human primates with safety findings related to engagement with normal tissue expressing lower levels of the antigen (Lutterbuese et al, *Proc Natl Acad Sci U S A*;107(28):12605-10 (2010)). Additionally, high affinity CD3-binders of HER-targeting TCEs were shown to distribute preferentially to secondary lymphatic tissues, reducing systemic exposure (Mandikian et al, *Mol. Cancer Ther*.;17 (4): 776 LP-785 (2018)). Antibody-based T cell engagers often show more than 1000-fold higher affinity for CD3, when compared to the natural TCR-MHC interaction (Wu et al, *Pharmacol Ther*;182:161–75 (2018); WO2014/167022; Junntila et al, Cancer Res.; 19:5561–71 (2014); Yang et al, J Immunol Aug.; 15 (137):1097–100 (1986)). This high affinity is correlated to lower efficiency in terms of T cell activation and tumor cell killing (Bortoletto et al, *Eur. J. Immunol*. 32; 11: 3102–3107 (2002); Ellerman, *Methods*;154:102-117(2019); Mandikian et al, *Mol. Cancer Ther*.;17 (4): 776 LP-785 (2018); Vafa et al, *Frontiers in Oncology*; 10: 446 (2020)).

Thus, there remains a need for new CD3-specific binding proteins with beneficial properties suitable for being used in T cell engager formats, and for therapeutic approaches for the treatment of diseases, including cancer and infectious diseases, benefitting from the CD3-specific binding.

SUMMARY

The present disclosure provides recombinant binding proteins comprising a designed ankyrin repeat domain with binding specificity for CD3. Further provided are such binding proteins linked to one or more binding agents, preferably ankyrin repeat domains, with binding specificity for a Disease-Associated Antigen (DAA), such as, e.g., an Infection Associated Antigen (IAA), preferably a Virus Associated Antigen (VAA), or a Tumor Associated Antigen (TAA), which facilitate DAA-dependent activation of T cells by the binding proteins. In addition, the disclosure provides nucleic acids encoding such binding proteins and pharmaceutical compositions comprising such binding proteins or nucleic acids. The disclosure also provides the use of such binding proteins, nucleic acids or pharmaceutical compositions in methods for localized activation of T cells, e.g., tumor-localized or infection-localized activation, and in methods of treating diseases, such as infectious diseases, preferably viral infectious diseases, or cancer, in a mammal, including a human.

Applicant carried out dedicated studies to design, generate and select CD3-specific binding domains suitable to be used in T cell engager pharmaceuticals and having beneficial properties (see below). As a result of a complex screening process followed by several rounds of affinity maturation combined with rational design, four CD3-specific ankyrin repeat proteins were engineered, namely DARPin® protein #1 (SEQ ID NO: 1), DARPin® protein #2 (SEQ ID NO: 2), DARPin® protein #3 (SEQ ID NO: 3) and DARPin® protein #4 (SEQ ID NO: 4). These recombinant binding proteins all comprise a designed ankyrin repeat domain with binding specificity for CD3 while displaying different binding affinities to CD3 and/or to T cells. The binding proteins have a binding affinity to CD3 and/or to T cells that is adequately high to achieve targeted T cell activation, and at the same time relatively low so that the risk of adverse effects (e.g. caused by too much T cell activation) is reduced. These recombinant binding proteins were found to be suitable to be linked to one or more DAA-specific binding agent(s) to form functional T cell engager (TCE) molecules, including multi-specific TCEs. Thus, binding proteins of the disclosure may further comprise a binding agent with binding specificity for a disease-associated antigen. Such binding proteins in TCE format are capable

of engaging the immune system, and more specifically T cells, in a localized and targeted fashion dependent on the presence of the respective DAA on disease-associated cells, such as tumor cells or infected cells. The binding proteins of the disclosure comprising an ankyrin repeat domain with binding specificity for CD3 (such as SEQ ID NO: 1, 2, 3 or 4 or variants thereof) thus represent a "toolbox" of CD3-specific binding proteins to be used, inter alia, in the generation of TCE drug candidates and in methods of treating diseases in human involving efficient activation of T cells while avoiding or reducing overstimulation of T cells and/or adverse effects.

