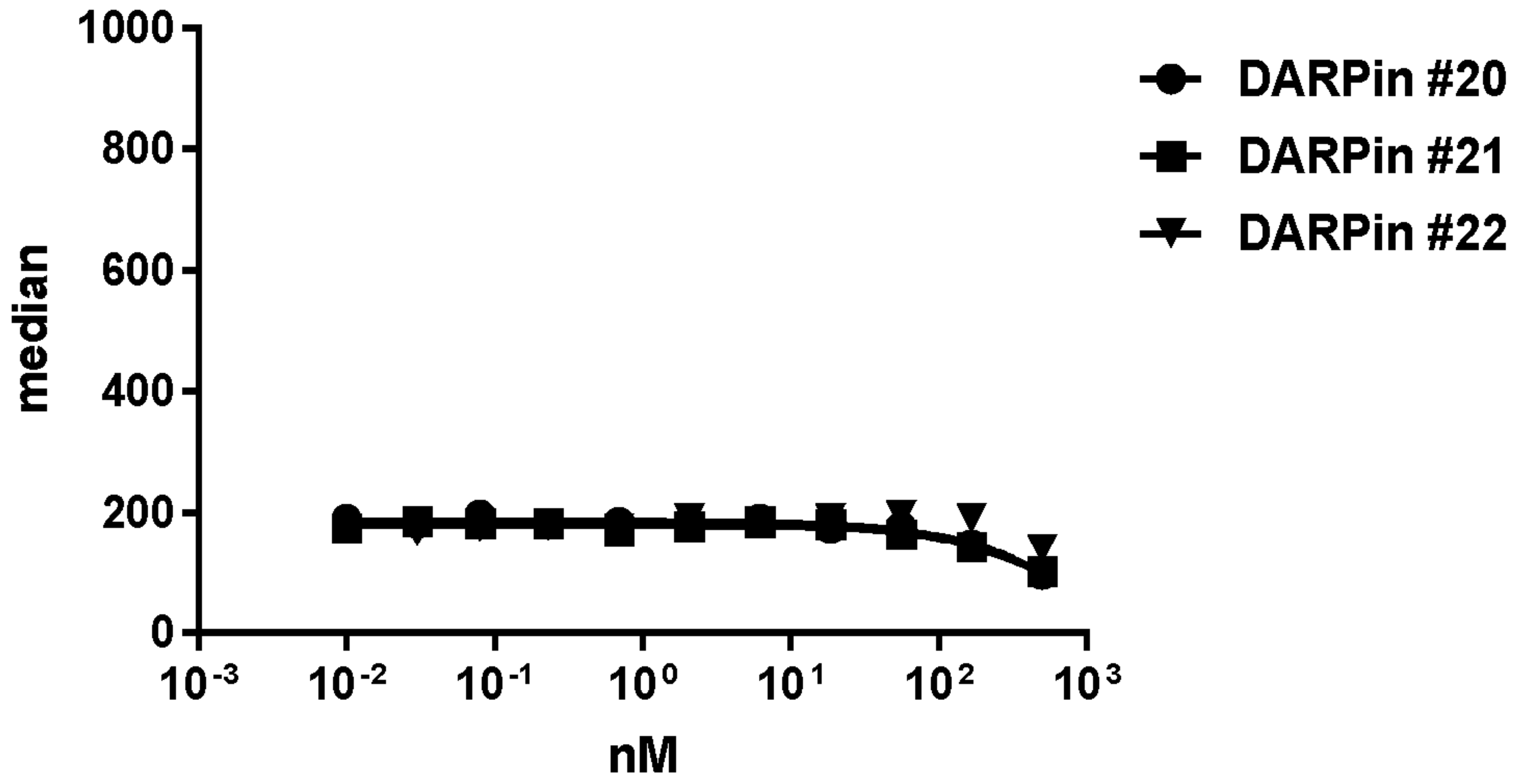

20181017 #7
X2C
MWD1D
mAU
120
100
80
60
40
20
0
-20
1 - 3.914
min
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00

FIGURE 5

A


12000
8000
4000
0
median
10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10² 10³
nM
DARPin #20
DARPin #21
DARPin #22

B


1000
800
600
400
200
0
median
10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10² 10³
nM
DARPin #20
DARPin #21
DARPin #22

C

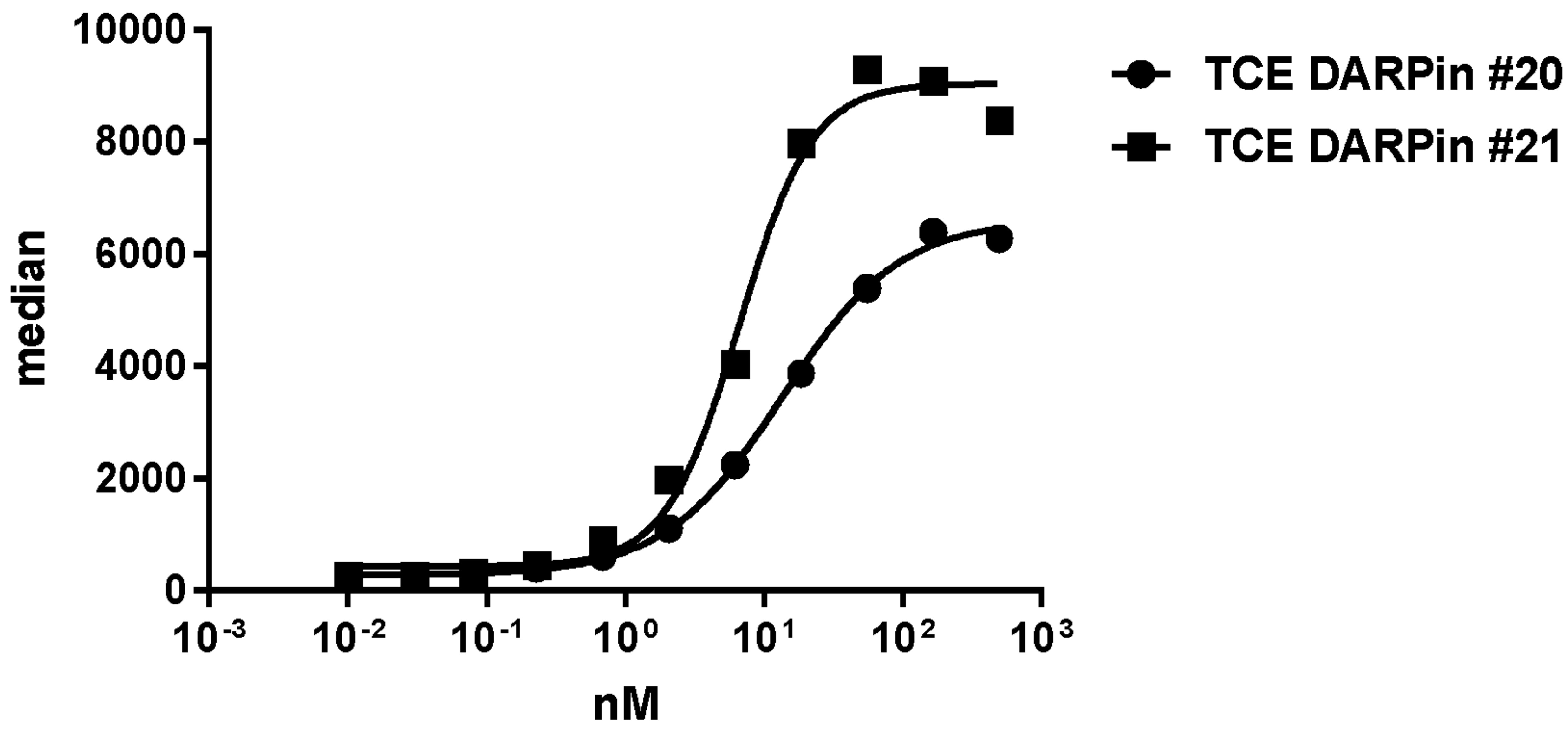
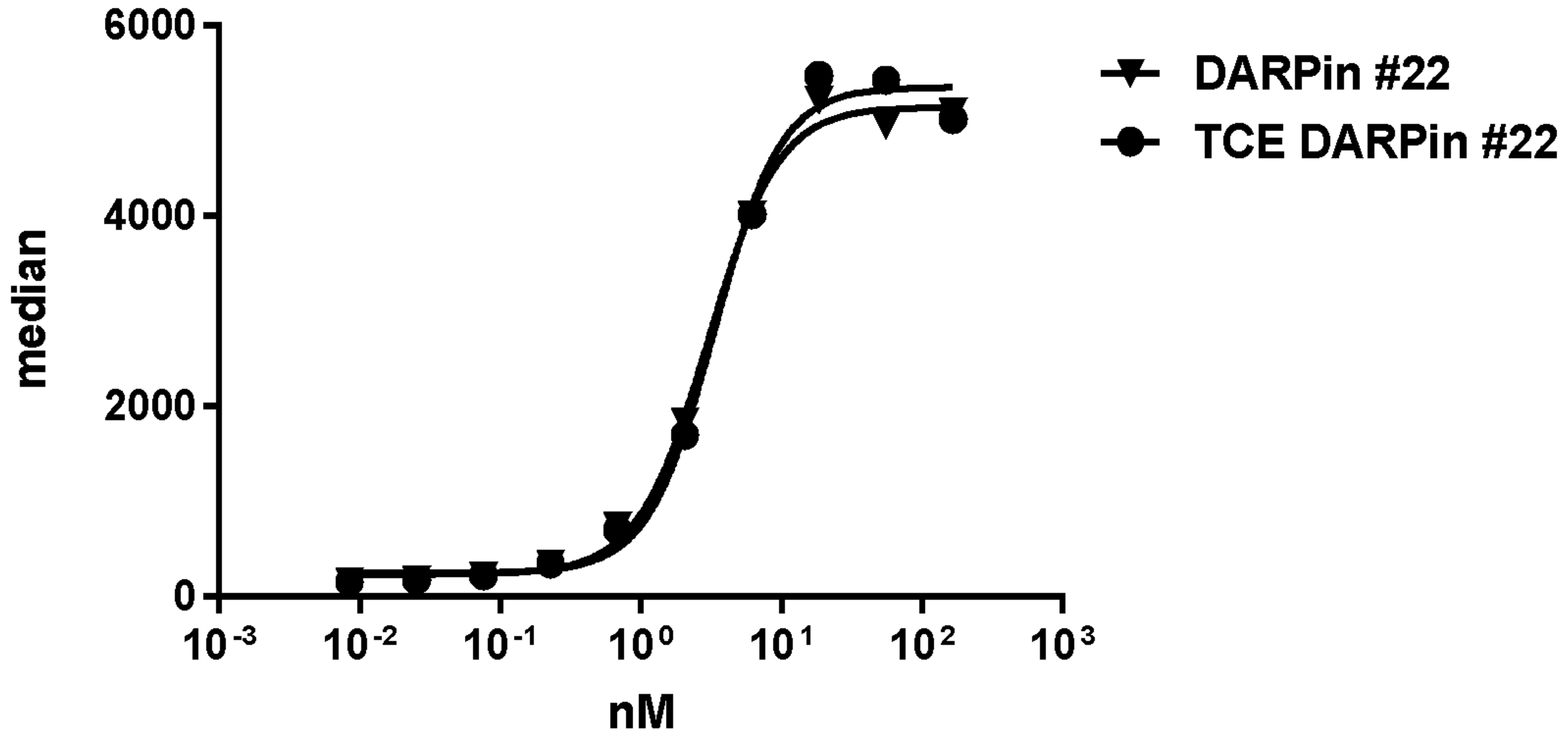
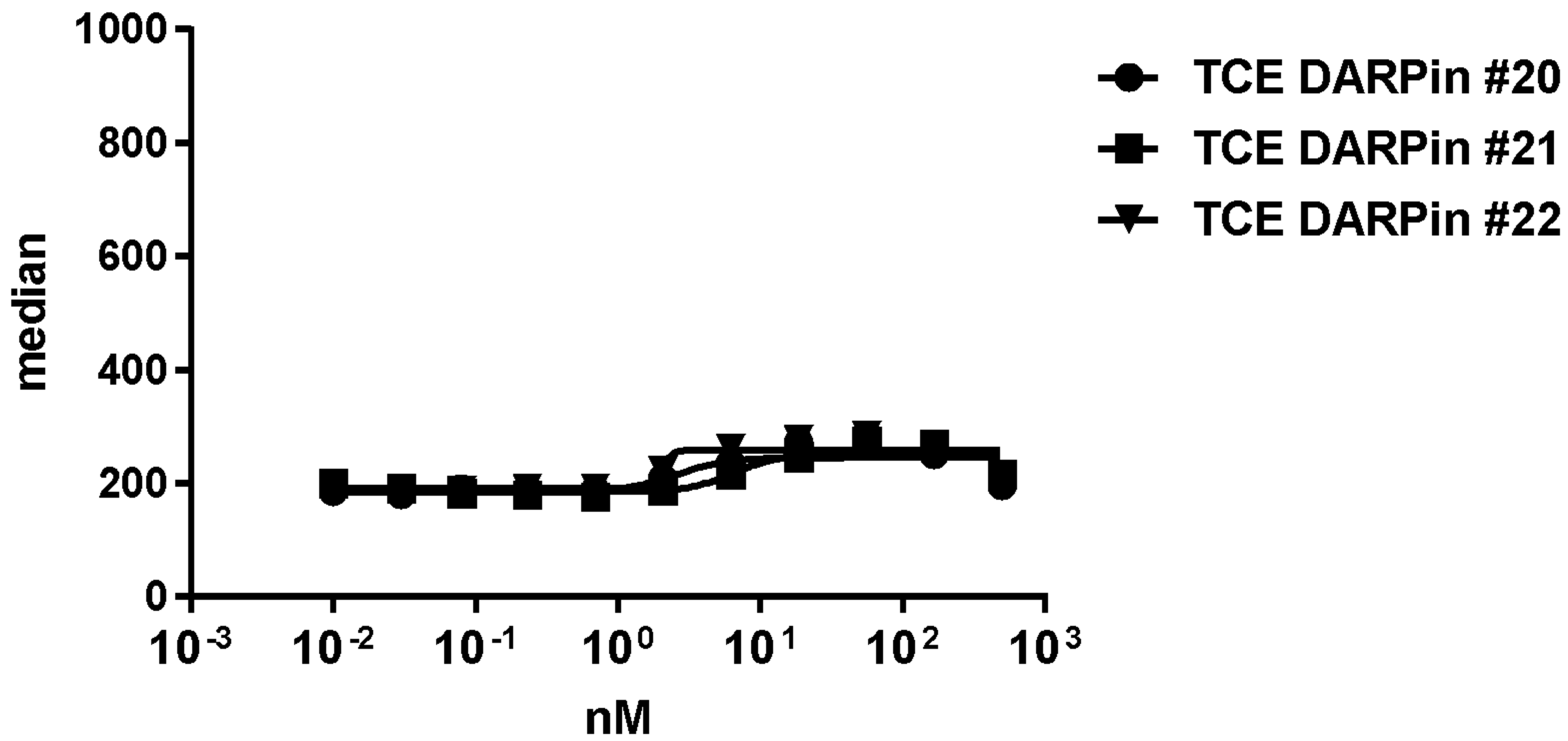

10000
8000
6000
4000
2000
0
median
10⁻³ 10⁻² 10⁻¹ 10⁰ 10¹ 10² 10³
nM
TCE DARPin #20
TCE DARPin #21

FIGURE 5 (cont.)

D



E



F

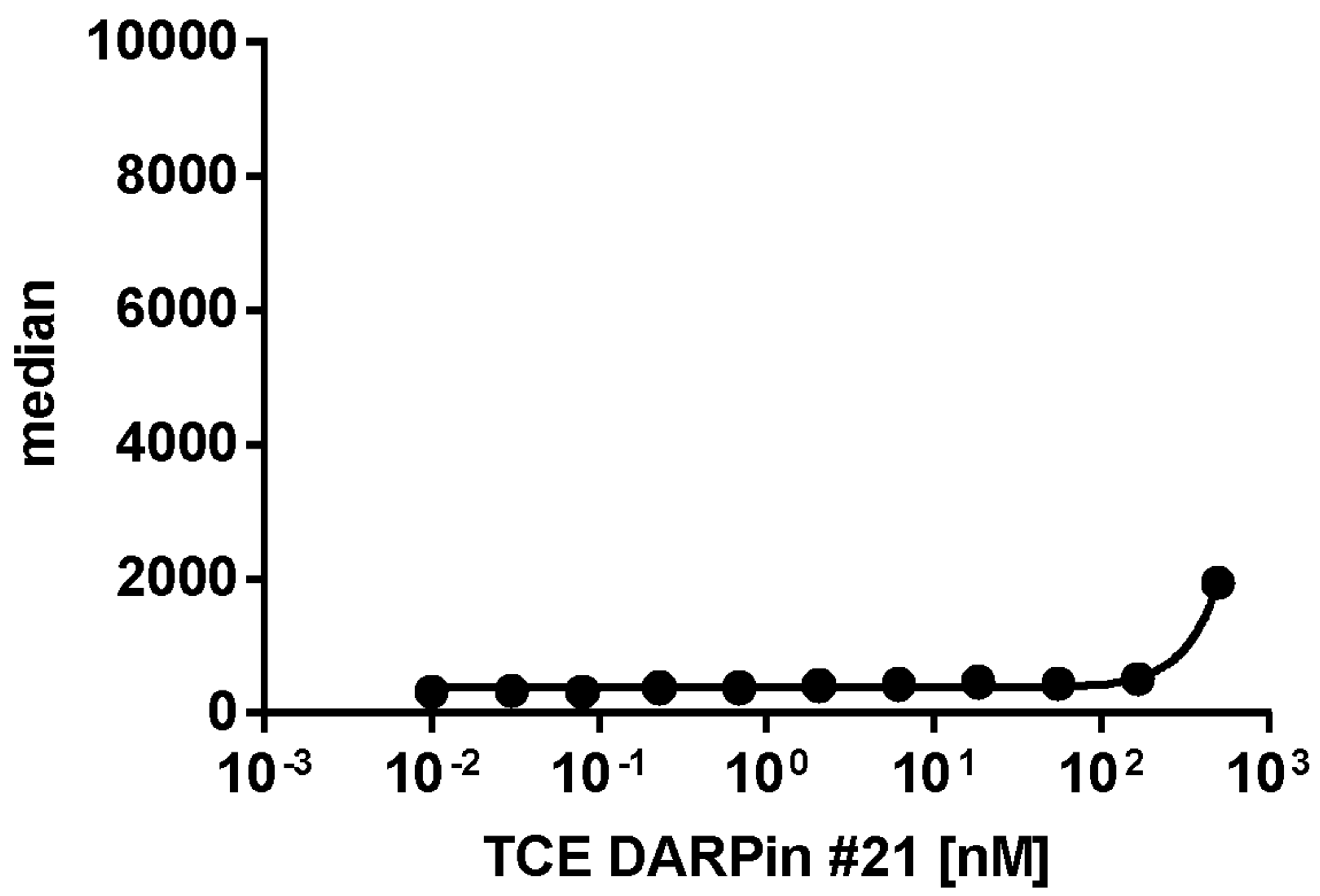
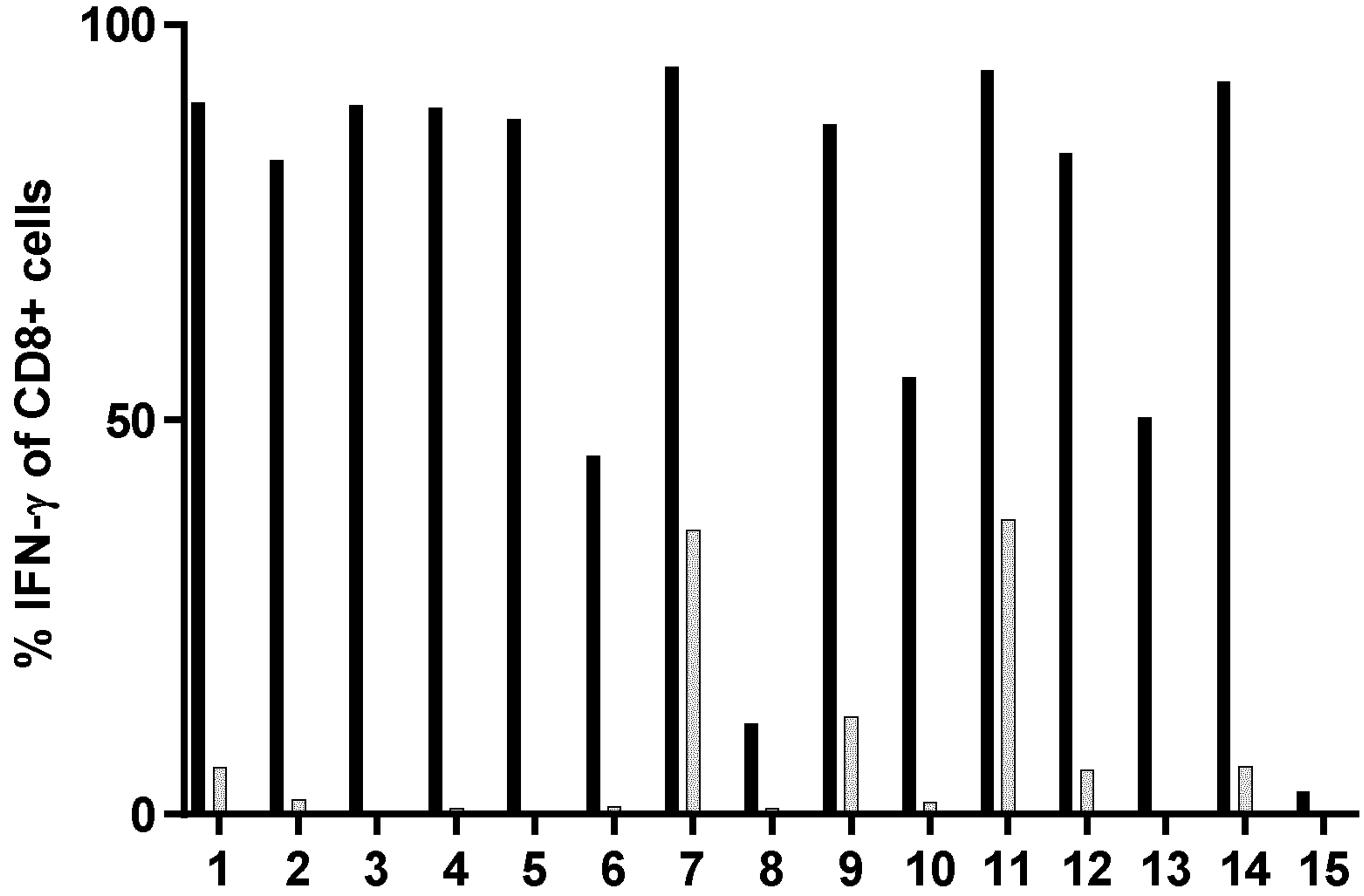


