

CD47-CD38 Bispecific Antibodies

Field of the Invention

The present invention relates to novel bispecific heterodimeric immunoglobulins that target both a component of the human CD47 antigen and human CD38 antigen. More specifically, the present invention relates to an anti-CD38 heavy chain variable region and a light chain variable region and an anti-CD47 heavy chain variable region and a light chain variable region. The present invention also relates to the use of this novel class of bispecific heterodimeric immunoglobulins to treat autoimmune and proliferative diseases and in particular cancers such as hematologic malignancies and solid tumors.

Background of the Invention

Multiple myeloma is a neoplastic plasma-cell disorder characterized by clonal proliferation of malignant plasma cells in the bone marrow (BM) microenvironment, monoclonal protein in the blood or urine and associated organ dysfunction. Multiple myeloma accounts for 1-2% of all new cancer diagnoses and approximately 20% of all deaths from blood malignancies. The disease is slightly more common in males and African- Americans. Multiple myeloma remains an incurable cancer, although recent improved understanding of pathogenesis of myeloma has led to the development of new treatments and improved survival.

The diagnosis of multiple myeloma requires the presence of one or more myeloma defining events (MDE) in addition to evidence of either 10% or more clonal plasma cells on BM examination or a biopsy-proven plasmacytoma. MDE include so-called CRAB (hypercalcemia, renal failure, anemia, or lytic bone lesions) features as well as three specific biomarkers: clonal BM plasma cells > 60%, serum free light chain (sFLC) ratio > 100 (provided involved sFLC level is > 100 mg/L), and more than 1 focal lesion on magnetic resonance imaging (MRI). Several genetic abnormalities that occur in tumor plasma cells play major roles in the pathogenesis of myeloma and determine disease prognosis.

The uncontrolled growth of myeloma cells has many consequences, including skeletal destruction, BM failure, increased plasma volume and viscosity, suppression of normal immunoglobulin production, and renal impairment.

Symptomatic (active) disease should be treated immediately, whereas asymptomatic (smoldering) myeloma requires only clinical observation, since early treatment with conventional chemotherapy has

- shown no clear benefit yet. Investigational trials are currently evaluating the ability of immunomodulatory drugs to delay the progression from asymptomatic to symptomatic myeloma. For active myeloma, current data support the initiation of induction therapy regimens including thalidomide, lenalidomide, and/or bortezomib followed by autologous hematopoietic stem-cell transplantation (HSCT) after major disease response for patients who can tolerate auto-HSCT conditioning regimens. Considerations of physiologic age, which may differ from chronologic age, and the presence of coexisting conditions drive decisions of treatment choice and drug dose. For example, less intensive approaches are desirable for patients with significant comorbidities, including cardiopulmonary or hepatic impairment, limiting treatment-related mortality and mitigating risk of treatment interruption.
- 10 Treatment of relapsed/refractory multiple myeloma presents a special therapeutic challenge, due to the heterogeneity of disease at relapse and the absence of clear biological based recommendations regarding the choice of salvage therapies at various time points of disease progression. With increasing recognition of the inherent clonal heterogeneity and genomic instability of the plasma cells influencing both inherent and acquired therapeutic resistance, the identification of the optimal choice and sequence of therapies
- 15 has become critical. New agents have gained approval by United States (US) Food and Drug Administration (FDA) for relapsed/refractory myeloma in recent years, adding to the complexity of these decisions, including proteasome inhibitors (carfilzomib and ixazomib), the thalidomide derivative pomalidomide, and the histone deacetylase inhibitor panobinostat. Other molecularly targeted therapies directed at specific cell signaling pathways, as well as survival and proliferation controls (including PI3K/AKT/mTOR
- 20 inhibitors, Hsp90 inhibitors, cyclin-dependent kinase inhibitors, kinesin spindle protein inhibitors) are currently in development. Despite advances in the management of multiple myeloma, relapse is inevitable in almost all patients. Recurrence of myeloma is typically more aggressive with each relapse, leading to the development of treatment refractory- disease, which is associated with a shorter survival. Thus, additional treatment options are needed.
- 25 Antibodies that target two different antigens within the same molecule offer an immunotherapeutic treatment strategy for numerous malignancies. The largest class of immune effector cell redirecting antibodies has been shown to mediate T cell redirection both in pre-clinical and clinical investigations (May C et al., (2012) *Biochem Pharmacol*, 84(9): 1105-12; Frankel SR & Baeuerle PA, (2013) *Curr Opin Chem Biol*, 17(3): 385-92; Nie et al., (2020) *Antibody Therapeutics*, 3(1), 17-62). All effector cell retargeting
- 30 bispecific antibodies or fragments thereof are engineered to have at least two antigen binding sites

wherein one site binds a surface antigen on a target cell and the other site binds an effector cell surface antigen.

Nevertheless, improvements in effector cell redirecting antibody-based therapeutics are still needed, particularly relating to the therapeutic window. The therapeutic window is defined as the range in concentration of a drug that exerts therapeutic efficacy with no or limited toxicity observed. (Handbook of Pharmacogenomics and Stratified Medicine, 2014). The aim of any therapy is to reach a dose where efficacy is seen in all patients, with no or manageable side effects in any of the patients (Principles of Clinical Pharmacology (Third Edition), 2012). To broaden a bispecific antibody's therapeutic window, the concentration of a drug that gives half-maximal therapeutic response (EC₅₀) can be decreased (improved efficacy), and/or the concentration that exerts toxic effects can be increased (improved on-target specificity).

Aiming to target a larger therapeutic window in comparison to other approved or candidate medicines for CD38-overexpressing cancers, such as multiple myeloma, the inventors have set out to provide improved effector cell redirecting antibody-based therapeutics, that specifically act on the tumor cells. Nevertheless, while it has been shown that monoclonal antibodies that binds CD38, such as daratumumab, may be used in combination with CD47-blocking scFv genetically fused in tandem to a CD20-targeting scFv to enhance phagocytosis of cancer cells by blocking the CD47/SIRP α signal (van Bommel, P. E. et al. (2017) CD20-selective inhibition of CD47-SIRP α "don't eat me" signaling with a bispecific antibody-derivative enhances the anticancer activity of daratumumab, alemtuzumab and obinutuzumab. ONCOIMMUNOLOGY, vol. 7, no. 2, 1 - 8), the possibility to block the CD47-SIRP α interaction selectively in CD38 expressing cancer cells, still remains an unmet need.

Detailed Description

The present invention relates to a bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47.

The present invention also relates to a bispecific antibody comprising at least one binding portion which binds to human CD47 and at least two binding portions which bind to human CD38.

The present invention also relates to a bispecific antibody comprising at least one binding portion which binds to human CD47 and at least two binding portions which bind to human CD38, wherein the at least two CD38 binding portions are monoparatopic.

The present invention also relates to a bispecific antibody comprising at least one binding portion which binds to human CD47 and at least two binding portions which bind to human CD38, wherein the at least two CD38 binding portions are biparatopic.

5 The present invention also relates to a bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47, wherein at least one of the binding portions which binds to human CD47 can also bind to cynomolgus CD47.

The present invention also relates to a bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47, wherein at least one of the binding portions which binds to human CD38 can also bind to cynomolgus CD38.

10 The present invention also relates to the bispecific antibody above for use as a medicament.

More in particular, for use in treating multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, lymphoma, breast cancer such as Her2+ breast cancer, prostate cancer, cervical cancer, germinal center B-cell lymphoma or B-cell acute lymphoblastic leukemia, Chronic lymphocytic leukemia (CLL), Myelodysplastic syndrome (MDS), Non-Hodgkin
15 lymphoma, diffuse large B-cell lymphoma, non small cell lung cancer (NSCLC), Hepatocellular carcinoma (HCC), High-grade serous ovarian carcinoma, peritoneal cancer.

According to one aspect of the present invention, the disclosed bispecific antibody comprises at least one binding portion which binds to human CD38 comprising a CDR set selected from the group comprising: SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ
20 ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241.

According to another aspect of the present invention, the disclosed bispecific antibody comprises at least one binding portion which binds to human CD47 comprising a CDR set comprising: SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195.

In certain embodiments, the bispecific antibody according to the present invention comprises a Fc region.

25 In particular, said Fc region is a variant which comprises at least one amino acid modification relative to the Fc region of the parent antibody, whereas the antibody comprising the variant Fc region exhibits altered effector function compared to the parent antibody.

In certain embodiments, the bispecific antibody according to the present invention comprises at least one binding portion which binds to human CD47 that has an affinity to human CD47 lower than the affinity of at least one binding portion which binds to human CD38 has to human CD38.

5 The present invention also relates to an epitope on the human CD38 extracellular domain which is bound by the bispecific antibody disclosed herein.

The present invention also relates to an epitope on the human CD47 extracellular domain which is bound by the bispecific antibody disclosed herein.

The present invention also relates to an isolated nucleic acid encoding the bispecific antibody disclosed herein.

10 The present invention also relates to a vector comprising an isolated nucleic acid encoding the bispecific antibody disclosed herein.

The present invention also relates to a host cell comprising an isolated nucleic acid encoding the bispecific antibody disclosed herein or a vector comprising an isolated nucleic acid encoding the bispecific antibody disclosed herein.

15 The present invention also relates to a composition comprising the bispecific antibody according to the present invention and a pharmaceutically acceptable carrier, and to said composition further comprising another pharmaceutically active agent.

The present invention also relates to an immunoconjugate comprising the bispecific antibody according to the present invention linked to a therapeutic agent.

20 As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a molecule" optionally includes a combination of two
25 or more such molecules, and the like.

It is understood that aspects and embodiments of the present disclosure described herein include "comprising," "consisting," and "consisting essentially of aspects and embodiments.

The term "polynucleotide" as used herein refers to single-stranded or double-stranded nucleic acid polymers of at least 10 nucleotides in length. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. Such modifications include base modifications such as bromuridine, ribose modifications such as arabinoside and 2',3'-dideoxyribose, and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate and phosphoroamidate. The term "polynucleotide" specifically includes single-stranded and double-stranded forms of DNA.

An "isolated polynucleotide" is a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, which: (1) is not associated with all or a portion of a polynucleotide in which the isolated polynucleotide is found in nature, (2) is linked to a polynucleotide to which it is not linked in nature, or (3) does not occur in nature as part of a larger sequence.

An "isolated polypeptide" is one that: (1) is free of at least some other polypeptides with which it would normally be found, (2) is essentially free of other polypeptides from the same source, e.g., from the same species, (3) is expressed by a cell from a different species, (4) has been separated from at least about 50 percent of polynucleotides, lipids, carbohydrates, or other materials with which it is associated in nature, (5) is not associated (by covalent or noncovalent interaction) with portions of a polypeptide with which the "isolated polypeptide" is associated in nature, (6) is operably associated (by covalent or noncovalent interaction) with a polypeptide with which it is not associated in nature, or (7) does not occur in nature. Such an isolated polypeptide can be encoded by genomic DNA, cDNA, mRNA or other RNA, of synthetic origin, or any combination thereof. Preferably, the isolated polypeptide is substantially free from polypeptides or other contaminants that are found in its natural environment that would interfere with its use (therapeutic, diagnostic, prophylactic, research or otherwise).

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragments or single chains thereof. Naturally occurring antibodies typically comprise a tetramer. Each such tetramer is typically composed of two identical pairs of polypeptide chains, each pair having one full-length "light" chain (typically having a molecular weight of about 25 kDa) and one full-length "heavy" chain (typically having a molecular weight of about 50-70 kDa). The terms "heavy chain" and "light chain" as used herein refer to any immunoglobulin polypeptide having sufficient variable domain sequence to confer specificity

for a target antigen. The amino-terminal portion of each light and heavy chain typically includes a variable domain of about 100 to 110 or more amino acids that typically is responsible for antigen recognition. The carboxy-terminal portion of each chain typically defines a constant domain responsible for effector function. Thus, in a naturally occurring antibody, a full-length heavy chain immunoglobulin polypeptide includes a variable domain (VH) and three constant domains (CH1, CH2, and CH3), wherein the VH domain is at the amino-terminus of the polypeptide and the CH3 domain is at the carboxyl-terminus, and a full-length light chain immunoglobulin polypeptide includes a variable domain (VL) and a constant domain (CL), wherein the VL domain is at the amino-terminus of the polypeptide and the CL domain is at the carboxyl-terminus.

Human light chains are typically classified as kappa and lambda light chains, and human heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. IgG has several subclasses, including, but not limited to, IgG1, IgG2, IgG3, and IgG4. IgM has subclasses including, but not limited to, IgM1 and IgM2. IgA is similarly subdivided into subclasses including, but not limited to, IgA1 and IgA2. Within full-length light and heavy chains, the variable and constant domains typically are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See, e.g., FUNDAMENTAL IMMUNOLOGY (Paul, W., ed., Raven Press, 2nd ed., 1989), which is incorporated by reference in its entirety for all purposes. The variable regions of each light/heavy chain pair typically form an antigen binding site. The variable domains of naturally occurring antibodies typically exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair typically are aligned by the framework regions, which may enable binding to a specific epitope. From the amino-terminus to the carboxyl-terminus, both light and heavy chain variable domains typically comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4.

The term "CDR set" refers to a group of three CDRs that occur in a single variable region capable of binding the antigen. The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat et al, SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST (National Institutes of Health, Bethesda, Md. (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as Kabat

CDRs. Chothia and coworkers (Chothia and Lesk, 1987, J. Mol Biol. 196: 901-17; Chothia et al, 1989, Nature 342: 877-83) found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as L1, L2, and L3 or H1, H2, and H3 where the "L" and the "H" designates the light chain and the heavy chain regions, respectively. These regions may be referred to as Chothia CDRs, which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been described by Padlan, 1995, FASEB J. 9: 133- 39; MacCallum, 1996, J. Mol. Biol. 262(5): 732-45; and Lefranc, 2003, Dev. Comp. Immunol. 27: 55-77. Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat or Chothia defined CDRs. Identification of predicted CDRs using the amino acid sequence is well known in the field, such as in Martin, A.C. "Protein sequence and structure analysis of antibody variable domains," In Antibody Engineering, Vol. 2. Kontermann R., Diibbel S., eds. Springer- Verlag, Berlin, p. 33-51 (2010). The amino acid sequence of the heavy and/or light chain variable domain may be also inspected to identify the sequences of the CDRs by other conventional methods, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. The numbered sequences may be aligned by eye, or by employing an alignment program such as one of the CLUSTAL suite of programs, as described in Thompson, 1994, Nucleic Acids Res. 22: 4673-80. Molecular models are conventionally used to correctly delineate framework and CDR regions and thus correct the sequence-based assignments. All such alternative definitions are encompassed by the current invention and the sequences provided in this specification are not intended to exclude alternatively defined CDR sequences which may only comprise a portion of the CDR sequences provided in the sequence listing

The term "Fc" as used herein refers to a molecule comprising the sequence of a non-antigen-binding fragment resulting from digestion of an antibody or produced by other means, whether in monomeric or multimeric form, and can contain the hinge region. The original immunoglobulin source of the native Fc is preferably of human origin and can be any of the immunoglobulins. Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent {i.e., disulfide bonds) and non- covalent association. The number of intermolecular disulfide bonds between monomeric

subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, and IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, IgGA2, and IgG4). One example of a Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG. The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

5 A F(ab) fragment typically includes one light chain and the VH and CH1 domains of one heavy chain, wherein the VH-CH1 heavy chain portion of the F(ab) fragment cannot form a disulfide bond with another heavy chain polypeptide. As used herein, a F(ab) fragment can also include one light chain containing two variable domains separated by an amino acid linker and one heavy chain containing two variable domains separated by an amino acid linker and a CH1 domain.

10 A F(ab') fragment typically includes one light chain and a portion of one heavy chain that contains more of the constant region (between the CH1 and CH2 domains), such that an interchain disulfide bond can be formed between two heavy chains to form a F(ab')₂ molecule.

The term "binding protein" as used herein refers to a non-naturally occurring or recombinant or engineered molecule, such as non-naturally occurring or recombinant or engineered antibody, that
15 specifically binds to at least one target antigen, e.g., a CD38 polypeptide, or a CD47 polypeptide of the present disclosure.

A "recombinant" molecule is one that has been prepared, expressed, created, or isolated by recombinant means.

One embodiment of the disclosure provides binding proteins having biological and immunological
20 specificity to between one and three target antigens. Another embodiment of the disclosure provides nucleic acid molecules comprising nucleotide sequences encoding polypeptide chains that form such binding proteins. Another embodiment of the disclosure provides expression vectors comprising nucleic acid molecules comprising nucleotide sequences encoding polypeptide chains that form such binding proteins. Yet another embodiment of the disclosure provides host cells that express such binding proteins
25 (i.e., comprising nucleic acid molecules or vectors encoding polypeptide chains that form such binding proteins).

The term "antigen" or "target antigen" or "antigen target" as used herein refers to a molecule or a portion of a molecule that is capable of being bound by a binding protein, and additionally is capable of being

used in an animal to produce antibodies capable of binding to an epitope of that antigen. A target antigen may have one or more epitopes. With respect to each target antigen recognized by a binding protein, the binding protein is capable of competing with an intact antibody that recognizes the target antigen. The antigen is bound by a binding protein, such as an antibody, via an antigen binding site, also referred herein as "binding portion" or "binding domain".

"CD38" is cluster of differentiation 38 polypeptide, a glycoprotein found on the surface of many immune cells. In some embodiments, a binding protein of the present disclosure binds the extracellular domain of one or more CD38 polypeptide. Exemplary CD38 extracellular domain polypeptide sequences include, but are not limited to, the extracellular domain of human CD38 (e.g., as represented by SEQ ID NO: 5) and the extracellular domain of cynomolgus monkey CD38 (e.g., as represented by SEQ ID NO: 6).

"CD47" is cluster of differentiation 47, a transmembrane protein that in humans is encoded by the CD47 gene. CD47 belongs to the immunoglobulin superfamily, it partners with membrane integrins and also binds the ligands thrombospondin-1 (TSP-1) and signal-regulatory protein alpha (SIRP α). CD47 acts as a don't eat me signal to macrophages of the immune system. Human CD47 is encoded by SEQ ID NO: 7 and cynomolgus monkey CD47 is encoded by SEQ ID NO: 8.

The term "monospecific binding protein" refers to a binding protein that specifically binds to one antigen target.

The term "monovalent binding protein" refers to a binding protein that has one antigen binding site.

The term "bispecific binding protein" refers to a binding protein that specifically binds to two different antigen targets. In some embodiments, a bispecific binding protein binds to two different antigens. In some embodiments, a bispecific binding protein binds to two different epitopes on the same antigen.

The term "bivalent binding protein" refers to a binding protein that has two antigen binding sites.

The term "trispecific binding protein" refers to a binding protein that specifically binds to three different antigen targets. In some embodiments, a trispecific binding protein binds to three different antigens. In some embodiments, a trispecific binding protein binds to one, two, or three different epitopes on the same antigen.

The term "trivalent binding protein" refers to a binding protein that has three binding sites. In particular embodiments the trivalent binding protein can bind to one antigen target (i.e. monospecific trivalent binding protein"). In other embodiments, the trivalent binding protein can bind to two antigen targets (i.e. bispecific trivalent binding protein"). In other embodiments, the trivalent binding protein can bind to three antigen targets (i.e. trispecific trivalent binding protein"). In a more particular embodiment, the trivalent binding protein that bind to two antigen targets comprises a binding portion that binds to a first antigen target and two binding portions that bind to the same epitope of a second antigen target; such binding protein is referred to as "monoparatopic". In another more particular embodiment, the trivalent binding protein that bind to two antigen targets comprises a binding portion that binds to a first antigen target and two binding portions that bind to two distinct epitopes of a second antigen target; such binding protein is referred to as "biparatopic". An "isolated" binding protein is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the binding protein, and may include enzymes, hormones, and other proteinaceous or non- proteinaceous solutes. In some embodiments, the binding protein will be purified: (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated binding proteins include the binding protein in situ within recombinant cells since at least one component of the binding protein's natural environment will not be present.

The terms "substantially pure" or "substantially purified" as used herein refer to a compound or species that is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition). In some embodiments, a substantially purified fraction is a composition wherein the species comprises at least about 50% (on a molar basis) of all macromolecular species present. In other embodiments, a substantially pure composition will comprise more than about 80%, 85%, 90%, 95%, or 99% of all macromolar species present in the composition. In still other embodiments, the species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

A nucleic acid is "isolated" or "substantially pure" when purified away from other cellular components or other contaminants, e.g., other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art, see e.g. F. Ausubel, et al., ed. (1987) Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York. A nucleic acid of the invention can be, for example, DNA or RNA and may or may not contain intron sequences.

The term "epitope" includes any determinant, preferably a polypeptide determinant, capable of specifically binding to an immunoglobulin or T-cell receptor. In certain embodiments, epitope determinants include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl groups, or sulfonyl groups, and, in certain embodiments, may have specific three-dimensional structural characteristics and/or specific charge characteristics. An epitope is a region of an antigen that is bound by an antibody or binding protein. In certain embodiments, a binding protein is said to specifically bind an antigen when it preferentially recognizes its target antigen in a complex mixture of proteins and/or macromolecules. In some embodiments, a binding protein is said to specifically bind an antigen when the equilibrium dissociation constant is $< 10^8\text{M}$, more preferably when the equilibrium dissociation constant is $< 10^9\text{M}$, and most preferably when the dissociation constant is $< 10^{10}\text{M}$.

The dissociation constant (KD) of a binding protein can be determined, for example, by surface plasmon resonance. Generally, surface plasmon resonance analysis measures real-time binding interactions between ligand (a target antigen on a biosensor matrix) and analyte (a binding protein in solution) by surface plasmon resonance (SPR) using the BIAcore system (GE). Surface plasmon analysis can also be performed by immobilizing the analyte (binding protein on a biosensor matrix) and presenting the ligand (target antigen). The term "KD," as used herein refers to the dissociation constant of the interaction between a particular binding protein and a target antigen.

The term "binds to" as used herein in reference to a binding protein refers to the ability of a binding protein or an antigen-binding fragment thereof to bind to an antigen containing an epitope with a Kd of at least about $1 \times 10^6\text{M}$, $1 \times 10^7\text{M}$, $1 \times 10^8\text{M}$, $1 \times 10^9\text{M}$, $1 \times 10^{10}\text{M}$, $1 \times 10^{11}\text{M}$, $1 \times 10^{12}\text{M}$, or more, and/or to bind to an epitope with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen. In some embodiments, a binding protein of the present disclosure binds to two or more antigens, e.g., a human and a cynomolgus monkey CD38 polypeptide.

In some embodiments, an antigen binding domain and/or binding protein of the present disclosure "cross reacts" with human and cynomolgus monkey CD38 polypeptides, e.g., CD38 extracellular domains, such as SEQ ID NO: 295 (human CD38 isoform 28907-1), SEQ ID NO: 294 (human CD38 isoform 28907-2), SEQ ID NO: 296 (human CD38 isoform 28907-E) and SEQ ID NO:6 (cynomolgus monkey CD38). In some
5 embodiments, a binding protein or antigen-binding fragment thereof cross-reacts with human CD47 (e.g., SEQ ID NO: 300 (human CD47 isoform OA3-323), SEQ ID NO: 301 (human CD47 isoform OA3-293), SEQ ID NO: 302 (human CD47 isoform OA3-305), and SEQ ID NO: 303 (human CD47 isoform OA3-312)) and cynomolgus monkey CD47. A binding protein binding to antigen 1 is "cross-reactive" to antigen 2 when the EC50s are in a similar range for both antigens.

10 The term "linker" as used herein refers to one or more amino acid residues inserted between immunoglobulin domains to provide sufficient mobility for the domains of the light and heavy chains to fold into cross over dual variable region immunoglobulins. A linker is inserted at the transition between variable domains or between variable and constant domains, respectively, at the sequence level. The transition between domains can be identified because the approximate size of the immunoglobulin
15 domains are well understood. The precise location of a domain transition can be determined by locating peptide stretches that do not form secondary structural elements such as beta-sheets or alpha-helices as demonstrated by experimental data or as can be assumed by techniques of modeling or secondary structure prediction.

The term "vector" as used herein refers to any molecule (e.g., nucleic acid, plasmid, or virus) that is used
20 to transfer coding information to a host cell. The term "vector" includes a nucleic acid molecule that is capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double- stranded DNA molecule into which additional DNA segments may be inserted. Another type of vector is a viral vector, wherein additional DNA segments may be inserted into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are
25 introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell and thereby are replicated along with the host genome. In addition, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression
30 vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms "plasmid" and "vector" may be used interchangeably herein, as a plasmid is the most

commonly used form of vector. However, the disclosure is intended to include other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses, and adeno-associated viruses), which serve equivalent functions.

5 The phrase "recombinant host cell" (or "host cell") as used herein refers to a cell into which a recombinant expression vector has been introduced. A recombinant host cell or host cell is intended to refer not only to the particular subject cell, but also to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but such cells are still included within the scope of the term "host cell" as used herein. A wide variety of host cell expression systems can be used to express the
10 binding proteins, including bacterial, yeast, baculoviral, and mammalian expression systems (as well as phage display expression systems). An example of a suitable bacterial expression vector is pUC19. To express a binding protein recombinantly, a host cell is transformed or transfected with one or more recombinant expression vectors carrying DNA fragments encoding the polypeptide chains of the binding protein such that the polypeptide chains are expressed in the host cell and, preferably, secreted into the
15 medium in which the host cells are cultured, from which medium the binding protein can be recovered.

The term "transformation" as used herein refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain a new DNA. For example, a cell is transformed where it is genetically modified from its native state. Following transformation, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be
20 maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been stably transformed when the DNA is replicated with the division of the cell. The term "transfection" as used herein refers to the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art. Such techniques can be
25 used to introduce one or more exogenous DNA molecules into suitable host cells.