In one aspect, the disclosure provides a recombinant binding protein comprising an ankyrin repeat domain with binding specificity for CD3, wherein said ankyrin repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 5 to 9 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 5 to 9 are substituted by other amino acids. In one example, said ankyrin repeat module is a first ankyrin repeat module and wherein said ankyrin repeat domain further comprises a second ankyrin repeat module having an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 5 to 9 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 5 to 9 are substituted by other amino acids. In one particular example, said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 5 and (2) sequences in which up to 9 amino acids in SEQ ID NO: 5 are substituted by other amino acids, and wherein said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 6 and (2) sequences in which up to 9 amino acids of SEQ ID NO: 6 are substituted by other amino acids. In another particular example, said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 7 and (2) sequences in which up to 9 amino acids in SEQ ID NO: 7 are substituted by other amino acids, and wherein said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 6 and (2) sequences in which up to 9 amino acids of SEQ ID NO: 6 are substituted by other amino acids. In a further particular example, said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 7 and (2) sequences in which up to 9 amino acids in SEQ ID NO: 7 are substituted by other amino acids, and wherein said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 8 and (2) sequences in which up to 9 amino acids of SEQ ID NO: 8 are substituted by other amino acids. In a further particular example, said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 7 and (2) sequences in which up to 9 amino acids in SEQ ID NO: 7 are substituted by other amino acids, and wherein said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 9 and (2) sequences in which up to 9 amino acids of SEQ ID NO: 9 are substituted by other amino acids. In one preferred example, said first ankyrin repeat module is located N-terminally of said second ankyrin repeat module within said ankyrin repeat domain.

In one aspect, the disclosure provides a recombinant binding protein comprising an ankyrin repeat domain with binding specificity for CD3, wherein said ankyrin repeat domain comprises an amino acid sequence with at least 80% amino acid sequence identity with any one of SEQ ID NOs: 1 to 4, and wherein A at the

second last position of SEQ ID NOs: 1 to 4 is optionally substituted by L, and/or A at the last position of SEQ ID NOs: 1 to 4 is optionally substituted by N.

In one example, any of said binding proteins binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M. In a further example, any of said binding proteins binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 5 to about 400 nM. In a further example, said ankyrin repeat domain with binding specificity for CD3 comprised in said binding proteins has a melting temperature (T_m) higher than 65^0C, preferably higher than 70^0C. In a further example, said ankyrin repeat domain with binding specificity for CD3 comprised in said binding proteins has an aggregation onset temperature (T_{agg}) higher than 70^0C, preferably higher than 75^0C. In one particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 1 and said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M and/or binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 5 to about 400 nM. In one particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 1, and wherein said ankyrin repeat domain has a melting temperature (T_m) higher than 75^0C, preferably higher than 80^0C, and/or said ankyrin repeat domain has an aggregation onset temperature (T_{agg}) higher than 75^0C, preferably higher than 80^0C, and/or said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, and/or said binding protein binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 5 to about 400 nM. In another particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 2 and said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, preferably of or below about 5 x 10^{-8}M, and/or binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 250 to about 400 nM. In another particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 2, and wherein said ankyrin repeat domain has a melting temperature (T_m) higher than 72^0C, preferably higher than 77^0C, and/or said ankyrin repeat domain has an aggregation onset temperature (T_{agg}) higher than 70^0C, preferably higher than 75^0C, and/or said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, preferably of or below about 5 x 10^{-8}M, and/or said binding protein binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 250 to about 400 nM. In an alternative particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 3 and said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, preferably of or below about 2 x 10^{-8}M, and/or binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 50 to about 100 nM. In another particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 3, and wherein said ankyrin repeat domain has a melting temperature (T_m) higher than 65^0C, preferably higher than 70^0C, and/or said ankyrin repeat domain has an aggregation onset temperature (T_{agg}) higher than 70^0C, preferably higher than 75^0C, and/or said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, preferably of or below about 2 x 10^{-8}M,

and/or said binding protein binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 50 to about 100 nM. In another alternative example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 4 and said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, preferably of or below about 10^{-8}M and/or binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 5 to about 15 nM. In another particular example, said ankyrin repeat domain with binding specificity for CD3 comprises an amino acid sequence with at least 80% amino acid sequence identity with SEQ ID NO: 4, and wherein said ankyrin repeat domain has a melting temperature (T_m) higher than 73^0C, preferably higher than 78^0C, and/or said ankyrin repeat domain has an aggregation onset temperature (T_{agg}) higher than 70^0C, preferably higher than 75^0C, and/or said binding protein binds human CD3 in PBS with a dissociation constant (K_D) below 10^{-7}M, preferably of or below about 10^{-8}M, and/or said binding protein binds human CD3 on T cells with an EC_{50} ranging from 2 to 900 nM, preferably from about 5 to about 15 nM.