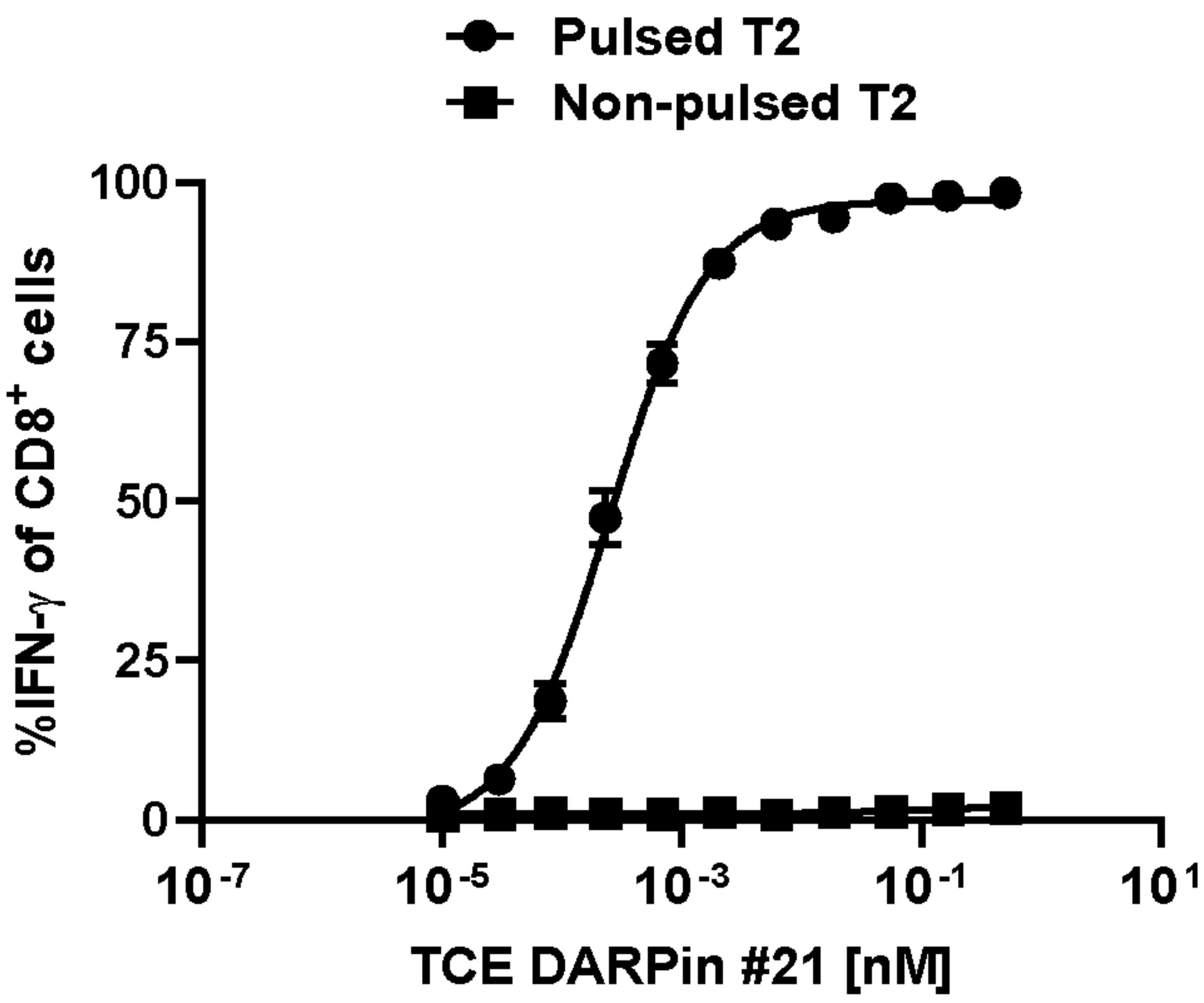
FIGURE 6

A



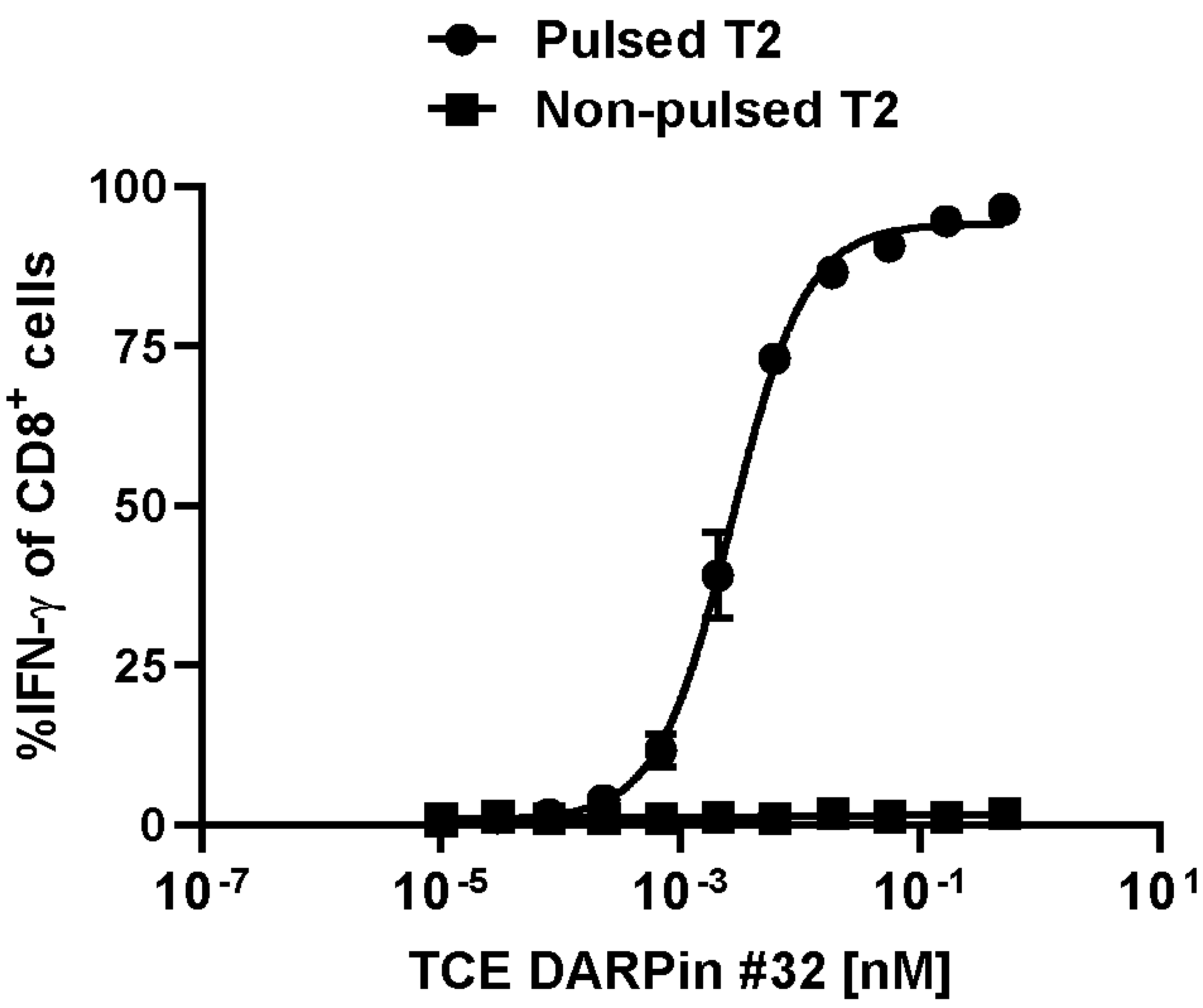
100
50
0
% IFN-γ of CD8+ cells
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B



Pulsed T2
Non-pulsed T2
100
75
50
25
0
%IFN-γ of CD8$^+$ cells
10^{-7} 10^{-5} 10^{-3} 10^{-1} 10^{1}
TCE DARPin #21 [nM]

C



Pulsed T2
Non-pulsed T2
100
75
50
25
0
%IFN-γ of CD8$^+$ cells
10^{-7} 10^{-5} 10^{-3} 10^{-1} 10^{1}
TCE DARPin #32 [nM]

FIGURE 7

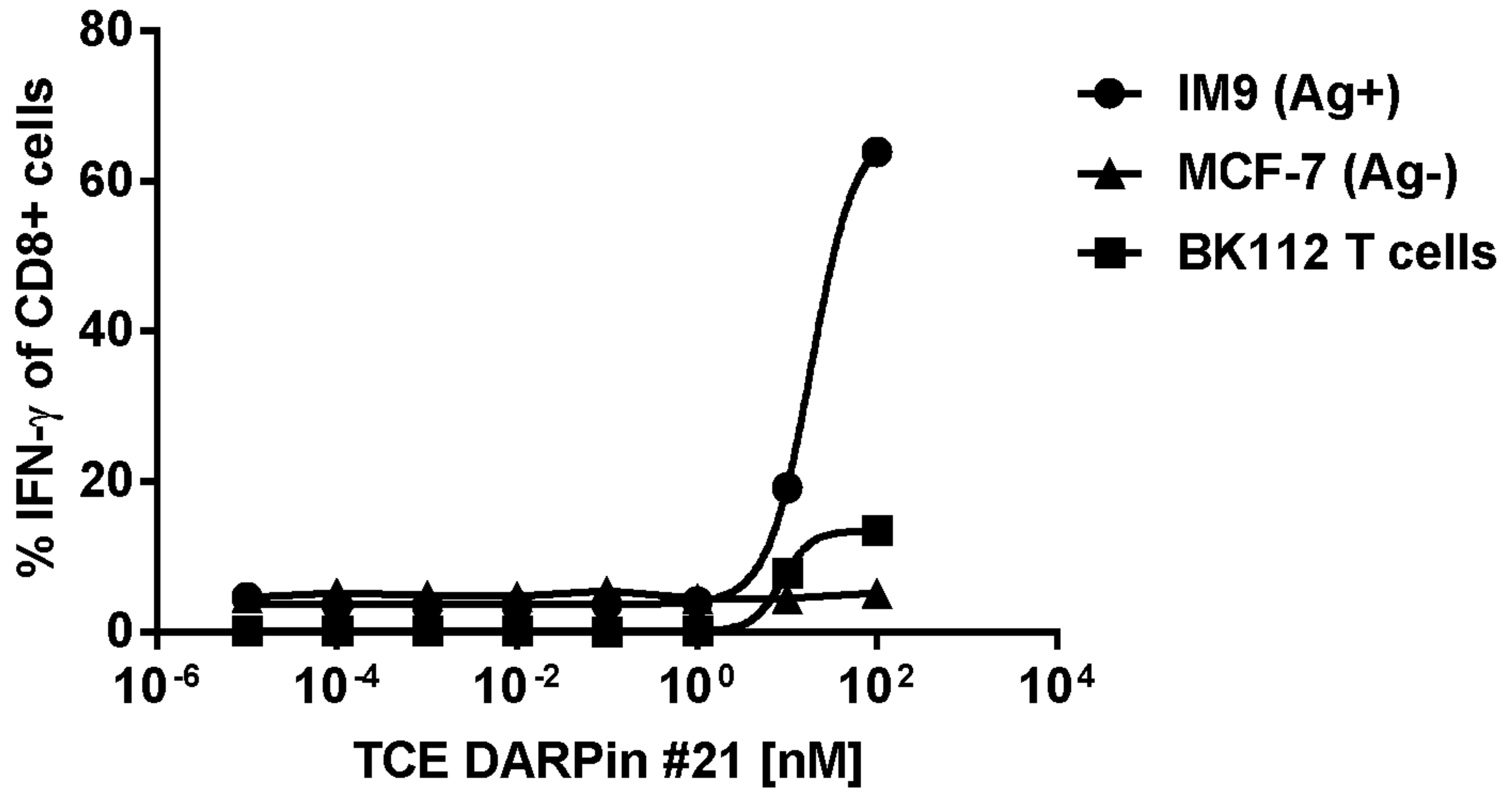
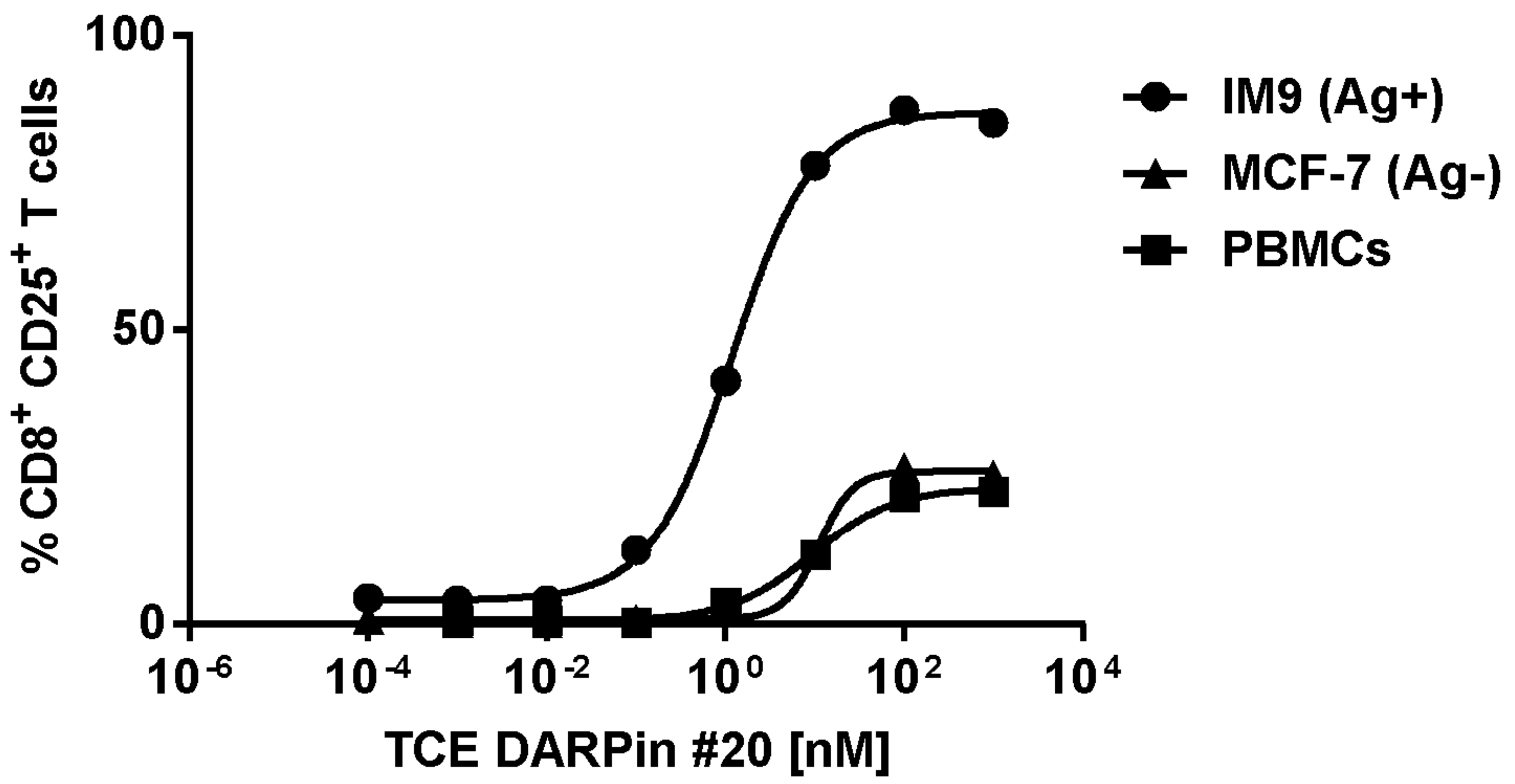