The term "naturally occurring" as used herein and applied to an object refers to the fact that the object can be found in nature and has not been manipulated by man. For example, a polynucleotide or polypeptide that is present in an organism (including viruses) that can be isolated from a source in nature and that has not been intentionally modified by man is naturally-occurring. Similarly, "non-naturally

occurring" as used herein refers to an object that is not found in nature or that has been structurally modified or synthesized by man.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids; unnatural amino acids and
5 analogs such as α -, α -di substituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for the polypeptide chains of the binding proteins. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ -N-methylarginine, and other similar amino acids and imino acids (e.g., 4-
10 hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxyl-terminal direction, in accordance with standard usage and convention.

Naturally occurring residues may be divided into classes based on common side chain properties:

- (1) hydrophobic: Met, Ala, Val, Leu, Ile, Phe, Trp, Tyr, Pro;
- 15 (2) polar hydrophilic: Arg, Asn, Asp, Gln, Glu, His, Lys, Ser, Thr ;
- (3) aliphatic: Ala, Gly, Ile, Leu, Val, Pro;
- (4) aliphatic hydrophobic: Ala, Ile, Leu, Val, Pro;
- (5) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (6) acidic: Asp, Glu;
- 20 (7) basic: His, Lys, Arg;
- (8) residues that influence chain orientation: Gly, Pro;
- (9) aromatic: His, Trp, Tyr, Phe; and
- (10) aromatic hydrophobic: Phe, Trp, Tyr.

Conservative amino acid substitutions may involve exchange of a member of one of these classes with
25 another member of the same class. Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class.

A skilled artisan will be able to determine suitable variants of the polypeptide chains of the binding proteins using well-known techniques. For example, one skilled in the art may identify suitable areas of a polypeptide chain that may be changed without destroying activity by targeting regions not believed to

be important for activity. Alternatively, one skilled in the art can identify residues and portions of the molecules that are conserved among similar polypeptides. In addition, even areas that may be important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity or without adversely affecting the polypeptide structure.

- 5 The term "patient" as used herein includes human and animal subjects.

The terms "treatment" or "treat" as used herein refer to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those having a disorder as well as those prone to have the disorder or those in which the disorder is to be prevented. In particular embodiments, binding proteins can be used to treat humans with cancer, or humans susceptible to cancer, or ameliorate cancer

10 in a human subject. The binding proteins can also be used to prevent cancer in a human patient. In particular embodiments, the cancer is multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, lymphoma, breast cancer such as Her2+ breast cancer, prostate cancer, cervical cancer, germinal center B-cell lymphoma or B-cell acute lymphoblastic leukemia, Chronic lymphocytic leukemia (CLL), Non-Hodgkin lymphoma, diffuse large B-cell lymphoma,

15 non small cell lung cancer (NSCLC), Hepatocellular carcinoma (HCC), High-grade serous ovarian carcinoma, peritoneal cancer.

The terms "pharmaceutical composition" or "therapeutic composition" as used herein refer to a compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient.

- 20 The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of a binding protein.

The terms "effective amount" and "therapeutically effective amount" when used in reference to a pharmaceutical composition comprising one or more binding proteins refer to an amount or dosage

25 sufficient to produce a desired therapeutic result. More specifically, a therapeutically effective amount is an amount of a binding protein sufficient to inhibit, for some period of time, one or more of the clinically defined pathological processes associated with the condition being treated. The effective amount may vary depending on the specific binding protein that is being used, and also depends on a variety of factors and conditions related to the patient being treated and the severity of the disorder. For example, if the

binding protein is to be administered in vivo, factors such as the age, weight, and health of the patient as well as dose response curves and toxicity data obtained in preclinical animal work would be among those factors considered. The determination of an effective amount or therapeutically effective amount of a given pharmaceutical composition is well within the ability of those skilled in the art.

- 5 One embodiment of the disclosure provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a binding protein.

Anti-CD38 Binding Proteins

Certain aspects of the present disclosure relate to binding proteins that comprise an antigen binding site that binds a CD38 polypeptide {e.g., human and cynomolgus monkey CD38 polypeptides). In some
10 embodiments, the binding proteins are monospecific and/or monovalent, bispecific and/or bivalent, trispecific and/or trivalent, or multispecific and/or multivalent.

A variety of features of exemplary monospecific, bispecific, or trispecific binding proteins are described herein. For example, in some embodiments, a binding protein or antigen-binding fragment thereof cross-
15 reacts with human CD38 (e.g., SEQ ID NO: 295 (human CD38 isoform 28907-1), SEQ ID NO: 294 (human CD38 isoform 28907-2), SEQ ID NO: 296 (human CD38 isoform 28907-E)) and cynomolgus monkey CD38. In some embodiments, a binding protein induces apoptosis of a CD38+ cell.

In some embodiments, the binding protein is bivalent and/or bispecific. In some embodiments, the
20 binding protein is multivalent, such as trivalent or tetravalent. In some embodiments, at least one of the antigen binding sites binds a CD38 polypeptide (e.g., the extracellular domain of human and/or cynomolgus monkey CD38 polypeptides).

In any of the bispecific binding proteins described supra, the target antigen other than CD38 can be any of the following exemplary antigen targets: A2AR, APRIL, ATPDase, BAFF, BAFFR, BCMA, BIYS, BTK, BTLA,
25 B7DC, B7H1, B7H4 (also known as VTCN1), B7H5, B7H6, B7H7, B7RP1, B7-4, C3, C5, CCL2 (also known as MCP-1), CCL3 (also known as MIP-1a), CCL4 (also known as MIP-1b), CCL5 (also known as RANTES), CCL7 (also known as MCP-3), CCL8 (also known as mcp-2), CCL11 (also known as eotaxin), CCL15 (also known as MIP-1d), CCL17 (also known as TARC), CCL19 (also known as MIP-3b), CCL20 (also known as MIP-3a), CCL21 (also known as MIP-2), CCL24 (also known as MIP-2/eotaxin-2), CCL25 (also known as TECK), CCL26
30 (also known as eotaxin-3), CCR3, CCR4, CD3, CCR7, CD19, CD20, CD23 (also known as FCER2, a receptor

for IgE), CD24, CD27, CD28, CD38, CD39, CD40, CD47, CD48, CD70, CD80 (also known as B7-1), CD86 (also known as B7-2), CD122, CD137 (also known as 41BB), CD137L, CD152 (also known as CTLA4), CD154 (also known as CD40L), CD160, CD272, CD273 (also known as PDL2), CD274 (also known as PDL1), CD275 (also known as B7H2), CD276 (also known as B7H3), CD278 (also known as ICOS), CD279 (also known as PD-1),

5 CDH1 (also known as E- cadherin), chitinase, CLEC9, CLEC91, CRTH2, CSF-1 (also known as M-CSF), CSF-2 (also known as GM-CSF), CSF-3 (also known as GCSF), CX3CL1 (also known as SCYD1), CXCL12 (also known as SDF1), CXCL13, CXCR3, DNGR-1, ectonucleoside triphosphate diphosphohydrolase 1, EGFR, ENTPD1, FCER1A, FCER1, FLAP, FOLH1, Gi24, GATR, GITRL, GM-CSF, GPRC5D, Her2, HHLA2, HMGB1, HVEM, ICOSLG, IDO, IFNa, IgE, IGF1R, IL2Rbeta, IL1, IL1RAP, IL1LIA, IL1B, IL1F10, IL2, IL4, IL4Ra, IL5, IL5R, IL6, IL7, IL7Ra, IL8,

10 IL9, RAP, IL9R, IL10, rIL10, IL12, IL13, IL13Ra1, IL13Ra2, IL15, IL17, IL17Rb (also known as a receptor for IL25), IL18, IL22, IL23, IL25, IL27, IL33, IL35, ITGB4 (also known as b4 integrin), ITK, KIR, LAG3, LAMP1, leptin, LPFS2, MHC class II, NCR3LG1, KG2D, NTPDase-1, OX40, OX40L, PD-1H, platelet receptor, PROM1, S152, SISP1, SLC, SPG64, ST2 (also known as a receptor for IL33), STEAP2, Syk kinase, TACI, TDO, T14, TIGIT, TIM3, TLR, TLR2, TLR4, TLR5, TLR9, TMEF1, TNFa, TFRSF7, Tp55, TREM1, TSLP (also known as a co-receptor

15 for IL7Ra), TSLPR, TWEAK, VEGF, VISTA, Vstm3, WUCAM, and XCR1 (also known as GPR5/CCXCR1), XCL1 and XCL2. In some embodiments, one or more of the above antigen targets are human antigen targets.

In any of the bispecific binding proteins described supra, any linker or combination of linkers described herein may be used.

20 In certain particular embodiments, the present invention relates to a bispecific antibody comprising at least one binding portion which binds to human CD38 comprising a CDR set selected from the group comprising: SEQ ID NO: 80, SEQ ID NO: 140, SEQ ID NO: 200; SEQ ID NO: 81, SEQ ID NO: 141, SEQ ID NO: 201; SEQ ID NO: 82, SEQ ID NO: 142, SEQ ID NO: 202; SEQ ID NO: 83, SEQ ID NO: 143, SEQ ID NO: 203; SEQ ID NO: 84, SEQ ID NO: 144, SEQ ID NO: 204; SEQ ID NO: 85, SEQ ID NO: 145, SEQ ID NO: 205; SEQ ID NO: 86, SEQ ID NO: 146, SEQ ID NO: 206; SEQ ID NO: 87, SEQ ID NO: 147, SEQ ID NO: 207, SEQ ID NO: 88, SEQ ID NO: 148, SEQ ID NO: 208; SEQ ID NO: 89, SEQ ID NO: 149, SEQ ID NO: 209; SEQ ID NO: 90, SEQ ID NO: 150, SEQ ID NO: 210; SEQ ID NO: 91, SEQ ID NO: 151, SEQ ID NO: 211; SEQ ID NO: 92, SEQ ID NO: 152, SEQ ID NO: 212; SEQ ID NO: 93, SEQ ID NO: 153, SEQ ID NO: 213; SEQ ID NO: 94, SEQ ID NO: 154, SEQ ID NO: 214; SEQ ID NO: 95, SEQ ID NO: 155, SEQ ID NO: 215; SEQ ID NO: 96, SEQ ID NO: 156, SEQ ID NO: 216; SEQ ID NO: 97, SEQ ID NO: 157, SEQ ID NO: 217; SEQ ID NO: 98, SEQ ID NO: 158, SEQ ID NO: 218; SEQ ID NO: 99, SEQ ID NO: 159, SEQ ID NO: 219; SEQ ID NO: 100, SEQ ID NO: 160, SEQ ID NO: 220; SEQ ID NO: 101,

30

SEQ ID NO: 161, SEQ ID NO: 221; SEQ ID NO: 102, SEQ ID NO: 162, SEQ ID NO: 222; SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223; SEQ ID NO: 104, SEQ ID NO: 164, SEQ ID NO: 224; SEQ ID NO: 105, SEQ ID NO: 165, SEQ ID NO: 225; SEQ ID NO: 106, SEQ ID NO: 166, SEQ ID NO: 226; SEQ ID NO: 107, SEQ ID NO: 167, SEQ ID NO: 227; SEQ ID NO: 108, SEQ ID NO: 168, SEQ ID NO: 228; SEQ ID NO: 109, SEQ ID NO: 169, SEQ ID NO: 229; SEQ ID NO: 110, SEQ ID NO: 170, SEQ ID NO: 230; SEQ ID NO: 111, SEQ ID NO: 171, SEQ ID NO: 231; SEQ ID NO: 112, SEQ ID NO: 172, SEQ ID NO: 232; SEQ ID NO: 113, SEQ ID NO: 173, SEQ ID NO: 233, SEQ ID NO: 114, SEQ ID NO: 174, SEQ ID NO: 234; SEQ ID NO: 115, SEQ ID NO: 176, SEQ ID NO: 235; SEQ ID NO: 116, SEQ ID NO: 175, SEQ ID NO: 236; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ ID NO: 118, SEQ ID NO: 178, SEQ ID NO: 238; SEQ ID NO: 119, SEQ ID NO: 179, SEQ ID NO: 239; SEQ ID NO: 120, SEQ ID NO: 180, SEQ ID NO: 240; SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; SEQ ID NO: 122, SEQ ID NO: 182, SEQ ID NO: 242; SEQ ID NO: 123, SEQ ID NO: 183, SEQ ID NO: 243; SEQ ID NO: 124, SEQ ID NO: 184, SEQ ID NO: 244; SEQ ID NO: 125, SEQ ID NO: 185, SEQ ID NO: 245; SEQ ID NO: 126, SEQ ID NO: 186, SEQ ID NO: 246; SEQ ID NO: 127, SEQ ID NO: 187, SEQ ID NO: 247; SEQ ID NO: 128, SEQ ID NO: 188, SEQ ID NO: 248; SEQ ID NO: 129, SEQ ID NO: 189, SEQ ID NO: 249; SEQ ID NO: 130, SEQ ID NO: 190, SEQ ID NO: 250. In a preferred embodiment, the bispecific antibody according to the present invention comprises at least one binding portion which binds to human CD38 comprising a CDR set selected from the group comprising SEQ ID NO: 112, SEQ ID NO: 172, SEQ ID NO: 232; SEQ ID NO: 115, SEQ ID NO: 176, SEQ ID NO: 235; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241.

20 Anti-CD47 Binding Proteins

Certain aspects of the present disclosure relate to binding proteins that comprise an antigen binding site that binds a CD47 polypeptide (e.g., human and cynomolgus monkey CD47 polypeptides). In some embodiments, the binding proteins are monospecific and/or monovalent, bispecific and/or bivalent, trispecific and/or trivalent, or multispecific and/or multivalent.

25 A variety of features of exemplary monospecific, bispecific, or trispecific binding proteins are described herein. For example, in some embodiments, a binding protein or antigen-binding fragment thereof cross-reacts with human CD47 (e.g., SEQ ID NO: 300 (human CD47 isoform OA3-323), SEQ ID NO: 301 (human CD47 isoform OA3-293), SEQ ID NO: 302 (human CD47 isoform OA3-305), and SEQ ID NO: 303 (human CD47 isoform OA3-312)) and cynomolgus monkey CD47.

In some embodiments, the binding protein is bivalent and/or bispecific. In some embodiments, the binding protein is multivalent, such as trivalent or tetravalent. In some embodiments, at least one of the antigen binding sites binds a CD47 polypeptide (e.g., the extracellular domain of human and/or cynomolgus monkey CD47 polypeptides).

5 In any of the bispecific binding proteins described supra, the target antigen other than CD47 can be any of the following exemplary antigen targets: A2AR, APRIL, ATPDase, BAFF, BAFFR, BCMA, BIYS, BTK, BTLA, B7DC, B7H1, B7H4 (also known as VTCN1), B7H5, B7H6, B7H7, B7RP1, B7-4, C3, C5, CCL2 (also known as MCP-1), CCL3 (also known as MIP-1a), CCL4 (also known as MIP-1b), CCL5 (also known as RANTES), CCL7 (also known as MCP-3), CCL8 (also known as mcp-2), CCL11 (also known as eotaxin), CCL15 (also known as MIP-1d), CCL17 (also known as TARC), CCL19 (also known as MIP-3b), CCL20 (also known as MIP-3a),
 10 CCL21 (also known as MIP-2), CCL24 (also known as MIP-2/eotaxin-2), CCL25 (also known as TECK), CCL26 (also known as eotaxin-3), CCR3, CCR4, CD3, CCR7, CD19, CD20, CD23 (also known as FCER2, a receptor for IgE), CD24, CD27, CD28, CD38, CD39, CD40, CD47, CD48, CD70, CD80 (also known as B7-1), CD86 (also known as B7-2), CD122, CD137 (also known as 41BB), CD137L, CD152 (also known as CTLA4), CD154 (also known as CD40L), CD160, CD272, CD273 (also known as PDL2), CD274 (also known as PDL1), CD275 (also known as B7H2), CD276 (also known as B7H3), CD278 (also known as ICOS), CD279 (also known as PD-1), CDH1 (also known as E-cadherin), chitinase, CLEC9, CLEC91, CRTH2, CSF-1 (also known as M-CSF), CSF-2 (also known as GM-CSF), CSF-3 (also known as G-CSF), CX3CL1 (also known as SCYD1), CXCL12 (also known as SDF1), CXCL13, CXCR3, DNCR-1, ectonucleoside triphosphate diphosphohydrolase 1, EGFR, ENTPD1,
 20 FCER1A, FCER1, FLAP, FOLH1, Gi24, GPCR, GPRC5D, Her2, HHLA2, HMGB1, HVEM, ICOSLG, IDO, IFN α , IgE, IGF1R, IL2R β , IL1, IL1RAP, IL1A, IL1B, IL1F10, IL2, IL4, IL4Ra, IL5, IL5R, IL6, IL7, IL7Ra, IL8, IL9, RAP, IL9R, IL10, rIL10, IL12, IL13, IL13Ra1, IL13Ra2, IL15, IL17, IL17Rb (also known as a receptor for IL25), IL18, IL22, IL23, IL25, IL27, IL33, IL35, ITGB4 (also known as β 4 integrin), ITK, KIR, LAG3, LAMP1, leptin, LPFS2, MHC class II, NCR3LG1, KG2D, NTPDase-1, OX40, OX40L, PD-1H, platelet receptor, PROM1, S152, SISP1, SLC, SPG64, ST2 (also known as a receptor for IL33), STEAP2, Syk kinase, TACI, TDO, T14, TIGIT, TIM3, TLR, TLR2, TLR4, TLR5, TLR9, TMEF1, TNF α , TFRSF7, Tp55, TREM1, TSLP (also known as a co-receptor for IL7Ra), TSLPR, TWEAK, VEGF, VISTA, Vstm3, WUCAM, and XCR1 (also known as GPR5/CCXCR1), XCL1 and XCL2. In some embodiments, one or more of the above antigen targets are human antigen targets.

In certain particular embodiments, the present invention relates to a bispecific antibody comprising a
 30 binding portion which binds to human CD47 comprising a CDR set selected from the group comprising: SEQ ID NO: 71, SEQ ID NO: 131, SEQ ID NO: 191; SEQ ID NO: 72, SEQ ID NO: 132, SEQ ID NO: 192; SEQ ID

NO: 73, SEQ ID NO: 133, SEQ ID NO: 193; SEQ ID NO: 74, SEQ ID NO: 134, SEQ ID NO: 194; SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195; SEQ ID NO: 76, SEQ ID NO: 136, SEQ ID NO: 196; SEQ ID NO: 77, SEQ ID NO: 137, SEQ ID NO: 197; SEQ ID NO: 78, SEQ ID NO: 138, SEQ ID NO: 198; SEQ ID NO: 79, SEQ ID NO: 139, SEQ ID NO: 199; SEQ ID NO: 80, SEQ ID NO: 140, SEQ ID NO: 200; SEQ ID NO: 81, SEQ ID NO: 141, SEQ ID NO: 201. In a preferred embodiment, the bispecific antibody according to the present invention comprises a binding portion which binds to human CD47 comprising a CDR set comprising SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195.

Bispecific anti-CD47 and anti-CD38 proteins

The present invention relates to a bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47.

In particular the at least one binding portion which binds to human CD38 comprises a CDR set selected from the group comprising: SEQ ID NO: 80, SEQ ID NO: 140, SEQ ID NO: 200; SEQ ID NO: 81, SEQ ID NO: 141, SEQ ID NO: 201; SEQ ID NO: 82, SEQ ID NO: 142, SEQ ID NO: 202; SEQ ID NO: 83, SEQ ID NO: 143, SEQ ID NO: 203; SEQ ID NO: 84, SEQ ID NO: 144, SEQ ID NO: 204; SEQ ID NO: 85, SEQ ID NO: 145, SEQ ID NO: 205; SEQ ID NO: 86, SEQ ID NO: 146, SEQ ID NO: 206; SEQ ID NO: 87, SEQ ID NO: 147, SEQ ID NO: 207, SEQ ID NO: 88, SEQ ID NO: 148, SEQ ID NO: 208; SEQ ID NO: 89, SEQ ID NO: 149, SEQ ID NO: 209; SEQ ID NO: 90, SEQ ID NO: 150, SEQ ID NO: 210; SEQ ID NO: 91, SEQ ID NO: 151, SEQ ID NO: 211; SEQ ID NO: 92, SEQ ID NO: 152, SEQ ID NO: 212; SEQ ID NO: 93, SEQ ID NO: 153, SEQ ID NO: 213; SEQ ID NO: 94, SEQ ID NO: 154, SEQ ID NO: 214; SEQ ID NO: 95, SEQ ID NO: 155, SEQ ID NO: 215; SEQ ID NO: 96, SEQ ID NO: 156, SEQ ID NO: 216; SEQ ID NO: 97, SEQ ID NO: 157, SEQ ID NO: 217; SEQ ID NO: 98, SEQ ID NO: 158, SEQ ID NO: 218; SEQ ID NO: 99, SEQ ID NO: 159, SEQ ID NO: 219; SEQ ID NO: 100, SEQ ID NO: 160, SEQ ID NO: 220; SEQ ID NO: 101, SEQ ID NO: 161, SEQ ID NO: 221; SEQ ID NO: 102, SEQ ID NO: 162, SEQ ID NO: 222; SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223; SEQ ID NO: 104, SEQ ID NO: 164, SEQ ID NO: 224; SEQ ID NO: 105, SEQ ID NO: 165, SEQ ID NO: 225; SEQ ID NO: 106, SEQ ID NO: 166, SEQ ID NO: 226; SEQ ID NO: 107, SEQ ID NO: 167, SEQ ID NO: 227; SEQ ID NO: 108, SEQ ID NO: 168, SEQ ID NO: 228; SEQ ID NO: 109, SEQ ID NO: 169, SEQ ID NO: 229; SEQ ID NO: 110, SEQ ID NO: 170, SEQ ID NO: 230; SEQ ID NO: 111, SEQ ID NO: 171, SEQ ID NO: 231; SEQ ID NO: 112, SEQ ID NO: 172, SEQ ID NO: 232; SEQ ID NO: 113, SEQ ID NO: 173, SEQ ID NO: 233, SEQ ID NO: 114, SEQ ID NO: 174, SEQ ID NO: 234; SEQ ID NO: 115, SEQ ID NO: 176, SEQ ID NO: 235; SEQ ID NO: 116, SEQ ID NO: 175, SEQ ID NO: 236; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ ID NO: 118, SEQ ID NO: 178, SEQ ID NO: 238; SEQ ID NO: 119, SEQ ID NO: 179, SEQ ID NO: 239;

SEQ ID NO: 120, SEQ ID NO: 180, SEQ ID NO: 240; SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; SEQ ID NO: 122, SEQ ID NO: 182, SEQ ID NO: 242; SEQ ID NO: 123, SEQ ID NO: 183, SEQ ID NO: 243; SEQ ID NO: 124, SEQ ID NO: 184, SEQ ID NO: 244; SEQ ID NO: 125, SEQ ID NO: 185, SEQ ID NO: 245; SEQ ID NO: 126, SEQ ID NO: 186, SEQ ID NO: 246; SEQ ID NO: 127, SEQ ID NO: 187, SEQ ID NO: 247; SEQ ID NO: 128, SEQ ID NO: 188, SEQ ID NO: 248; SEQ ID NO: 129, SEQ ID NO: 189, SEQ ID NO: 249; SEQ ID NO: 130, SEQ ID NO: 190, SEQ ID NO: 250; and the at least one binding portion which binds to human CD47 comprises a CDR set selected from the group comprising: SEQ ID NO: 71, SEQ ID NO: 131, SEQ ID NO: 191; SEQ ID NO: 72, SEQ ID NO: 132, SEQ ID NO: 192; SEQ ID NO: 73, SEQ ID NO: 133, SEQ ID NO: 193; SEQ ID NO: 74, SEQ ID NO: 134, SEQ ID NO: 194; SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195; SEQ ID NO: 76, SEQ ID NO: 136, SEQ ID NO: 196; SEQ ID NO: 77, SEQ ID NO: 137, SEQ ID NO: 197; SEQ ID NO: 78, SEQ ID NO: 138, SEQ ID NO: 198; SEQ ID NO: 79, SEQ ID NO: 139, SEQ ID NO: 199; SEQ ID NO: 80, SEQ ID NO: 140, SEQ ID NO: 200; SEQ ID NO: 81, SEQ ID NO: 141, SEQ ID NO: 201.

More specifically, the at least one binding portion which binds to human CD38 comprises a CDR set selected from the group comprising: SEQ ID NO: 112, SEQ ID NO: 172, SEQ ID NO: 232; SEQ ID NO: 115, SEQ ID NO: 176, SEQ ID NO: 235; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; and the at least one binding portion which binds to human CD47 comprises a CDR set comprising SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195. Even more specifically, the at least one binding portion which binds to human CD38 comprises a CDR set comprising SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; and the at least one binding portion which binds to human CD47 comprises a CDR set selected from the group comprising set comprising SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195.

In exemplary embodiments, the present invention relates to bispecific antibodies comprising at least one binding portion which binds to human CD47 and at least two binding portions which bind to human CD38. The at least one binding portion which binds to human CD47 may comprise any of the CDRs set described above and in paragraph "Anti-CD47 Binding Proteins". The at least two binding portions which bind to human CD38 may comprise any of the CDRs set described above and in paragraph "Anti-CD38 Binding Proteins".