In another example, the disclosure provides a recombinant binding protein comprising an ankyrin repeat domain with binding specificity for CD3, wherein said binding protein further comprises a binding agent with binding specificity for a disease-associated antigen. In one example, said binding agent is a designed ankyrin repeat domain with binding specificity for a disease-associated antigen. In a further example, said disease-associated antigen is an Infection Associated Antigen (IAA), preferably a Virus Associated Antigen (VAA). In another example, said disease-associated antigen is Tumor Associated Antigen (TAA). In one example of the disclosure, the binding agent with binding specificity for a disease-associated antigen is covalently linked to or fused to the designed ankyrin repeat domain with binding specificity for CD3. In one particular example, the binding agent with binding specificity for a disease-associated antigen is covalently linked to the ankyrin repeat domain with binding specificity for CD3 with a peptide linker, preferably a proline-threonine rich peptide linker. In one example, the amino acid sequence of said peptide linker has a length from 1 to 50 amino acids, preferably from 6 to 38 amino acids. In one preferred example, said binding agent with binding specificity for a disease-associated antigen is located N-terminally of said ankyrin repeat domain with binding specificity for CD3 within said binding protein.

In another example, the disclosure provides a recombinant binding protein comprising an ankyrin repeat domain with binding specificity for CD3, wherein said binding protein further comprises a half-life extending moiety. In one example, said half-life extending moiety comprises a binding agent with binding specificity for human serum albumin. In one example, said binding agent with binding specificity for human serum albumin is a designed ankyrin repeat domain with binding specificity for human serum albumin. In one particular example, said binding agent with binding specificity for human serum albumin is a designed ankyrin repeat domain with binding specificity for human serum albumin comprising the amino acid sequence of any one of SEQ ID NOs: 28 to 30, preferably SEQ ID NO: 29. In one preferred example, said half-life extending moiety is located N-terminally of said ankyrin repeat domain with binding specificity for CD3 within said binding protein.

In another example, the disclosure provides nucleic acids encoding the recombinant binding proteins of the disclosure and pharmaceutical compositions comprising the binding protein of the disclosure or the nucleic acid of the disclosure and optionally a pharmaceutically acceptable carrier and/or diluent. In one aspect, the present disclosure refers to a nucleic acid encoding any one of the binding proteins disclosed herein. In another aspect, the present disclosure refers to a pharmaceutical composition comprising any one of the binding proteins disclosed herein or the nucleic acid disclosed herein, and optionally a pharmaceutically acceptable carrier and/or diluent.

In one aspect, the present disclosure refers to use of any one of the binding proteins disclosed herein, the nucleic acid disclosed herein or the pharmaceutical composition disclosed herein, in the manufacture of a medicament for treating a medical condition.

In one aspect, the present disclosure refers to any one of the binding proteins disclosed herein, the nucleic acid disclosed herein or the pharmaceutical composition disclosed herein, for use in therapy.

In another example, the disclosure provides the binding protein of the disclosure, the nucleic acid of the disclosure or the pharmaceutical composition of the disclosure for use in a method of treating a medical condition. In one example, said medical condition is an infectious disease, preferably a viral infectious disease. In another example, said medical condition is a cancer.

In another example, the disclosure provides a method of tumor-localized activation of T cells in a mammal, preferably a human, the method comprising administering to said mammal the binding protein of the disclosure, the nucleic acid of the disclosure or the pharmaceutical composition of the disclosure. In one particular example, such method comprises administering the binding protein of the disclosure further comprising a binding agent with binding specificity for a tumor-associated antigen to a mammal, including a human patient with a tumor, resulting in localized T-cell activation in the tumor tissue.