FIGURE 8

A



B

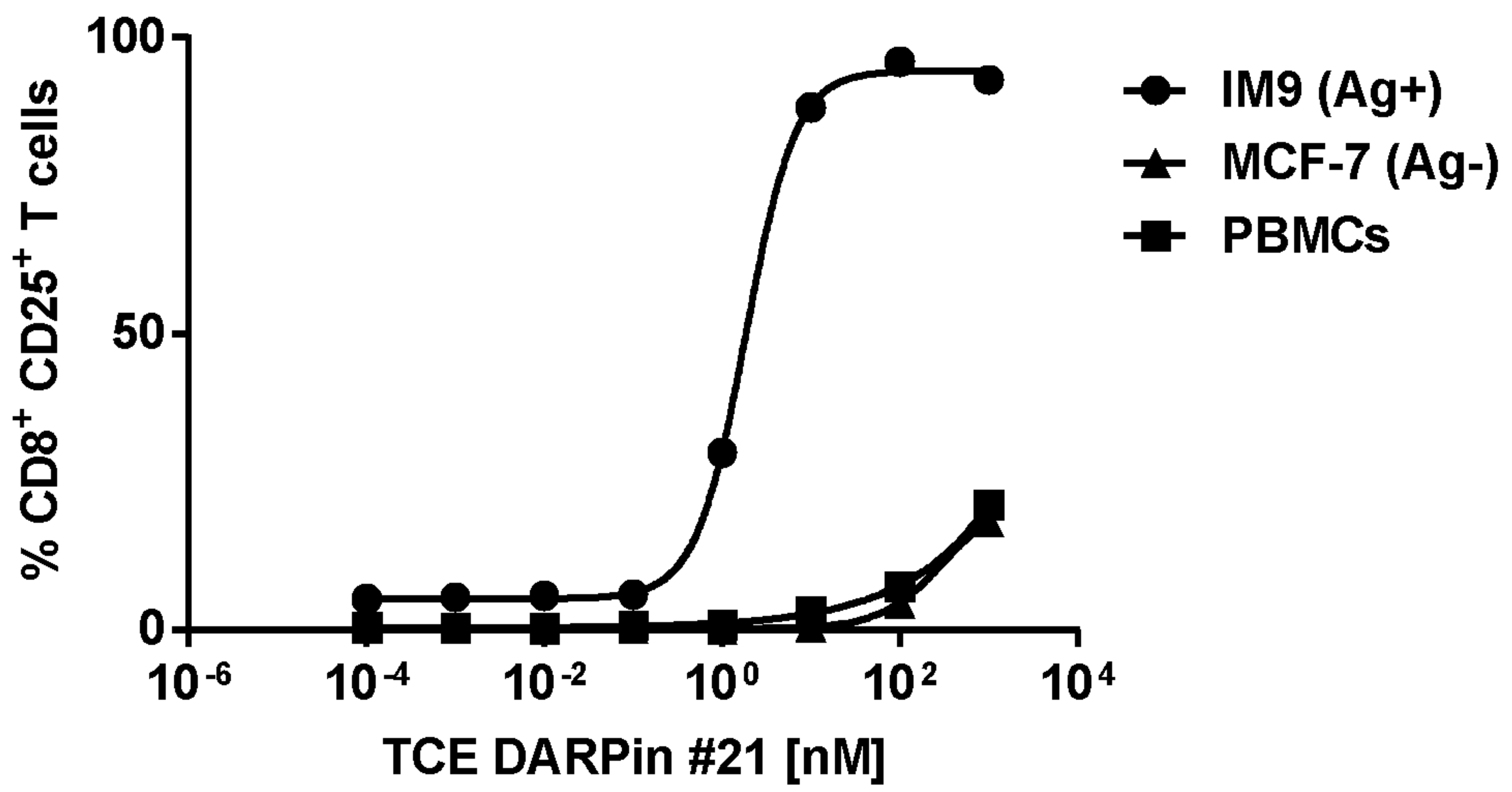
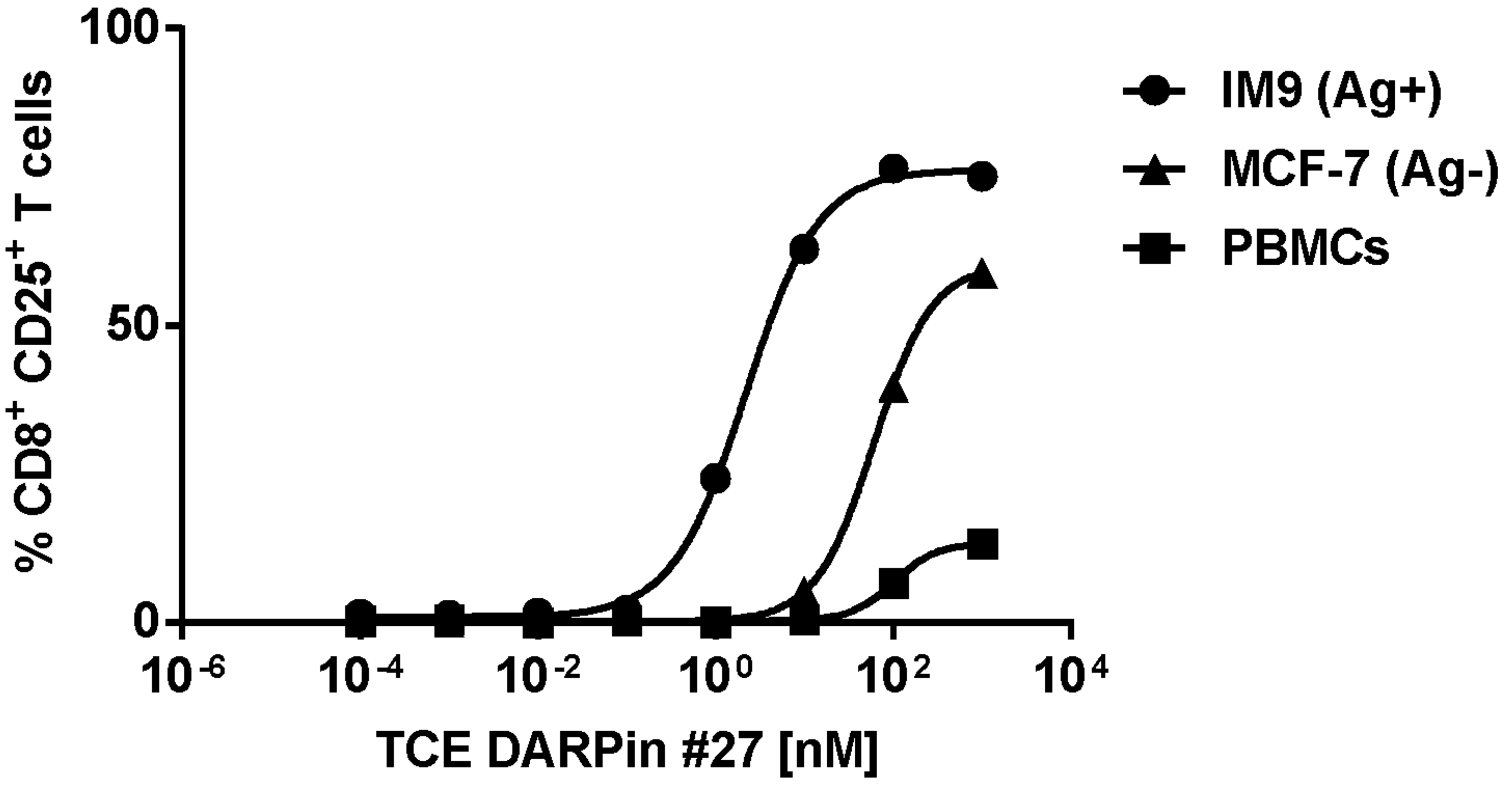
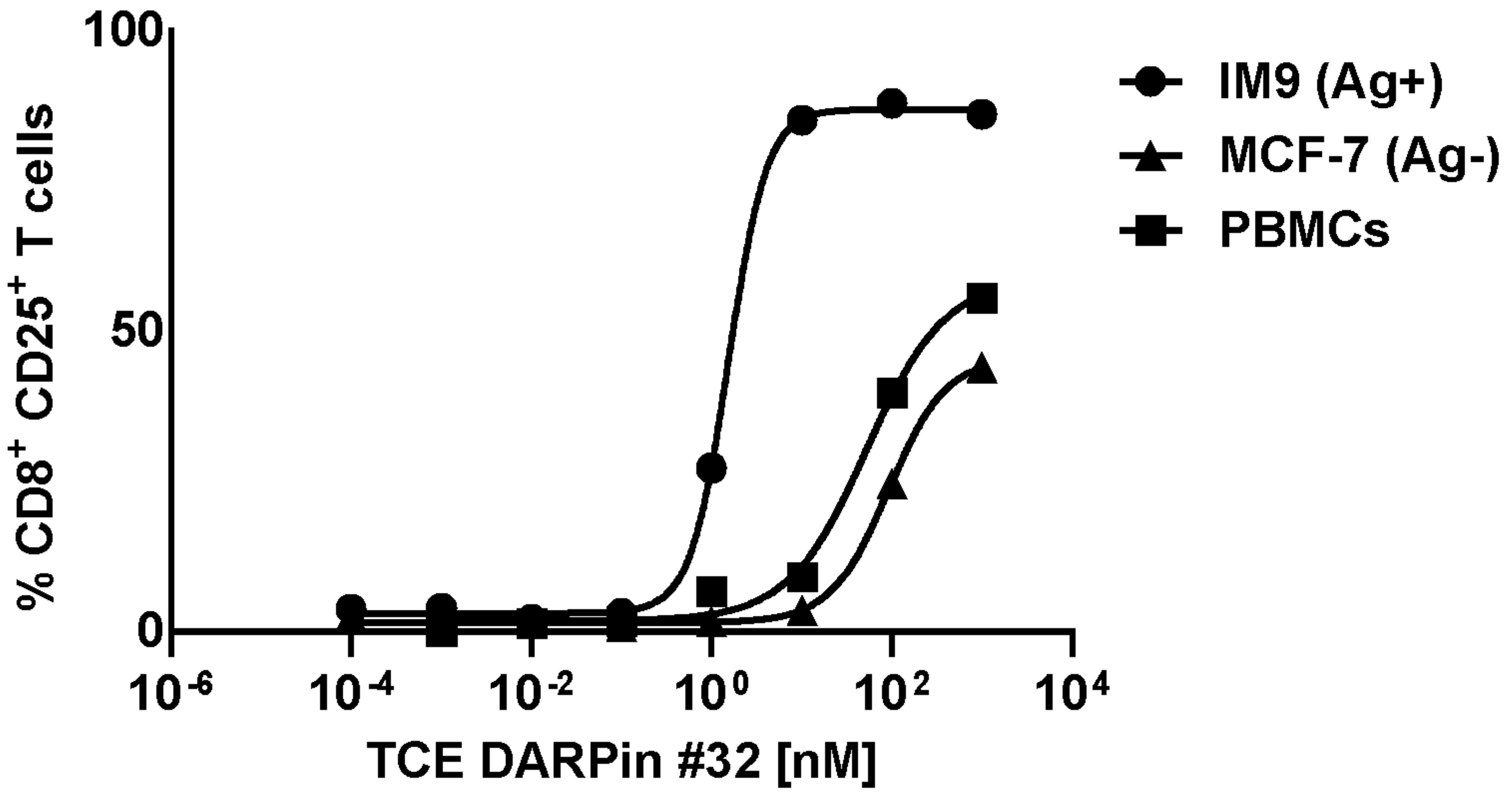


FIGURE 8 (cont.)

C



D



E

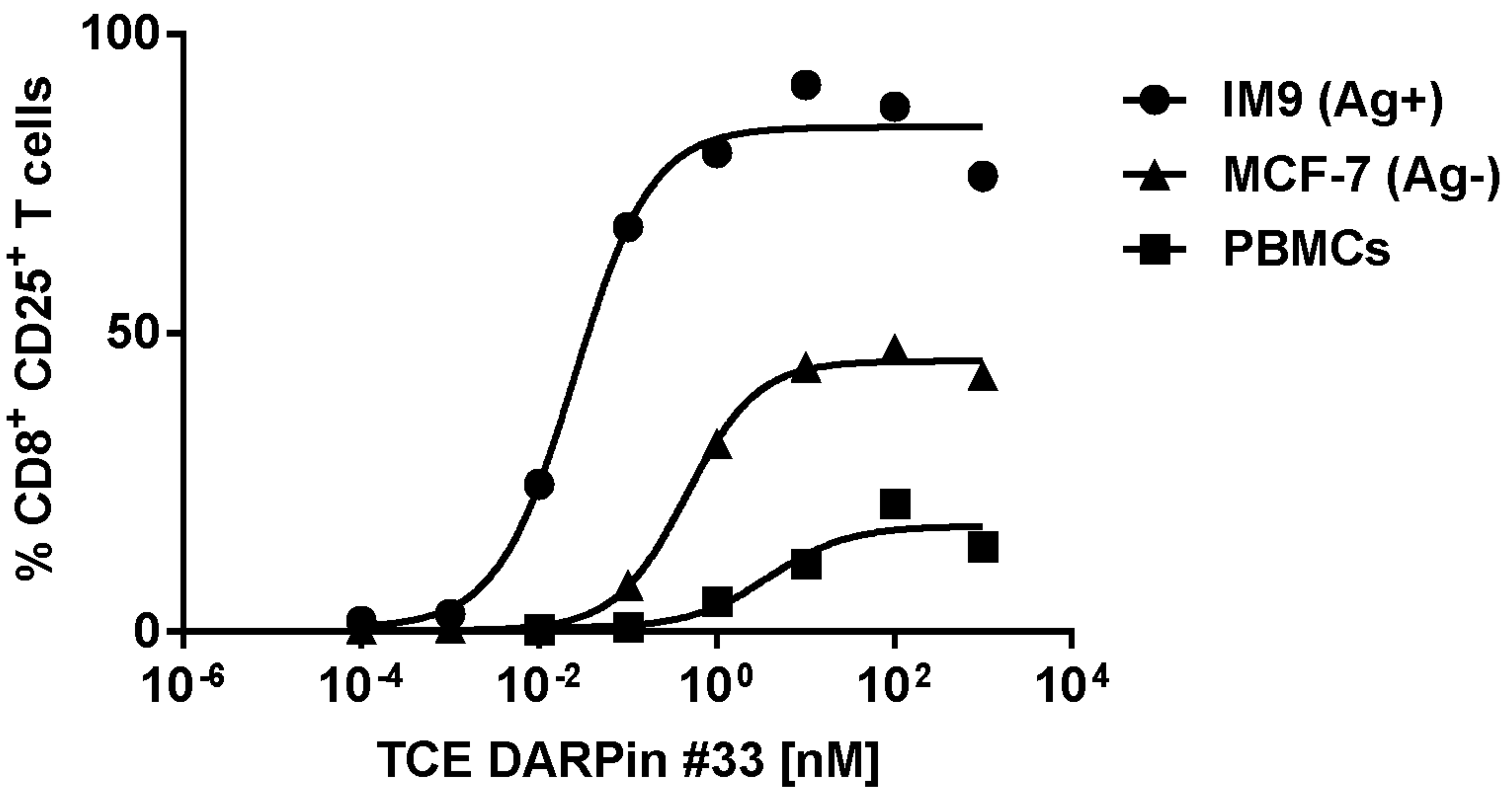


FIGURE 9

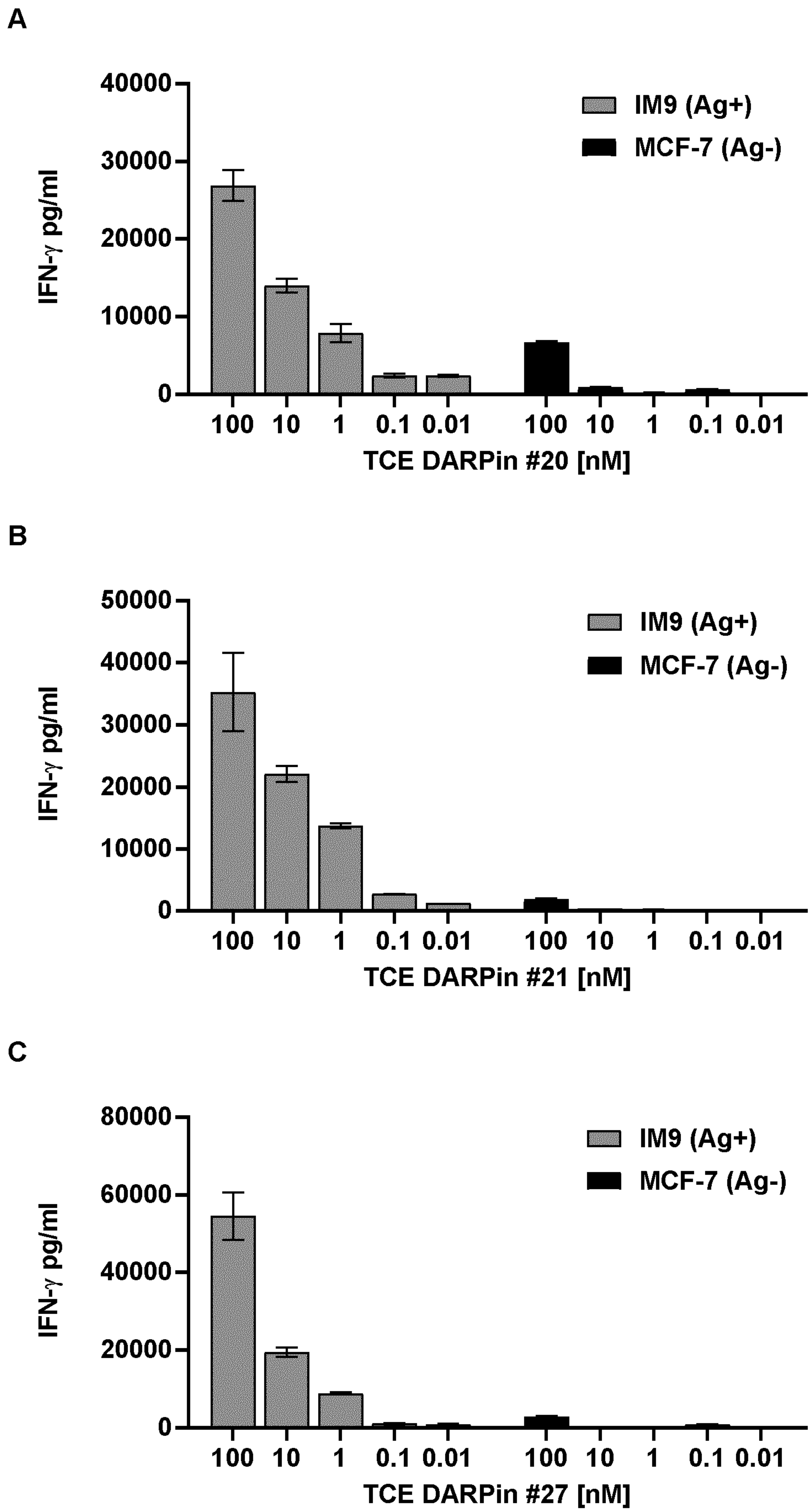
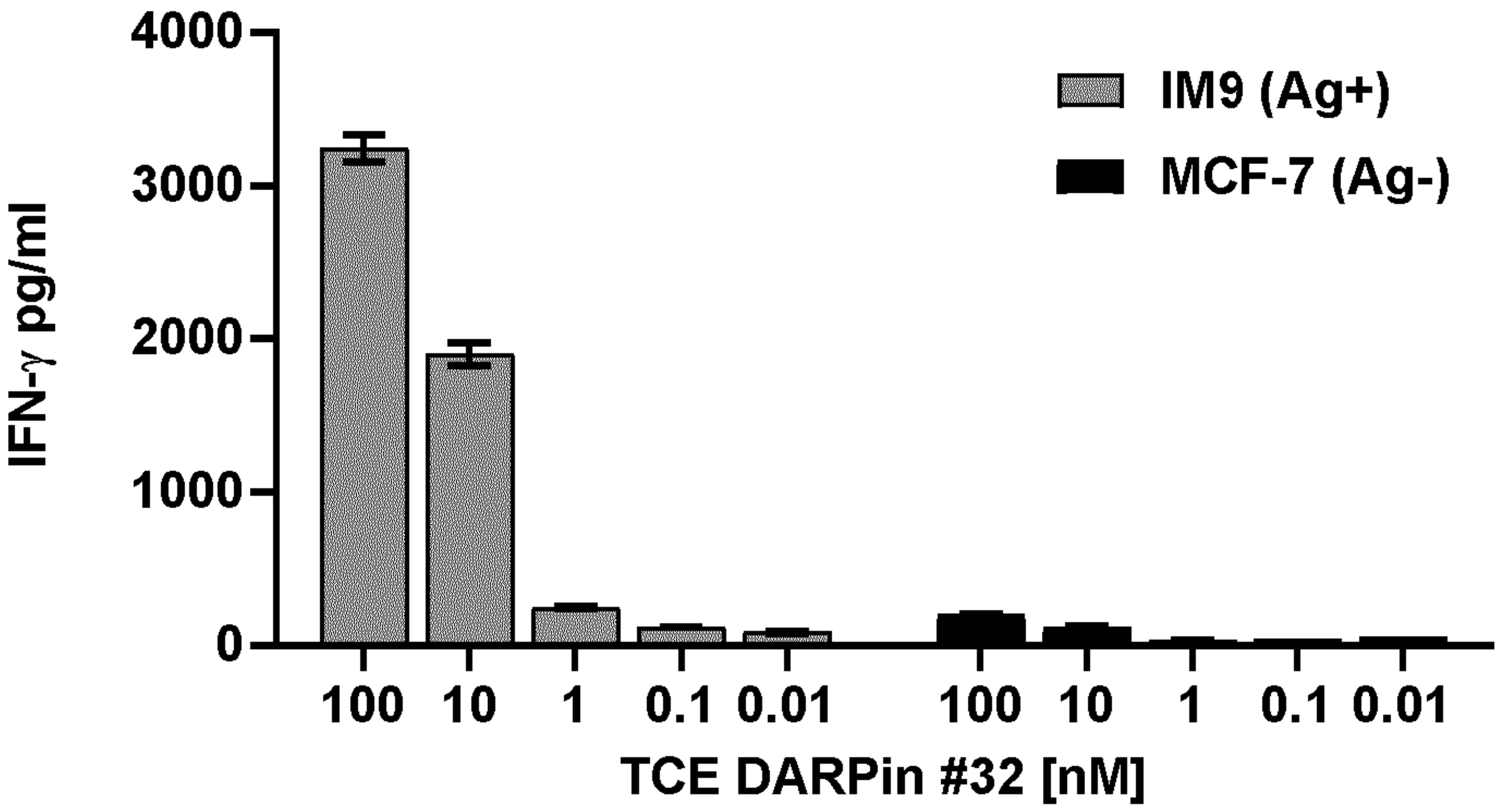

A
40000
30000
20000
10000
0
IFN-γ pg/ml
IM9 (Ag+)
MCF-7 (Ag-)
100 10 1 0.1 0.01 100 10 1 0.1 0.01
TCE DARPin #20 [nM]
B
50000
40000
30000
20000
10000
0
IFN-γ pg/ml
IM9 (Ag+)
MCF-7 (Ag-)
100 10 1 0.1 0.01 100 10 1 0.1 0.01
TCE DARPin #21 [nM]
C
80000
60000
40000
20000
0
IFN-γ pg/ml
IM9 (Ag+)
MCF-7 (Ag-)
100 10 1 0.1 0.01 100 10 1 0.1 0.01
TCE DARPin #27 [nM]

FIGURE 9 (cont.)