In certain embodiments, the at least two CD38 binding portions are monoparatopic, i.e. they bind to the same epitope of their antigen target. In other embodiments, the at least two CD38 binding portions are biparatopic, i.e. they bind to different epitopes their antigen target. In particular, the antibody according

to certain embodiments of the present invention comprises at least two binding portions which binds to human CD38: one Fc proximal binding portion which binds to a first epitope of human CD38, and one Fc distal binding portion which binds to a second epitope of human CD38. In particular embodiments, at least one of the at least two binding portions which binds to human CD38 comprises a CDR set selected

5 from the group comprising SEQ ID NO: 112, SEQ ID NO: 172, SEQ ID NO: 232; SEQ ID NO: 115, SEQ ID NO: 176, SEQ ID NO: 235; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241. In more specific embodiments, at least one of the at least two binding portions which binds to human CD38 comprises a CDR set selected

10 from the group comprising SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; and at least one of the at least two binding portions which binds to human CD38 comprises the CDR set SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237. In a preferred embodiment, the Fc proximal binding portion which binds to a first epitope of human CD38 comprises a CDR set selected

15 from the group comprising SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; and the Fc distal binding portion which binds to a second epitope of human CD38 comprises the CDR set SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237. In a more preferred embodiment, the Fc proximal binding portion which binds to a first epitope of human CD38 comprises a CDR set selected from the group comprising SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; the Fc distal binding portion which binds to a second epitope

20 of human CD38 comprises the CDR set SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; further the bispecific antibody described above comprises at least one binding portion which binds to human CD47 comprising a CDR set comprising SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195. Preferably, the bispecific antibody of the present invention comprises at least one binding portion which binds to human CD38 comprises a CDR set comprising SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237, at least a second binding

25 portion which binds to human CD38 comprises a CDR set selected from the group comprising SEQ ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223, and SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241; and at least one binding portion which binds to human CD47 comprising a CDR set comprising SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195.

In certain embodiments of the present invention, the bispecific antibody is a full-length antibody, wherein the at least one binding portion which binds to human CD38 and/or the at least one of binding portion

30 which binds to human CD47 is a Fab region. The term "Fab" or "Fab region" or "Fab domain" as used herein includes the polypeptides that comprise the VH, CH1, VL, and CL immunoglobulin domains. Fab

- may refer to this region in isolation, or this region in the context of a full-length antibody or antibody fragment. The present invention also provides an antibody fragment that binds to a human CD38 or to a human Cd47. Antibody fragments include, but are not limited to, (i) the Fab fragment consisting of VL, VH, CL and CH1 domains, including Fab' and Fab'-SH, (ii) the Fd fragment consisting of the VH and CH1 domains, (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward ES et al., (1989) Nature, 341: 544-546) which consists of a single variable, (v) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vi) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird RE et al., (1988) Science 242: 423-426 ; Huston JS et al., (1988) Proc. Natl. Acad. Sci. USA, 85: 5879-83), (vii) bispecific single chain Fv dimers (PCT/US92/09965), (viii) "diabodies" or "triabodies", multivalent or multispecific fragments constructed by gene fusion (Tomlinson I & Hollinger P (2000) Methods Enzymol. 326: 461-79; WO94/13804 ; Holliger P et al., (1993) Proc. Natl. Acad. Sci. USA, 90: 6444-48) and (ix) scFv genetically fused to the same or a different antibody (Coloma MJ & Morrison SL (1997) Nature Biotechnology, 15(2): 159-163).
- 15 In certain embodiments the bispecific antibody of the present invention comprises at least one binding portion which binds to human CD47 and it is a Fab comprising an amino acid sequence selected from the group comprising SEQ ID NOs: 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 299; in a particular embodiment the Fab which binds to human CD47 comprises a VH chain with a G65S mutation (Kabat numbering), in more particular embodiments the Fab which binds to human CD47 comprises an amino acid sequence of
- 20 SEQ ID NO: 15 or 299. In a preferred embodiment, the bispecific antibody of the present invention comprises at least a Fab which binds to human CD47 having SEQ ID NO: 15 and comprising a VH chain with a G65S mutation (Kabat numbering), in particular the bispecific antibody of the present invention comprises at least a Fab which binds to human CD47 comprising an amino acid sequence of SEQ ID NO: 299. In certain embodiments the bispecific antibody of the present invention comprises at least one
- 25 binding portion which binds to human CD38 and is a Fab comprising an amino acid sequence selected from the group comprising SEQ ID NOs: 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70. Preferably the amino acid sequence of the Fab which binds to human CD38 is selected from the group comprising SEQ ID NOs: 43, 52, 55, 57 and 61.
- 30 In particular embodiments of the present invention, the bispecific antibody is constructed using the BEAT® heavy chain (Hc) heterodimerization technology previously described (Skegrod et al., (2017) J Biol Chem

292(23): 9745-9759 and Stutz et al., (2020) J Biol Chem 295(28): 9392-9408, WO2012131555), wherein the BEAT(A) chain, also referred herein to as BEAT(A), and the BEAT (B) chain, also referred herein to as BEAT(B). More specifically, the BEAT (A) Hc encompasses a VH domain with mutation G65S (Kabat numbering) a CH1 γ 1 region, a γ 1 hinge region, a γ 1 CH2 region, and a γ 3 based BEAT (A) CH3 domain assembled with a common light chain (cLc). BEAT (B) Hc encompasses a VH domain, a CH1 γ 1 region, a γ 1 hinge region, a γ 1 CH2 region, and a γ 1 based BEAT (B) CH3 domain assembled with a cLc. According to the present invention the BEAT(A) heavy chain (Hc) comprises an amino acid sequence selected from the group comprising SEQ ID NOs: 259, 274, 276, 280, 281, 283, 285 and 278; the BEAT(B) Hc comprises an amino acid sequence selected from the group comprising SEQ ID NOs: 260, 261, 262, 275, 277, 279, 282, 284, 286, 287, 288, 289, 290, 291, 292 and 293. The cLc comprises an amino acid sequence of SEQ ID NO: 10. In preferred embodiments the bispecific antibody according to the present invention comprises a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 259, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 260, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 259, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 261, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 259, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 262, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 274, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 275, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 276, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 277, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 278, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 279, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 280, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 277, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 281, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 282, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 283, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 284, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 285, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 286, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 281, a BEAT(B) chain comprising

the amino acid sequence of SEQ ID NO: 287, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 278, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 288, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 281, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 290, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 278, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 291, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 280, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 292, and a cLc comprising the amino acid sequence of SEQ ID NO: 10; or a BEAT(A) chain comprising the amino acid sequence of SEQ ID NO: 280, a BEAT(B) chain comprising the amino acid sequence of SEQ ID NO: 293, and a cLc comprising the amino acid sequence of SEQ ID NO: 10. In preferred embodiments, the bispecific antibody disclosed herein comprises a BAET(A) chain that binds to CD47 and comprises the amino acid sequence of SEQ ID NO: 280; a BEAT(B) chain that binds to CD38 and comprises an amino acid sequence of SEQ ID NOs: 292 or 293, more preferably the amino acid sequence of SEQ ID NO: 293; and a cLc comprising the amino acid sequence of SEQ ID NO: 10.

In certain aspects the present invention discloses a bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47 wherein the at least one binding portion which binds to human CD47 has an affinity to human CD47 lower than the affinity that the at least one binding portion which binds to human CD38 has against human CD38. In particular, the at least one binding portion which binds to human CD47 has an affinity to human CD47 of about 1000 nM or less, or about 900 nM or less, or about 800 nM or less, or about 850 nM or less, or about 700 nM or less, or about 600 nM or less, or about 500 nM or less, or about 400 nM or less, or about 300 nM or less, 200 nM or less, or of about 150 nM or less, or of about 140 or less, or of about 130 nM or less, or of about 120 nM or less, preferably of about 110 nM or less, more preferably of 105 nM or less, for instance of about 104 nM; or preferably between about 700 nM and about 1000 nM, more preferably between about 798 nM and about 958 nM, more preferably about 878.3 nM. In particular, the at least one binding portion which binds to human CD38 has an affinity to human CD38 of about 100 nM or less, or of about 50 nM or less, or of about 30 or less, or of about 20 or less, or of about 10 or less, preferably of about 10 nM or less, of about 9 nM or less, of about 8 nM or less, of about 7 nM or less, of about 6 nM or less, of about 5 nM or less, of about 4 nM or less, of about 3 nM or less, of about 2 nM or

less, preferably of about 1 nM or less, for instance of about 16.22 nM, about 17.6 nM, about 6.13 nM, about 2.3 nM, about 3.5 nM, about 0.55 nM, about 0.9 nM.

The present invention also discloses bispecific antibodies comprising at least one binding portion which binds to human CD47 and at least two binding portions which bind to human CD38, particular biparatopic bispecific antibodies wherein the affinity of the Fc proximal binding portion to a first epitope of human CD38 is about the same, or the same or different than the affinity of the Fc distal binding portion to a second epitope of human CD38.

Nucleic acids

Standard recombinant DNA methodologies are used to construct the polynucleotides that encode the polypeptides which form the binding proteins, incorporate these polynucleotides into recombinant expression vectors, and introduce such vectors into host cells. See e.g., Sambrook et al. , 2001 , MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Laboratory Press, 3rd ed.). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclature utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Similarly, conventional techniques may be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, delivery, and treatment of patients.

Other aspects of the present disclosure relate to isolated nucleic acid molecules comprising a nucleotide sequence encoding any of the binding proteins described herein. In some embodiments, the isolated nucleic acid molecules comprise a sequence that is at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical.

Certain aspects of the present disclosure relate to kits of polynucleotides. In some embodiments, one or more of the polynucleotides is a vector {e.g., an expression vector}. The kits may find use, inter alia, in producing one or more of the binding proteins described herein, e.g., a bi-, or trispecific binding protein of the present disclosure. In some embodiments, the kit comprises one, two, three, or four polynucleotides.

In some embodiments, the isolated nucleic acid is operably linked to a heterologous promoter to direct transcription of the binding protein-coding nucleic acid sequence. A promoter may refer to nucleic acid control sequences which direct transcription of a nucleic acid. A first nucleic acid sequence is operably linked to a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence of a binding protein if the promoter affects the transcription or expression of the coding sequence. Examples of promoters may include, but are not limited to, promoters obtained from the genomes of viruses (such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus, Simian Virus 40 (SV40), and the like), from heterologous eukaryotic promoters (such as the actin promoter, an immunoglobulin promoter, from heat-shock promoters, and the like), the CAG-promoter (Niwa et al., Gene 108(2): 193-9, 1991), the phosphoglycerate kinase (PGK)-promoter, a tetracycline-inducible promoter (Masui et al., Nucleic Acids Res. 33 :e43, 2005), the lac system, the tip system, the tac system, the trc system, major operator and promoter regions of phage lambda, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, and the promoter of the yeast alpha-mating factors. Polynucleotides encoding binding proteins of the present disclosure may be under the control of a constitutive promoter, an inducible promoter, or any other suitable promoter described herein or other suitable promoter that will be readily recognized by one skilled in the art.

In some embodiments, the isolated nucleic acid is incorporated into a vector. In some embodiments, the vector is an expression vector. Expression vectors may include one or more regulatory sequences operatively linked to the polynucleotide to be expressed. The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Examples of suitable enhancers may include, but are not limited to, enhancer sequences from mammalian genes (such as globin, elastase, albumin, alpha-fetoprotein, insulin and the like), and enhancer sequences from a eukaryotic cell virus (such as SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, adenovirus enhancers, and the like). Examples of suitable vectors may include, for example, plasmids, cosmids, episomes, transposons, and viral vectors (e.g., adenoviral, vaccinia viral, Sindbis-viral, measles, herpes viral, lentiviral, retroviral, adeno-associated viral vectors, etc.). Expression vectors can be used to transfect host cells, such as, for example, bacterial cells, yeast cells, insect cells, and mammalian cells.

Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art and can be used to transfect any cell of interest.

Other aspects of the present disclosure relate to a vector system comprising one or more vectors encoding a first, second, third, and fourth polypeptide chain of any of the binding proteins described herein. In some embodiments, the vector system comprises a first vector encoding the first polypeptide chain of the binding protein, a second vector encoding the second polypeptide chain of the binding protein, a third vector encoding the third polypeptide chain of the binding protein, and a fourth vector encoding the fourth polypeptide chain of the binding protein. In some embodiments, the vector system comprises a first vector encoding the first and second polypeptide chains of the binding protein, and a second vector encoding the third and fourth polypeptide chains of the binding protein. In some embodiments, the vector system comprises a first vector encoding the first and third polypeptide chains of the binding protein, and a second vector encoding the second and fourth polypeptide chains of the binding protein. In some embodiments, the vector system comprises a first vector encoding the first and fourth polypeptide chains of the binding protein, and a second vector encoding the second and third polypeptide chains of the binding protein. In some embodiments, the vector system comprises a first vector encoding the first, second, third, and fourth polypeptide chains of the binding protein. The one or more vectors of the vector system may be any of the vectors described herein. In some embodiments, the one or more vectors are expression vectors.

Isolated host cells

Other aspects of the present disclosure relate to an isolated host cell comprising one or more isolated polynucleotides, polynucleotide kits, vectors, and/or vector systems described herein. In some embodiments, the host cell is a bacterial cell (e.g., a *E. coli* cell). In some embodiments, the host cell is a yeast cell (e.g., an *S. cerevisiae* cell). In some embodiments, the host cell is an insect cell. Examples of insect host cells may include, for example, *Drosophila* cells (e.g., S2 cells), *Trichoplusia ni* cells (e.g., High Five™ cells), and *Spodoptera frugiperda* cells (e.g., Sf21 or Sf9 cells). In some embodiments, the host cell is a mammalian cell. Examples of mammalian host cells may include, for example, human embryonic kidney cells (e.g., 293 or 293 cells subcloned for growth in suspension culture), Expi293™ cells, CHO cells, baby hamster kidney cells (e.g., BHK, ATCC CCL 10), mouse Sertoli cells (e.g., TM4 cells), monkey kidney cells (e.g., CV1 ATCC CCL 70), African green monkey kidney cells (e.g., VERO-76, ATCC CRL-1587), human cervical carcinoma cells (e.g., HELA, ATCC CCL 2), canine kidney cells (e.g., MDCK, ATCC CCL 34), buffalo

rat liver cells (e.g., BRL 3A, ATCC CRL 1442), human lung cells (e.g., W138, ATCC CCL 75), human liver cells (e.g., Hep G2, HB 8065), mouse mammary tumor cells (e.g., MMT 060562, ATCC CCL51), TRI cells, MRC 5 cells, FS4 cells, a human hepatoma line (e.g., Hep G2), and myeloma cells (e.g., NS0 and Sp2/0 cells).

Other aspects of the present disclosure relate to a method of producing any of the binding proteins described herein. In some embodiments, the method includes a) culturing a host cell (e.g., any of the host cells described herein) comprising an isolated nucleic acid, vector, and/or vector system (e.g., any of the isolated nucleic acids, vectors, and/or vector systems described herein) under conditions such that the host cell expresses the binding protein; and b) isolating the binding protein from the host cell. Methods of culturing host cells under conditions to express a protein are well known to one of ordinary skill in the art. Methods of isolating proteins from cultured host cells are well known to one of ordinary skill in the art, including, for example, by affinity chromatography (e.g., two step affinity chromatography comprising protein A affinity chromatography followed by size exclusion chromatography).

In some embodiments, a binding protein of the present disclosure is purified by protein A affinity chromatography.

15 Linkers

In some embodiments, the linkers L1, L2, L3 and L4 range from no amino acids (length=0) to about 100 amino acids long, or less than 100, 50, 40, 30, 20, or 15 amino acids or less. The linkers can also be 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids long. L1, L2, L3 and L4 in one binding protein may all have the same amino acid sequence or may all have different amino acid sequences.

20 Examples of suitable linkers include a single glycine (Gly) residue; a diglycine peptide (Gly-Gly); a tripeptide (Gly-Gly-Gly); a peptide with four glycine residues; a peptide with five glycine residues; a peptide with six glycine residues; a peptide with seven glycine residues; and a peptide with eight glycine residues. Other combinations of amino acid residues may be used such as the peptide GGGT (SEQ ID NO: 297), or the GGGGS, or repetitions of said peptides, such as the peptide GGGGS GGGGS GGGGS (SEQ ID NO: 298). In a preferred embodiment, the linker has an amino acid sequence of SEQ ID NO: 298.

The examples listed above are not intended to limit the scope of the disclosure in any way, and linkers comprising randomly selected amino acids selected from the group consisting of valine, leucine, isoleucine, serine, threonine, lysine, arginine, histidine, aspartate, glutamate, asparagine, glutamine,

glycine, and proline have been shown to be suitable in the binding proteins. For additional descriptions of linker sequences, see, e.g., WO2012135345 and International Application No. PCT/US2017/027488.

The identity and sequence of amino acid residues in the linker may vary depending on the type of secondary structural element necessary to achieve in the linker. For example, glycine, serine, and alanine are best for linkers having maximum flexibility. Some combination of glycine, proline, threonine, and serine are useful if a more rigid and extended linker is necessary. Any amino acid residue may be considered as a linker in combination with other amino acid residues to construct larger peptide linkers as necessary depending on the desired properties.

Fc regions and constant domains

The present invention also relates to a bispecific antibody comprising an Fc region. In some embodiments, a binding protein of the present disclosure comprises an antibody fragment, including but not limited to antibody F(ab), F(ab')₂, Fab'-SH, Fv, or scFv fragments. In some embodiments, a binding protein of the present disclosure comprises an antibody fragment, including but not limited to antibody F(ab), F(ab')₂, Fab'-SH, Fv, or scFv fragments, comprising an Fc region.

In some embodiments, a binding protein of the present disclosure comprises a full-length antibody heavy chain or a polypeptide chain comprising an Fc region. In some embodiments, the Fc region is a human Fc region, e.g., a human IgG1, IgG2, IgG3, or IgG4 Fc region. In some embodiments, the Fc region includes an antibody hinge, CH1, CH2, CH3, and optionally CH4 domains. In some embodiments, the Fc region is a human IgG1 Fc region. In some embodiments, the Fc region is a human IgG4 Fc region. In some embodiments, the Fc region includes one or more of the mutations described herein.

In some embodiments, a binding protein of the present disclosure includes one or two Fc variants. The term "Fc variant" as used herein refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn (neonatal Fc receptor). Exemplary Fc variants, and their interaction with the salvage receptor, are known in the art. Thus, the term "Fc variant" can comprise a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises regions that can be removed because they provide structural features or biological activity that are not required for the antibody-like binding proteins of the invention. Thus, the term "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues, or in which one or more Fc sites or residues has been modified, that affect or are involved in: (1) disulfide bond formation, (2)

incompatibility with a selected host cell, (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody- dependent cellular cytotoxicity (ADCC).

5 The term "parent antibody" or "parent immunoglobulin" as used herein includes an unmodified antibody that is subsequently modified to generate a variant. Said parent antibody may be a naturally occurring antibody, a non-naturally occurring antibody, or a variant or engineered version of a naturally occurring antibody. Parent antibody may refer to the antibody itself, compositions that comprise the parent antibody, or the amino acid sequence that encodes it. In preferred embodiments of the present invention, the parent antibody comprises an Fc region. More specifically, the Fc region of the parent antibody
10 according to the present invention is a human IgG1, IgG2, IgG3, or IgG4 Fc region; in some embodiments the Fc region of the parent antibody according to the present invention is a modified or not modified IgG1 Fc region.

In certain aspects, the bispecific antibody of the present invention comprises a variant Fc region which comprises at least one amino acid modification relative to the Fc region of the parent antibody, whereas
15 the antibody comprising the variant Fc region exhibits altered effector function compared to the parent antibody. More specifically the CH2 domain of the Fc region comprises at least one amino acid modification.

In particular embodiments of the present invention, the bispecific antibody is constructed using the BEAT® heavy chain (Hc) heterodimerization technology previously described (Skegro *et al.*, (2017) J Biol Chem
20 292(23): 9745-9759 and Stutz *et al.*, (2020) J Biol Chem 295(28): 9392-9408, WO2012131555), wherein the BEAT(A) chain, also referred herein to as BEAT(A), and the BEAT (B) chain, also referred herein to as BEAT(B), have been engineered to as to increase the Fc effector function. More specifically the CH2 domain of the Fc region has been engineered so as to comprise at least one amino acid modification. More specifically BEAT(A) comprises one or more substitutions at a position selected from the group
25 comprising: 324, 334, 269, 298, 239, 332 and 333; and BEAT(B) comprises one or more substitutions at a position selected from the group comprising: 324, 334, 269, 298, 239, 332 and 333; preferably comprising 324, 334, 269, 289, 298, 333. Even more specifically BEAT(A) comprises one or more substitutions selected from the group comprising: S324N, K334E, K334A, E269D, S298A, S239D, I332E and E333A; and BEAT(B) comprises one or more substitutions at a position selected from the group comprising: S324N, K334E,
30 K334A, E269D, S289A, K334A, E333A. In certain particular embodiments, BEAT(A) comprises a set of

mutations selected from the group comprising: S324N; or S324N and K334E; or E269D, S298A, S324N and K334A; or S239D, I332E and S324N; or E269D, S298A, S324N and E334A; or S298A, S324N and E333A; or S298A, S324N and K334A; or S324N, S298A, E269D and E333A; or S324N, S298A, E269D and K334A. In other particular embodiments, BEAT(B) comprises a set of mutations selected from the group comprising:

5 S324N; or S324N and K334E; or E269D, S298A, S324N and K334A; or S239D, I332E and S324N; or E269D; or E269D, S298A, S324N and E334A; or S298A, S324N and E333A; or S298A, S324N and K334A; or S324N, S298A, E269D and E333A; or S324N, S298A, E269D and K334A. In certain preferred embodiments, BEAT(A) and BEAT(B) comprises the mutation S324N; or the mutations S324N and K334E; or the mutations E269D, S298A, S324N and K334A; or the mutations E269D, S298A, S324N and E334A; or the mutations S298A, S324N and E333A; or the mutations S298A, S324N and K334A; or the mutations S324N and K334E. In particularly preferred embodiments, BEAT(A) comprises the mutations S239D, I332E and S324N, and BEAT(B) comprises the mutation S324N.

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In some embodiments, the Fc region comprises one or more mutations that reduce or eliminate Fc receptor binding and/or effector function of the Fc region (e.g., Fc receptor- mediated antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), and/or antibody-dependent cellular cytotoxicity (ADCC)).

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In some embodiments, the Fc region is a human IgG1 Fc region comprising one or more amino acid substitutions at positions corresponding to positions 234, 235, and/or 329 of human IgG1 according to EU Index. In some embodiments, the amino acid substitutions are L234A, L235A, and/or P329A. In some

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embodiments, the Fc region is a human IgG1 Fc region comprising amino acid substitutions at positions corresponding to positions 298, 299, and/or 300 of human IgG1 according to EU Index. In some embodiments, the amino acid substitutions are S298N, T299A, and/or Y300S.

In some embodiments, the Fc region is a human IgG4 Fc region comprising one or more mutations that reduce or eliminate FcγI and/or FcγII binding. In some embodiments, the Fc region is a human IgG4 Fc region comprising one or more mutations that reduce or eliminate FcγI and/or FcγII binding but do not affect FcRn binding. In some embodiments, the Fc region is a human IgG4 Fc region comprising amino acid substitutions at positions corresponding to positions 228 and/or 409 of human IgG4 according to EU Index. In some embodiments, the amino acid substitutions are S228P and /or R409K. In some embodiments, the Fc region is a human IgG4 Fc region comprising amino acid substitutions at positions corresponding to

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positions 234 and/or 235 of human IgG4 according to EU Index. In some embodiments, the amino acid

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substitutions are F234A and/or L235A. In some embodiments, the Fc region is a human IgG4 Fc region comprising amino acid substitutions at positions corresponding to positions 228, 234, 235, and/or 409 of human IgG4 according to EU Index. In some embodiments, the amino acid substitutions are S228P, F234A, L235A, and /or R409K. In some embodiments, the Fc region is a human IgG4 Fc region comprising amino acid substitutions at positions corresponding to positions 233-236 of human IgG4 according to EU Index. In some embodiments, the amino acid substitutions are E233P, F234V, L235A, and a deletion at 236. In some embodiments, the Fc region is a human IgG4 Fc region comprising amino acid mutations at substitutions corresponding to positions 228, 233-236, and/or 409 of human IgG4 according to EU Index. In some embodiments, the amino acid mutations are S228P; E233P, F234V, L235A, and a deletion at 236; and /or R409K.

In some embodiments, a binding protein of the present disclosure comprises one or more mutations to improve purification, e.g., by modulating the affinity for a purification reagent. For example, it is known that heterodimeric binding proteins can be selectively purified away from their homodimeric forms if one of the two Fc regions of the heterodimeric form contains mutation(s) that reduce or eliminate binding to Protein A, because the heterodimeric form will have an intermediate affinity for Protein A-based purification than either homodimeric form and can be selectively eluted from Protein A, e.g., by use of a different pH (See e.g., Smith, E.J. et al. (2015) Sci. Rep. 5: 17943). In some embodiments, the mutation comprises substitutions at positions corresponding to positions 435 and 436 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are H435R and Y436F. In some embodiments, the binding protein comprises a second polypeptide chain further comprising a first Fc region linked to CH1, the first Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains, and a third polypeptide chain further comprising a second Fc region linked to CH1, the second Fc region comprising an immunoglobulin hinge region CH2 and CH3 immunoglobulin heavy chain constant domains; and wherein only one of the first and the second Fc regions comprises amino acid substitutions at positions corresponding to positions 435 and 436 of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are H435R and Y436F. In some embodiments, a binding protein of the present disclosure comprises knob and hole mutations and one or more mutations to improve purification. In some embodiments, the first and/or second Fc regions are human IgG1 Fc regions. In some embodiments, the first and/or second Fc regions are human IgG4 Fc regions.

To improve the yields of some binding proteins (e.g., bispecific or trispecific binding proteins), the CH domains can be altered by the BEAT technology which is described in detail with several examples in and in International Publication No. WO2012131555.