In another example, the disclosure provides a method of infection-localized activation of T cells in a mammal, preferably a human, the method comprising administering to said mammal the binding protein of the disclosure, the nucleic acid of the disclosure or the pharmaceutical composition of the disclosure. In one particular example, such method comprises administering the binding protein of the disclosure further comprising a binding agent with binding specificity for a virus-associated antigen to a mammal, including a human patient with a viral infection, resulting in localized T-cell activation in the infected tissue.

In another example, the disclosure provides a method for treating a medical condition in a human patient, the method comprising administering to said patient a therapeutically effective amount of the binding protein of the disclosure, the nucleic acid of the disclosure or the pharmaceutical composition of the disclosure. In one particular example, such method comprises administering to said patient a therapeutically effective amount of a binding protein of the disclosure further comprising a binding agent with binding specificity for a disease-associated antigen, a nucleic acid encoding such a binding protein of the disclosure or a pharmaceutical composition comprising such a binding protein of the disclosure. In one example said medical condition is cancer. In another example, said medical condition is an infectious disease, preferably a viral infectious disease.

In one particular example, the medical condition is cancer, wherein the cancer or tumor tissue comprises cells that express or display a tumor-associated antigen, and said binding agent with binding specificity for a disease-associated antigen binds said tumor-associated antigen expressed or displayed in said cells. In one particular example, said disease-associated antigen is the extracellular domain of a cell surface protein expressed or overexpressed in said cancer or tumor tissue. In another particular example, said binding agent with binding specificity for a tumor-associated antigen binds to a peptide-MHC complex, wherein said peptide is derived from a protein expressed in a tumor cell (such as, e.g. NY-ESO-1 or MAGE-A3). In one particular example, said MHC is MHC class I. In one example, said cancer is selected from carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed type-cancers.

In another particular example, the medical condition is an infectious disease, preferably a viral infectious disease, wherein the infected tissue comprises cells that express or display an infection-associated antigen, preferably a virus-associated antigen, and said binding agent with binding specificity for a disease-associated antigen binds said infection-associated antigen expressed or displayed in said cells. In one particular example, said disease-associated antigen is the extracellular domain of a virus protein expressed or overexpressed in said infected tissue. In another particular example, said binding agent with binding specificity for a virus-associated antigen binds to a peptide-MHC complex, wherein said peptide is derived from a protein of an infectious agent, such as a bacterial infectious agent or a viral infectious agent, preferably a viral infectious agent (such as, e.g. HBcAg or EBNA-1). In one particular, example, said MHC is MHC class I.

The disclosure further provides a kit comprising the recombinant binding protein of the disclosure, the nucleic acid of the disclosure or the pharmaceutical composition of the disclosure. The disclosure further provides a method for producing the recombinant binding protein of the disclosure, the method comprising the steps of (i) expressing said recombinant binding protein in a suitable host cell (prokaryotic or eukaryotic cell) , and (ii) purifying said recombinant binding protein (e.g., using chromatography).

Based on the disclosure provided herein, those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific examples of the disclosure described herein. Such equivalents are intended to be encompassed by the following examples (E).

E1. A recombinant binding protein comprising an ankyrin repeat domain with binding specificity for CD3, wherein said ankyrin repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 5 to 9 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 5 to 9 are substituted by other amino acids.

E2. The binding protein of E1, wherein said ankyrin repeat module is a first ankyrin repeat module and wherein said ankyrin repeat domain further comprises a second ankyrin repeat module having an amino acid sequence selected from the group consisting of (1) SEQ ID NOs: 5 to 9 and (2) sequences in which up to 9 amino acids in any of SEQ ID NOs: 5 to 9 are substituted by other amino acids.

E3. The binding protein of E2, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 5 and (2) sequences in which up to 9 amino acids in SEQ ID NO: 5 are substituted by other amino acids, and wherein said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 6 and (2) sequences in which up to 9 amino acids of SEQ ID NO: 6 are substituted by other amino acids.

E4. The binding protein of E2, wherein said first ankyrin repeat module comprises an amino acid sequence selected from the group consisting of (1) SEQ ID NO: 7 and (2) sequences in which up to 9 amino acids in SEQ ID NO: 7 are substituted by other amino acids, and wherein said second ankyrin repeat module comprises an amino acid sequence selected from the group consisting of