D



E

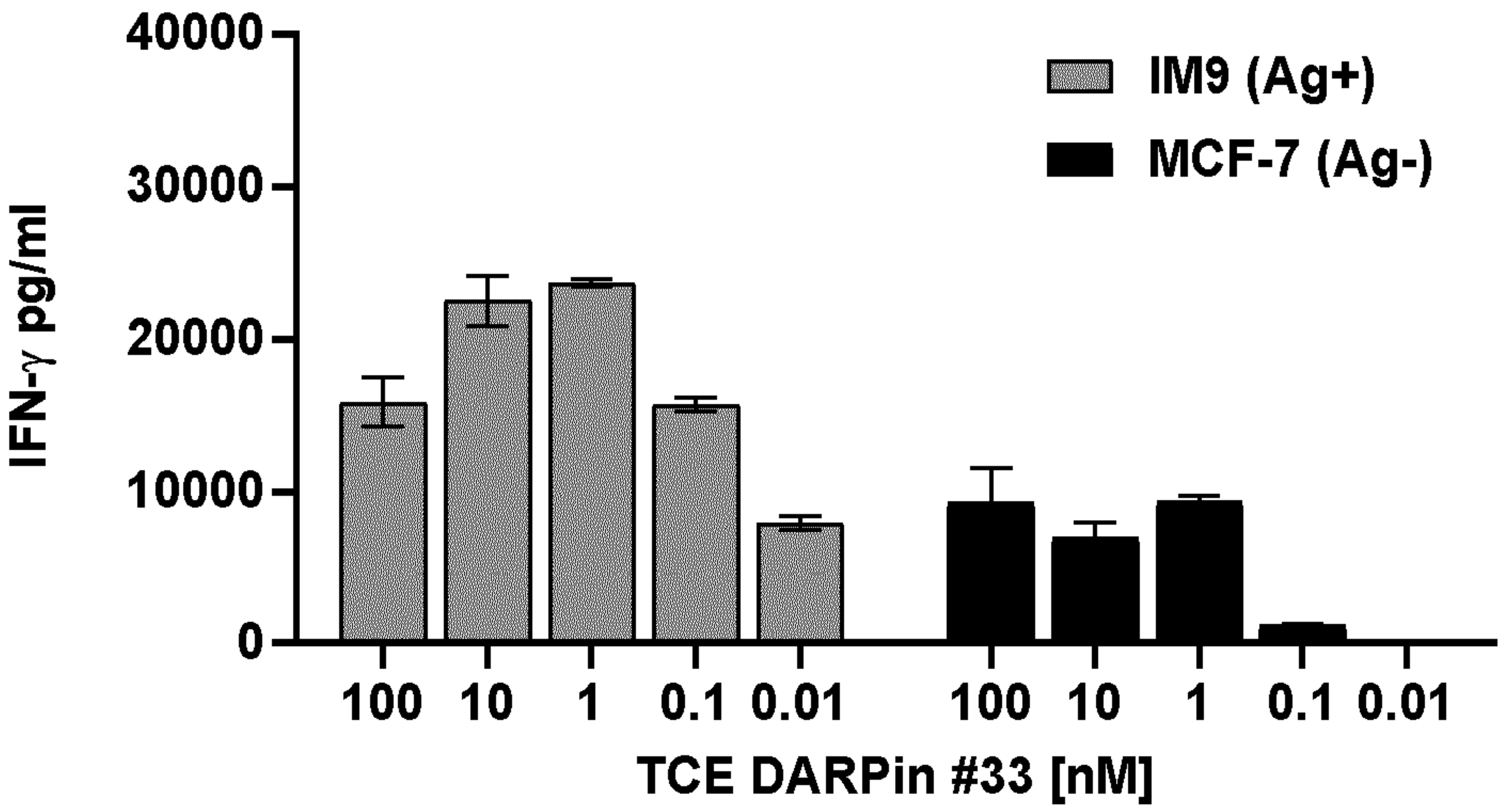
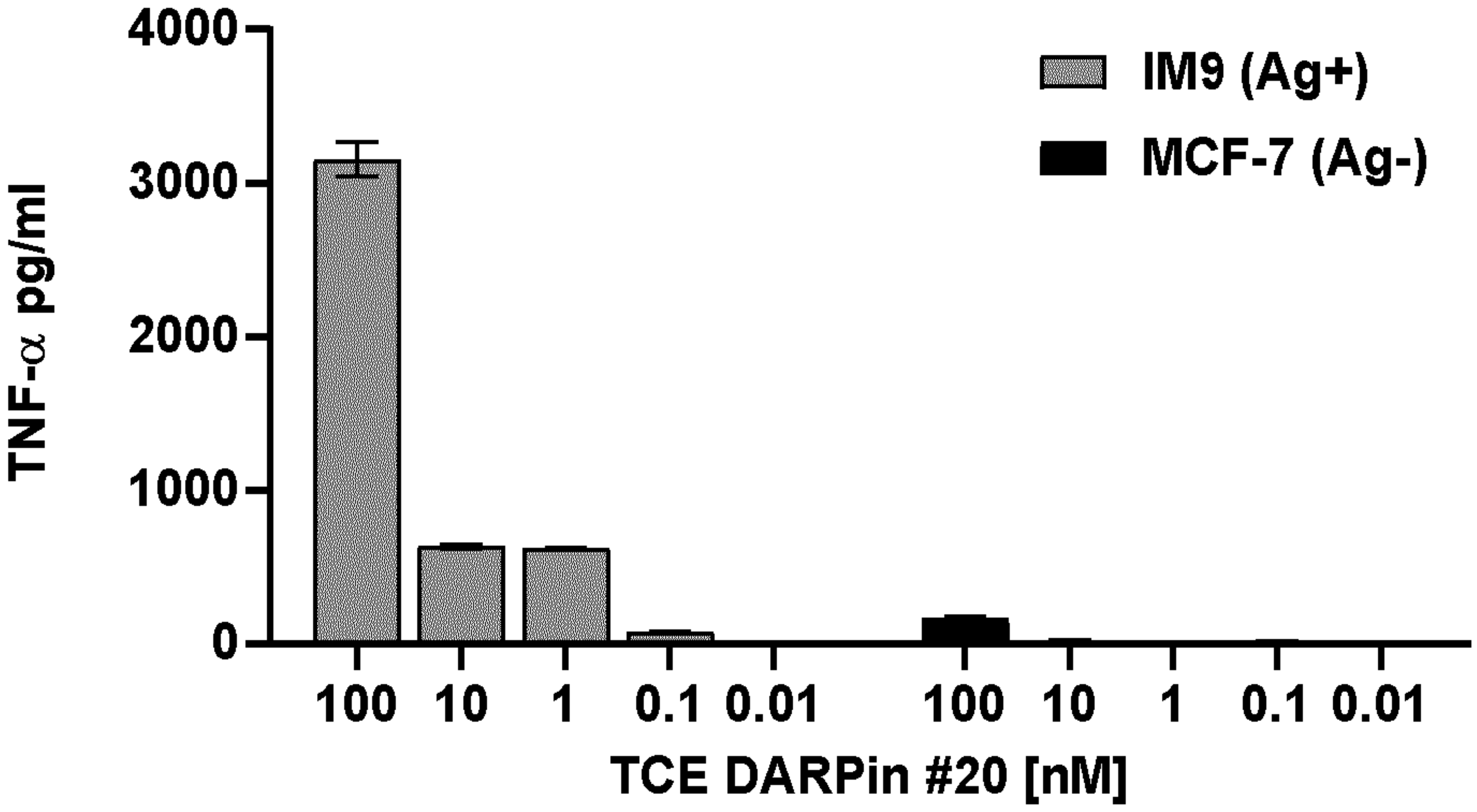
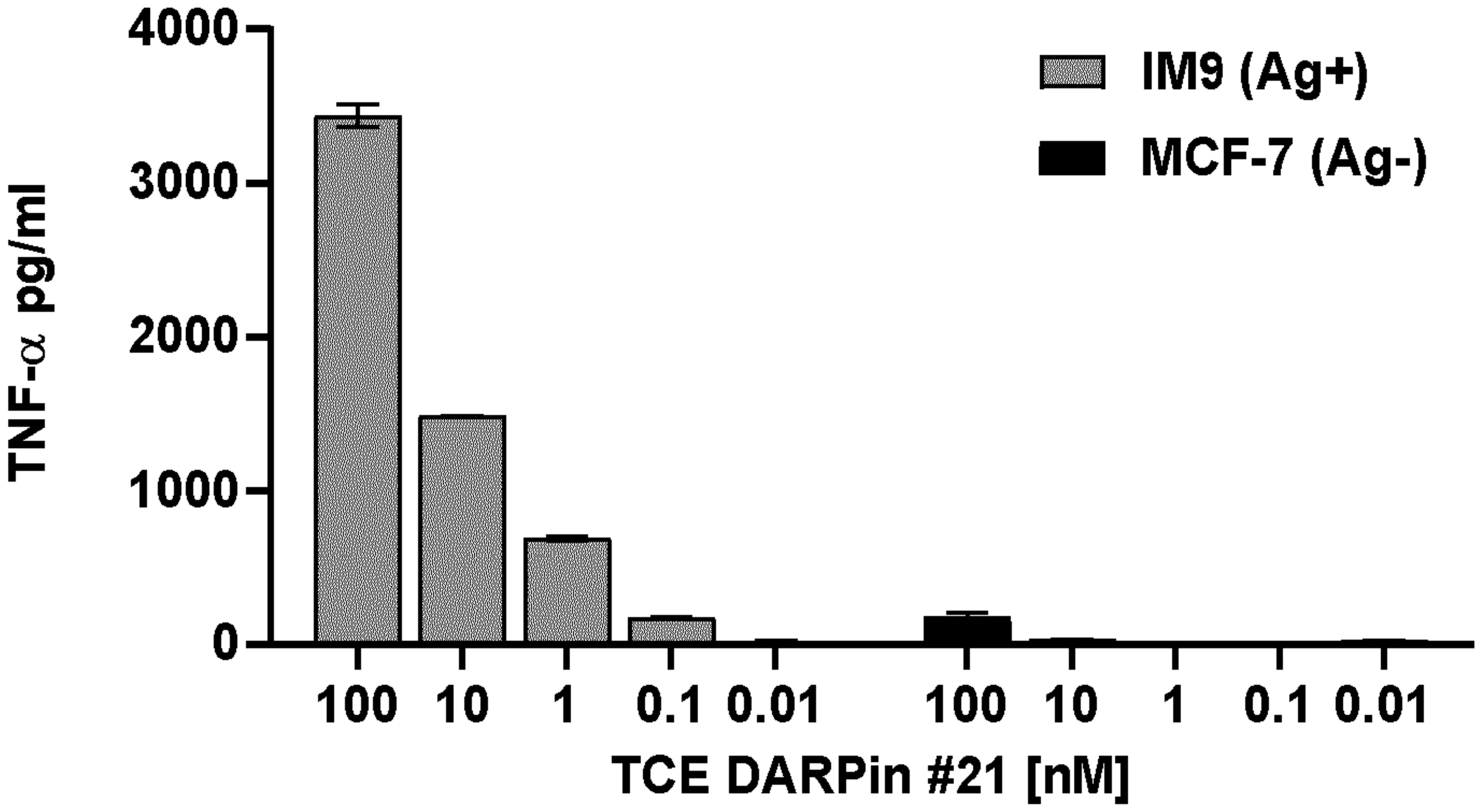


FIGURE 10

A



B



C

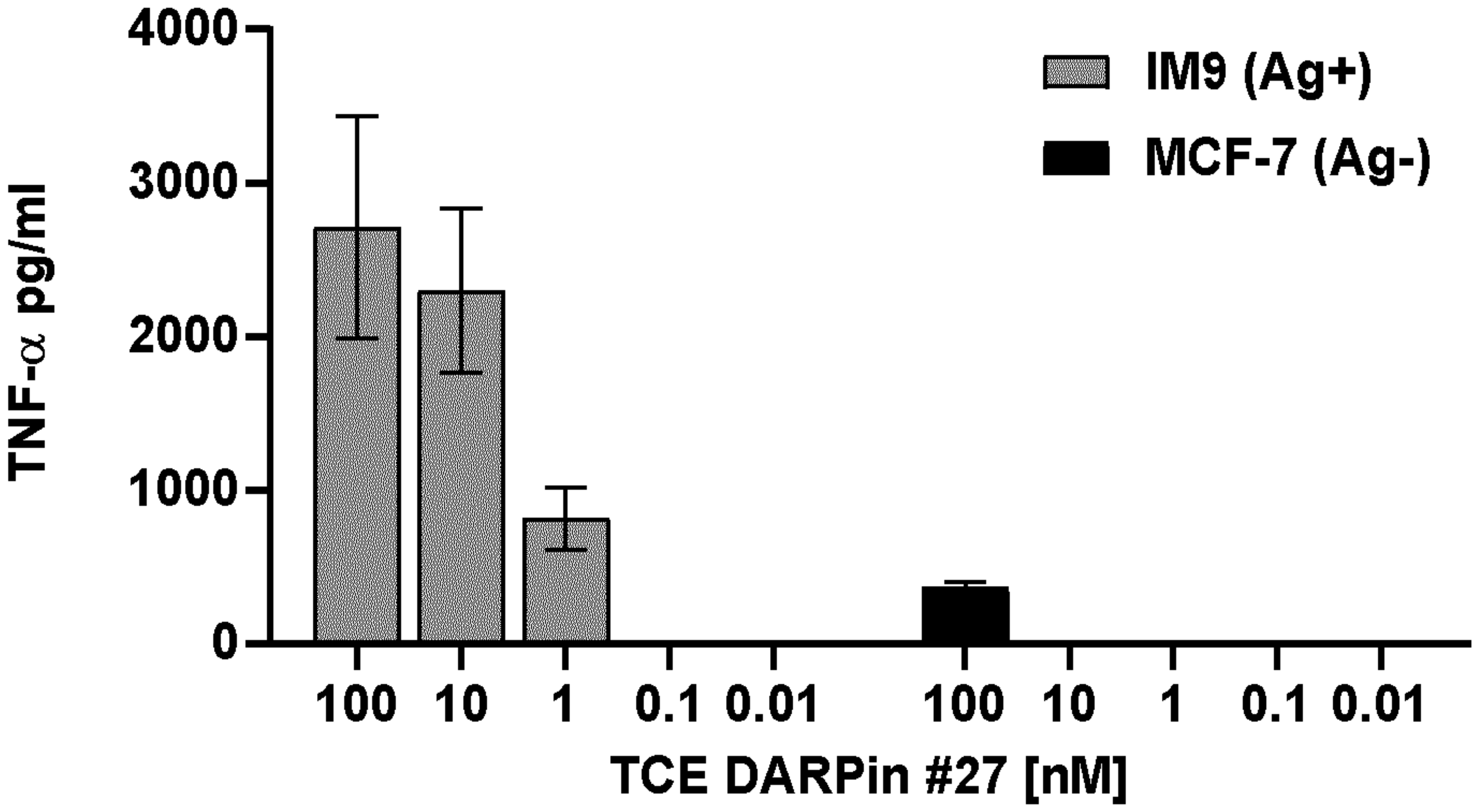
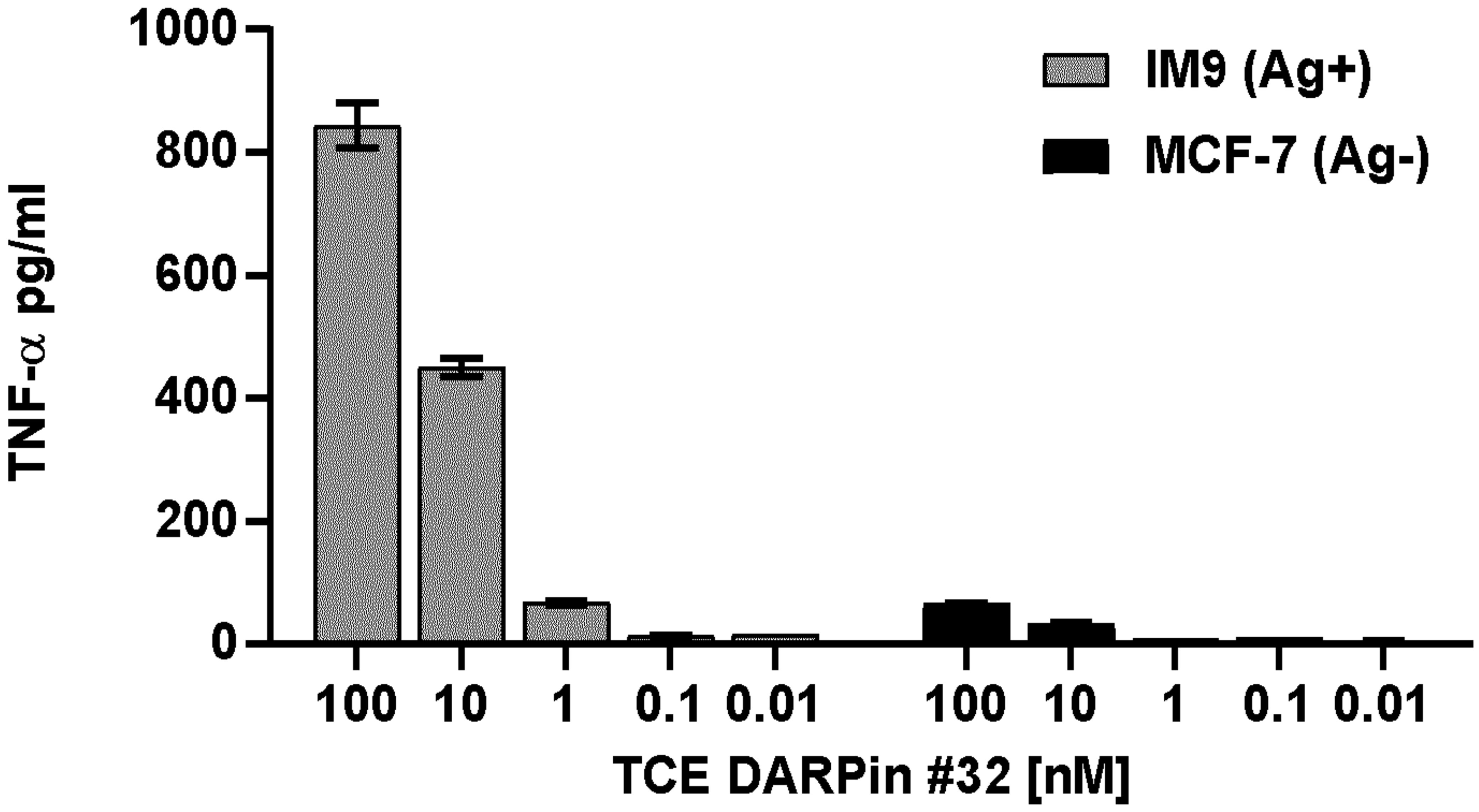


FIGURE 10 (cont.)

D



E

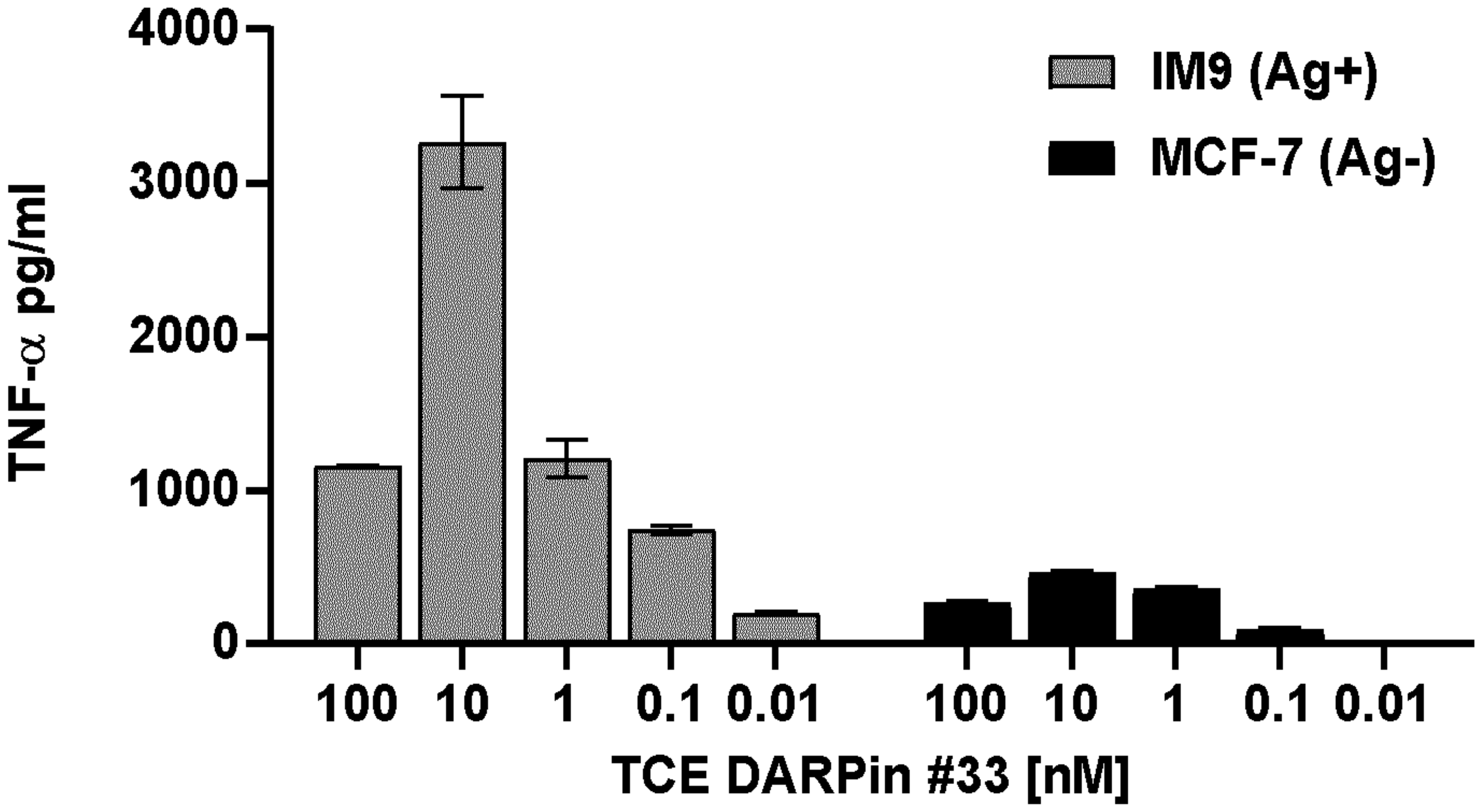
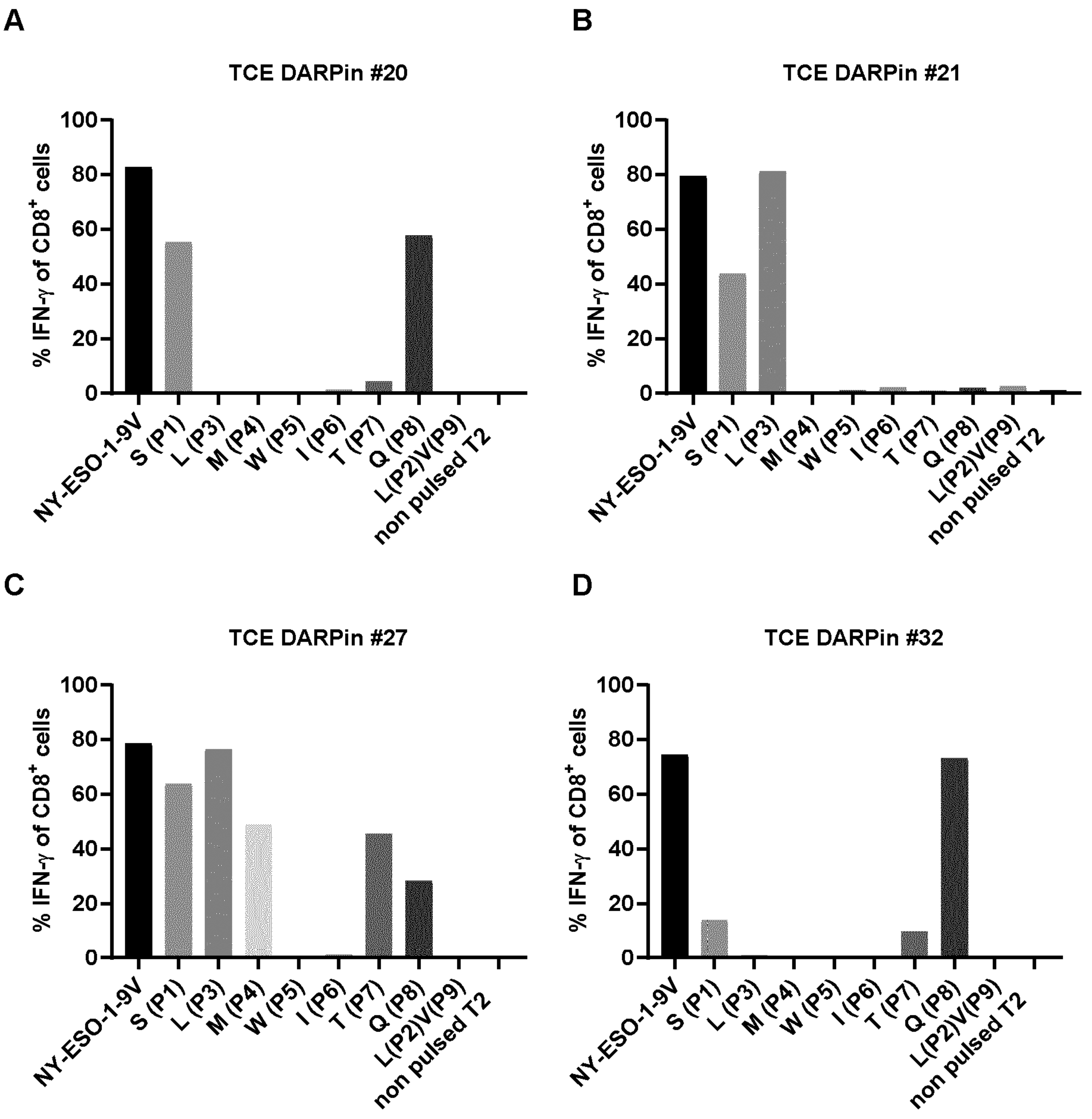