In some embodiments, a binding protein of the present disclosure comprises one or more mutations to
5 improve serum half-life (See e.g., Hinton, P R. et al. (2006) J. Immunol. 176(l):346-56). In some
embodiments, the mutation comprises substitutions at positions corresponding to positions 428 and 434
of human IgG1 or IgG4 according to EU Index, wherein the amino acid substitutions are M428L and N434S.
In some embodiments, the binding protein comprises a second polypeptide chain further comprising a
first Fc region linked to CH1, the first Fc region comprising an immunoglobulin hinge region and CH2 and
10 CH3 immunoglobulin heavy chain constant domains, and a third polypeptide chain further comprising a
second Fc region linked to CH1, the second Fc region comprising an immunoglobulin hinge region and CH2
and CH3 immunoglobulin heavy chain constant domains, wherein the first and/or second Fc regions
comprise amino acid substitutions at positions corresponding to positions 428 and 434 of human IgG1 or
IgG4 according to EU Index, wherein the amino acid substitutions are M428L and N434S. In some
15 embodiments, a binding protein of the present disclosure comprises knob and hole mutations and one or
more mutations to improve serum half-life. In some embodiments, the first and/or second Fc regions are
human IgG1 Fc regions. In some embodiments, the first and/or second Fc regions are human IgG4 Fc
regions.

In some embodiments, a binding protein of the present disclosure comprises one or more mutations to
20 reduce effector function, e.g., Fc receptor-mediated antibody -dependent cellular phagocytosis (ADCP),
complement-dependent cytotoxicity (CDC), and/or antibody- dependent cellular cytotoxicity (ADCC). In
some embodiments, the second polypeptide chain further comprises a first Fc region linked to CH1, the
first Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain
constant domains; wherein the third polypeptide chain further comprises a second Fc region linked to
25 CH1, the second Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin
heavy chain constant domains; wherein the first and second Fc regions are human IgG1 Fc regions; and
wherein the first and the second Fc regions each comprise amino acid substitutions at positions
corresponding to positions 234 and 235 of human IgG1 according to EU Index, wherein the amino acid
substitutions are L234A and L235A. In some embodiments, the Fc regions of the second and the third
30 polypeptide chains are human IgG1 Fc regions, and wherein the Fc regions each comprise amino acid
substitutions at positions corresponding to positions 234 and 235 of human IgG1 according to EU Index,

wherein the amino acid substitutions are L234A and L235A. In some embodiments, the second polypeptide chain further comprises a first Fc region linked to CH1, the first Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; wherein the third polypeptide chain further comprises a second Fc region linked to CH1, the second Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; wherein the first and second Fc regions are human IgG1 Fc regions; and wherein the first and the second Fc regions each comprise amino acid substitutions at positions corresponding to positions 234, 235, and 329 of human IgG1 according to EU Index, wherein the amino acid substitutions are L234A, L235A, and P329A. In some embodiments, the Fc regions of the second and the third polypeptide chains are human IgG1 Fc regions, and wherein the Fc regions each comprise amino acid substitutions at positions corresponding to positions 234, 235, and 329 of human IgG1 according to EU Index, wherein the amino acid substitutions are L234A, L235A, and P329A. In some embodiments, the Fc regions of the second and the third polypeptide chains are human IgG4 Fc regions, and the Fc regions each comprise amino acid substitutions at positions corresponding to positions 234 and 235 of human IgG4 according to EU Index, wherein the amino acid substitutions are F234A and L235A. In some embodiments, the binding protein comprises a second polypeptide chain further comprising a first Fc region linked to CH1, the first Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains, and a third polypeptide chain further comprising a second Fc region linked to CH1, the second Fc region comprising an immunoglobulin hinge region and CH2 and CH3 immunoglobulin heavy chain constant domains; and wherein the first and the second Fc regions each comprise amino acid substitutions at positions corresponding to positions 234 and 235 of human IgG4 according to EU Index, wherein the amino acid substitutions are F234A and L235A.

In some embodiments, a binding protein of the present disclosure comprises knob and hole mutations and one or more mutations to reduce effector function. In some embodiments, the first and/or second Fc regions are human IgG1 Fc regions. In some embodiments, the first and/or second Fc regions are human IgG4 Fc regions. For further description of Fc mutations at position 329, see, e.g., Shields, R.L. et al. (2001) J. Biol. Chem. 276:6591-6604 and WO 1999051642.

Application of the binding protein

The binding proteins can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays for the detection and quantitation

of one or more target antigens. The binding proteins will bind the one or more target antigens with an affinity that is appropriate for the assay method being employed.

For diagnostic applications, in certain embodiments, binding proteins can be labeled with a detectable moiety. The detectable moiety can be any one that is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety can be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , ^{125}I , ^{99}Tc , ^{111}In , or ^{67}Ga ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, β -galactosidase, or horseradish peroxidase.

The binding proteins are also useful for in vivo imaging. A binding protein labeled with a detectable moiety can be administered to an animal, preferably into the bloodstream, and the presence and location of the labeled antibody in the host assayed. The binding protein can be labeled with any moiety that is detectable in an animal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

For clinical or research applications, in certain embodiments, binding proteins can be conjugated to a cytotoxic agent. A variety of antibodies coupled to cytotoxic agents (i.e., antibody-drug conjugates) have been used to target cytotoxic payloads to specific tumor cells. Cytotoxic agents and linkers that conjugate the agents to an antibody are known in the art; see, e.g., Parslow, A.C. et al. (2016) *Biomedicines* 4: 14 and Kalim, M. et al. (2017) *Drug Des. Devel. Ther.* 11 :2265-2276.

The disclosure also relates to a kit comprising a binding protein and other reagents useful for detecting target antigen levels in biological samples. Such reagents can include a detectable label, blocking serum, positive and negative control samples, and detection reagents. In some embodiments, the kit comprises a composition comprising any binding protein, polynucleotide, vector, vector system, and/or host cell described herein. In some embodiments, the kit comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing a condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). In some embodiments, the label or package insert indicates that the composition is used for

preventing, diagnosing, and/or treating the condition of choice. Alternatively, or additionally, the article of manufacture or kit may further comprise a second (or third) container comprising a pharmaceutically acceptable buffer, such as bacteriostatic water for injection (BWI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In some embodiments, a binding protein of the present disclosure is administered to a patient in need thereof for the treatment or prevention of cancer. In some embodiments, the present disclosure relates to a method of preventing and/or treating a proliferative disease or disorder (e.g., cancer). In some embodiments, the method comprises administering to a patient a therapeutically effective amount of the heterodimeric antibody, or pharmaceutical compositions related thereto, described herein. In some embodiments, the present disclosure relates to uses of the heterodimeric antibody, or pharmaceutical compositions related thereto, described herein for preventing and/or treating a proliferative disease or disorder (e.g., cancer) in a patient in need thereof. In some embodiments, the present disclosure relates to the heterodimeric antibody, or pharmaceutical compositions related thereto, described herein for use in the manufacture of a medicament for preventing and/or treating a proliferative disease or disorder (e.g., cancer) in a patient in need thereof. In some embodiments, the patient is a human. In some embodiments, the binding protein comprises one antigen binding site that binds a T-cell surface protein and another antigen binding site that binds the extracellular domain of a human CD38 polypeptide. In some embodiments, the binding protein comprises an antigen binding site that binds the extracellular domain of a human CD38 polypeptide and an antigen binding site that binds a human CD47 polypeptide.

In some embodiments, cells of the cancer express a human CD38 1 polypeptide on their cell surface (e.g., comprising the amino acid sequence of SEQ ID NO: 1). In some embodiments, cells of the cancer express a human CD38 isoform SEQ ID NO: 295 (human CD38 isoform 28907-1), SEQ ID NO: 294 (human CD38 isoform 28907-2), SEQ ID NO: 296 (human CD38 isoform 28907-E). In some embodiments, the patient is selected for treatment on the basis that the cells of the cancer express a human CD38 isoform SEQ ID NO: 295 (human CD38 isoform 28907-1), SEQ ID NO: 294 (human CD38 isoform 28907-2), SEQ ID NO: 296 (human CD38 isoform 28907-E).

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In certain embodiments, the cancer is selected from the group comprising: for use in treating multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia,

lymphoma, breast cancer such as Her2+ breast cancer, prostate cancer, cervical cancer, germinal center B-cell lymphoma or B-cell acute lymphoblastic leukemia, Chronic lymphocytic leukemia (CLL), Myelodysplastic syndrome (MDS), Non-Hodgkin lymphoma, diffuse large B-cell lymphoma, non small cell lung cancer (NSCLC), Hepatocellular carcinoma (HCC), High-grade serous ovarian carcinoma, peritoneal cancer. In a specific embodiment the cancer is multiple myeloma. Anti-CD38 antibodies have been tested for the treatment of multiple myeloma, such as Daratumumab, also called herein Darzalex® and isatuximab. However, while multiple myeloma is considered treatable, relapse is inevitable in almost all patients, leading to the development of treatment-refractory disease. In some embodiments, the cancer is relapsed or refractory multiple myeloma. In some embodiments, the patient has been treated with a prior multiple myeloma treatment. In some embodiments, a binding protein of the present disclosure is administered to the patient as a 1st, 2nd, or 3rdline treatment for multiple myeloma.

In certain embodiments, the bispecific antibody according to the present invention which has at least one binding portion that binds to CD47 allows blocking the interaction between CD47 and SIRPα, avoiding the transmission of a “Don’t eat me” signal due to CD47-SIRPα interaction, which inhibits macrophages phagocytosis. An exemplary clinical benchmark monoclonal antibody which binds to CD47 is Magrolimab, also called herein 5F9. In the present invention the terms “Magrolimab” and “5F9” are used interchangeably. In certain embodiments the tested molecule is a “59F-like” (also called herein “Magrolimab-like”) antibody which is the same as the clinical Magrolimab molecule but it is produced in house. In the present invention the terms “Magrolimab”, “5F9”, “59F-like” and “Magrolimab-like” are interchangeable. More in particular the bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47, blocks the CD47-SIRPα interaction and therefore the “Don’t eat me” signal selectively in CD38 expressing cells.

In certain embodiments, the bispecific antibody according to the present invention shows higher binding to CD38 high tumor cell lines (such as Daudi, Raji, KMS-12-PE) as compared to benchmark Darzalex and 5F9, *in vitro*. In other embodiments the bispecific antibody according to the present invention shows higher binding to CD38 low tumor cell lines (NCI-H929 and KMS-12-BM) as compared to Darzalex. In certain embodiments, the bispecific antibodies according to the present invention show a comparable efficiency as that of 5F9 at inducing phagocytosis of both CD38^{high} or CD38^{low} expressing tumor cells, *in vitro*. In certain embodiments, the bispecific antibodies according to the present invention show higher tumor cells killing by CDC as compared to Darzalex, *in vitro*. In certain embodiments, the bispecific antibodies according to the present invention show comparable killing by ADCC of CD38^{high} or CD38^{low}

expressing tumor cells as that induced by Darzalex and on CD38^{low} expressing tumor cells higher potency, *in vitro*. In certain embodiments, the bispecific antibodies according to the present invention show higher potency as compared to the combination of Darzalex and 5F9, *in vitro*.

5 Binding protein therapeutic compositions and administration thereof

Therapeutic or pharmaceutical compositions comprising binding proteins are within the scope of the disclosure. Such therapeutic or pharmaceutical compositions can comprise a therapeutically effective amount of a binding protein, or binding protein-drug conjugate, in admixture with a pharmaceutically or physiologically acceptable formulation agent selected for suitability with the mode of administration.

- 10 Acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed.

The pharmaceutical composition can contain formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine, or lysine), antimicrobials, antioxidants (such as ascorbic acid, sodium sulfite, or sodium hydrogen-sulfite), buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids), bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)), complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin, or hydroxypropyl-beta-cyclodextrin), fillers, monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose, or dextrans), proteins (such as serum albumin, gelatin, or immunoglobulins), coloring, flavoring and diluting agents, emulsifying agents, hydrophilic polymers (such as polyvinylpyrrolidone), low molecular weight polypeptides, salt-forming counterions (such as sodium), preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, or hydrogen peroxide), solvents (such as glycerin, propylene glycol, or polyethylene glycol), sugar alcohols (such as mannitol or sorbitol), suspending agents, surfactants or wetting agents (such as pluronics; PEG; sorbitan esters; polysorbates such as polysorbate 20 or polysorbate 80; triton; tromethamine; lecithin; cholesterol or tyloxapal), stability enhancing agents (such as sucrose or sorbitol), tonicity enhancing agents (such as alkali metal halides - preferably sodium or potassium chloride - or mannitol sorbitol), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants (see, e.g., REMINGTON'S PHARMACEUTICAL SCIENCES (18th Ed., A.R. Gennaro, ed., Mack

Publishing Company 1990), and subsequent editions of the same, incorporated herein by reference for any purpose).

The optimal pharmaceutical composition will be determined by a skilled artisan depending upon, for example, the intended route of administration, delivery format, and desired dosage. Such compositions
5 can influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the binding protein.

The primary vehicle or carrier in a pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier for injection can be water, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for
10 parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles.

Other exemplary pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute. In one embodiment of the disclosure, binding protein compositions can be prepared for storage by mixing the selected composition
15 having the desired degree of purity with optional formulation agents in the form of a lyophilized cake or an aqueous solution. Further, the binding protein can be formulated as a lyophilizate using appropriate excipients such as sucrose.

The pharmaceutical compositions of the disclosure can be selected for parenteral delivery or subcutaneous. Alternatively, the compositions can be selected for inhalation or for delivery through the
20 digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

25 When parenteral administration is contemplated, the therapeutic compositions for use can be in the form of a pyrogen-free, parenterally acceptable, aqueous solution comprising the desired binding protein in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which a binding protein is formulated as a sterile, isotonic solution, properly preserved.

Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which can then be delivered via a depot injection. Hyaluronic acid can also be used, and this can have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In one embodiment, a pharmaceutical composition can be formulated for inhalation. For example, a binding protein can be formulated as a dry powder for inhalation. Binding protein inhalation solutions can also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions can be nebulized.

It is also contemplated that certain formulations can be administered orally. In one embodiment of the disclosure, binding proteins that are administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the binding protein. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Another pharmaceutical composition can involve an effective quantity of binding proteins in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions of the disclosure will be evident to those skilled in the art, including formulations involving binding proteins in sustained- or controlled-delivery formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. Additional examples of sustained-release preparations include semipermeable polymer

matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides, copolymers of L-glutamic acid and gamma ethyl -L-glutamate, poly(2-hydroxyethyl-methacrylate), ethylene vinyl acetate, or poly-D(-)-3-hydroxybutyric acid. Sustained-release compositions can also include liposomes, which can be prepared by any of several methods known in the art.

Pharmaceutical compositions to be used for in vivo administration typically must be sterile. This can be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using this method can be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration can be stored in lyophilized form or in a solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. Such formulations can be stored either in a ready-to-use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration.

The disclosure also encompasses kits for producing a single-dose administration unit. The kits can each contain both a first container having a dried protein and a second container having an aqueous formulation. Also included within the scope of this disclosure are kits containing single and multi-chambered pre-filled syringes (e.g., liquid syringes and lyosyringes).

The effective amount of a binding protein pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the binding protein is being used, the route of administration, and the size (body weight, body surface, or organ size) and condition (the age and general health) of the patient. Accordingly, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect.

Dosing frequency will depend upon the pharmacokinetic parameters of the binding protein in the formulation being used. Typically, a clinician will administer the composition until a dosage is reached that

achieves the desired effect. The composition can therefore be administered as a single dose, as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed
5 by them. Appropriate dosages can be ascertained through use of appropriate dose-response data.

The route of administration of the pharmaceutical composition is in accord with known methods, e.g., orally; through injection by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, intraocular, intraarterial, intraportal, or intralesional routes; by
10 sustained release systems; or by implantation devices. Where desired, the compositions can be administered by bolus injection or continuously by infusion, or by implantation device.

The composition can also be administered locally via implantation of a membrane, sponge, or other appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed- release bolus, or continuous administration.

15 The disclosure also provides a method of treating multiple myeloma in a subject in need thereof. The method employs a heterodimeric antibody that co-engages CD47 and CD38 in such a manner so as to transiently connect malignant cells with T cells, thereby inducing T cell mediated killing of the bound malignant cell. The method described herein utilizes a heterodimeric antibody that binds CD47 and CD38 in such a manner so as to maximize destruction of target cells while reducing unwanted side effects (e.g.,
20 uncontrolled cytokine release).

Therapeutic regimen

The heterodimeric antibody is administered to a subject in need thereof, e.g., a human subject suffering from multiple myeloma, such as relapsed/refractory multiple myeloma. Relapsed myeloma is characterized as a recurrence of disease after prior response. Examples of laboratory and radiological
25 criteria signaling the disease include, but are not limited to, > 25% increase of the serum or urine monoclonal protein (M-protein) or > 25% difference between involved and uninvolved serum free light chains from nadir, respectively, or the development of new plasmacytomas or hypercalcemia. Sonneveld et al., Haematologica. 2016 Apr; 101(4): 396-406.

In non-secretory disease patients, relapse is characterized by an increase of the bone marrow plasma cells. A signal for relapsed disease also is characterized by the appearance or reappearance of one or more CRAB criteria or a rapid and consistent biochemical relapse. Refractory myeloma is myeloma that is not responsive to treatment. Relapsed/refractory multiple myeloma refers to the disease which becomes
5 non-responsive or progressive on therapy or within 60 days of the last treatment in patients who previously achieved at least a minimal response on previous therapy. Sonneveld, supra; Anderson et al., Leukemia. 2008;22(2):231-239.

The method of the disclosure comprises administering to the subject a dose of about 0.05 mg to about 200 mg of the heterodimeric antibody. The dose is, in various embodiments, about 0.5 mg to about 200
10 mg, about 0.5 to about 150 mg, about 1 mg to about 150 mg, about 10 mg to about 100 mg, about 10 mg to about 200 mg, about 4 mg to about 200 mg, about 12 mg to about 200 mg, about 12 mg to about 100 mg, about 36 mg to about 200 mg, about 36 mg to about 100 mg, or about 100 mg to about 200 mg. In various aspects of the method, the dose administered to the subject is about 0.05 mg, about 0.15 mg, about 0.45 mg, about 1.35 mg, about 4 mg, about 12 mg, about 36 mg, about 100 mg, or about 200 mg.

15 In alternative aspects, a single dose of heterodimeric antibody is at least about 0.05 mg, at least about 0.15 mg, at least about 0.45 mg, at least about 1.35 mg, at least about 4 mg, at least about 12 mg, at least about 36 mg, or at least about 100 mg. In various aspects, a single dose of heterodimeric antibody is no more than about 200 mg (e.g., no more than about 100 mg or no more than about 36 mg). It will be appreciated that a single dose may be administered via multiple administrations (i.e., a divided dose),
20 such that the multiple administrations combine to the dose recited herein. For example, multiple administrations (e.g., two or more injections) combine to be at least about 0.05 mg, at least about 0.15 mg, at least about 0.45 mg, at least about 1.35 mg, at least about 4 mg, at least about 12 mg, at least about 36 mg, or at least about 100 mg. In various aspects, the multiple administrations of a single dose of heterodimeric antibody combine to be no more than about 200 mg (e.g., no more than about 100 mg or
25 no more than about 36 mg).

In various aspects of the method, the dose is adjusted over the course of treatment. For example, the subject is administered an initial dose at one or more administrations, and a higher dose is used in one or more subsequent administrations. Put another way, the disclosure contemplates increasing the dose of heterodimeric antibody at least once over the course of treatment. Alternatively, the dose may be

decreased over the course of treatment, such that amount of heterodimeric antibody is reduced as treatment progresses.

The disclosure contemplates a method wherein multiple (i.e., two or more) doses of the heterodimeric antibody are administered over the course of a treatment period. The individual doses may be administered at any interval, such as once a week, twice a week, three times a week, four times a week, or five times a week. Individual doses may be administered every two weeks, every three weeks, or every four weeks. In other words, in some aspects, a waiting period of at two weeks passes between heterodimeric antibody administrations to the subject. The waiting period between administrations of the doses need not be consistent over the course of the treatment period. In other words, the interval between doses can be adjusted over the course of treatment. In some aspects, the method comprises administering two doses of heterodimeric antibody per week to the subject in the first and second weeks of treatment (i.e., twice a week for weeks 1 and 2), administering one dose of heterodimeric antibody per week to the subject in the third and fourth weeks of treatment (i.e., once a week for weeks 3 and 4), and administering one dose of heterodimeric antibody every two weeks starting in week 5 through the end of treatment (i.e., there is a waiting period of two weeks between doses starting in week 5 through the end of treatment). While not wishing to be bound to any particular theory, the shorter interval between doses for the first administrations (e.g., two doses per week) promotes rapid target cell clearance. Increasing the interval between doses as set forth herein maintains cell clearance while minimizing unwanted side effects associated with immunotherapy.

Alternatively, in various aspects, the method comprises administering one dose of heterodimeric antibody per week for weeks 1-4 of treatment, and optionally administering one dose of the heterodimeric antibody every two weeks starting in week 5 through the end of treatment.

The multiple doses of heterodimeric antibody are administered over treatment period of, e.g., three months to about 18 months, or about three months to about 12 months, or about three months to about nine months, or about three months to about six months, or about three months to about eight months, or about six months to about 18 months, or about six months to about 12 months, or about eight months to about 12 months, or about six months to about eight months, or about eight months to about 12 months (e.g., about eight months). Optionally, the multiple (i.e., two or more) doses of the heterodimeric antibody are administered over a treatment period of about 12 weeks to about 52 weeks, or about 12

weeks to about 36 weeks, or about 24 weeks to about 32 weeks, with doses administered twice a week, once a week, once every two weeks, or once every four weeks.

By "treating" multiple myeloma is meant achievement of any positive therapeutic response with respect to the disease. For example, a positive therapeutic response includes one or more of the following improvements in the disease: (1) a reduction in the number of neoplastic cells; (2) an increase in neoplastic cell death; (3) inhibition of neoplastic cell survival; (4) reduction in paraprotein production by tumor cells; (5) inhibition (i.e., slowing to some extent, preferably halting) of tumor growth; (6) an increased patient survival rate; and (7) some relief from one or more symptoms associated with the disease or condition. Tumor response can be assessed for changes in tumor morphology (i.e., overall tumor burden, tumor size, and the like) using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, bone scan imaging, endoscopy, and tumor biopsy sampling including bone marrow aspiration (BMA) and counting of tumor cells in the circulation. A complete therapeutic response (i.e., absence of clinically detectable disease with normalization of any previously abnormal radiographic studies, bone marrow, and cerebrospinal fluid (CSF) or abnormal monoclonal protein) is not required; any degree of improvement is contemplated. Various additional parameters associated with disease treatment and improvement are set forth in the Examples.

The heterodimeric antibody may be administered via any suitable means to the subject, e.g., via intravenous, intraarterial, intralymphatic, intrathecal, intracerebral, intraperitoneal, intracerebrospinal, intradermal, subcutaneous, intraarticular, intrasynovial, oral, topical, or inhalation routes. For example, the heterodimeric antibody may be administered via intravenous administration as a bolus or by continuous infusion over a period of time. In various aspects, the method comprises administering the heterodimeric antibody via intravenous infusion over a period of about 30 minutes to about four hours. Optionally, the time for infusion is decreased in subsequent administrations. For example, in one embodiment, the first dose of heterodimeric antibody is administered over a period of about four hours, and subsequent doses are administered over a period of two hours or less. In this regard, the first dose of heterodimeric antibody is optionally administered over a period of about four hours, the second dose of heterodimeric antibody is optionally administered over a period of about two hours, and subsequent doses are optionally administered over a period of about 30 minutes.

In some instances, the subject has previously been treated for multiple myeloma. For example, the subject may have previously been administered an immunomodulatory drug (thalidomide, lenalidomide,

pomalidomide), a proteasome inhibitor (such as pomalidomide, bortezomib, or carfilzomib), dexamethasone, doxorubicin, or combinations thereof.

Optionally, the subject was previously treated with an anti-CD38 monospecific antibody, such as daratumumab (DARZALEX®). In various embodiments, the subject is relapsed or refractory with prior anti-
5 CD38 monospecific antibody treatment. When the patient has been treated with anti-CD38 monospecific antibody, the initial dose of the heterodimeric antibody is preferably administered following a wash-out period sufficient to reduce systemic concentration of the anti-CD38 monospecific antibody to 0.2 pg/ml or less. Put another way, the method comprises a waiting period between the previous administration of anti-CD38 monospecific antibody and administration of the heterodimeric antibody. In various
10 embodiments, the method comprises ceasing treatment with the anti-CD38 monospecific antibody for at least 12 weeks (e.g., about 13 to about 15 weeks) prior to administering an initial dose of the heterodimeric antibody.

Co-therapy

Optionally, the heterodimeric antibody is part of a therapeutic regimen that comprises administration of
15 one or more other therapeutic agents, radiation therapy, stem cell transplantation, and the like.

The method of the disclosure optionally further comprises administering dexamethasone to the subject. The dexamethasone may be administered by any route, such as the routes described here. Preferably, the dexamethasone is administered intravenously or orally. When the dexamethasone is administered intravenously, it is optionally administered to the subject within one hour prior to administration of the
20 heterodimeric antibody. The dexamethasone is optionally administered in an amount of about 8 mg or about 4 mg.

In various embodiments, the method of the disclosure further comprises administering a chemotherapeutic agent. Non-limiting examples of DNA damaging chemotherapeutic agents include topoisomerase I inhibitors (e.g., irinotecan, topotecan, camptothecin and analogs or metabolites thereof,
25 and doxorubicin); topoisomerase II inhibitors (e.g., etoposide, teniposide, and daunorubicin); alkylating agents (e.g., melphalan, chlorambucil, busulfan, thiopeta, ifosfamide, carmustine, lomustine, semustine, streptozocin, decarbazine, methotrexate, mitomycin C, and cyclophosphamide); DNA intercalators (e.g., cisplatin, oxaliplatin, and carboplatin); DNA intercalators and free radical generators such as bleomycin;

and nucleoside mimetics (e.g., 5-fluorouracil, capecitabine, gemcitabine, fludarabine, cytarabine, mercaptopurine, thioguanine, pentostatin, and hydroxyurea).

Chemotherapeutic agents that disrupt cell replication include: paclitaxel, docetaxel, and related analogs; vincristine, vinblastin, and related analogs; thalidomide, lenalidomide, and related analogs (e.g., CC-5013 and CC-4047); protein tyrosine kinase inhibitors (e.g., imatinib mesylate and gefitinib); proteasome inhibitors (e.g., bortezomib, CEP-18770, MG132, peptide vinyl sulfones, peptide epoxyketones (such as epoxomicin and carfilzomib), beta-lactone inhibitors (such as lactacystin, MLN 519, NPI-0052, Salinosporamide A), compounds that create dithiocarbamate complexes with metals (such as Disulfiram), and certain antioxidants (such as Epigallocatechin-3-gallate, catechin-3-gallate, and Salinosporamide A); NF- κ B inhibitors, including inhibitors of I κ B kinase; antibodies which bind to proteins overexpressed in cancers and thereby downregulate cell replication (e.g., trastuzumab, rituximab, cetuximab, and bevacizumab); and other inhibitors of proteins or enzymes known to be upregulated, over-expressed or activated in cancers, the inhibition of which downregulates cell replication.

The therapeutic regimen may comprise administration of other antibody therapeutics, such as elotuzumab (a humanized monoclonal against SLAMF7; Tai et al., Blood, 2008;112:1329-37); daratumumab, MOR202, and isatuximab that target CD38; nBT062-SMCC-DML, nBT062-SPDB-DM4, and nBT062-SPP-DML that target CD138; lucatumumab (also known as HCD122) and dacetuzumab (also known as SGN-40) that target CD40; Lorvotuzumab which targets CD56. For a review of antibody therapeutics for the treatment of multiple myeloma, see, e.g., Tandon et al., Oncology & Hematology Review, 2015;11(2):115-21, and Sondergeld et al., Clinical Advances in Hematology & Oncology, 2015; 13(9), 599, both incorporated by reference.

In some embodiments, the heterodimeric antibody is administered prior to, concurrent with, or after treatment with Velcade® (bortezomib), Thalomid™ (thalidomide), Aredia™ (pamidronate), or Zometa™ (zoledronic acid).

All cited references are herein expressly incorporated by reference in their entirety. Whereas particular embodiments of the invention have been described above for purposes of illustration, it will be appreciated by those skilled in the art that numerous variations of the details may be made without departing from the invention as described in the appended claims.

Figures

Figure 1. Blocking of the human CD47/human SIRP α interaction upon binding of anti-CD47-H2 Fab to human CD47 assessed by Octet Bio-Layer Interferometry. Biotinylated human CD47-avi-his protein was loaded on a streptavidin SA Biosensor. Anti-CD47-H2 Fab was injected over immobilized human CD47 at 1 μ M in kinetic buffer to reach saturation of the surface. Then, a pre-mixed solution of anti-CD47 Fab at 1 μ M and human SIRP α at 2 μ M final concentration was injected over saturated surface. Plot shows binding to the sensor tip as a wavelength shift (in nm; Y axis) vs. time (X axis). Curves are labelled by Fab clone name.

Figure 2. Epitope binning of anti-CD38-E2 and anti-CD38-B6 clones to human CD38 using Octet Bio-Layer Interferometry Biotinylated human CD38-avi-his protein was loaded on a streptavidin SA Biosensor. Anti-CD38-E2 Fab was injected over immobilized human CD38 at 200 nM in kinetic buffer to reach saturation of the surface. Then, a pre-mixed solution of anti-CD38-E2 Fab and anti-CD38-B6 Fab at 200 nM final concentration each was injected over saturated surface. Same experimental procedure was performed using anti-CD38-H5 Fab instead of anti-CD38-B6 Fab, as competing control. Plot shows binding to the sensor tip as a wavelength shift (in nm; Y axis) vs. time (X axis). Curves are labelled by competing Fab clone name.

Figure 3. Schematic drawing of a BEAT CD38/CD47 bispecific antibody. A(-) means no PA binding site while A(+) means a PA binding site is present.

Figure 4. CDC assay on Daudi cells comparing Fc engineered variants to the non-enhanced control.

Figure 5. ADCP assay on Raji cells comparing Fc engineered variants to the non-enhanced control. A horizontal line is drawn through the average fold change in phagocytosis for non-enhanced BEAT CD38/CD47-19 to visualize the impact of Fc engineering on ADCP potency.

Figure 6. Schematic drawing of a "2+1" BEAT CD38/CD47 bispecific antibody. "2+1" BEAT bispecific antibodies were engineered analogously to those in the "1+1" format. The "Fc distal" anti-CD38 Fab domain was fused to the N-terminus of the VH domain of the "Fc proximal" anti-CD38 Fab.

Figure 7. CDC assay on Daudi cells comparing CD38/CD47 BEAT antibodies in the "2+1" and "1+1" formats.

Figure 8. ADCP assay on Raji cells comparing CD38/CD47 BEAT antibodies in the “2+1” and “1+1” formats. A horizontal line is drawn through the average fold change in phagocytosis for non-enhanced “1+1” BEAT CD38/CD47-19 to visualize the impact of the “2+1” format and Fc engineering on ADCP potency.

Figure 9. CDC, ADCC and ADCP assay comparing BEAT CD38/CD47-42, -48 and -60.

- 5 Figure 10. Thermostability analysis of CD38/CD47 bispecific antibodies by DSC. A, BEAT CD38/CD47-42. B, BEAT CD38/CD47-48. C, BEAT CD38/CD47-60.

Figure 11. Binding of BEAT CD38/47-48 and 60 was evaluated by flow cytometry on a panel of lymphoma, Daudi (A) and Raji (B), and multiple myeloma cells, KMS-12-PE (C), NCI-H929 (D) and KMS-12-BM (E). BEAT CD38/47-48 and BEAT CD38/47-60 show superior dose dependent binding to tumor cell lines with
10 different level of CD38 and CD47 expression as compared to benchmarks.

Figure 12. (A) BEAT CD38/47-48 and BEAT CD38/47-60 block CD47/SIRPα interaction comparable to high affinity anti-CD47 antibody benchmark, isotype control background displayed as a dotted line; (B) Cumulative analysis of Maximal CD47/SIRPα inhibition and EC50.

Figure 13. ADCP assay performed with CD38^{high} (Daudi) or CD38^{low} (KMS12-BM) tumor cells. (A-C) Two
15 representative curves of phagocytosis of Daudi (A) or KMS12-BM (C) tumor cells. Mean of duplicates +/- Standard Deviations (SD). (B-D) Cumulative analyses of Maximum phagocytosis and EC50 in multiple experiments Daudi (B) or KMS12-BM (D) tumor cells. Mean of n=9 experiments +/- SD is represented. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Figure 14. ADCP assay performed with CD38^{low} (KMS12-BM) tumor cells. (A) Schematic of molecules
20 structures and Fab arms affinities. (B-C) Cumulative analyses of Maximum phagocytosis (B) and EC50 (C) in multiple experiments of KMS12-BM tumor cells. Mean of n=7 experiments +/- SD is represented.

Figure 15. CDC assay performed with CD38 high expressing tumor cells (Daudi). (A) Representative curve
of specific killing. Mean of duplicates is represented here +/- Standard Deviations (SD). (B) Cumulative
25 analyses of Maximum killing and EC50 in multiple experiments. Mean of n=5 experiments +/- SD is represented. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Figure 16. ADCC assay performed with CD38^{high} (Raji) or CD38^{low-int} (NCI-H929) tumor cells. (A-C) Two representative curves of specific killing of Raji (A) or NCI-H929 (C) tumor cells. Mean of duplicates +/- Standard Deviations (SD). (B-D) Cumulative analyses of ADCC Maximum killing and EC50 in multiple experiments in Raji (B) or NCI-H929 (D) tumor cells. (Daudi). Mean of n=3 experiments +/- SD is represented. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Figure 17. MMoAK assay performed with CD38^{low-int} (NCI-H929) tumor cells. (A) Schematic of the assay (B) A representative curve of tumor cell killing by MMoAK. Mean of duplicates +/- Standard Deviations (SD). (C-D) Cumulative analyses of Maximal killing (C) and EC50 (D) in multiple experiments using CD38^{low-int} (NCI-H929) as target cells. Mean of n=9 experiments +/- SD is represented. ADCC = antibody dependent cell cytotoxicity; ADCP = antibody dependent cell phagocytosis; CDC = complement dependent cytotoxicity. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Figure 18. MMoAK assay performed with CD38^{low-int} (NCI-H929) tumor cells in the presence of antigen sink. (A-D) Cumulative analyses of Maximal killing (A-B) and EC50 (C-D) in multiple experiments using CD38^{low-int} (NCI-H929) in the presence of 5 million/ml of RBCs (A-C) or 2.8 ng/ml of sCD38 (B-D) as antigen sink. Mean of n= 3-5 experiments +/- SD is represented. Statistics: paired T-test. NS = not significant RBCs = Red Blood Cells; sCD38 = soluble CD38.

Figure 19. (A-B) MMoAK assay performed with CD38^{low-int} (NCI-H929) tumor cells. (A) Representative curve of phagocytosis of tumor cells and (B) the cumulative analyses of Maximum phagocytosis and EC50 in multiple experiments are represented. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Figure 20. (A-C) Representative experiment showing (A) binding of Darzalex (AF647 conjugated), (B) BEAT 38/47-60 (AF488 conjugated) or (C) a mix of conjugated Darzalex and BEAT 38/47-60 in Raji-CD47KO cells. (D) Percentage of competition with BEAT 38/47-60 (AF488 conjugated) mediated by Control antibody (Cntrl IgG1), Darzalex or BEAT 38/47-60 purified antibodies in Raji-CD47KO cells.

Figure 21. (A-B) ADCP assay performed with CD38^{high} (Daudi) or CD38^{low} (KMS12-BM) tumor cells. Two representative curves of phagocytosis of Daudi (A) or KMS12-BM (B) tumor cells and the cumulative analyses of Maximum phagocytosis and EC50 in multiple experiments Daudi (A) or KMS12-BM (B) tumor cells. (C-D) ADCC assay performed with CD38^{high} (Raji) or CD38^{low-int} (NCI-H929) tumor cells. Two

representative curves of specific killing of Raji (C) or NCI-H929 (D) tumor cells. Graphs show the cumulative analyses of ADCC Maximum killing and EC50 in multiple experiments. (E-F) CDC assay performed with CD38^{high} expressing tumor cells (Daudi). (E) Rerepresentative curve of specific killing and (F) Cumulative analyses of Maximum killing and EC50 in multiple experiments. Mean of duplicates is represented in all
5 representative curves graphs +/- Standard Deviations.

Figure 22. (A) Representative nonlinear sigmoidal regression binding curves (Relative Geometric Mean Fluorescence Intensity) in human healthy donor RBCs analyzed by flow cytometry (n=6). (B) Hemagglutination as measured by Coombs assay (n=6). Extent of agglutination was scored after centrifugation from 0 (no agglutination) to 4 (complete agglutination). (C) Induction of RBCs depletion
10 evaluated in-house using a hematology analyzer (Sigma 5H). (D) Hemolysis quantified by measuring the absorbance of hemoglobin in plasma at 414nm using spectrometry. Results were normalized in GraphPad Prism software to no antibody treatment condition (negative control) and triton X-100 (positive control). (E) Platelet aggregation relative to LeoA1 positive control analyzed by flow cytometry.

Figure 23. Representative nonlinear sigmoidal regression binding curves (Relative Geometric Mean
15 Fluorescence Intensity) in human healthy donor analyzed by flow cytometry upon gating of cell types as depicted on each graph: (A) CD8+T cells, (B) CD4+ T cells, (C) B cells, (D) Granulocytes and (E) Myeloid cells. Binding was evaluated on six different donors.

Figure 24. BEAT CD38/47-48 controlled Raji tumor growth in therapeutic treatment at 10mg/kg. A. Tumor
20 growth (+/- SEM) over time in mice xenografted subcutaneously with Raji tumors (n=5 mice / group) and treated with PBS (black), BEAT CD38/47-48 at 10 mg/kg (dark grey), BEAT CD38/47-48 at 1 mg/kg (grey), BEAT CD38/47-48 at 0,1 mg/kg (light grey). Treatments were administered when tumor reached around 100 mm³. B. Tumor volume (+/-SEM) at day 27. Raj_9 study.

Figure 25. BEAT CD38/47-60 controlled Raji tumor growth in therapeutic treatment at 10mg/kg. A. Tumor
25 growth (+/- SEM) over time in mice xenografted subcutaneously with Raji tumors (n=5 mice / group) and treated with PBS (black), BEAT CD38/47-60 at 10 mg/kg (dark grey), BEAT CD38/47-60 at 1 mg/kg (grey), BEAT CD38/47-60 at 0,1 mg/kg (light grey). Treatments were administered when tumor reached around 100 mm³. B. Tumor volume (+/-SEM) at day 27. Raj_9 study.

Figure 26. BEAT CD38/47-48 controlled Raji tumor growth in therapeutic treatment. A. Tumor growth (+/- SEM) over time in mice xenografted subcutaneously with Raji tumors (n=10 mice / group; 5 mice were

used for ex vivo studies after day 15) and treated with PBS (black), BEAT CD38/47-48 at 10 mg/kg (dark grey), BEAT CD38/47-59 at 10 mg/kg (grey) and Darzalex (square - dotted lines). Treatments were administered when tumor reached around 100 mm³. B. Tumor volume (+/-SEM) at day 36, Statistical analysis performed: one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparisons. P<0.05 was considered as statistically significant. Level of significance is represented by asterisks. Raj_10 study.

Figure 27. BEAT CD38/47-60 controlled Raji tumor growth in therapeutic treatment. A. Tumor growth (+/-SEM) over time in mice xenografted subcutaneously with Raji tumors (n=10 mice / group; 5 mice were used for ex vivo studies after day 15) and treated with PBS (black), BEAT CD38/47-60 at 10 mg/kg (dark grey), BEAT CD38/47-79 at 10 mg/kg (grey) and Darzalex (square - dotted lines). Treatments were administered when tumor reached around 100 mm³. B. Tumor volume (+/-SEM) at day 36, Statistical analysis performed: one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparisons. P<0.05 was considered as statistically significant. Level of significance is represented by asterisks. Raj_10 study.

Figure 28. BEAT CD38/47-48 and BEAT CD38/47-60 both controlled Raji tumor growth in therapeutic treatment. A. Tumor growth (+/-SEM) over time in mice xenografted subcutaneously with Raji tumors (n=10 mice / group; 5 mice were used for ex vivo studies after day 15) and treated with PBS (black), BEAT CD38/47-48 at 10 mg/kg (dark grey), BEAT CD38/47-60 at 10 mg/kg (grey) and Darzalex (square - dotted lines). Treatments were administered when tumor reached around 100 mm³. B. Tumor volume (+/-SEM) at day 36, Statistical analysis performed: one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparisons. P<0.05 was considered as statistically significant. Level of significance is represented by asterisks. Raj_10 study.

Figure 29. IL-1b levels and MHCII+ macrophages in tumors. 16 days after the xenograft, 5 animals per group were sacrificed. Mice serum and tumors were harvested and analyzed ex vivo by Luminex (A) and FACS (B). BEAT CD38/CD47-48 and BEAT CD38/CD47-60 showed a trend of IL-1b decrease level in the tumors (A). BEAT CD38/CD47-60 showed a significant decrease of MHCII+ macrophages in the tumors (B). Statistical analysis performed: one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparisons. P<0.05 was considered as statistically significant. Level of significance is represented by asterisks. Raj_10 study.

Figure 30. BEAT CD38/47-48 controlled KMS-12-BM tumor growth in therapeutic treatment at 10mg/kg. A. Tumor growth (+/- SEM) over time in mice xenografted subcutaneously with KMS-12-BM tumors (n=5 mice / group) and treated with PBS (black), BEAT CD38/47-48 at 10 mg/kg (dark grey), BEAT CD38/47-48 at 1 mg/kg (grey), BEAT CD38/47-48 at 0,1 mg/kg (light grey) and Darzalex (square - dotted lines).
 5 Treatments were administered when tumor reached around 100 mm³. B. Tumor growth since randomization (ΔT) (+/-SEM) at day 37. Statistical analysis performed: one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparisons. P<0.05 was considered as statistically significant. Level of significance is represented by asterisks. KMS_7 study.

Figure 31. BEAT CD38/47-60 controlled KMS-12-BM tumor growth in therapeutic treatment at 10mg/kg. A. Tumor growth (+/- SEM) over time in mice xenografted subcutaneously with KMS-12-BM tumors (n=5 mice / group) and treated with PBS (black), BEAT CD38/47-60 at 10 mg/kg (dark grey), BEAT CD38/47-60 at 1 mg/kg (grey), BEAT CD38/47-60 at 0,1 mg/kg (light grey) and Darzalex (square - dotted lines).
 10 Treatments were administered when tumor reached around 100 mm³. B. Tumor growth since randomization (ΔT) (+/-SEM) at day 37. Statistical analysis performed: one-way analysis of variance (ANOVA) followed by Dunnett's post hoc for multiple comparisons. P<0.05 was considered as statistically significant. Level of significance is represented by asterisks. KMS_7 study.

Example 1: Generation of anti-CD47 antibodies

Material and methods

Recombinant antigens

20 Human codon-optimized sequences encoding the full-length human CD47 (UniProt accession no: Q08722; SEQ ID NO: 7) and full-length cynomolgus monkey CD47 (accession no: A0A2K5X4I2, SEQ ID NO: 8) were synthesized by Twist Biosciences (San Francisco, USA). The soluble extracellular regions of human CD47 (residues 19 to 141; SEQ ID NO: 3) and cynomolgus monkey CD47 (residues 19 to 141; SEQ ID NO: 4) were cloned in a modified pcDNATM3.1 plasmid (ThermoFisher Scientific, catalog NO: V79020) to generate a
 25 protein with a C-terminal AvitagTM (Avidity LLC) followed by a 10-His tag with a Gly₃ linker sequence between the two tags (abbreviated human CD47-ECD-Avi-His and cynomolgus CD47-ECD-Avi-His). For both human and cynomolgus monkey proteins, the cysteine located at position 33 was mutated to a serine in order to prevent multimerization. The expression vector was carrying the murine VJ2C leader peptide to drive product secretion as well as the OriP sequence. For protein expression, the plasmids coding for
 30 human CD47-ECD-Avi-His and cynomolgus CD47-ECD-Avi-His (residues 19 to 141; SEQ ID NO: 3 and SEQ

ID NO: 4, respectively) and a non-coding plasmid were co-transfected into suspension-adapted HEK293-EBNA cells (ATCC-LGL standards; Cat. No: CRL-10852) using Polyethyleneimine (PEI; Polysciences). Briefly, cells were prepared at 8 million cells per ml in RPMI 1640 (Biowest) supplemented with 0.1% Pluronic F-68 (Gibco). Cells were then transfected with a DNA-PEI mixture at 37 °C. Four hours post-transfection, the cell culture was diluted 1:1 in EX-CELL® 293 (Sigma Aldrich) supplemented with Phenol Red and 4 mM L-Glutamine and incubated for 5 days with orbital shaking at 37 °C, 5% CO₂ and 80% humidity. Post-expression, clarified supernatants were prepared by centrifugation and filtration, pH was adjusted at 7.4 (4 °C) using 1 M sodium hydroxide. Ni-Sepharose Excell beads (GE Healthcare) were added to the clarified supernatant and incubated overnight at 4 °C under gentle agitation. Next, the mixtures were loaded on Econo-Columns (Bio-Rad Laboratories) for gravity-flow purification. The beads were first washed in 1X PBS, pH 7.4 (10 CV), then 1X PBS supplemented with 20 mM imidazole (10 CV) and the proteins were eluted, either with a single elution step at 500 mM imidazole (9x1 CV) or following a step-elution protocol using, sequentially, 1X PBS, pH 7.4 supplemented with 40 mM (9x1 CV), 80 mM (11x1 CV), 250 mM (5x1 CV) and 500 mM (3x1 CV) imidazole. Fractions were pooled and dialyzed twice against 1X PBS, pH 7.4 at 4 °C. Protein quality was assessed by SDS-PAGE, SE-HPLC, endotoxin measurement and SPR as described above. Human and cynomolgus monkey CD47-ECD-Avi-His tagged fusion proteins as described herein have SEQ ID NO: 3 and 4, respectively.

Human codon-optimized sequence encoding the full-length human SIRPα (UniProt accession no: P78324-2) was synthesized by Twist Bioscience. The soluble extracellular regions of human SIRPα (residues 31 to 352; SEQ ID NO: 9) was cloned in a modified pcDNA™3.1 plasmid (ThermoFisher Scientific, catalog NO: V79020) to generate a protein with a C-terminal Avitag™ (Avidity LLC) followed by a 10-His tag with a Gly₃ linker sequence between the two tags (abbreviated human SIRPα -ECD-Avi-His). The expression vector was carrying the murine VJ2C leader peptide to drive product secretion as well as the OriP sequence. For protein expression, the plasmids coding for human SIRPα -ECD-Avi-His (residues 31 to 352; SEQ ID NO: 9) and a non-coding plasmid were co-transfected into suspension-adapted HEK293-EBNA cells (ATCC-LGL standards; Cat. No: CRL-10852) using Polyethyleneimine (PEI; Polysciences) as described above. Post expression, Ni-Sepharose Excell beads (GE Healthcare) were added to the clarified supernatant and incubated overnight at 4 °C under gentle agitation. Next, the mixtures were loaded on Econo-Columns (Bio-Rad Laboratories) for gravity-flow purification. The beads were first washed in 1X PBS, pH 7.4 (10 CV), then 1X PBS supplemented with 20 mM imidazole (10 CV) and the proteins were eluted with a single elution step at 500 mM imidazole (6x2 CV). Buffer exchange to 1X PBS, pH 7.4 was performed on PD-10

columns (GE). Protein quality was assessed by SDS-PAGE, SE-HPLC, endotoxin measurement and SPR as described above. Human SIRP α -ECD-Avi-His tagged fusion proteins as described herein have SEQ ID NO: 9.

Recombinant cell lines

5 The human codon-optimized sequences of the full-length human CD47 (UniProt sequence ID Q08722; residues 1-323; SEQ ID NO: 7) and full-length cynomolgus monkey CD47 (accession number A0A2K5X4I2; residues 1-323; SEQ ID NO: 8) were cloned in a modified pcDNA™3.1 plasmid (ThermoFisher Scientific, catalog NO: V79020). The vector also contained the enhanced Green Fluorescent Protein (eGFP) and puromycin resistance genes (with intercalated intraribosomal entry sites (IRES) elements). For protein
10 expression, the aforementioned plasmids were transfected into suspension-adapted CHO-S cells (cGMP banked, Invitrogen, Cat.-NO:A1136401) using Polyethyleneimine (PEI; Polysciences). Briefly, cells were prepared at 2 million cells per ml in CD CHO (Gibco). Cells were then transfected with a DNA-PEI mixture at 37 °C. Four hours post-transfection, the cell culture was diluted 1:1 PowerCHO 2 (Lonza) supplemented with 4mM L-Glutamine and incubated with orbital shaking at 37 °C, 5% CO₂ and 80% humidity. The
15 expression of the target antigens was assessed by monitoring the expression of the eGFP reporter protein with a fluorescence microscope. Human and cynomolgus monkey CD47 proteins as described herein have SEQ ID NO: 7 and 8, respectively.

Library generation

The library used herein was from synthetic origin with a diversity restricted to the heavy chain (CDR-H1, CDR-H2 and CDR-H3) and a fixed V κ 3-15/J κ 1 light chain (SEQ ID NO: 10). The library contained 4 different
20 sub libraries based on VH1-69, VH3-23, VH3-15 and VH3-53 antibody germlines. CDRs have been randomized using Trimer oligonucleotides. Primers used for diversifying CDR-H1 and CDR-H2 were designed for each sub libraries and encoded germline-specific naturally occurring diversity at Kabat residues 27-35 and 50-58, respectively. CDR-H3 has been randomized using a pool of oligonucleotides
25 encoding 15 CDR-H3 lengths (6-20) and length-specific naturally occurring diversity at Kabat residues 95-102. Diversified scFv fragments have been pooled to mimic natural CDR-H3 length distribution and cloned into the pNGLEN (in-house modified pUC119 phagemid vector) and the resulting ligation reaction electroporated into *E. coli* TG1 cells. Each sub-library had diversity between 1.2×10^{10} and 1.7×10^{10} , the four sub-libraries reached a total diversity of 5.6×10^{10} .

Library selection

Each sub-library were either selected individually or as a pool. The first panning strategy consisted in two rounds of selection using recombinant human CD47 protein followed by 2 rounds using cynomolgus monkey CD47-expressing CHO cells. The second panning strategy consisted in one round of selection using recombinant human CD47 protein followed by 3 rounds using human CD47-expressing CHO cells. The third panning strategy consisted in one round of selection using human CD47-expressing CHO cells followed by 3 rounds using recombinant human CD47 protein.