FIGURE 11


A
TCE DARPin #20
B
TCE DARPin #21
C
TCE DARPin #27
D
TCE DARPin #32
% IFN-γ of CD8⁺ cells
100
80
60
40
20
0
NY-ESO-1-9V
S (P1)
L (P3)
M (P4)
W (P5)
I (P6)
T (P7)
Q (P8)
L(P2)V(P9)
non pulsed T2

E

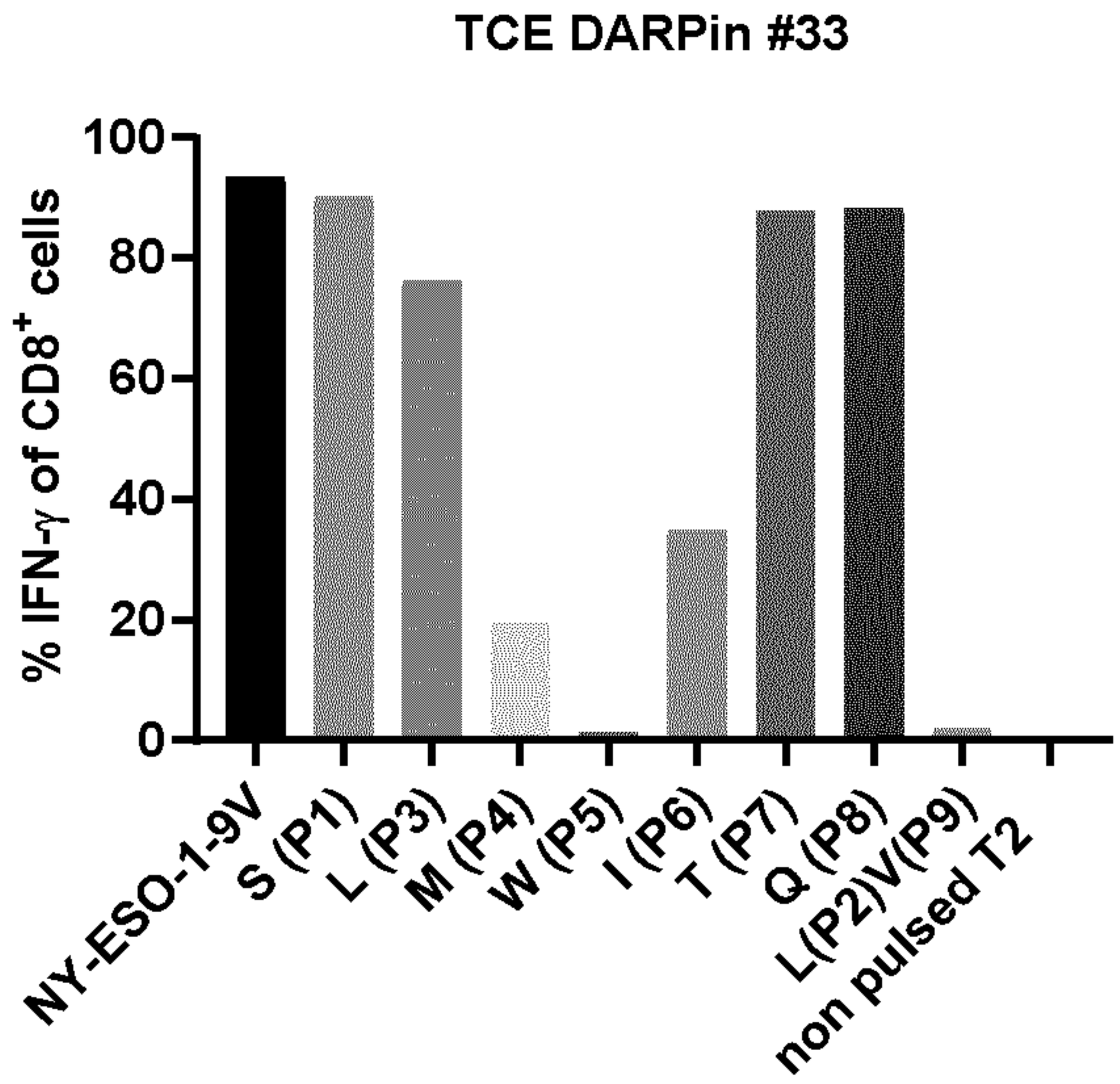

TCE DARPin #33
% IFN-γ of CD8+ cells
100
80
60
40
20
0
NY-ESO-1-9V
S (P1)
L (P3)
M (P4)
W (P5)
I (P6)
T (P7)
Q (P8)
L(P2)V(P9)
non pulsed T2

FIGURE 12

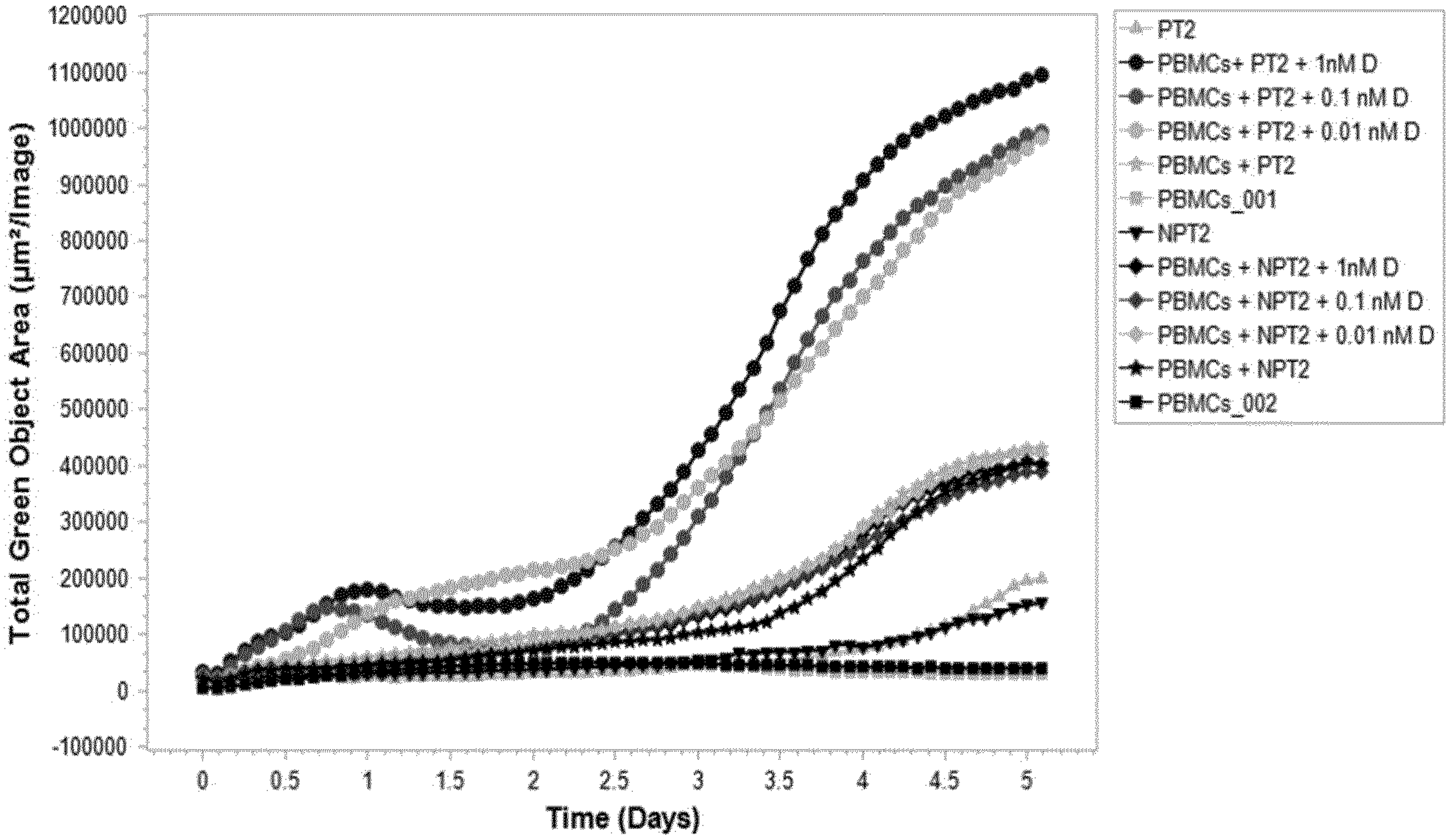


FIGURE 13

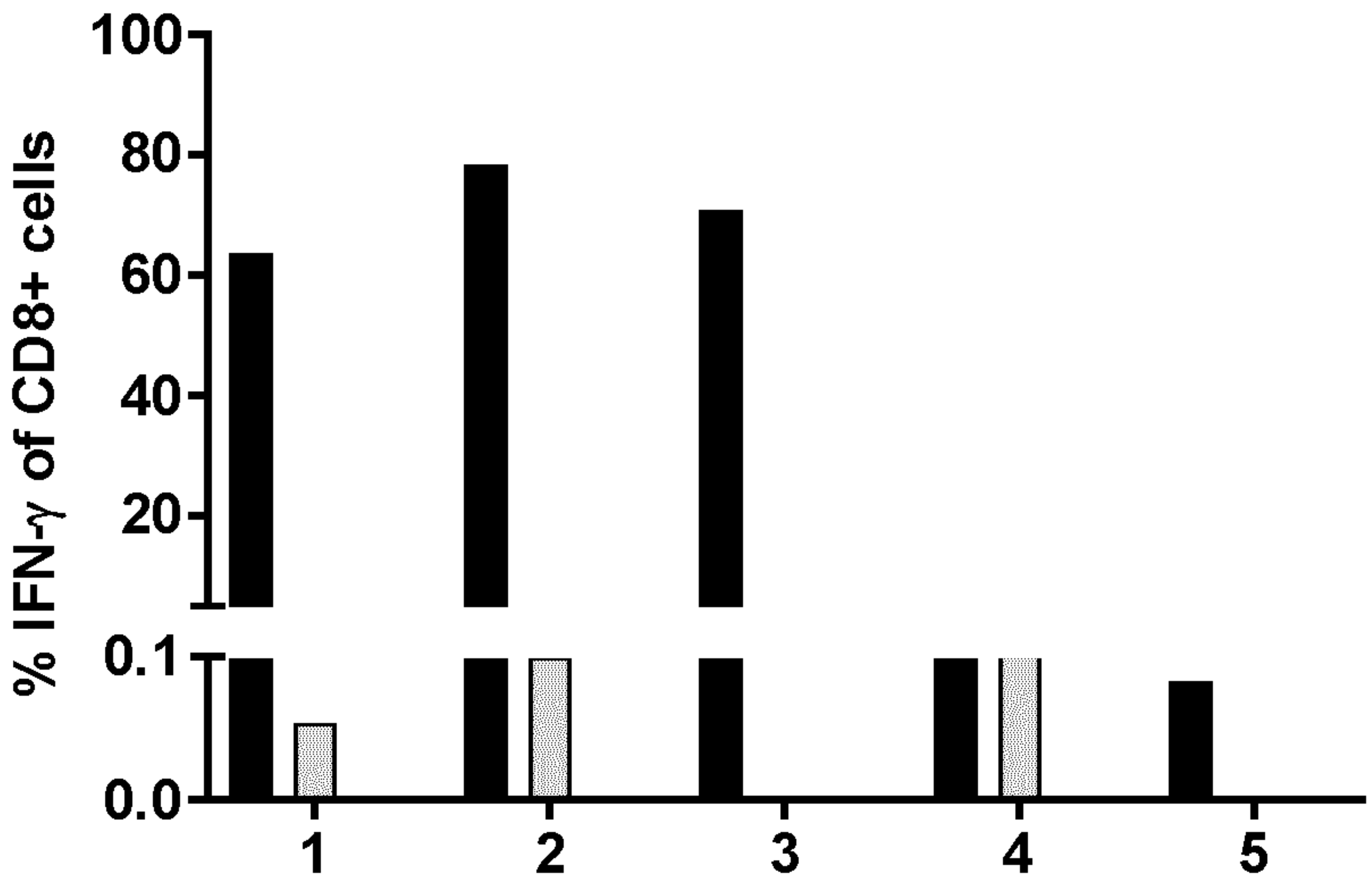
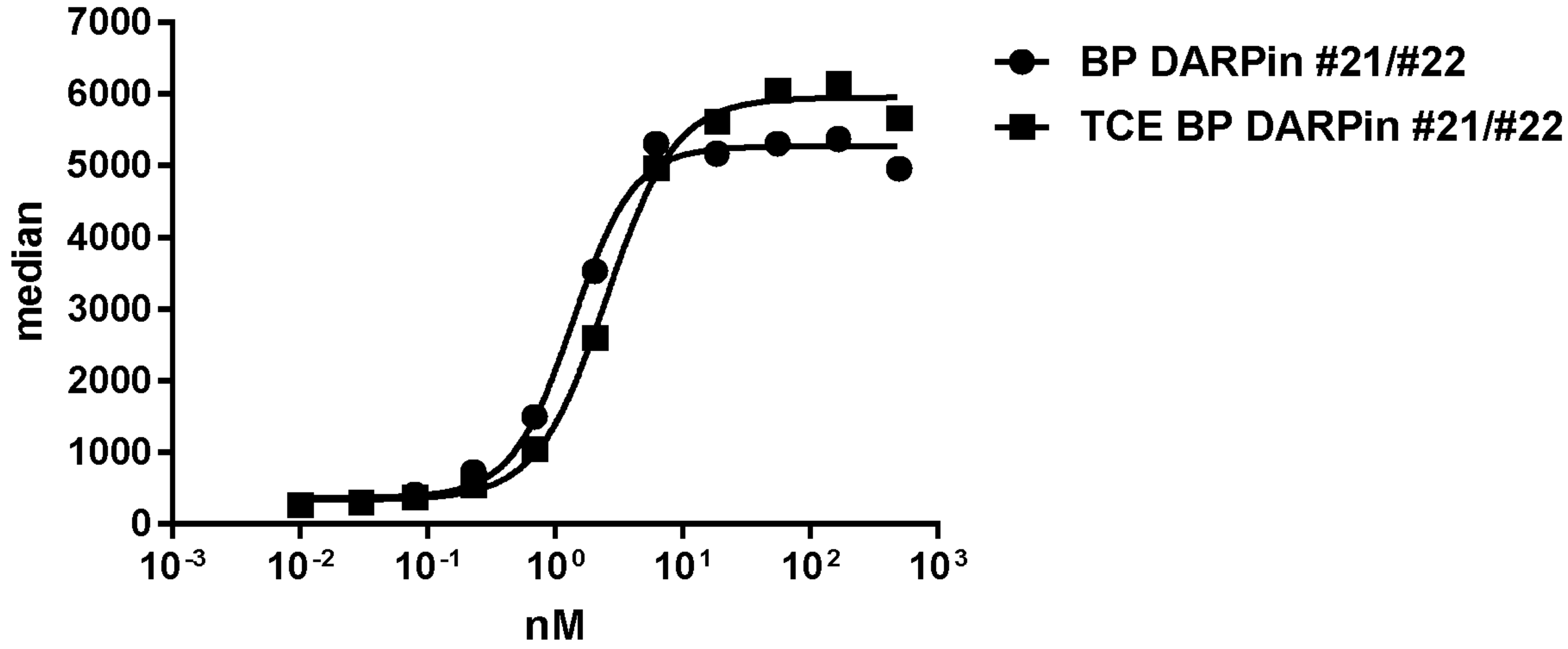


FIGURE 14

A


7000
6000
5000
4000
3000
2000
1000
0
median
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
nM
BP DARPin #21/#22
TCE BP DARPin #21/#22