For the panning strategies using recombinant proteins, purified phage particles (10^{12} plaque-forming units) and magnetic Dynabeads™ MyOne™ Streptavidin C1 beads (Invitrogen, catalog NO: 65002) were blocked with phosphate buffered saline (PBS) containing 3% (w/v) skimmed milk (3% MPBS) for 1h at room temperature (RT). Phages were deselected against pre-blocked beads for 1h at RT. Deselected phages were incubated with 100 nM of biotinylated recombinant human CD47-avi-his protein produced in house (SEQ ID NO: 3) for 2h at RT. Antigen bound phages were captured on streptavidin beads for 30 min at RT and beads were washed five times with PBS containing 0.1% (v/v) Tween (PBS-Tween 0.1%) and twice with PBS. Phages were eluted with 100 mM triethylamine for 10 min at RT and neutralized using Tris-HCl 1 M pH 8. Eluted phages were used to infect 10 ml of exponentially growing *E. coli* TG1 cells. Infected cells were grown in 2YT medium for 1 h at 37 °C and 100 rotation per minute (RPM), then spread on 2YTAG (2TY medium supplemented with 100 µg/ml ampicillin and 2% glucose) agar plates and incubated overnight (ON) at 30 °C. Colonies were scrapped off the plates into 10 ml of 2YT and 15% glycerol (v/v) was added for storage at -80 °C. TG1 cells from glycerol stocks were grown at 37 °C and 240 RPM in 2YTAG medium until OD at 600 nm reached 0.5. Cells were then superinfected with the M13K07 helper phage using a multiplicity of infection (MOI) of 10 for 1 h at 37 °C and 100 RPM. Culture medium was then changed for 2YTAK (2YT medium supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin) and cells were further cultured ON at 30 °C and 280 RPM. The next day, 10 µl of phage containing cell-free supernatant were used for the subsequent round of selection.

For the panning strategies using CHO cells, purified phage particles were blocked with phosphate buffered saline (PBS) containing 3% (w/v) Bovine Serum Albumin (PBS/BSA 3%) for 1h at RT. 2×10^7 non-transfected CHO cells and 2×10^7 CHO cells transiently expressing human CD47 protein (SEQ ID NO: 7) or cynomolgus monkey CD47 protein (SEQ ID NO: 8) were blocked with PBS/BSA 3% supplemented with 0.1% azide to avoid receptor internalization for 1h at RT. Phage were deselected against non-transfected cells for 1h at

RT. The deselected phages were then incubated with the transfected cells for 2 h at RT. In order to remove non-specific phages, cells were washed four times with PBS-Tween 0.1% and twice with PBS. Phages were eluted with citric acid 76 mM, pH 2.0 for 10 min at RT and neutralized using Tris-HCl 1 M pH 8. Eluted phages were used to infect 10 ml of exponentially growing *E. coli* TG1 cells. Phage amplification was performed as described above.

scFv screening by SPR

Surface Plasmon Resonance (SPR) analysis was used to confirm specific binding activity of the scFv clones. Measurements were performed on a Biacore 2000 instrument (Biacore, GE Healthcare) using the Biacore 2000 Control Software v3.2 at room temperature and analyzed with the Biacore T200 Evaluation Software (v3.1) from the same manufacturer. Recombinant human CD47-avi-his protein produced in house (SEQ ID NO: 3) and recombinant cynomolgus monkey CD47-avi-his protein produced in house (SEQ ID NO: 4) were individually diluted to a final concentration of 200 nM in acetate buffer pH 4.5 (Biacore, GE Healthcare, catalog NO: BR100350) and subsequently immobilized on flow-path 2 and 3 respectively, to a level of about one 1500 resonance units (abbreviated RU) on CM5 Sensor Chips (Biacore, GE Healthcare, catalog NO: BR100012) using an amine coupling kit (Biacore, GE Healthcare, catalog NO: BR100050). Flow-path 4 was similarly immobilized with an irrelevant protein. HBS-EP (Biacore, GE Healthcare, catalog NO: BR100188) was used as running buffer. Filtered periplasmic extracts were injected directly on the covalently coupled human CD47 and cynomolgus CD47 CM5 Sensor Chip. Samples were injected on the flow-path 1, 2, 3 and 4 (flow-path 1 being used as reference) at a 30 µl/min flow rate for 3 min, followed by a dissociation time of 3 min in running buffer. After each binding event, surface was regenerated with 10 mM Glycine pH 1.5 solution (Biacore, GE Healthcare, catalog NO: BR100354) injected for 1 min at 30 µl/min. Each measurement included zero-concentration samples as well as irrelevant scFv periplasmic extracts for referencing and specificity, respectively.

scFv screening by flow cytometry

The binding of scFv clones to CHO cells transiently expressing human CD47 protein (SEQ ID NO: 7) was assessed by flow cytometry. Individual *E. coli* colonies from the third or fourth round of selection were picked and grown in 2TY medium supplemented with 100 µg/ml ampicillin and 0.1% glucose in 96-well deepwell plates. scFv expression was induced by addition of 0.02 mM of IPTG and incubation ON at 30 °C and 250 RPM. Cells were centrifuged and periplasmic extracts were obtained by resuspending the

bacterial pellets in TES buffer (50 mM Tris-HCl pH 8; 1 mM EDTA pH 8; 20% sucrose) followed by incubation on ice for 30 min. Cellular debris were removed by centrifugation, and the scFv containing supernatants were used in flow cytometry experiment. CD47-expressing and non-transfected CHO cells were seeded at a density of 10^5 cells/well in microtiter plates. Next, the plates were centrifuged to remove the cell supernatant and 100 μ l of periplasmic extract previously diluted 1:1 in PBS containing 3% (w/v) bovine serum albumin (PBS-BSA 3%) was added to each well and the plates further incubated for 30 min at 4 °C. Cells were then washed with PBS-BSA 3% and incubated with a biotin-chicken anti-c-Myc antibody (Gallus Immunotech catalog NO: ACMYC-B) diluted at 1:200 in PBS-BSA 3% for 30 min at 4 °C. Next, cells were washed with PBS-BSA 3% and incubated with streptavidin APC (eBioscience, catalog NO: 17-4317) diluted at 1:100 in PBS-BSA 3% for 30 min at 4 °C. Finally, cell fluorescence was measured using a FACSCalibur flow cytometer (BD biosciences).

Fab expression

cDNAs encoding the different antibody constant regions were gene synthesized by GENEART AG (Regensburg, Germany) and modified using standard molecular biology techniques. PCR products were digested with appropriate DNA restriction enzymes, purified and ligated in modified pcDNA3.1 plasmids (Invitrogen) which carried a CMV promoter and a bovine hormone poly-adenylation (poly(A)). The expression vectors also carried oriP, which is the origin of plasmid replication of Epstein-Barr virus, and the murine VJ2C leader peptide for secretion of the encoded polypeptide chain. For reformatting scFv library clones into human IgG1 Fab fragments, each scFv clone in its phage library vector was used to amplify its individual VH cDNAs by PCR, next the VH PCR product was cloned in the modified pcDNA 3.1 vector described above upstream of a cDNA encoding a human IgG1 heavy chain CH1 domain, whereas the fixed Vk3-15/Jk1 light chain (SEQ ID NO: 10) was cloned in the modified pcDNA 3.1 vector described above upstream of a cDNA encoding a human kappa constant light chain domain.

For Fab expression, equal quantities of heavy chain and light chain vectors were co-transfected into suspension-adapted HEK293-EBNA cells (ATCC, cat no CRL-10852) using PEI. Typically, cells were prepared at 8 million cells per ml in RPMI supplemented with 0.1% Pluronic F-68. Cells were then transfected with a DNA-PEI mixture. Four hours post-transfection, the cell culture was diluted 1:1 in EX-CELL® 293 supplemented with Phenol Red and 4 mM L-Glutamine and incubated for 5 days with orbital shaking at 37 °C, 5% CO₂ and 80% humidity. Cell-free culture supernatants containing the recombinant proteins were prepared by centrifugation followed by filtration, and used for further purification. Fab proteins were

purified using CaptureSelect™ IgG-CH1 Affinity Matrix (ThermoScientific, catalog NO: 194320050). Affinity resin was added to the filtered culture supernatants and incubated ON at 4 °C with gentle mixing. The next day, resin beads were collected into Poly-Prep columns (Bio-Rad Laboratories), washed with PBS, and the recombinant proteins eluted with an acidic buffer (typically glycine 0.1 M pH 3). After neutralization with 1/10 volume of Tris-HCl pH 8, preparations were buffer-exchanged into PBS.

Fab binding affinities for CD47

Surface plasmon resonance (SPR) was used to measure the binding affinities of the Fab fragments for human and cynomolgus monkey CD47. Affinities were measured on a Biacore T200 instrument (Biacore, GE Healthcare) at 25 °C and analyzed with the Biacore T200 Evaluation Software (v3.1). Measurements were performed on Series S CM5 Sensor Chips (Biacore, GE Healthcare, catalog NO: BR100530) using recombinant human CD47-avi-his and recombinant cynomolgus monkey CD47-avi-his proteins produced in house (SEQ ID NO: 3 and SEQ ID NO: 4, respectively). Recombinant human CD47 protein and recombinant cynomolgus monkey CD47 protein were covalently immobilized to around 50 RU on flow-path 2 and flow-path 4 respectively of the Sensor Chip surface using a commercially available amine coupling kit (Biacore, GE Healthcare, catalog NO: BR100050). Fab fragments were injected in single cycle kinetic at different concentrations ranging from 15.6 to 2000 nM, in HBS-EP+ buffer (Biacore, GE Healthcare, catalog NO: BR100669) at a flow rate of 30 µl/min for 3 min on flow-path 1, 2, 3 and 4 (flow-path 1 and 3 being used as reference). Dissociation was monitored for 5 min. After each cycle, the Series S CM5 Sensor Chip surface coated with recombinant CD47 proteins was regenerated with 30 µl of 10 mM Glycine pH 1.5 (Biacore, GE Healthcare, catalog NO: BR100354). Experimental data were processed using the 1:1 Langmuir kinetic fitting model. Measurements included zero-concentration samples for referencing. Chi², U- and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models.

CD47/SIRPα blocking assay

The ability of the Fab fragments to block the interaction between human CD47 and human SIRPα was assessed by Bio-Layer Interferometry (BLI). Measurements were done on an OctetRED96e instrument (ForteBio) and analyzed using the Data Analysis HT version 11.1 software (Octet, ForteBio). Biotinylated recombinant human CD47-avi-his protein produced in house (SEQ ID NO: 3) was loaded at 1 µg/ml in kinetic buffer (ForteBio, catalog NO: 18-1105) on a streptavidin SA Biosensor (ForteBio, catalog NO: 15-5019) for 100 sec. Streptavidin biosensor coated with biotinylated human CD47 antigen was dipped into

a solution of 1 μ M of Fab for 10 min to achieve saturation of the CD47 coated surface, followed by a successive dip into a mixed solution of 1 μ M of Fab 1 and 2 μ M of recombinant human SIRP α protein produced in house (SEQ ID NO: 9) for 5 min. All steps were performed at 25 °C and 1000 RPM shaking. Fresh streptavidin biosensors were coated with biotinylated human CD47 protein before each cycle.

5 Results and conclusions

ScFv clones showing specific binding to recombinant human CD47 protein and to recombinant cynomolgus monkey CD47 protein by SPR, as well as, for those tested, showing specific binding to CD47-expressing CHO cells, were sequenced and unique sequences were reformatted in Fab fragment for further characterization. Biochemical characterization included assessment of binding affinities and functional assessment was performed using human CD47/SIRP α blocking assays.

Fab binding affinities for CD47

Twenty one Fab clones showed binding affinities to human and cynomolgus monkey CD47 with an equilibrium dissociation constant (KD) below 2 μ M as determined by SPR. Clone anti-CD47-UCP01-H2, hereafter also referred to as anti-CD47-H2, showed affinities to human CD47 of 104 nM and to cynomolgus monkey CD47 of 142 nM. Heavy chain sequence identification numbers and binding affinities of the mentioned clones are reported in Table 1.

Table 1. Overview of the developed Fab clones and their relative affinity to human and cynomolgus monkey CD47.

Clone name	Clone heavy chain SEQ ID NO	Human CD47 KD (nM)	Cynomolgus monkey CD47 KD (nM)
anti-CD47-UCP01-A1 FAB	SEQ ID NO: 11	292	15.9
anti-CD47-UCP01-G1 FAB	SEQ ID NO: 12	136	214
anti-CD47-UCP01-A2 FAB	SEQ ID NO: 13	822	1070
anti-CD47-UCP01-G2 FAB	SEQ ID NO: 14	183	190
anti-CD47-UCP01-H2 FAB	SEQ ID NO: 15	878.3	939.3
anti-CD47-UCP01-A3 FAB	SEQ ID NO: 16	1170	1260
anti-CD47-UCP01-B3 FAB	SEQ ID NO: 17	316	340
anti-CD47-UCP02-C1 FAB	SEQ ID NO: 18	193	113
anti-CD47-UCP02-C2 FAB	SEQ ID NO: 19	1100	1230
anti-CD47-UCP02-B3 FAB	SEQ ID NO: 20	1010	1120
anti-CD47-UCP02-H3 FAB	SEQ ID NO: 21	455	17.8

CD47/SIRPα blocking assay

Fab fragments were tested at blocking the interaction between human CD47 and human SIRPα using Bio-Layer Interferometry. All developed Fab fragments tested to block the interaction between human CD47 and human SIRPα. Blocking of the interaction between recombinant human CD47 protein and recombinant human SIRPα protein with clone anti-CD47-H2 is illustrated in Figure 1.

Example 2: Generation of anti-CD38 antibodiesMaterial and methods*Recombinant target antigens*

cDNA for human and cynomolgus monkey CD38 were obtained from Source Biosciences (Erwin-Negelein-Haus, Germany, Cat. NO.: IRAU37D11, 4309086), their extracellular regions (UniProt accession No: P28907 residues 43-300 and Uniprot accession No: Q5VAN0, residues 43-301 (residue 44 was deleted), respectively) were PCR amplified and cloned into an in-house expression vector derived from pcDNA3.1 (Invitrogen AG). This expression vector encompassed a Kozak sequence and a start codon followed by the murine IgGκ light chain leader peptide at the 5' end and a 6-His-tag at the 3' end of its multiple cloning site. The soluble extracellular region of human CD38 (residues 43 to 300, SEQ ID NO: 1) and cynomolgus monkey CD38 (residues 43-301, SEQ ID NO: 2) fused to a 6-His-tag were expressed as follows. Briefly, one volume of RPMI 1640 medium (PAA Laboratories) containing HEK cells, 0.1% pluronic acid (Invitrogen AG), expression vector and polyethylenimine (JetPEI®, Polyplus-transfection, Illkirch, France) was incubated in a shake flask at 37 °C, 5% CO₂ and 80% humidity. One volume of Ex-Cell 293 medium supplemented with 6 mM glutamine was added to the mixture after 4 hours and incubation continued further for a total of 5 days. Post production, clarified supernatant was obtained by centrifugation and filtrated using 0.2 µm filters, pH was adjusted at 7.4 (4 °C) using Tris 1 M pH 8.7. Ni-Sepharose Excell beads (GE Healthcare) were added to the clarified supernatant and incubated overnight at 4 °C under agitation. The solution was loaded on an Econo-Column (Bio-Rad Laboratories) for gravity-flow purification. The beads were subsequently washed with 1X PBS, pH 7.4 (1 or 2X 10 CV) and 1X PBS, pH 7.4 supplemented with 20 mM imidazole (10 CV). The protein was, depending on the batch, either eluted with 1X PBS, pH 7.4 supplemented with 500 mM imidazole or eluted by increasing stepwise the concentration in imidazole (40 mM, 80 mM and 250 mM imidazole). Fractions of interest were pooled and dialyzed twice against 1X PBS, pH 7.4 at 4 °C. The protein was concentrated and sterile-filtered using 0.22 µm filters. Protein quality was assessed by SDS-PAGE, SE-HPLC, endotoxin measurement and ELISA. Briefly, SE-HPLC was performed

using a Tosoh Bioscience TSKgel G3000SWxl column (catalog NO: 08541, Tosoh Bioscience) at room temperature with 0.1 M sodium phosphate buffer, 0.15 M sodium chloride, pH 6.8 as eluent at 1 ml/min flow rate, on a Waters Alliance 2695 HPLC system with a Waters 2998 PDA detector (Waters), monitoring at 214 nm and 280 nm. The Multi-Cartridge System Endosafe-MCS from Charles River utilizing a Limulus ameocyte lysate (LAL)-based assay was used to confirm a bacterial endotoxin level inferior to 0.5 EU/mg. These proteins are referred herein as human CD38-C-His and cyno CD38-C-His and are described as SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

Recombinant cell lines

The human codon-optimized sequences of the full-length human CD38 (UniProt sequence ID P28907; residues 1-300; SEQ ID NO: 5), of the full-length cynomolgus monkey CD38 (accession number: Q5VAN0; residues 1-301; SEQ ID NO: 6) were cloned in a modified pcDNA™3.1 plasmid (ThermoFisher Scientific, catalog NO: V79020). Transfection and protein expression were done in suspension-adapted CHO-S cells (cGMP banked, Invitrogen, Cat.-NO:A1136401) as described in Example 1. Human and cynomolgus monkey CD38 proteins as described herein have SEQ ID NO: 5 and 6, respectively.

Library selection

The selections have been performed as described in the Example 1 with the following modifications. The first panning strategy consisted in three rounds of selection using biotinylated recombinant human CD38 protein (Acrobiosystems, catalog NO: CD8-H82E7). The second panning strategy consisted in two rounds of selection using biotinylated recombinant human CD38 protein followed by two rounds using CHO cells transiently expressing human CD38 protein (SEQ ID NO: 5) or cynomolgus monkey CD38 protein (SEQ ID NO: 6).

scFv screening by SPR

Surface Plasmon Resonance (SPR) analysis was used to confirm specific binding activity of the scFv clones. Measurements were performed on a Biacore 2000 instrument (Biacore, GE Healthcare) using the Biacore 2000 Control Software v3.2 at room temperature and analyzed with the Biacore T200 Evaluation Software (v3.1) from the same manufacturer. Commercially available recombinant human CD38 protein (Acrobiosystems, catalog NO: CD8-H224) and recombinant cynomolgus monkey CD38 protein (R&D Systems, catalog NO: 9834-AC-050), or recombinant human CD38 extracellular domain (ECD)-C-his (SEQ ID NO: 1) and recombinant cynomolgus CD38-ECD-C-his (SEQ ID NO: 2) proteins produced in house were

individually diluted to a final concentration of 200 nM in acetate buffer pH 4.5 (Biacore, GE Healthcare, catalog NO: BR100350) and subsequently immobilized on flow-path 2 and 3 respectively, to a level of about one 1500 resonance units (abbreviated RU) on CM5 Sensor Chips (Biacore, GE Healthcare, catalog NO: BR100012) using an amine coupling kit (Biacore, GE Healthcare, catalog NO: BR100050). Flow-path 4 was similarly immobilized with commercially available recombinant mouse CD38 protein (Creative Biomart, catalog NO: CD38-3112M) or with an irrelevant antigen. HBS-EP (Biacore, GE Healthcare, catalog NO: BR100188) was used as running buffer. Filtered periplasmic extracts were injected directly on the covalently coupled human CD38-his and cynomolgus CD38-his CM5 Sensor Chip. Samples were injected on the flow-path 1, 2, 3 and 4 (flow-path 1 being used as reference) at a 30 µl/min flow rate for 3 min, followed by a dissociation time of 5 min in running buffer. After each binding event, surface was regenerated with 10 mM Glycine pH 1.5 solution (Biacore, GE Healthcare, catalog NO: BR100354) injected for 1 min at 30 µl/min. Each measurement included zero-concentration samples as well as irrelevant scFv periplasmic extracts for referencing and specificity, respectively.

scFv screening by flow cytometry

The binding of scFv clones to CHO cells transiently expressing human CD38 protein (SEQ ID NO: 5) was assessed by flow cytometry as described in Example 1.

Fab binding affinities for CD38

Human IgG1 Fab fragments were expressed and purified as described in Example 1. Surface plasmon resonance (SPR) was used to measure the binding affinities of the Fab fragments for human and cynomolgus monkey CD38. Affinities were measured on a Biacore T200 instrument (Biacore, GE Healthcare) at 25 °C and analyzed with the Biacore T200 Evaluation Software (v3.1). Measurements to human CD38 were performed on Series S Sensor Chips SA (Biacore, GE Healthcare, catalog NO: BR100531) or on Series S BiotinCAPture Chips (Biacore, GE Healthcare, catalog NO: 28920234) using commercially available recombinant biotinylated human CD38 Avitag™, His Tag (Acrobiosystems, catalog NO: CD8-H82E7). Measurements to cynomolgus monkey CD38 were performed on Series S CM5 sensor chips (Biacore, GE Healthcare, catalog NO: BR100530) previously immobilized with anti-histidin antibody (Biacore, GE Healthcare, catalog NO: 28995056) using recombinant cynomolgus monkey CD38-ECD-C-his protein produced in house (SEQ ID NO: 2).

The affinities to human and cynomolgus monkey CD38 were assessed by immobilizing CD38 and using Fab fragments as analyte. Biotinylated recombinant human CD38 protein was immobilized to around 160 RU

on flow-path 2 of a Series S Sensor Chip SA or to around 100 RU on flow-path 2 of a Series S BiotinCAPture Chip and recombinant cynomolgus monkey CD38-his protein was immobilized to around 50-100 RU on flow-path 2 of a Series S CM5 Sensor Chip previously coated with anti-histidin antibody. Fab fragments were injected in single cycle kinetic at different concentrations ranging from 7.8 to 2000 nM, in HBS-EP+ buffer (Biacore, GE Healthcare, catalog NO: BR100669) at a flow rate of 30 µl/min for 3 min on flow-path 1 and 2 (flow-path 1 being used as reference). Dissociation was monitored for 5 min. After each cycle, the Series S Sensor Chip SA coated with biotinylated recombinant human CD38 protein surface was regenerated with 60 µl of 10 mM Glycine pH1.5 (Biacore, GE Healthcare, catalog NO: BR100354) while Series S BiotinCAPture Chip and Series S CM5 Sensor Chip coated with anti-histidin antibody surfaces were regenerated using regeneration solution provided with BiotinCAPture kit (Biacore, GE Healthcare, catalog NO: 28920234) and His Capture kit (Biacore, GE Healthcare, catalog NO: 28995056) respectively. Experimental data were processed using the 1:1 Langmuir kinetic fitting model. Measurements included zero-concentration samples for referencing. Chi², U- and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models

15 *Epitope binning*

Epitope binning of Fab fragments on human CD38 was assessed using Bio-Layer Interferometry (BLI). Measurements were done on an OctetRED96e instrument (ForteBio) and analyzed using the Data Analysis HT version 11.1 software (Octet, ForteBio). Biotinylated human CD38-avi-his protein (Acrobiosystems, catalog NO: CD8-H82E7) was loaded at 1 µg/ml in kinetic buffer (ForteBio, catalog NO: 18-1105) on a streptavidin SA Biosensor (ForteBio, catalog NO: 15-5019) for 5 min. Streptavidin biosensor coated with biotinylated human CD38 antigen was dipped into a solution of 200 nM of Fab 1 (saturating Fab) for 10 min, followed by a successive dip into a mixed solution of 200 nM of Fab 1 and 200 nM of Fab 2 (competing Fab) for 5 min. All steps were performed at 25 °C and 1000 RPM shaking. Fresh streptavidin biosensors were coated with biotinylated human CD38 before each cycle.

25 Results and conclusions

ScFv clones showing specific binding to both recombinant human CD38 and recombinant cynomolgus monkey CD38 proteins by SPR as well as specific binding to human CD38 CHO cells were sequenced and unique sequences showing the best binding profiles on SPR were reformatted in Fab fragment for binding affinity measurement.

Fab binding affinities to CD38

Thirty-five Fab clones showed binding affinities to recombinant human and cynomolgus monkey CD38 proteins with an equilibrium dissociation constant (KD) below 10 μ M as determined by SPR. Clone anti-CD38-UCP01-E2, hereafter also referred to as anti-CD38-E2 (heavy chain SEQ ID NO: 28) showed affinities to human CD38 and cynomolgus monkey CD38 of 110 nM and 89 nM respectively. Clone anti-CD38-UCP03-B6, hereafter also referred to as anti-CD38-B6 (heavy chain SEQ ID NO: 43) showed affinities to human CD38 and cynomolgus monkey CD38 of 225 nM and 829 nM respectively. Heavy chain sequence identification numbers and binding affinities of the mentioned clones are reported in Table 2.

- 10 Table 2. Overview of the developed Fab clones and their relative affinity to human and cynomolgus monkey CD38. NB = no detectable binding; NT = not tested.

Clone name	Clone heavy chain SEQ ID NO	Human CD38 KD (nM)	Cynomolgus monkey CD38 KD (nM)
Anti-CD38-UCP01-B1 Fab	SEQ ID NO: 22	193	102
Anti-CD38-UCP01-D1 Fab	SEQ ID NO: 23	666	NB
Anti-CD38-UCP01-F1 Fab	SEQ ID NO: 24	429	393
Anti-CD38-UCP01-G1 Fab	SEQ ID NO: 25	1124	283
Anti-CD38-UCP01-H1 Fab	SEQ ID NO: 26	437	352
Anti-CD38-UCP01-C2 Fab	SEQ ID NO: 27	85.3	53.5
Anti-CD38-UCP01-E2 Fab	SEQ ID NO: 28	110	88.9
Anti-CD38-UCP02-A7 Fab	SEQ ID NO: 29	71.14	low binding
Anti-CD38-UCP02-B1 Fab	SEQ ID NO: 30	595	low binding
Anti-CD38-UCP02-B7 Fab	SEQ ID NO: 31	1709	NT
Anti-CD38-UCP02-C3 Fab	SEQ ID NO: 32	305.1	187.7
Anti-CD38-UCP02-D1 Fab	SEQ ID NO: 33	132.9	78
Anti-CD38-UCP02-D5 Fab	SEQ ID NO: 34	192	33.5
Anti-CD38-UCP02-E5 Fab	SEQ ID NO: 35	0.03	low binding
Anti-CD38-UCP02-F2 Fab	SEQ ID NO: 36	14.16	13.96
Anti-CD38-UCP02-F5 Fab	SEQ ID NO: 37	71.08	55.22
Anti-CD38-UCP02-F6 Fab	SEQ ID NO: 38	389.2	low binding
Anti-CD38-UCP02-H1 Fab	SEQ ID NO: 39	153.9	2.7

Anti-CD38-UCP03-B3 Fab	SEQ ID NO: 40	115	170
Anti-CD38-UCP03-C3 Fab	SEQ ID NO: 41	117	465
Anti-CD38-UCP03-H5 Fab	SEQ ID NO: 42	141.6	111
Anti-CD38-UCP03-B6 Fab	SEQ ID NO: 43	225	829
Anti-CD38-UCP03-E3 Fab	SEQ ID NO: 44	300	195
Anti-CD38-UCP03-A6 Fab	SEQ ID NO: 45	410	525
Anti-CD38-UCP03-B1 Fab	SEQ ID NO: 46	882	462
Anti-CD38-UCP03-C2 Fab	SEQ ID NO: 47	1388	628
Anti-CD38-UCP03-H2 Fab	SEQ ID NO: 48	1850	672
Anti-CD38-UCP03-H3 Fab	SEQ ID NO: 49	810	178

Epitope binning

To assess competition of anti-CD38 clones on CD38, epitope binning assay using anti-CD38 Fab fragments and recombinant human CD38 protein was performed using Octet Bio-Layer Interferometry. Figure 2 shows that anti-CD38-E2 and anti-CD38-B6 clones do not compete with each other and thereby do not recognize overlapping epitopes on CD38.