B

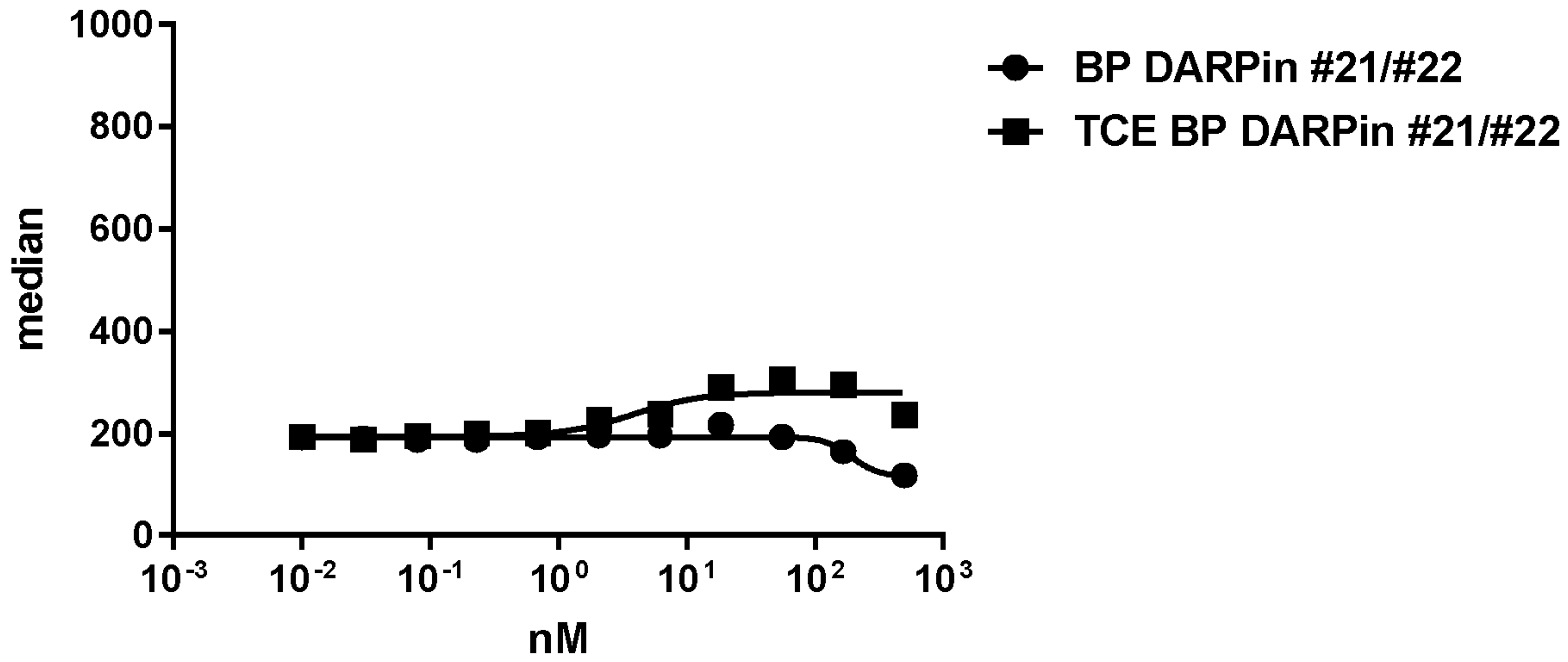
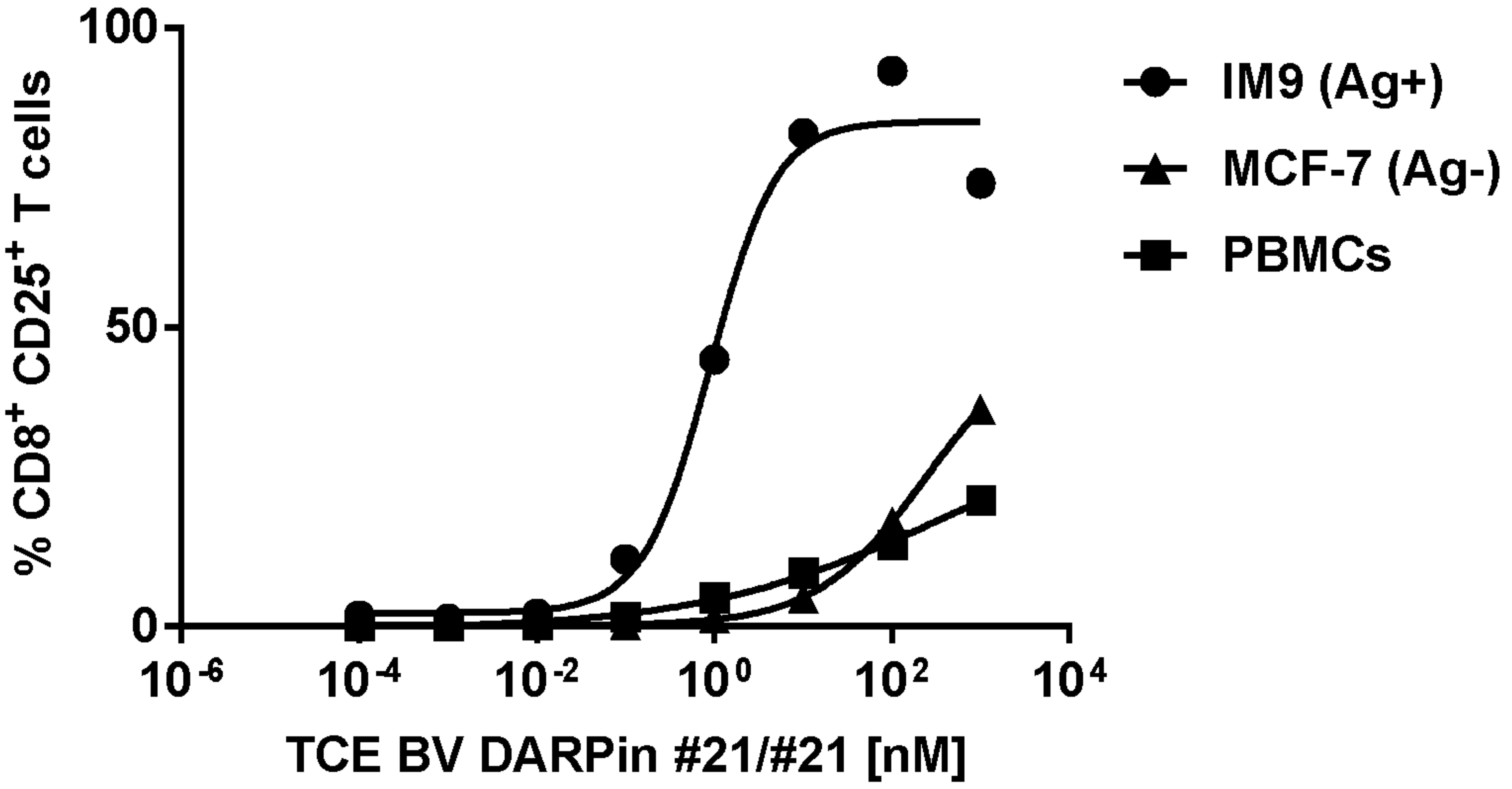

1000
800
600
400
200
0
median
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3}
nM
BP DARPin #21/#22
TCE BP DARPin #21/#22

FIGURE 15

A


100
50
0
% CD8+ CD25+ T cells
10-6
10-4
10-2
100
102
104
TCE BV DARPin #21/#21 [nM]
IM9 (Ag+)
MCF-7 (Ag-)
PBMCs

B

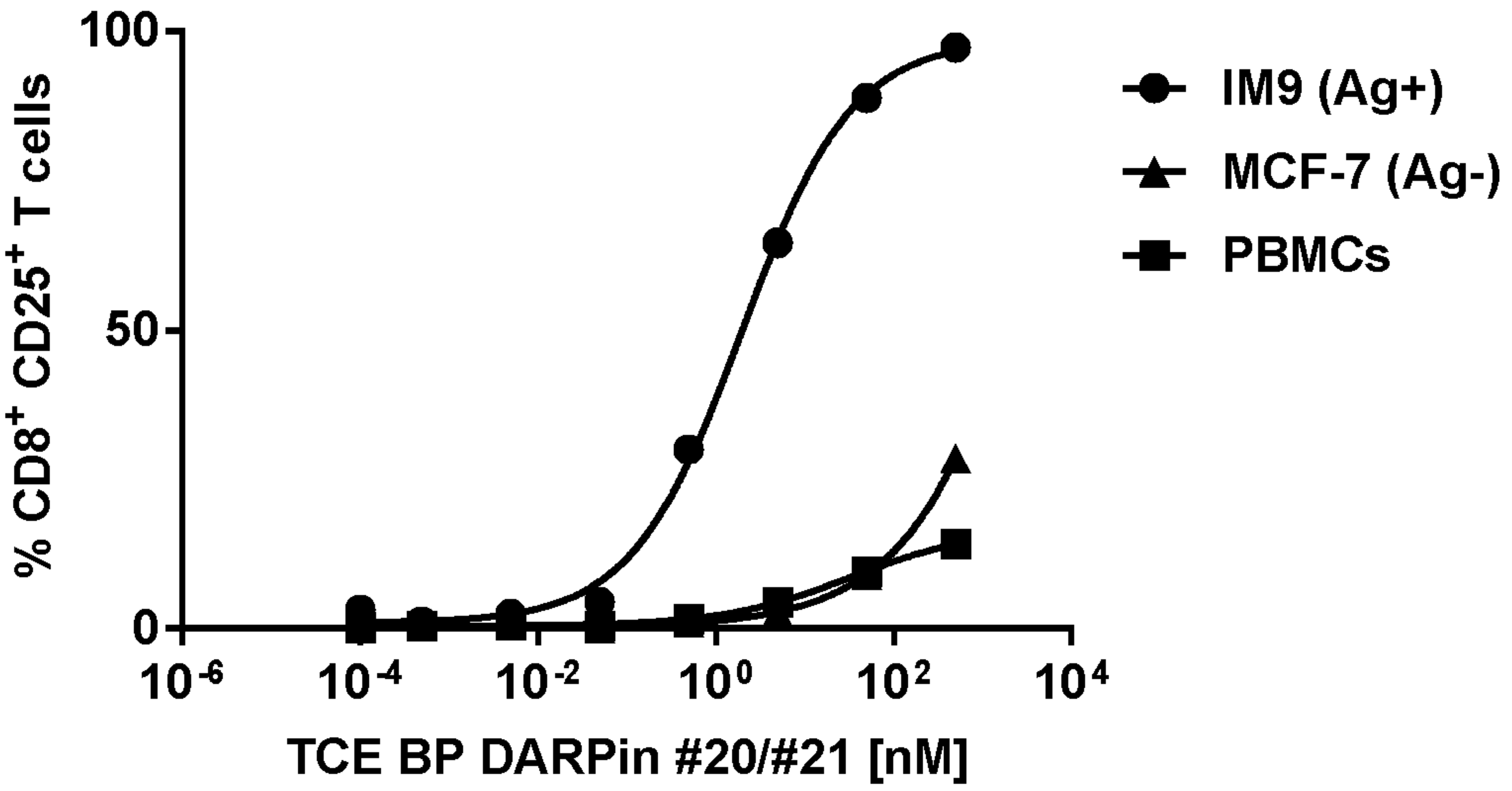
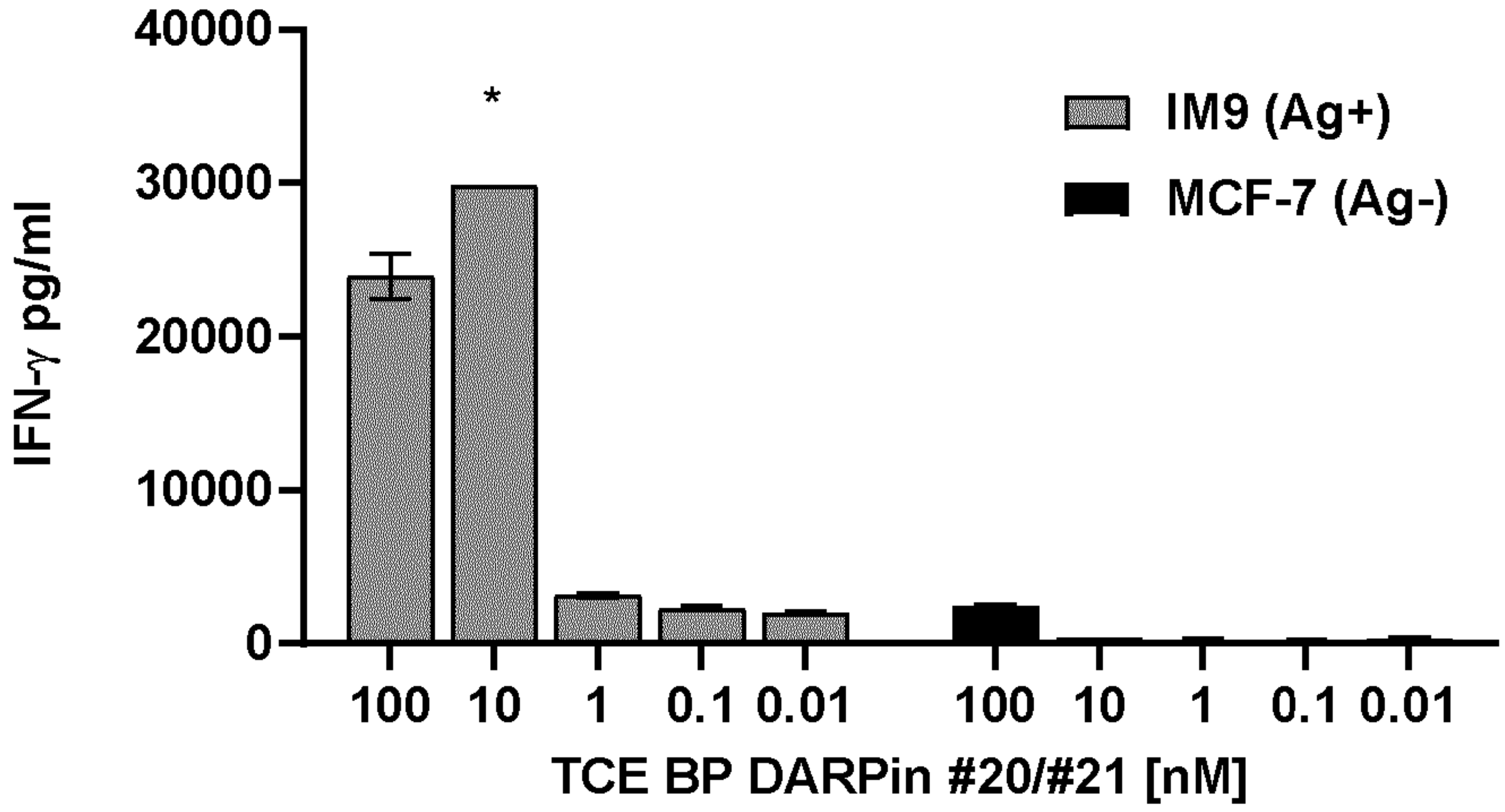

100
50
0
% CD8+ CD25+ T cells
10-6
10-4
10-2
100
102
104
TCE BP DARPin #20/#21 [nM]
IM9 (Ag+)
MCF-7 (Ag-)
PBMCs

FIGURE 16

A


40000
30000
20000
10000
0
IFN-γ pg/ml
*
IM9 (Ag+)
MCF-7 (Ag-)
100 10 1 0.1 0.01 100 10 1 0.1 0.01
TCE BP DARPin #20/#21 [nM]

B

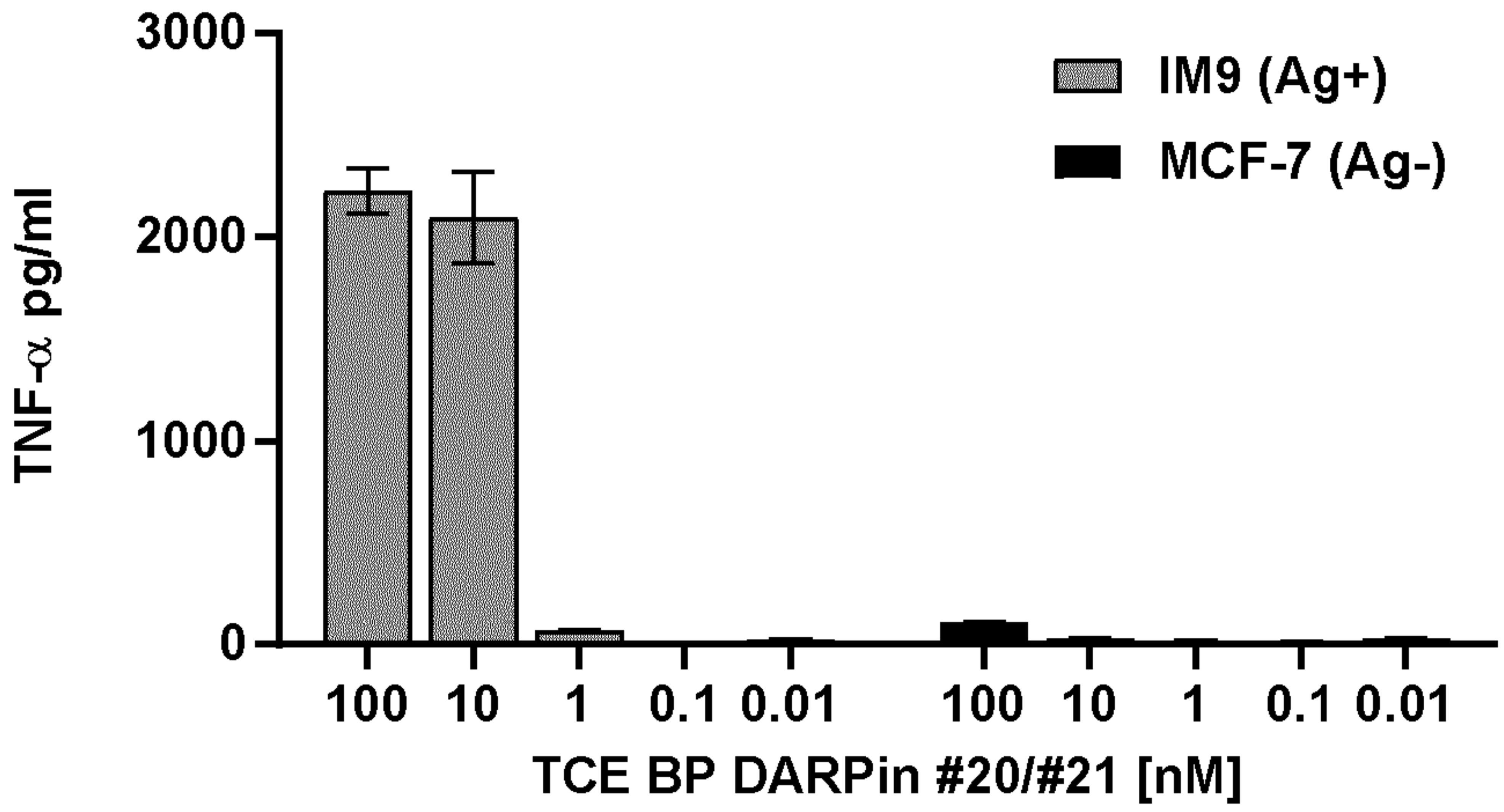

3000
2000
1000
0
TNF-α pg/ml
IM9 (Ag+)
MCF-7 (Ag-)
100 10 1 0.1 0.01 100 10 1 0.1 0.01
TCE BP DARPin #20/#21 [nM]

FIGURE 17

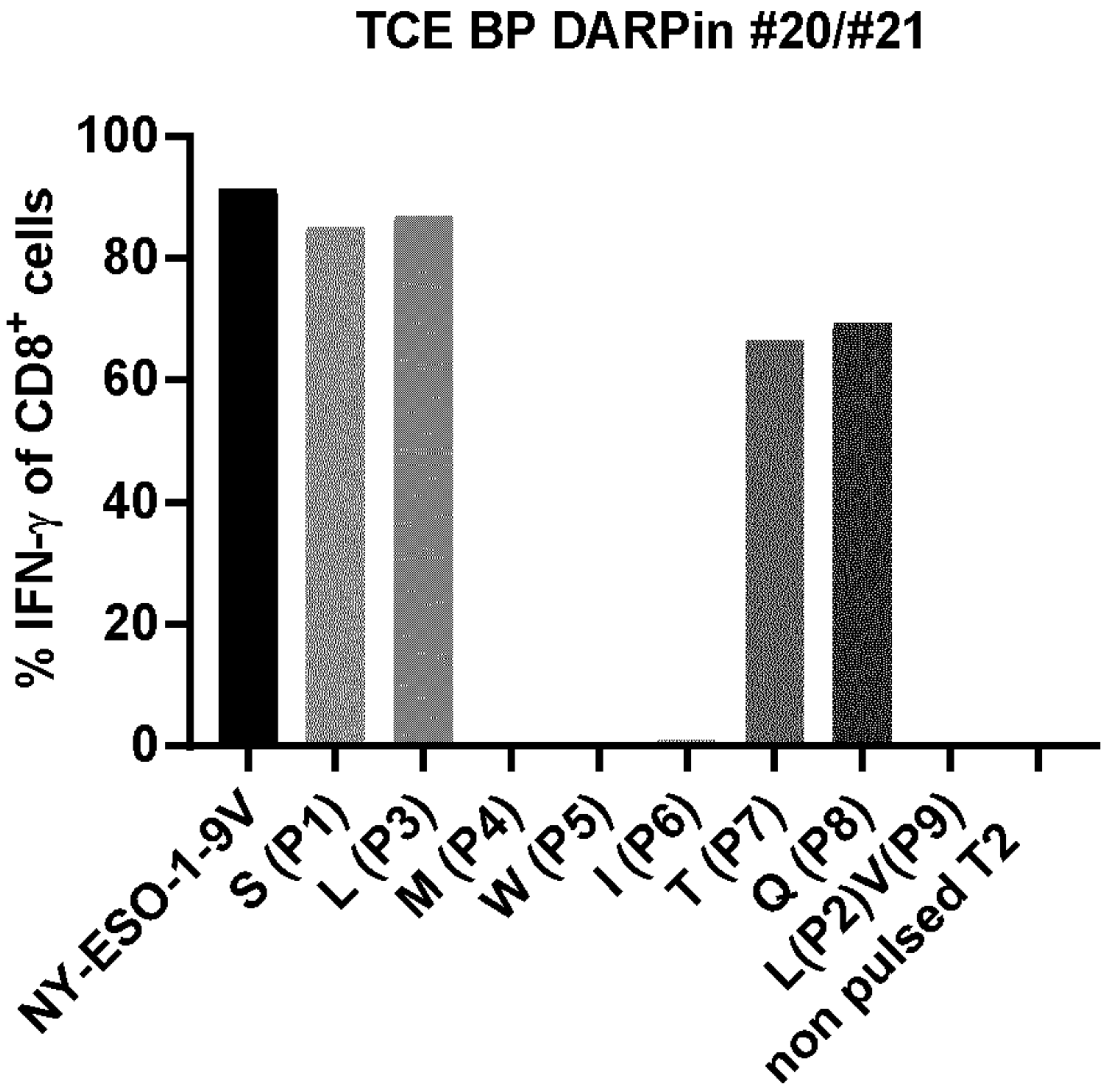

TCE BP DARPin #20/#21
% IFN-γ of CD8+ cells
100
80
60
40
20
0
NY-ESO-1-9V
S (P1)
L (P3)
M (P4)
W (P5)
I (P6)
T (P7)
Q (P8)
L(P2)V(P9)
non pulsed T2

FIGURE 18

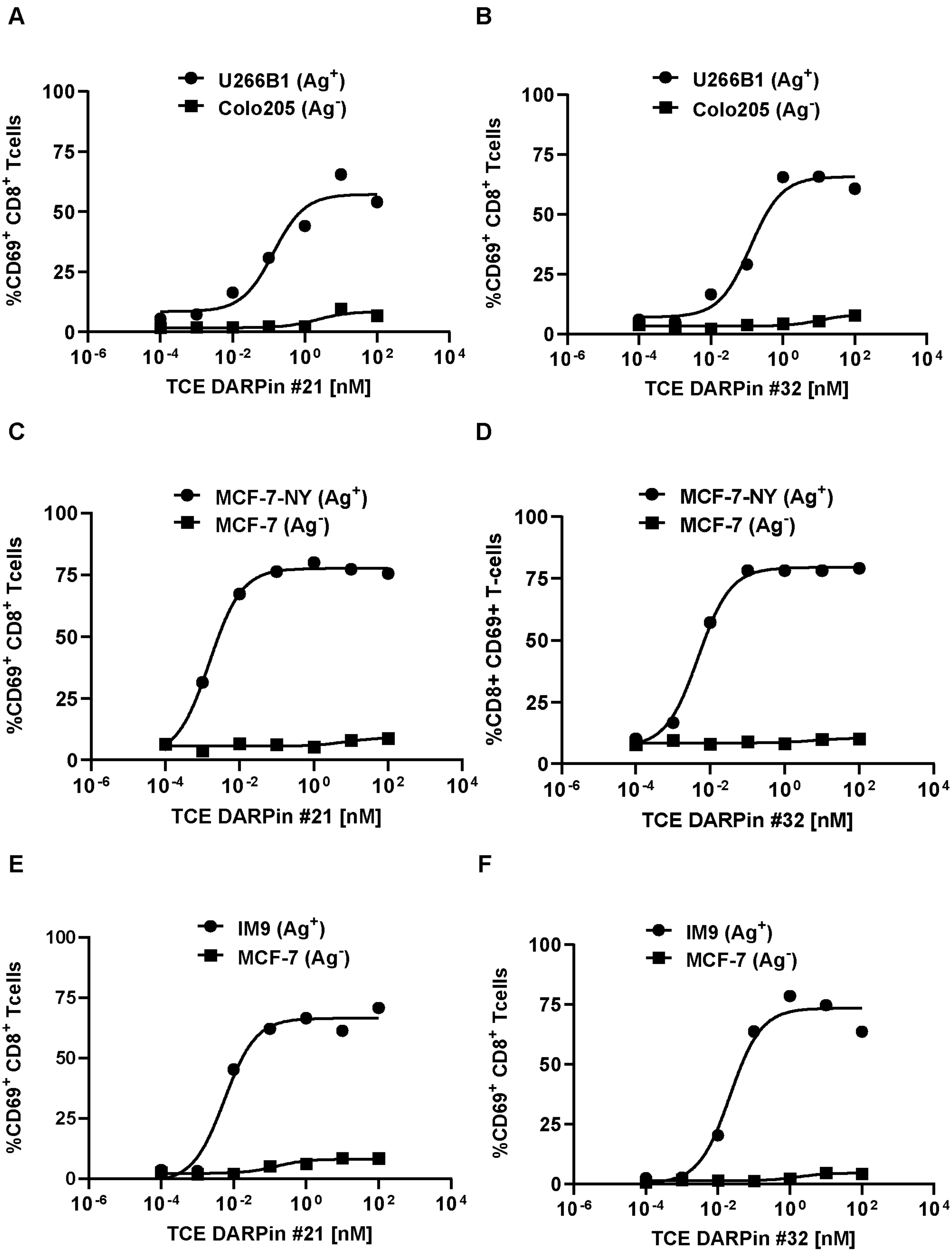

A
U266B1 (Ag+)
Colo205 (Ag-)
%CD69+ CD8+ Tcells
TCE DARPin #21 [nM]
B
U266B1 (Ag+)
Colo205 (Ag-)
%CD69+ CD8+ Tcells
TCE DARPin #32 [nM]
C
MCF-7-NY (Ag+)
MCF-7 (Ag-)
%CD69+ CD8+ Tcells
TCE DARPin #21 [nM]
D
MCF-7-NY (Ag+)
MCF-7 (Ag-)
%CD8+ CD69+ T-cells
TCE DARPin #32 [nM]
E
IM9 (Ag+)
MCF-7 (Ag-)
%CD69+ CD8+ Tcells
TCE DARPin #21 [nM]
F
IM9 (Ag+)
MCF-7 (Ag-)
%CD69+ CD8+ Tcells
TCE DARPin #32 [nM]

FIGURE 19

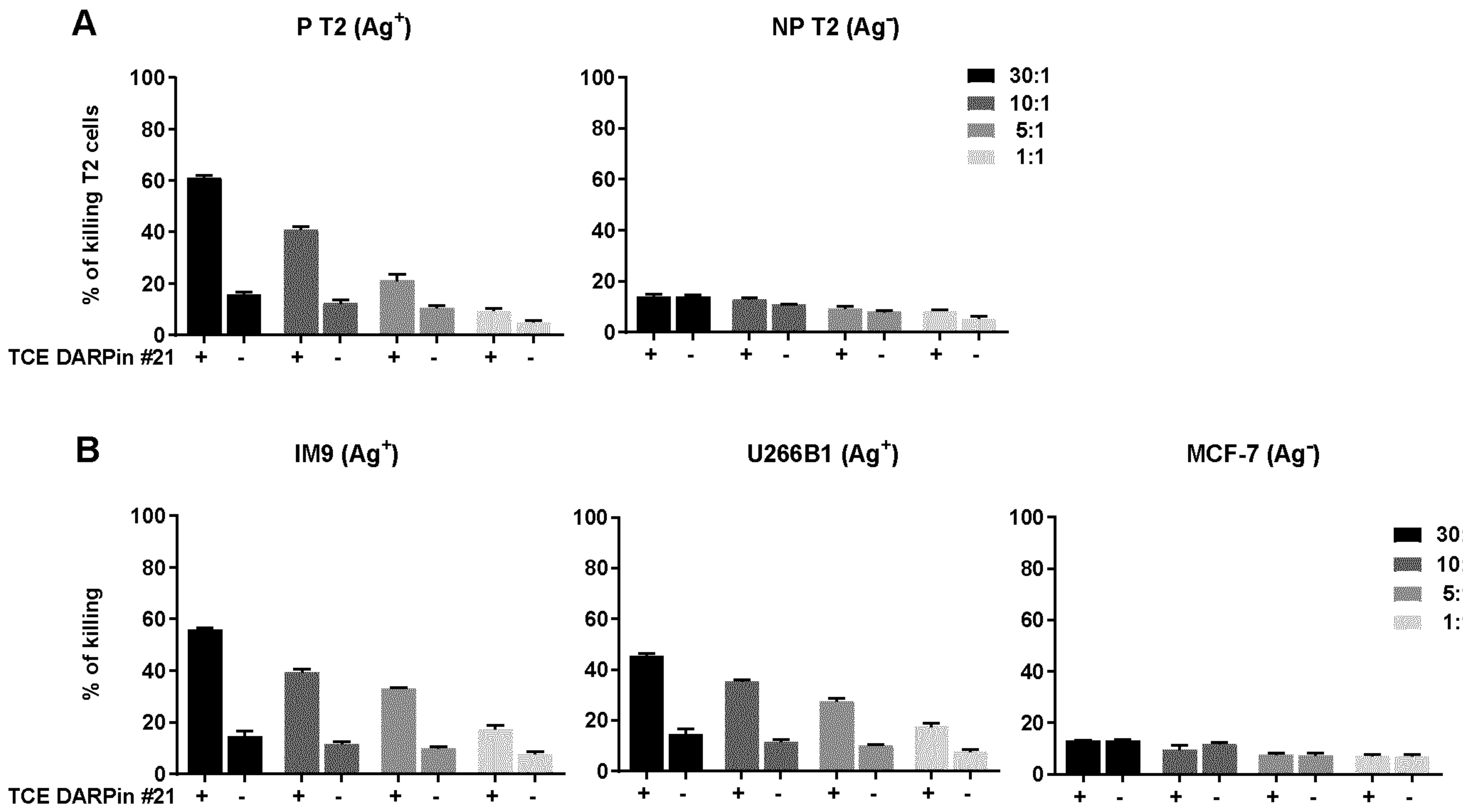


FIGURE 20

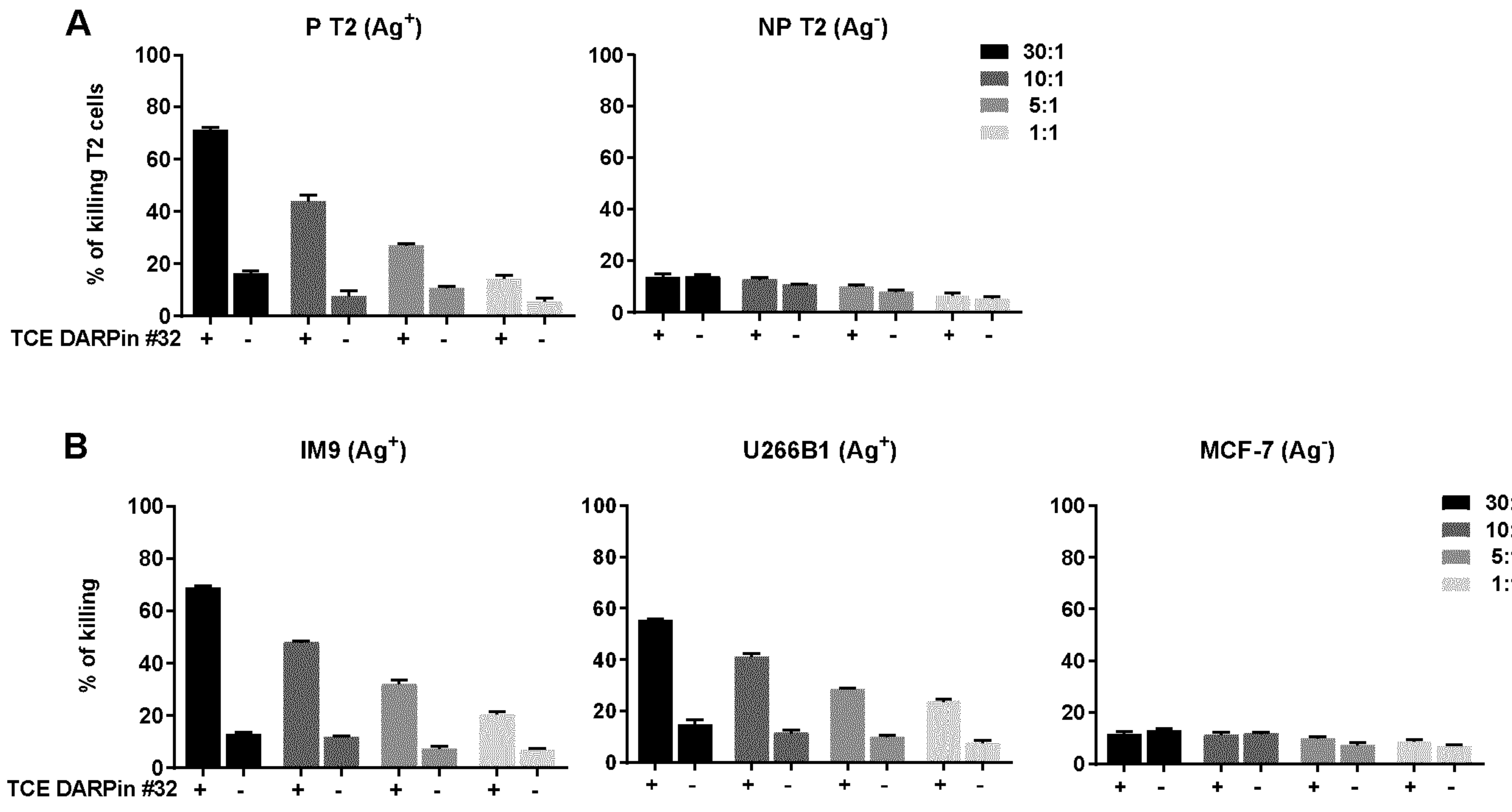


FIGURE 21

A

Position	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P1	S	89.7	94.3	0.5	27.4	72.5	103.1	43.0	69.0	13.3	30.1	79.3	77.9	9.3	69.2	76.3	100.0	107.5	113.8	114.4	111.7
P2	L	53.0	81.8	2.5	0.3	12.1	0.2	0.3	101.3	6.5	100.0	105.4	0.1	0.4	59.9	0.4	0.9	15.6	90.6	0.3	0.3
P3	L	96.1	52.9	96.7	3.5	70.4	39.0	70.0	98.7	2.8	100.0	97.0	93.9	44.2	95.9	0.1	37.7	86.8	75.4	102.6	4.6
P4	M	0.3	1.3	0.1	19.0	34.3	0.4	0.0	95.7	3.0	82.9	100.0	1.1	0.3	13.8	11.2	0.1	98.3	29.3	24.5	2.2
P5	W	0.1	0.2	0.1	0.2	16.4	0.1	0.1	0.3	0.3	0.4	1.0	0.4	0.1	0.1	0.0	0.1	0.2	0.0	100.0	0.2
P6	I	0.1	1.3	0.0	0.0	84.1	0.1	0.3	100.0	0.6	45.4	54.7	0.0	1.0	0.1	0.0	0.4	17.4	113.8	0.2	6.7
P7	T	5.5	32.1	0.1	24.8	114.6	0.3	13.0	95.3	1.0	93.3	102.4	14.1	1.6	78.1	82.3	75.2	100.0	74.8	7.3	52.7
P8	Q	23.1	0.2	0.0	17.8	0.1	20.2	0.7	0.2	0.6	3.4	0.9	0.1	0.1	100.0	0.1	12.6	57.1	3.7	0.3	0.2
P9	V	89.4	39.7	0.4	0.7	0.3	11.2	0.7	94.6	1.0	21.0	1.0	1.0	1.1	0.4	0.7	5.3	42.3	100.0	0.8	0.4

B

Position	WT	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
P1	S	78.1	73.6	27.7	29.5	68.3	2.0	57.2	69.6	0.6	13.1	81.1	109.9	64.9	97.9	35.2	100.0	110.7	108.4	106.8	93.2
P2	L	0.4	0.8	0.2	0.3	39.4	0.2	0.5	105.1	0.1	100.0	52.5	0.1	2.2	0.3	0.6	0.3	1.8	79.9	57.2	0.2
P3	L	0.3	0.1	0.4	0.2	0.6	0.3	0.1	110.2	0.0	100.0	83.4	0.1	2.5	48.6	0.5	0.6	0.2	71.3	20.2	0.1
P4	M	0.4	0.5	0.2	0.3	2.0	0.1	0.4	65.5	0.3	16.8	100.0	0.3	1.1	1.0	0.5	0.5	0.6	0.5	0.5	0.4
P5	W	0.3	0.1	0.4	0.3	13.7	0.6	0.4	1.3	1.5	1.0	2.1	0.7	0.3	0.3	0.3	0.3	0.3	0.5	100.0	0.7
P6	I	2.1	4.8	0.1	0.2	74.6	1.6	0.8	100.0	1.3	102.3	75.0	1.2	107.4	0.1	0.6	4.8	0.2	98.5	36.6	0.0
P7	T	57.7	93.2	0.3	11.4	106.5	107.8	0.4	96.0	0.3	108.5	124.0	76.2	15.1	108.5	102.2	92.0	100.0	100.8	19.6	9.4
P8	Q	122.6	95.6	120.6	122.2	43.9	126.9	124.2	13.4	123.8	98.1	107.1	100.8	113.3	100.0	51.9	113.0	120.7	104.6	108.9	99.6
P9	V	104.1	86.1	16.8	0.4	76.4	56.3	1.9	104.0	1.8	95.1	24.5	27.4	4.6	10.3	0.4	56.8	56.2	100.0	0.9	0.1