Example 3: Optimization of anti-CD38-E2 and anti-CD38-B6

Material and methods

Recombinant target antigens

- 10 Recombinant human CD38-ECD-C-his protein (SEQ ID NO: 1), hereafter also referred to as recombinant human CD38 protein, and recombinant cynomolgus monkey CD38-ECD-C-his protein (SEQ ID NO: 2), hereafter also referred to as recombinant cynomolgus monkey CD38 protein, were produced in house as described in Example 2.

Library generation and selection

- 15 Five affinity maturation libraries were generated for both anti-CD38-E2 (SEQ ID NO: 28) and anti-CD38-B6 (SEQ ID NO: 43) clones by introducing diversity in CDRs' heavy chain. CDR-H1, CDR-H2 and CDR-H3 were randomized using degenerated NNK codon oligonucleotides (wherein N is any of the four deoxyribonucleotides and K is G or T) at Kabat residues 27-35, 50-58, 95-101 minus 2, respectively. Each library was generated using a pool of overlapping oligonucleotides containing 5 consecutive degenerated

codons. CDR-H1 and CDR-H2 were also diversified using Trimer oligonucleotide at position Kabat 27-35 and 50-58 respectively. The resulting five library PCR products were cloned into the pNGLEN (in-house modified pUC119 phagemid vector) and the resulting ligation reaction electroporated into *E. coli* TG1 cells. Transformed cells were spread on 2YTAG plates and incubated ON at 30 °C. Colonies were scrapped off
5 the plates into 10 ml of 2YT medium and 15% glycerol (final concentration) was added for storage at -80 °C. Phages were produced and purified by two precipitations steps with one-third v/v of 20% PEG-6000, 2.5 M NaCl and resuspended in PBS.

Phage display selections were performed using recombinant proteins as described in Example 1 with the following modifications. Deselected phage were incubated with 50 nM, 5 nM and 0.5 nM of biotinylated
10 recombinant human CD38 protein (Acrobiosystems, catalog NO: CD8-H82E7) for round 1, round 2 and round 3, respectively. After 1 h incubation, 1 µM of recombinant human CD38 protein produced in house (non-biotinylated, SEQ ID NO: 1) was added for 3h at RT during rounds 2 and 3.

Affinity screening by SPR

SPR analysis was used to confirm specific binding activity of the new scFv clones and rank the positive
15 clones according to their binding profile. Measurements were performed as described in Example 2 with the following modifications. Measurements were performed on a Biacore T200 instrument (Biacore, GE Healthcare) at 25 °C and analyzed with the Biacore T200 Evaluation Software (v3.1). Commercially available recombinant human CD38 protein (Acrobiosystems, catalog NO: CD8-H224) was diluted to a final concentration of 200 nM in acetate buffer pH 4.5 (Biacore, GE Healthcare, catalog NO: BR100350) and
20 subsequently immobilized on flow-path 2 to a level of about 1300 RU on a Series S CM5 sensor CHIP (Biacore, GE Healthcare, catalog NO: BR100530) using an amine coupling kit (Biacore, GE Healthcare, catalog NO: BR100050). Filtered periplasmic extracts were injected directly on the covalently coupled human CD38 CM5 Sensor Chip on the flow-path 1 and 2 (flow-path 1 being used as reference). Dissociation in HBS-EP+ buffer was monitored for 10 min. The scFv clones showing the best binding profiles were
25 reformatted in Fab fragments as described in Example 1.

Fab binding affinities to CD3

Affinities of the Fab to human CD38 using Fab as analyte were performed on Series S BiotinCAPture Chips (Biacore, GE Healthcare, catalog NO: 28920234) using commercially available recombinant biotinylated human CD38 Avitag™, His Tag (Acrobiosystems, catalog NO: CD8-H82E7) as described in Example 2, with
30 Fab injection concentrations ranging from 1.95 nM to 500 nM. Measurements to cynomolgus monkey

CD38 using Fab as analyte were performed on Series S CM5 Sensor Chips (Biacore, GE Healthcare, catalog NO: BR100530) previously immobilized with anti-histidin antibody (Biacore, GE Healthcare, catalog NO: 28995056) using recombinant cynomolgus monkey CD38-ECD-C-his protein produced in house (SEQ ID NO: 2) as described in Example 2, with Fab injection concentrations ranging from 1.48 nM to 120 nM.

- 5 Affinities of the Fab to human and cynomolgus CD38 using the Fab as ligand were measured on a Biacore T200 instrument (Biacore, GE Healthcare) at 25 °C and analyzed with the Biacore T200 Evaluation Software (v3.1). Measurements were performed on Series S CM5 Sensor Chips (Biacore T200, Biacore, GE Healthcare, catalog NO: BR100530) coupled with anti-human Fab antibodies using a commercial kit (Human Fab Capture Kit, Biacore, GE Healthcare, catalog NO: 28958325). SPR measurements were
- 10 performed with recombinant human CD38 protein or recombinant cynomolgus monkey CD38 protein produced in house (SEQ ID NO: 1 and SEQ ID NO: 2, respectively). The affinities to human and cynomolgus monkey CD38 were assessed by immobilizing Fab fragments and using human or cynomolgus monkey CD38 protein as analyte. Around 50-200 RU of Fab was captured on flow-path 2 of a Series S CM5 Sensor Chip coupled with anti-human Fab antibodies. Human or cynomolgus monkey CD38 was injected in multi
- 15 cycle kinetic at different concentrations ranging from 0.2 to 100 nM, in HBS-EP+ buffer (Biacore, GE Healthcare, catalog NO: BR100669) at a flow rate of 30 µl/min for 5 min on flow-path 1 and 2 (flow-path 1 being used as reference). Dissociation was monitored for 10 min. After each cycle, the surface was regenerated with 60 µl of regeneration solution provided with Human Fab Capture Kit (Biacore, GE Healthcare, catalog NO: 28958325). Experimental data were processed using the 1:1 Langmuir kinetic
- 20 fitting model. Measurements included zero-concentration samples for referencing. Chi², U- and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models.

Results and conclusions

- Affinity maturation of anti-CD38-E2 clone (heavy chain SEQ ID NO: 28) and of anti-CD38-B6 clone (heavy
- 25 chain SEQ ID NO: 43) involved diversification of CDR-H1 (Kabat positions 27-35), CDR-H2 (Kabat positions 50-58) and CDR-H3 (Kabat positions 95-101 minus 2) in five individual libraries. ScFv clones having a slower off-rate than their respective parental clone (anti-CD38-E2 or anti-CD38-B6) as measured by SPR were isolated. ScFv clones were reformatted and expressed as Fab fragments and affinities to human and cynomolgus CD38 were measured by SPR. From this assessment, selected mutations originating from the
- 30 CDR-H1 and CDR-H2 libraries were combined for further off-rate improvement; one clone referred to as

Fab clone anti-CD38-E2-RecA (SEQ ID NO: 57) had the highest affinities to human and cynomolgus monkey CD38 with KD measured at 0.9 nM and 3.7 nM respectively using Fab as ligand. Clones anti-CD38-E2-UCP02-F3 (SEQ ID NO: 52), hereafter also referred to as anti-CD38-E2-F3, and anti-CD38-E2-UCP02-F8 (SEQ ID NO: 55), hereafter also referred to as anti-CD38-E2-F8, showed affinities to human CD38 of 3.5 nM and 17.6 nM respectively and to cynomolgus monkey CD38 of 2.4 nM and 24.5 nM respectively, measured using the Fabs as analyte. One clone referred to as Fab clone anti-CD38-B6-D9 (SEQ ID NO: 61) showed highest increase in affinity to human and cynomolgus CD38 from affinity maturation of anti-CD38-B6, with KD values measured at 2.3 nM and 5.2 nM respectively, using Fab as ligand and of 0.55 and 3.3 nM respectively, using Fab as analyte. Heavy chain sequence identification numbers and binding affinity of the mentioned clones to human and cynomolgus monkey CD38 are reported in Table 3.

Table 3. Overview of the developed Fab clones and their relative affinity to human and cynomolgus monkey CD38 measured with Fab as analyte and with Fab as ligand. NB = no detectable binding; NT = not tested.

Library	Clone name	Clone heavy chain SEQ ID NO	Human CD38 KD (nM), Fab as analyte	Cynomolgus monkey CD38 KD (nM), Fab as analyte	Human CD38 KD (nM), Fab as ligand	Cynomolgus monkey CD38 KD (nM), Fab as ligand
CDR-H1	anti-CD38-E2-UCP01-A10 FAB	SEQ ID NO: 50	9.4	8.6	16.89	NT
CDR-H2	anti-CD38-E2-UCP02-E7 FAB	SEQ ID NO: 51	2.2	1.8	4.66	NT
CDR-H2	anti-CD38-E2-UCP02-F3 FAB	SEQ ID NO: 52	3.5	2.4	6.13	NT
CDR-H2	anti-CD38-E2-UCP02-D6 FAB	SEQ ID NO: 53	4.9	1.1	5.35	NT
CDR-H2	anti-CD38-E2-UCP02-G8 FAB	SEQ ID NO: 54	5.2	4.2	7.7	NT
CDR-H2	anti-CD38-E2-UCP02-F8 FAB	SEQ ID NO: 55	17.6	24.5	16.22	NT
CDR-H2	anti-CD38-E2-UCP02-A5 FAB	SEQ ID NO: 56	23.6	4.4	35.03	NT
Combination	anti-CD38-420-E2-RecA FAB	SEQ ID NO: 57	NT	NT	0.9	3.7

Combination	anti-CD38-420-E2-RecB FAB	SEQ ID NO: 58	NT	NT	1.6	4.4
Combination	anti-CD38-420-E2-RecC FAB	SEQ ID NO: 59	NT	NT	1.3	3.9
Combination	anti-CD38-420-E2-RecD FAB	SEQ ID NO: 60	NT	NT	6.7	22.1
CDR-H1	anti-CD38-B6-MP01-D9 FAB	SEQ ID NO: 61	0.55	3.33	2.3	5.2
CDR-H1	anti-CD38-B6-MP01-B4 FAB	SEQ ID NO: 62	11.9	45.4	51.6	167
CDR-H1	anti-CD38-B6-MP02-C1 FAB	SEQ ID NO: 63	12	67.2	97.7	333
CDR-H1	anti-CD38-B6-MP02-B10 FAB	SEQ ID NO: 64	20.1	81.2	108	528
CDR-H1	anti-CD38-B6-MP01-H3a FAB	SEQ ID NO: 65	28.3	120	89.4	389
CDR-H1	anti-CD38-B6-MP01-H3b FAB	SEQ ID NO: 66	28.9	130	171	330
CDR-H1	anti-CD38-B6-MP01-A4 FAB	SEQ ID NO: 67	84.9	229	227	828
CDR-H1	anti-CD38-B6-MP01-C2 FAB	SEQ ID NO: 68	209	NT	345	NT
CDR-H1	anti-CD38-B6-MP02-C2 FAB	SEQ ID NO: 69	101	NT	404	342
CDR-H1	anti-CD38-B6-MP02-G11 FAB	SEQ ID NO: 70	425	262	128	534

Example 4: Engineering CD38/CD47 bispecific antibodies

Material and methods

Construction of expression vectors for transient mammalian cell expression

- 5 cDNAs encoding the different polypeptide chains in part or in full were first gene synthesized by Twist Biosciences (San Francisco, USA) and modified using standard molecular biology techniques. PCR products were inserted in a modified pcDNA3.1 plasmid (Invitrogen AG, Zug, Switzerland) carrying a CMV promoter and a bovine hormone poly-adenylation signal (poly(A)) using standard molecular biology techniques. All

polypeptide chains were independently cloned in an expression vector where secretion was driven by the murine IgGκ light chain leader peptide.

Expression of antibodies in HEK-293-EBNA cells

BEAT bispecific antibodies and control antibodies were expressed as follows unless otherwise indicated.

- 5 For transient expression, equal quantities of each engineered chains vectors were co-transfected into suspension-adapted HEK293-EBNA cells (ATCC-LGL standards, Teddington, UK; Cat. No: CRL-10852) using polyethyleneimine (PEI; Polysciences). Typically, cells were prepared at 8 million cells per ml in RPMI 1640 (Biowest) supplemented with 0.1% Pluronic F-68 (Gibco). Cells were then transfected with a DNA-PEI mixture at 37 °C. 4 hours post-transfection, the cell culture was diluted 1:1 in EX-CELL® 293 supplemented
- 10 with Phenol Red or BalanCD HEK293 (Irvine Scientific) and 4 mM L-Glutamine and incubated for 5 days with orbital shaking at 37 °C, 5% CO₂ and 80% humidity. Cell-free culture supernatants containing the recombinant proteins were prepared by centrifugation followed by filtration, and used for further purification.

Purification of BEAT CD38/CD47 bispecific antibodies by differential Protein A affinity chromatography

- 15 Supernatants from HEK293-EBNA were optionally conditioned with 0.1 volume (V) of sodium phosphate 1 M, pH 6.0 prior purification or used directly without conditioning. KanCapA resin (KANEKA, Belgium) was added to conditioned supernatants. Mixtures were incubated overnight at 4 °C with stirring. After incubation, bound proteins were washed with 10 column volumes (CVs) of PBS pH 7.4, followed by 2 CVs of sodium acetate 170 mM, pH 5.0, then eluted in multiple steps of 50 mM sodium acetate, pH 4.3, 4.1
- 20 and/or pH 3.9 and neutralised with 0.1V of 1 M Tris-HCl pH 8.0. The elution fractions were dialyzed to PBS, pH 7.4, sterile-filtered and analyzed by SE-HPLC (TSKgel G3000SWXL, 5 µm, 7.8 mm x 30 cm L, 5 µm particles and 250 Å pores (Cat N° 08541, TOSOH BIOSCIENCE) at room temperature with 0.1 M sodium phosphate buffer, 0.15 M sodium chloride, pH 6.8 as eluent at 1 ml/min flow rate on HPLC Alliance 2695 (Waters) or Acquity Arc HPLC (Waters) with column heater and either UV/Vis detector (2487 or 2489 from
- 25 Waters) or PDA detector (2996 or 2998 from Waters)) and SDS-PAGE (NuPAGE Bis-Tris 4-12% acrylamide, Invitrogen AG, Basel, Switzerland). The Multi-Cartridge System Endosafe-MCS from Charles River utilizing a Limulus amoebocyte lysate (LAL)-based assay was used to confirm a bacterial endotoxin level inferior to 5 EU/mg. Typically, for purified antibodies the content of aggregated forms was lower than 5% and monodispersity by SE-HPLC was superior to 95%. Samples were further analysed for heterodimerization

levels by reverse phase high performance liquid chromatography coupled to mass spectrometry (RP-HPLC-MS) as described below.

Heterodimerization level determination by RP-HPLC-MS

Samples were deglycosylated overnight with PNGase F. RP-HPLC-MS was performed on a Vanquish chromatography system (ThermoFisher Scientific) coupled to an extended mass range hybrid electrospray-quadrupole_Orbitrap mass spectrometer (QExactive Plus BioPharma, ThermoFisher Scientific). Purified proteins were injected on a polyphenyl reverse phase column (Bio BioResolve RP mAb 2.1 × 150 mm 450 Å, 2.7 µm column, Waters UK). Column temperature was set to 70 °C. The mobile phase consisted of 0.08% formic acid and 0.02% trifluoroacetic acid in water (solvent A) and 0.08% formic acid and 0.02% trifluoroacetic acid in acetonitrile (solvent B). The proteins were loaded at 5% solvent B with a 0.4 ml/min flow rate. Elution was performed with a linear 30% to 38% gradient of solvent B. The mass spectrometer was operated in positive ion mode scanning the m/z 1500– 5000 range with a resolution of 17500 at m/z 200. In-source collision energy was set to 60 eV to avoid TFA adducts. Acquired data was processed using the BioPharma Finder software (ThermoFisher Scientific). Peak detection was performed using the sliding window with deconvolution performed using the ReSpect® algorithm. Identifications were performed by comparing the measured masses with those calculated for the different expected species. Purity was assessed by integrating the areas of the extracted ion chromatograms for the detected protein species.

Results and conclusion

Bispecific antigen-binding molecules that bind CD38 and CD47 were constructed using the BEAT® heavy chain (Hc) heterodimerization technology previously described (Skegro *et al.*, (2017) J Biol Chem 292(23): 9745-9759 and Stutz *et al.*, (2020) J Biol Chem 295(28): 9392-9408); such bispecific antigen-binding molecules are referred to herein as BEAT CD38/CD47. The anti-CD38 portion of the BEAT CD38/CD47 molecule is useful for targeting tumor cells that express CD38, and the anti-CD47 portion of the bispecific molecule is useful for blocking the CD47 mediated phagocytosis inhibition signal which is driven through the interaction of CD47 with SIRPα (Signal regulatory protein α) on immune cells (mainly macrophages, Willingham *et al.*, (2012) Proc Natl Acad Sci USA 109(17): 6662-6667). The interaction of SIRPα with its ligand, CD47, a widely expressed transmembrane protein, transmits a “don't eat me” signal by initiating signalling cascades that ultimately inhibit phagocytosis. The presence of this inhibition signal allows cancer cells to escape in part macrophage mediated control mechanisms of the immune system. CD47 is

expressed on most cell types and thus in order to avoid binding CD47 expressing cells that do not express CD38, a weak affinity for CD47 was deemed suitable, *i.e.* letting the anti-CD38 domain drive the binding to the target cells which subsequently allows binding of a proximal CD47 receptor on the same cell by avidity. Importantly, CD47 is expressed on red blood cells (RBC) and platelets that express CD38. Critical to the on-target specificity of the bispecific antibody is mitigating the crosslinking of RBCs as well as platelets by bridging of CD47 and CD38, which would be induced by a bispecific antibody construct with two high affinity binding arms. We envisaged a low affinity of the CD47 binding arm to minimize the binding to CD47 expressing cells that do not express CD38 and to avoid hemagglutination and platelet-aggregation, but to efficiently block the CD47-SIRP α interaction. Blocking of CD47 in combination with a high specificity for CD38 will therefore allow disabling CD47 mediated phagocytosis inhibition specifically for cancer cells expressing CD38. As a result, target cells will be susceptible to antibody-dependent cellular phagocytosis (ADCP). Further effector mechanisms, *i.e.* antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), will contribute to the mechanism of action.

Bispecific antibodies comprising an anti-CD38-specific binding domain and an anti-CD47-specific binding domain were constructed using standard methodologies, wherein the anti-CD38 antigen binding domain and the anti-CD47 antigen binding domain each comprise different, distinct Hc variable regions (VH) paired with a common light chain (cLc) (Figure 3). The cLc circumvents any possible light chain mispairing that is commonly observed when incorporating two different VH domains as well as two different cognate light chains in a bispecific antibody. Bispecific antibodies contained the heterodimeric BEAT Fc region (SEQ ID NOs: 257 and 258) and Hcs were engineered with different avidity for Protein A (PA), thereby allowing separation of homo- and heterodimer species as previously reported (Skegro *et al.*, (2017) *supra* and Ollier *et al.*, (2019) MABs 11(8): 1464-1478). More specifically, the anti-CD47 arm of the hetero-dimeric immunoglobulin consisted of a BEAT (A) Hc encompassing a VH with mutation G65S (Kabat numbering) selected from Table 1 (anti-CD47-H2), a CH1 γ 1 region, a γ 1 hinge region, a γ 1 CH2 region, and a γ 3 based BEAT (A) CH3 domain assembled with a cLc (SEQ ID NO: 10). This Hc encompassed part of a human IgG3 Fc region and therefore had no binding to PA. However, since the Hc used herein had its VH domain originating from the VH3-subclass, the VH domain was mutated to include the G65S substitution, thereby removing any additional PA binding sites within the Hc (Ollier *et al.*, (2019) *supra*). Importantly, a number of additional constructs not shown here, with alternative anti-CD47 VH domains with varying affinities, were designed and tested after which anti-CD47-H2 was deemed the most suitable. The anti-CD38 arm of the heterodimeric immunoglobulin consisted of a BEAT (B) Hc encompassing a VH domain selected from

Table 3 (anti-CD38-E2-F3, anti-CD38-E2-F8 or anti-CD38-E2-RecA), a CH1 γ 1 region, a γ 1 hinge region, a γ 1 CH2 region, and a γ 1 based BEAT (B) CH3 domain assembled with a cLc. The bispecific antibodies resulting from the assembly of the BEAT (A) and BEAT (B) Hcs as well as the cLc are referred to herein as BEAT CD38/CD47-X where X is the construct number consisting of a particular set of variable domains (Table 4).

- 5 Bispecific antibodies were transiently expressed in HEK and purified by differential PA chromatography. For all antibodies, monodispersity of >95% was observed by SE-HPLC with <1% homodimeric contaminants as determined by RP-HPLC-MS. Endotoxin levels were inferior to 5 EU/mg. BEAT CD38/CD47 bispecific antibodies were further tested for their biological activity below.

10

Table 4. List of BEAT CD38/CD47 bispecific antibody constructs with their associated SEQ ID NOs.

Construct	BEAT (A) Hc	BEAT (B) Hc	cLc
BEAT CD38/CD47-3	SEQ ID NO: 259	SEQ ID NO: 260	SEQ ID NO: 10
BEAT CD38/CD47-6	SEQ ID NO: 259	SEQ ID NO: 261	SEQ ID NO: 10
BEAT CD38/CD47-19	SEQ ID NO: 259	SEQ ID NO: 262	SEQ ID NO: 10

Example 5: Engineering antibodies with increased effector function

Material and methods

Cloning, expression and purification

- 15 Bispecific antibodies were produced in HEK293-EBNA cells as described above. BEAT CD38/CD47-60 as well as -42 and -48 used in assays described in Figure 9 and after, were expressed in CHO-S cells. Engineered chains vectors and a vector encoding Epstein-Barr Virus (EBV) nuclear antigen-1 (EBNA-1) were co-transfected into CHO-S cells (cGMP banked, Invitrogen, Cat.-No.A1136401), using polyethyleneimine (PEI; Polysciences). Typically, cells were prepared at 8 million cells per ml in CD-CHO
- 20 media (Gibco). Cells were then transfected with a DNA-PEI mixture at 37 °C. Four hours post-transfection, the cell culture was diluted 1:1 in PowerCHO™ 2 (Lonza) supplemented with 4 mM L-Glutamine and incubated for 14 days with orbital shaking at 32 °C, 5% CO₂ and 80% humidity. Clarified cell culture supernatants containing the recombinant proteins were prepared by centrifugation followed by filtration. Purification of BEAT CD38/CD47 bispecific antibodies from supernatant was performed by differential PA
- 25 chromatography as described above.

Trastuzumab control IgG1 antibody (SEQ ID NOs: 263 and 264) was expressed in HEK293-EBNA or CHO-S as described above and purified as follows: clarified cell culture supernatants containing the recombinant proteins were prepared by centrifugation followed by filtration. Supernatants were used directly without conditioning. CaptivA resin (Repligen, Waltham, USA) was added to supernatant and incubated overnight at 4 °C under constant agitation. After incubation, bound protein was washed with 10 column volumes (CV) of PBS pH 7.4, followed by an additional 10 CV of PBS pH 7.4. Protein was then eluted in multiple steps of 4 CV of 100 mM Glycine, pH 3.0 and neutralized with 0.1V of 1 M Tris-HCl pH 8.0.

For expression of the isotype control antibody (ABC IgG1, SEQ ID NOs: 265 and 266), equal quantities of each engineered chains vectors, and vectors encoding enhanced Green Fluorescent Protein (eGFP), puromycin N-acetyl-transferase (PAC) and neomycin resistance gene, were co-transfected into CHO-S cells (cGMP banked, Invitrogen, Cat.-No.A1136401), using linear polyethyleneimine (PEI) in Opti-MEM medium (ThermoFisher). Four hours post-transfection, the cell culture was diluted 1:1 in PowerCHO2 CD medium (Lonza) with 4 mM L-Glutamine. The following day, cells were diluted to 2x10⁶ cells/ml and subcloned using limiting dilution (from 1:10 to 1:50) in 96-well plates (100 µl per well) in PowerCHO2 medium with 4 mM L-Glutamine supplemented with puromycin (5 µg/ml) and geneticine (200 µg/ml). Selection pressure was renewed after one week by addition of 100 µl of PowerCHO2 medium with 4 mM L-Glutamine and puromycin. The selection of stable clone was done 14 days post-transfection and based on the strength of the eGFP signal as judged by flow cytometry. Clones with the strongest eGFP expression were amplified. Stable CHO cell lines expressing ABC IgG1 antibody were subcultured in PowerCHO2 medium supplemented with 4 mM L-Glutamine, 5 µg/ml puromycin and 200 µg/ml geneticin in TubeSpin bioreactors (TPP), with orbital shaking at 200 RPM, at 37 °C, 5% CO₂ and 80% humidity. Purification was performed analogously to that of trastuzumab control IgG1 antibody described above.