FIGURE 22

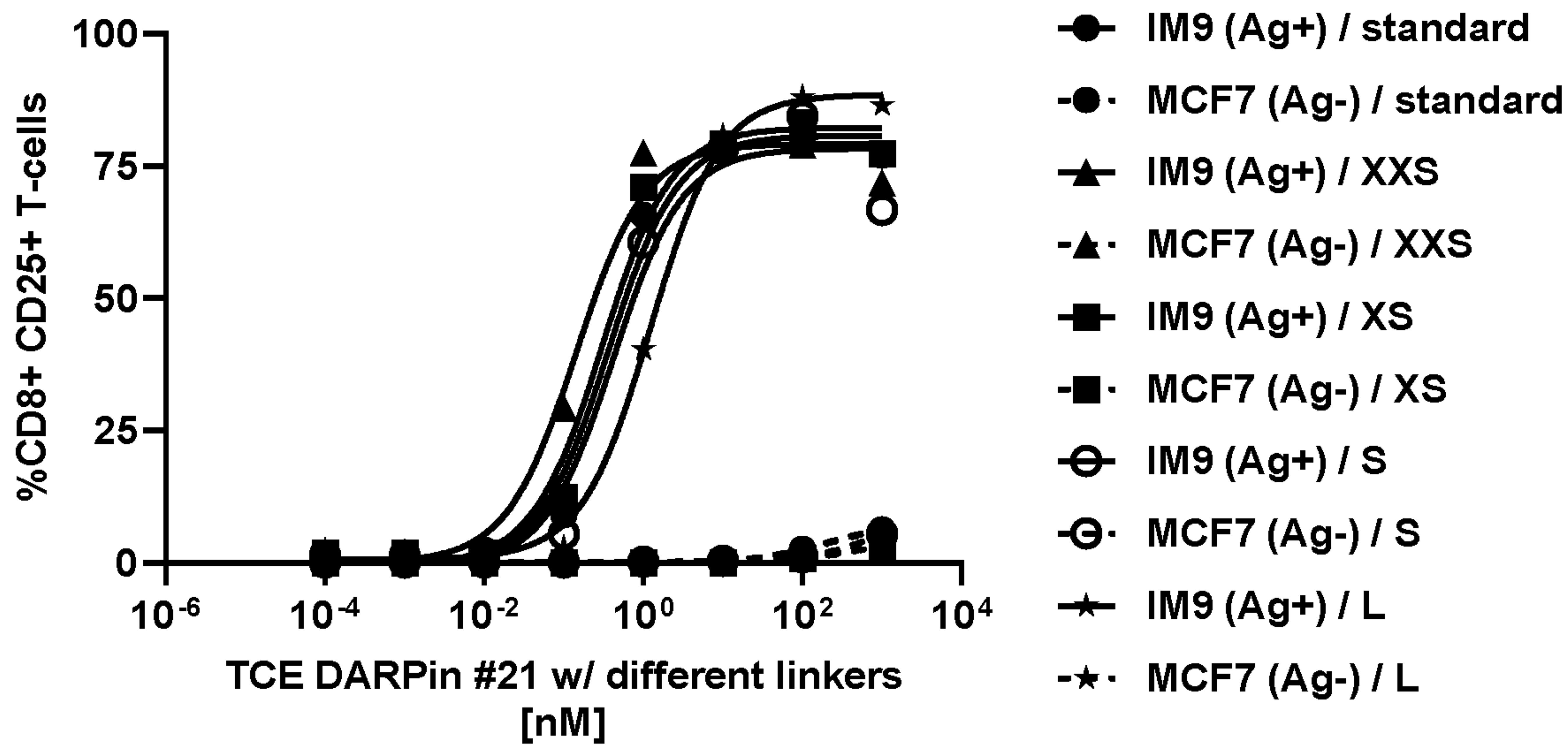


FIGURE 23

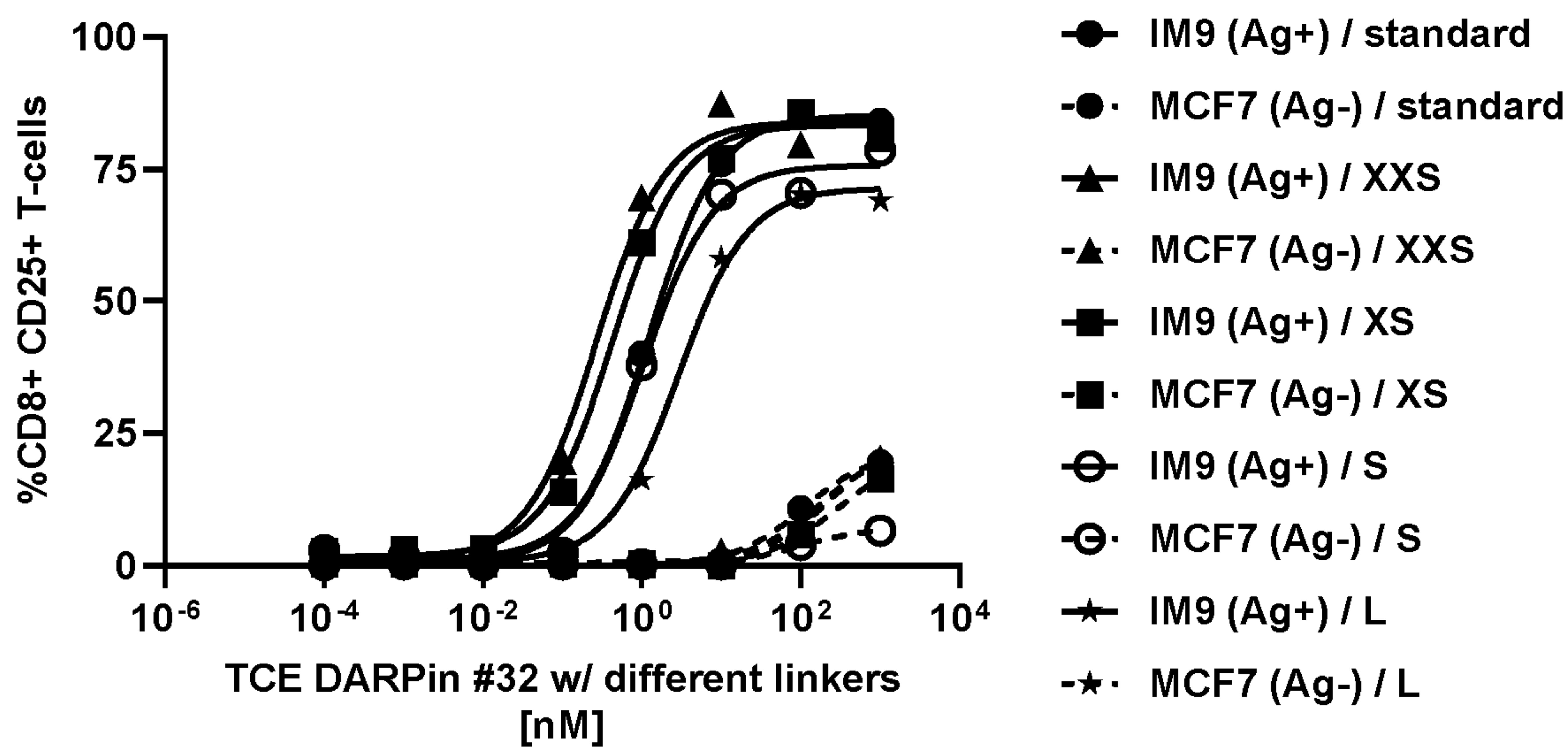


FIGURE 24

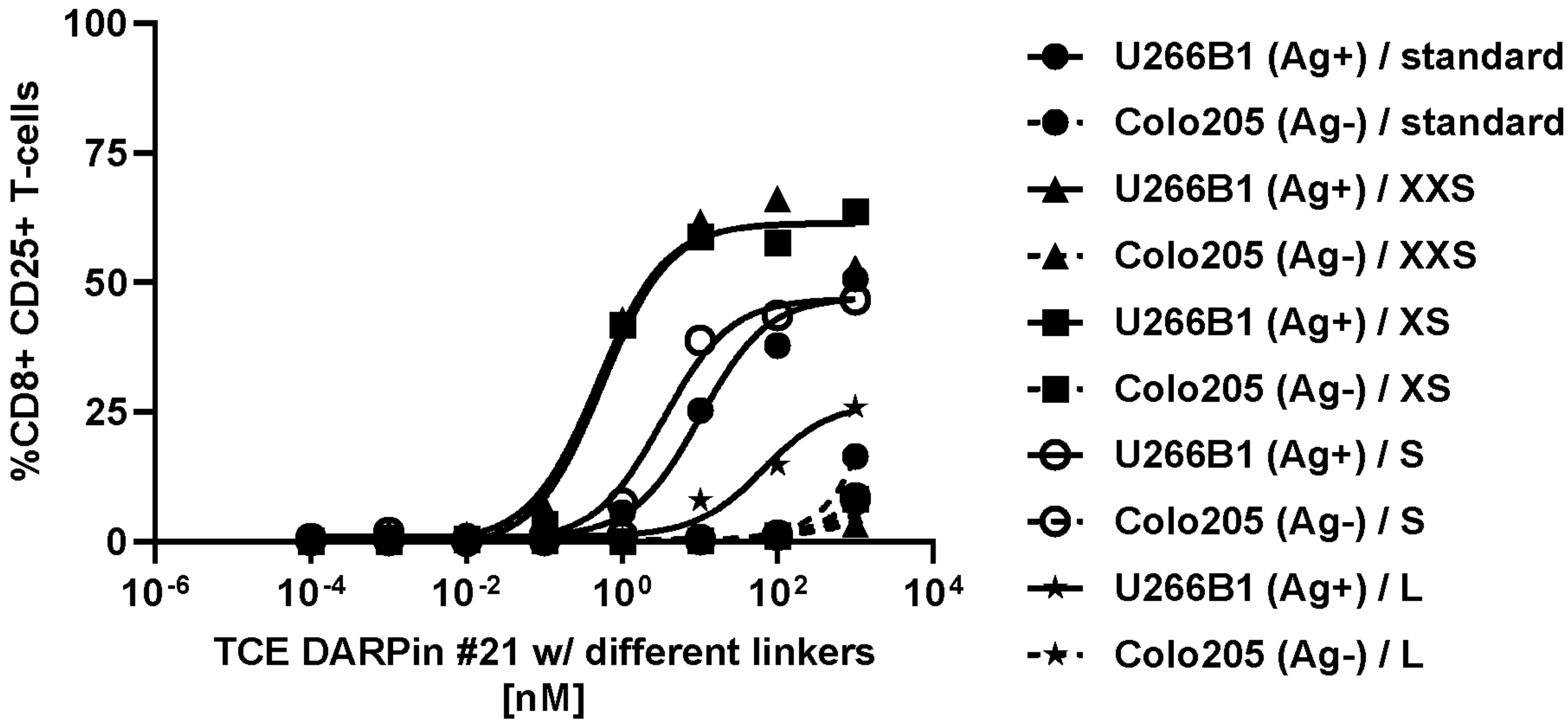


FIGURE 25

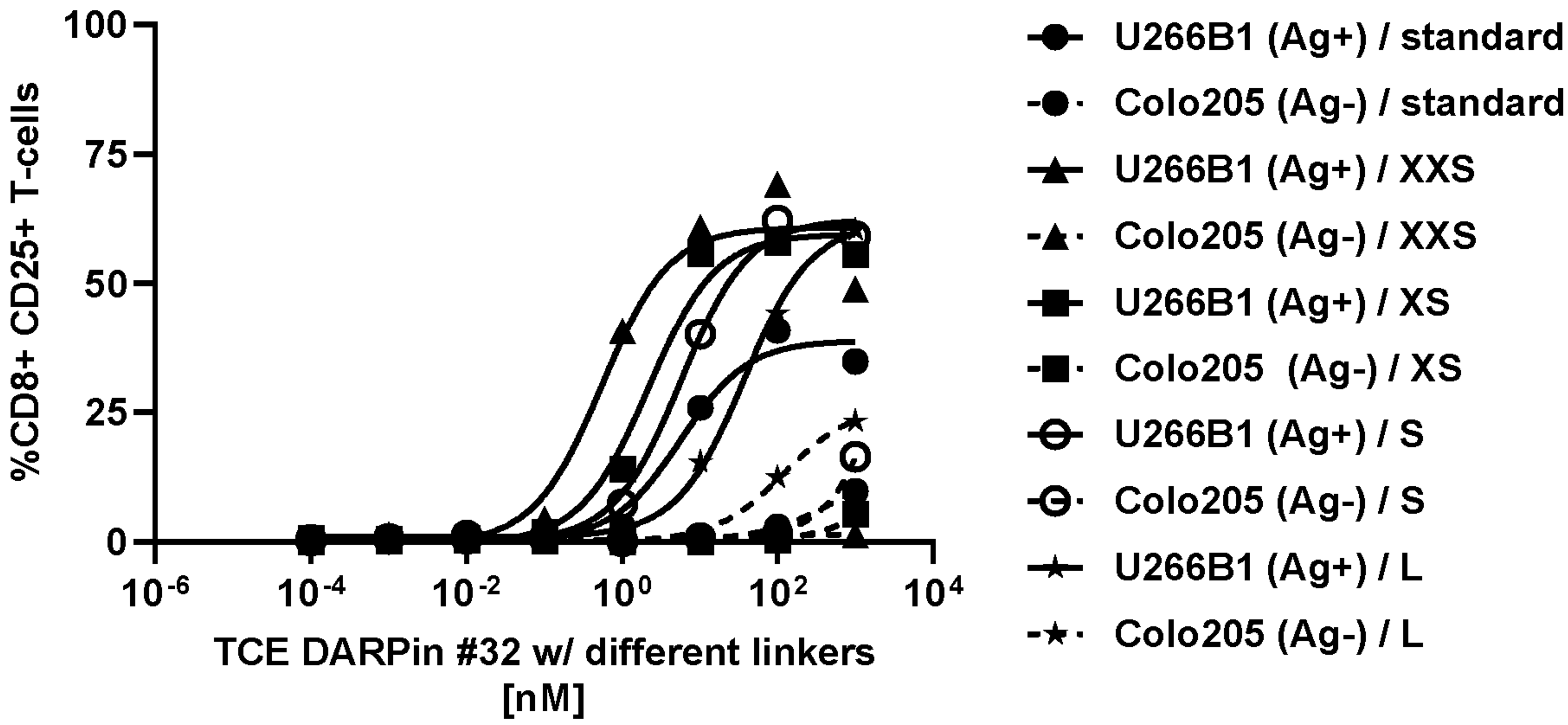


Fig. 26a

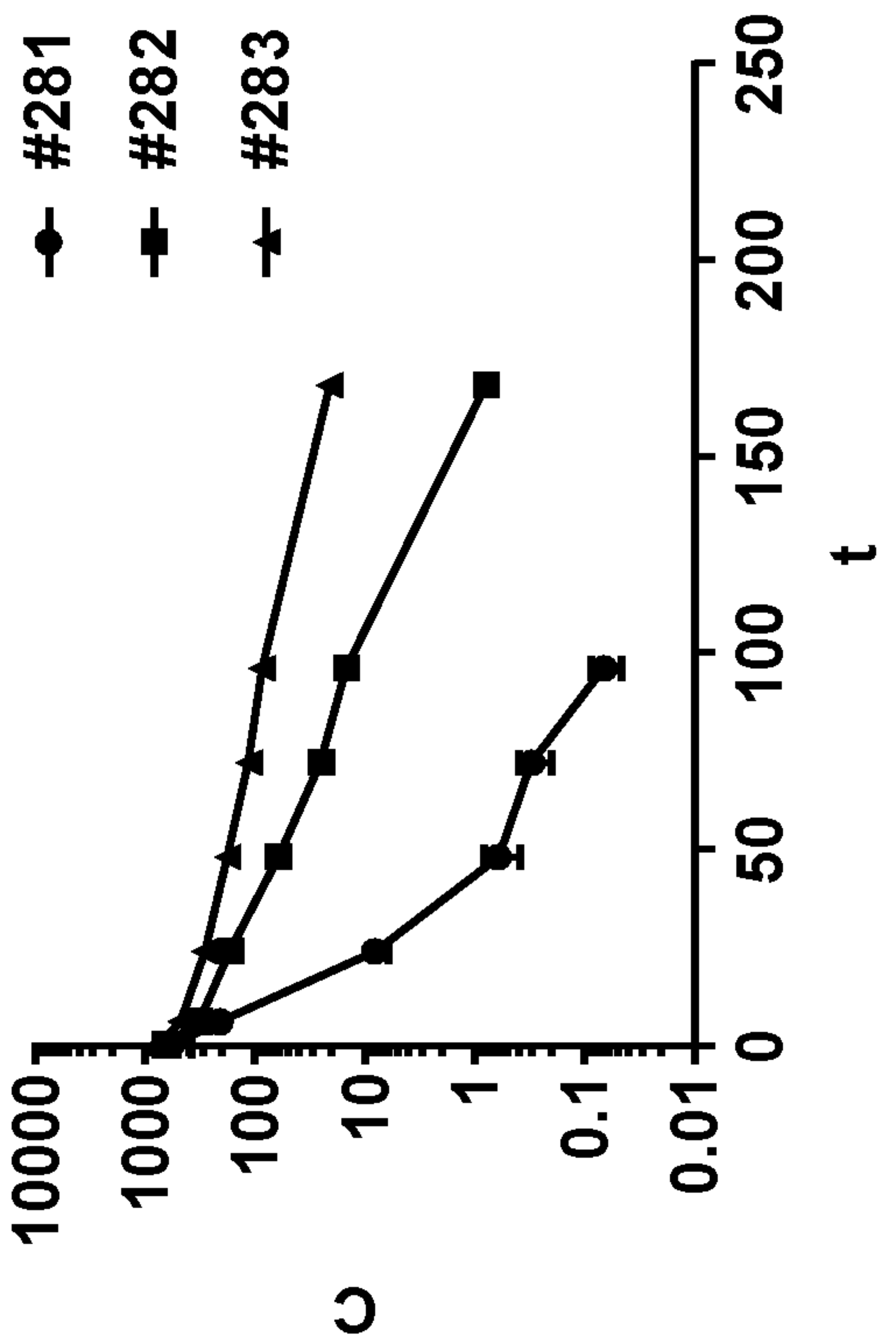


Fig. 26b

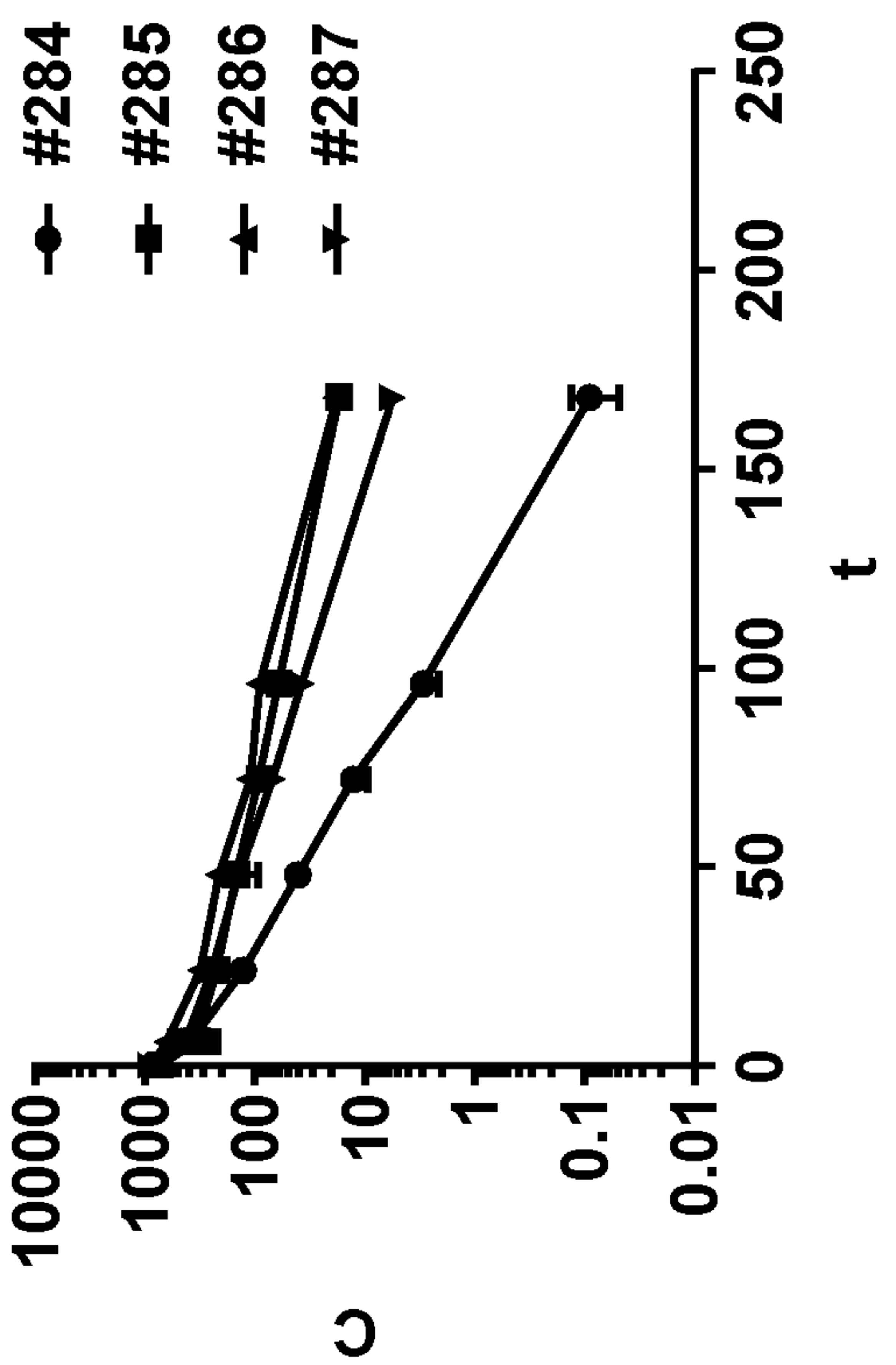


Fig. 26c

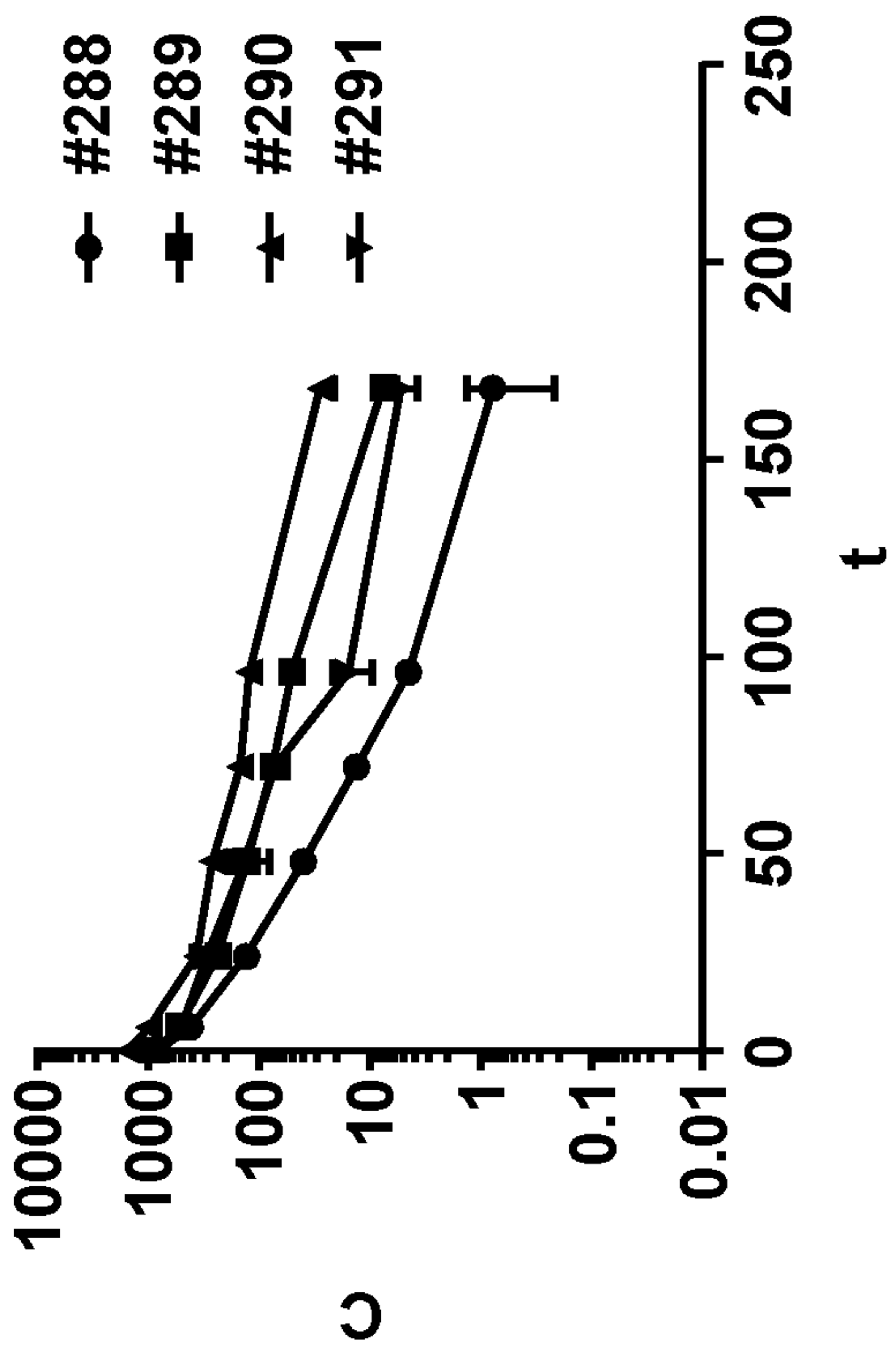


Fig. 26d