5F9 IgG4 hinge-stabilized antibody, referred hereafter as 5F9-G4 (SEQ ID NOs: 267 and 268), was expressed in HEK293-EBNA as described above and purified as follows: clarified cell culture supernatants containing the recombinant proteins were prepared by centrifugation followed by filtration. KANEKA KanCapATM resin (Kaneka) was added to supernatant and incubated overnight at 4 °C under constant agitation. After incubation, bound protein was washed with 1X PBS, pH 7.4 (1x10 CV) and eluted using a step-elution protocol with, sequentially, 50 mM sodium acetate pH 4.3, pH 4.1, pH 3.9 and glycine 0.1 M pH 3.5.

All purified samples were analysed by SDS-PAGE, SE-HPLC and optionally RP-HPLC-MS as described above.

Recombinant human Neonatal Fc Receptor (FcRn) extracellular domain Avitag™-poly-histidine tagged fusion protein

The soluble extracellular regions of human FcRn (Uniprot accession No: P55899; residues 24-297; SEQ ID NO: 269) and the full-length Beta 2-microglobulin (B2M; Uniprot accession No: P61769, residues 1-119; SEQ ID NO: 270) were cloned as described above. B2M was cloned without any tag and FcRn was cloned to generate a protein with a C-terminal Avitag™ (Avidity LLC) followed by a 10-His tag with a gly₃ linker sequence between the two tags (abbreviated human SIRPα -ECD-Avi-His). The expression vector coding for human FcRn was carrying the murine VJ2C leader peptide to drive product secretion as well as the OriP sequence. The expression vector coding for human B2M was carrying the B2M native leader peptide. For the expression of the human FcRn/B2M complex, the plasmids coding for human FcRn-ECD-Avi-His and B2M were co-transfected into suspension-adapted HEK293-EBNA cells as described above. Post expression, Ni-Sepharose Excell beads (GE Healthcare) were added to the clarified supernatant and incubated overnight at 4 °C under gentle agitation. Next, the mixtures were loaded on Econo-Columns (Bio-Rad Laboratories) for gravity-flow purification. The beads were first washed in 1X PBS, 20 mM imidazole, pH 7.4 (10 CV), then 1X PBS supplemented with 40 mM imidazole, pH 7.4 (10 CV) and the protein was eluted using 1X PBS supplemented with 250 mM imidazole (1x1 CV and 5x2 CV). Buffer exchange to 20 mM sodium phosphate, 100 mM NaCl, pH 6.0 was performed on PD-10 columns (GE). Protein quality was assessed by SDS-PAGE, SE-HPLC, endotoxin measurement as described above. Human FcRn-ECD-Avi-His tagged fusion protein as described herein have SEQ ID NO: 269.

Recombinant human Fcγ receptors (FcγRs) extracellular domain poly-histidine tagged fusion protein

The extracellular regions of FcγR2a (UniProt accession No: P12318 residues 34-208), of FcγR2b (UniProt accession No: P31994 residues 46-217) and FcγR3a (Uniprot accession No: P08637, residues 17-193) were cloned as described above. The expression vector encompassed a Kozak sequence and a start codon followed by the murine IgGκ light chain leader peptide at the 5' end and a 6 or 10-His-tag at the 3' end of its multiple cloning site. The soluble extracellular regions of human FcγR2a, FcγR2b and FcγR3a fused to a 6 or 10-His-tag were expressed as described above. After expression and harvest, the clarified supernatant was supplemented with 20 mM imidazole and the pH adjusted at 7.4 before adding the resin, except for FcγR3a-ECD-His. The supernatant was incubated with the resin overnight. The next day, the flow through was collected by gravity flow and the resin was washed with 2x10 CVs of 1X PBS, 20 mM imidazole, pH 7.4 (FcγR2a-ECD-His and FcγR2b-ECD-His) or 10 CV of 1X PBS, pH 7.4, followed by a second wash step with 10 CVs of 1X PBS, 20 mM imidazole, pH 7.4 (for FcγR3a-ECD-His). The protein was eluted with 5x1 CV and 2x2

CVs of 1X PBS, 500 mM imidazole, pH 7.4 (FcγR2a-ECD-His and FcγR2b-ECD-His) or 8x1 CV of 1X PBS, 250 mM imidazole, pH 7.4 (FcγR3a-ECD-His). The purified protein was then dialyzed against 1X PBS, pH 7.4. Protein quality was assessed by SDS-PAGE, SE-HPLC, endotoxin measurement as described above. Human FcγR2a, FcγR2b and FcγR3a His tagged fusion proteins as described herein have SEQ ID NO: 271, SEQ ID NO: 272 and SEQ ID NO: 273, respectively.

FcγRIa affinity measurements by SPR

SPR analysis was used to measure the association and dissociation rate constants for the binding kinetics of the different bispecific antibodies using single cycle kinetics. The binding kinetics of antibodies were measured on a BIAcore 8K+ instrument (BIAcore-GE Healthcare Europe GmbH, Glattbrugg, Switzerland) at 25 °C and analysed with the Biacore Insight Evaluation software (version 2.0.15.12933, BIAcore-GE Healthcare Europe GmbH).

Measurements were performed on Series S CAP sensor chips (Cytiva Life Sciences, formerly GE Healthcare Europe GmbH, Cat. No: 28920234) coupled with Avi-Tagged FcγRIa (Acrobiosystems, Cat. No: FCA-H82E8) using a commercial Biotin CAPture Kit (Cytiva Life Sciences, formerly GE Healthcare Europe GmbH, Cat. No: 28920234) to a final level of 50-150 RU. Different dilutions of bispecific antibody candidates and trastuzumab control IgG1 (0, 1.2, 3.7, 11.1, 33.3, 100 nM) were injected over the sensor chip for 2 min at 30 μl/min. After ten minutes of dissociation, a CAP regeneration solution was injected for 2 min at 10 μl/min for regeneration followed by a two-minute stabilization period. Data (sensorgram: fc2-fc1) were fitted with a 1:1 Langmuir model without mass transfer using global fitting. Dissociation times were of at least 600 seconds. Measurements were performed in duplicate and included zero-concentration samples for referencing. Both Chi2 and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models.

FcγRIIa, IIb, and IIIa affinity measurements by SPR

SPR analysis was used to measure the steady state affinity for the binding of the different bispecific antibodies. The steady state affinity of antibodies was measured on a BIAcore 8K+ instrument (BIAcore-GE Healthcare Europe GmbH, Glattbrugg, Switzerland) at 25 °C and analysed with the Biacore Insight Evaluation software (version 2.0.15.12933, BIAcore-GE Healthcare Europe GmbH).

Measurements were performed on Series S protein G sensor chips (Cytiva Life Sciences, formerly GE Healthcare Europe GmbH, Cat. No: 29179315). Bispecific candidate molecules and trastuzumab control IgG1 were captured to give a final R_{max} of approximately 80-100 RU.

5 Different dilution of Fc-receptors IIa, IIb, and IIIa (0, 39, 78, 156, 312, 625, 1250, 2500, 5000 nM) were injected over the sensor chip for 2 minutes at a flow rate of 30 µl/min. After 2 min of dissociation, the next concentration of analyte was injected. The chip was regenerated at the end of a concentration series, 10 mM glycine pH 1.5 was injected for 1 min at a flow rate of 30 µl/min, followed by a 2 min stabilization period. Data (sensorgram: fc2-fc1) were fitted with a steady state affinity model using global fitting. Dissociation times were of at least 120 seconds. Measurements were performed in duplicate and
10 included zero-concentration samples for referencing. Both Chi² and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models.

FcRn affinity measurements by SPR

SPR analysis was used to measure the steady state affinity of binding of the different bispecific antibodies. The steady state affinity of antibodies was measured on a BIAcore 8K+ instrument (BIAcore-GE Healthcare
15 Europe GmbH, Glattbrugg, Switzerland) at 25 °C and analysed with the Biacore Insight Evaluation software (version 2.0.15.12933, BIAcore-GE Healthcare Europe GmbH).

Measurements were performed on Series S CAP sensor chips (Cytiva Life Sciences, formerly GE Healthcare Europe GmbH, Cat. No: 28920234) coupled with Avi-Tagged FcRn (Acrobiosystems, Cat. No: FCM-H82W2) using a commercial Biotin CAPture Kit (Cytiva Life Sciences, formerly GE Healthcare Europe GmbH, Cat.
20 No: 28920234) to a final level of 50-150 RU. Different dilutions of bispecific antibody candidates trastuzumab control IgG1 (4, 12, 37, 111, 333 and 1000 nM) were injected over the chip for 2 min at a flow rate of 10 µl/min. Following 2 min dissociation, the chip was regenerated with an injection of HBS-EP+ pH 8.5, followed by a stabilization period of 2 min. Data (sensorgram: fc2-fc1) were fitted with a steady state affinity model using global fitting. Measurements were performed in duplicate and included zero-
25 concentration samples for referencing. Both Chi² and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models.

Alternatively (for BEAT CD38/CD47-60), measurements were performed on CM5 sensor chips (Cytiva Life Sciences, formerly GE Healthcare Europe GmbH, Cat. No: 29104988) coupled with a mixture of monoclonal antibodies that recognize kappa and lambda subtypes of human Fab light chains using a

commercial Human Fab Capture kit (Cytiva Life Sciences, formerly, GE Healthcare Europe GmbH, Cat. No: 28958325). Antibody candidate molecules and trastuzumab control IgG1 were coupled to give a final RU of approximately 20 RU. Different dilutions of FcRn (0, 7.2, 15.6, 31.3, 62.5, 125, 250, 500, 1000 nM) were injected over the chip for 3 min at a flow rate of 30 µl/min. Following 10 min dissociation, the chip was regenerated with an injection of 10 mM glycine pH 2.0 for 2 min at a flow rate of 30 µl/min, followed by a stabilization period of 1 min. Data (sensorgram: fc2-fc1) were fitted with a steady state affinity model using global fitting. Dissociation times were of at least 600 seconds. Measurements were performed in duplicate and included zero-concentration samples for referencing. Both Chi2 and residual values were used to evaluate the quality of a fit between the experimental data and individual binding models.

10 *Cell culture*

Raji (DSMZ, ACC-319) and Daudi (DSMZ, ACC-78) were cultured in RPMI1640. Cell culture media were supplemented with 10% heat-inactivated fetal calf serum (BioWest, cat# S1816), L-Glutamine (Biowest, cat# X0550), 1 mM sodium pyruvate (Dutscher, cat# L064213), 1% Nonessential Amino Acid (Gibco, cat# 11140-035), and 1% penicillin-streptomycin solution (Dutscher, cat# L0022). Medium was further supplemented with 1% HEPES (Dutscher, cat# L0180-100) for Raji and NCI-H929, and with 0.05 mM 2-mercaptoethanol (Gibco, cat# 31350-010) for NCI-H929.

Complement-Dependent Cytotoxicity (CDC) assay

Tumor cells were labelled with 5 µM calcein AM (ThermoFisher, cat #C1430) and plated in ultra-low attachment 96-well plates (Corning, cat# 3474) with increasing concentration of test antibody and in presence of human serum (Sigma Aldrich, H4522) for 4h30. Darzalex used here and in all other assays below was commercially obtained (conc perf 100 mg/5ml, Janssen). Triton X-100 was used as a positive control for maximum tumor cell killing. After the completion of the assay, cells were centrifuged at 350g for 5 minutes and cell supernatant was transferred in a 96well plate with clear bottom. Tumor cell killing was measured by quantifying calcein released in the supernatant using Synergy plate reader. Specific killing percentage was calculated according to the formula [(release in test condition – spontaneous release)/(maximum release – spontaneous release)].

Macrophage differentiation

PBMC were isolated by Ficoll gradient from fresh buffy coat. Human monocytes were subsequently isolated from PBMC using EasySep CD14+ monocytes isolation kit (Stemcell, cat# 19359). Isolated

monocytes were differentiated into macrophages by culturing them in complete medium containing 50ng/mL M-CSF (Peprotech, cat# 300-25) for 7 days.

Antibody-Dependent Cell Phagocytosis (ADCP) assay

ADCP assay shown in Figure 5 and Figure 8 was performed as follows: differentiated macrophages were collected using Detachin (AMSBio, cat# T00-100). 5 x10⁴ macrophages were plated in a 96-well flat bottom plate and incubated for 2h at 37 °C to let macrophages re-adhere. 1x10⁵ Raji cells labelled with phRodo (Sartorius, cat# 4649) following manufacturer's instructions were subsequently added to the plate (Effector: Target ratio 1:2) with 80 nM of test antibody. Plates were transferred into the IncuCyte cell analysis system (Sartorius) housed inside a cell incubator at 37 °C for 5h. Two images per well were taken every 30 min using a 10× objective with red channel acquisition time set to 800 ms. Plates were analyzed using IncuCyte software. Phagocytosis was quantified as the area under the curve measured for phRodo signal over 5h.

Assessment of phagocytosis by automated high throughput quantitative microscopy

ADCP assay shown in Figure 9 was performed as follows: 1.5 x10⁴ tumor cells labelled with 1 µg/mL pHrodo Red SE (ThermoFisher, P36600) were plated with 3x10³ monocytes-derived-macrophages stained with 2.5 µM Cell trace violet (ThermoFisher, C34557) (effector: target ratio = 5:1) in a 384-well clear-bottom plates (Corning, cat# 3770). Increasing concentration of test antibody was subsequently added to the well, and incubated for 2h30 at 37 °C. Phagocytosis was then evaluated using CellInsightCX5 High Content Screening Platform (ThermoFisher) and analyzed using HCS Studio Cell Analysis Software. Phagocytosis was quantified using image based analysis as the average number of pHrodo-bright tumor cells for 100 cell trace violet-positive macrophages.

Antibody-Dependent Cell Cytotoxicity (ADCC) assay

PBMC were isolated from fresh buffy coat by Ficoll gradient. NK cells were subsequently purified using NK cell isolation kit (Miltenyi, cat# 130-092-657). Purified NK cells were then incubated with 100 U/mL recombinant human IL-2 (Novartis, cat# L03AC01) overnight at 37 °C.

On the day of the experiment, 5x10⁴ IL-2 stimulated NK cells were plated with 1x10⁴ tumor cells (effector: target ratio = 5: 1) stained with 5 µM calcein AM (ThermoFisher, cat #C1430) in ultra-low attachment 96-well plates (Corning, cat# 3474). Increasing concentration of test antibody was subsequently added to the well and incubated for 4h30 at 37 °C. After the completion of the assay, cells were centrifuged at 350g for

5 minutes and resuspended in FACS Buffer containing Sytox dead cell stain (Thermofisher, cat# S11348). Samples were acquired using Cytoflex flow cytometer (Beckman Coulter) and analyzed using Flowjo (Tree Star). Viable tumor cells were identified as positive for calcein AM and negative for Sytox dead cell stain. Killing was quantified using the formula: $100 - [(count\ in\ test\ condition)/(count\ in\ no\ antibody\ condition)]$.

5 *Differential Scanning Calorimetry (DSC)*

The thermal stabilities of antibodies were compared using calorimetric measurements. Calorimetric measurements were carried out on a VP-DSC differential scanning calorimeter or a MicroCal PEAQ-DSC differential scanning calorimeter (Malvern Instruments, UK). The cell volume was 0.128 ml, the heating rate was 1 °C/min and the excess pressure was kept at 64 p.s.i. All protein fragments were used at a concentration of 1-2 mg/ml in PBS (pH 7.4). The molar heat capacity of each protein was estimated by comparison with duplicate samples containing identical buffer from which the protein had been omitted. The partial molar heat capacities and melting curves were analysed using standard procedures. Thermograms were baseline corrected and concentration normalized before being further analysed using a Non-Two State model in the manufacturer supplied software.

15 *Forced degradation*

Antibodies were diluted to 0.5 mg/mL in PBS, sterile filtered and incubated at 40 °C for a period of 3 to 14 days and then stored at -80 °C until analysis. Following the incubation, samples were analysed by SDS-PAGE and SE-HPLC as described above.

Results and conclusion

20 As discussed above, effector function is critical to the mode of action of BEAT CD38/CD47 bispecific antibodies. Mutations introduced in the Fc portion have previously been reported to increase the effector function of antibodies (e.g. Shields *et al.*, (2001) J Biol Chem 276(9): 6591-6604, Lazar *et al.*, (2006) Proc Natl Acad Sci U S A 103(11): 4005-4010). Accordingly, the Fc region of BEAT CD38/CD47 bispecific antibodies was further engineered to enhance multiple modes of action as described below.

25 *Antibody variants with enhanced complement-mediated effector function and enhanced antibody-dependent cellular phagocytosis*

A number of variants of the BEAT Fc were designed with the goal of enhancing CDC as well as ADCP. Interaction of the Fc portion of a target bound antibody with C1q, the first subcomponent of the C1

complement, activates the complement pathway and ultimately induces CDC. Several combinations of amino acid substitutions in the C1q binding region of the CH2 domain of IgG1 antibodies have previously been reported to enhance CDC (WO2011104604). ADCP conversely, depends on the interaction of the antibody Fc with the Fc gamma receptors (FcγRs) on effector cells. These are expressed on macrophages and FcγRIIa in particular has been implicated in ADCP, with FcγRIa and FcγRIIIa providing a lower but yet significant and complimentary contribution (Richards *et al.*, (2008) Mol Cancer Ther 7(8): 2517-2527). A plethora of mutations introduced into the CH2 domains of IgG antibodies have previously been reported to increase the affinities to the FcγRs to varying extents and to modulate FcγR mediated effector function (Shields *et al.*, (2001) *supra*, Lazar *et al.*, (2006) *supra*).

- 10 Here we aimed at introducing a combination of amino acid substitutions into the CH2 domains of the heterodimeric BEAT Fc with the goal of enhancing CDC and ADCP mediated by BEAT CD38/CD47 bispecific antibodies in cooperation with macrophage enabling CD47-SIRPα blockade.

We previously reported substitution S324N as well as combinations of S324N with E269D and S298A to greatly increase complement-induced lysis of target cells by an IgG1 antibody. Notably, substitution S298A has also been reported to increase affinity to FcγRIIIa and can thus be considered for CDC as well as ADCP enhancement (Shields *et al.*, (2001) *supra*). An important consideration to keep in mind when engineering ADCP enhancement in CD47 targeting antibodies, is the presence of CD47 as well as FcγRIIIa on the cell surface of platelet cells. The concern would be that CD38/CD47 bispecific antibodies with strongly increased affinity for FcγRIIIa could bind platelet cells via the Fc and by simultaneously interacting with CD47 could potentially induce cross-linking and aggregation of platelets, which would affect on-target specificity. To that extent, our engineering efforts were focused on increasing FcγRIIIa binding affinity, while limiting the increase in affinity to FcγRIIIa. Taken together, the aim was to explore various levels of increases in affinity to the different FcγRs in cooperation with CDC enhancing mutations, with the goal of finding the optimal balance between killing potency and on-target specificity. Accordingly, various combinations of CDC enhancing mutations S324N, E269D and S298A, and FcγR affinity modulating mutations S298A, K334E, K334A, S239D and I332E, were introduced into BEAT CD38/CD47-19 resulting in constructs -21, -22, -24 and -42 (Table 5). Substitutions were generally introduced symmetrically into both

heavy chains. Mutations S239D and I332E however, were introduced asymmetrically into the BEAT (A) Hc only, as symmetric engineering thereof abolished the expression of the BEAT bispecific antibodies.

Table 5. List of CD38/CD47 BEAT bispecific antibody constructs with effector function enhancing Fc mutations and their associated SEQ ID NOs.

Construct	Fc mutations	BEAT (A) Hc	BEAT (B) Hc	cLc
BEAT CD38/CD47-19	None	SEQ ID NO: 259	SEQ ID NO: 262	SEQ ID NO: 10
BEAT CD38/CD47-21	BEAT (A): S324N + K334E BEAT (B): S324N + K334E	SEQ ID NO: 274	SEQ ID NO: 275	SEQ ID NO: 10
BEAT CD38/CD47-22	BEAT (A): S324N BEAT (B): S324N	SEQ ID NO: 276	SEQ ID NO: 277	SEQ ID NO: 10
BEAT CD38/CD47-24	BEAT (A): E269D + S298A + S324N + K334A BEAT (B): E269D + S298A + S324N + K334A	SEQ ID NO: 278	SEQ ID NO: 279	SEQ ID NO: 10
BEAT CD38/CD47-42	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	SEQ ID NO: 280	SEQ ID NO: 277	SEQ ID NO: 10

5

Bispecific antibodies were produced and characterized as described above. Monodispersity of >95% was observed by SE-HPLC with <1% homodimeric contaminants as determined by RP-HPLC-MS. Endotoxin levels were inferior to 5 EU/mg. Their capacity to mediate CDC was assessed in a cell-based assay on Daudi cells. All further engineered variants showed superior CDC activity over the non-enhanced BEAT CD38/CD47-19, confirming that the mutations are functional in the context of the heterodimeric BEAT Fc, as they were in the context of wild-type IgG1 (Figure 4). S324N alone (BEAT CD38/CD47-22) mediated most of the gain in CDC. Additional mutations K334E in BEAT CD38/CD47-21 and E269D + S298A + K334A in BEAT CD38/CD47-24 mediated minimal additional gain over S324N alone at lower antibody concentrations. S239D + I332E did not mediate any additional gain in CDC over S324N alone (BEAT CD38/CD47-42).

15

Next, the capacity to mediate ADCP was assessed for BEAT CD38/CD47 bispecific antibodies in a cell-based assay on Raji cells. Additional constructs to those in tables 4 and 5 were assessed, where further FcγR affinity modulation was explored (Table 6). Removal of substitution E269D from BEAT CD38/CD47-23 and -24 was assessed in constructs BEAT CD38/CD47-25 and -26 with the hypothesis that while E269D is beneficial for CDC it could potentially disturb interaction with FcγRs as previously observed for mutation

20

E269A (Shields *et al.*, (2000) *supra*). Constructs were produced and characterized as described above. Monodispersity of >95% was observed by SE-HPLC with <1% homodimeric contaminants as determined by RP-HPLC-MS. Endotoxin levels were inferior to 5 EU/mg.

Table 6. List of further CD38/CD47 BEAT bispecific antibody constructs with effector function enhancing Fc mutations and their associated SEQ ID NOs.

Construct	Fc mutations	BEAT (A) Hc	BEAT (B) Hc	cLc
BEAT CD38/CD47-23	BEAT (A): E269D + S298A + S324N + E333A BEAT (B): E269D + S298A + S324N + E333A	SEQ ID NO: 281	SEQ ID NO: 282	SEQ ID NO: 10
BEAT CD38/CD47-25	BEAT (A): S298A + S324N + E333A BEAT (B): S298A + S324N + E333A	SEQ ID NO: 283	SEQ ID NO: 284	SEQ ID NO: 10
BEAT CD38/CD47-26	BEAT (A): S298A + S324N + K334A BEAT (B): S298A + S324N + K334A	SEQ ID NO: 285	SEQ ID NO: 286	SEQ ID NO: 10

Non-enhanced antibodies BEAT CD38/CD47-3, -6 and -19, which all share the same anti-CD47 VH, showed similar levels of ADCP, regardless of the anti-CD38 affinity, which was lowest for construct -6 and highest for construct -19 (Figure 5). Mutation K334E significantly enhanced ADCP for BEAT CD38/CD47-21. Removing substitution E269D mildly improved ADCP in BEAT CD38/CD47-25 and 26 over BEAT CD38/CD47-23 and -24. Mutations S239D + I332E introduced in BEAT CD38/CD47-42 mediated the largest gain in ADCP potency over any other construct. FcγR affinities for favorable constructs were measured by SPR and compared to those of the trastuzumab control IgG1 (Table 7). Strongest FcγR affinities correlated with largest gain in ADCP potency.

Table 7. Modulation of FcγR affinities as measured by SPR.

Construct	Fc mutations	Ia [fold gain in affinity vs control]	IIa [fold gain in affinity vs control]	IIb [fold gain in affinity vs control]	IIIa [fold gain in affinity vs control]
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BEAT CD38/CD47-21	BEAT (A): S324N + K334E BEAT (B): S324N + K334E	1027	1	0.1	2
BEAT CD38/CD47-42	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	3750	6	14	11

For T cell redirecting bispecific antibodies, Bluemel *et al.*, (2010) Cancer Immunol Immunother 59(8): 1197-1209 showed that potency can be affected by the targeted epitope and thus by the geometrical arrangement created on the cell surface. Accordingly, we hypothesized that the geometrical positioning of the Fc upon target engagement will determine the efficiency of C1q binding and in turn the extent of complement activation. Furthermore, we postulated that similarly, the geometry and distances created between the BEAT CD38/CD47 bispecific antibody and the FcγRs on immune cells will affect the potency of effector function. To that end, we explored various binding epitopes in CD38 as well as the “2+1” antibody format. “2+1” BEAT bispecific antibodies have an additional Fab domain fused to a first Fab domain via a flexible linker, commonly a glycine-serine sequence (Figure 6). The Fab domain that is located between the antibody Fc and the second Fab domain is termed the “Fc proximal” binding unit, while the Fab domain that is fused to the N-terminus of the VH of the “Fc proximal” Fab is termed the “Fc distal” binding unit. Analogously to the traditional BEAT antibody format described in Figure 3, which is now termed the “1+1” format, light chain mis-pairing in the “2+1” architecture is avoided by use of a cLc, which essentially allows the addition of an unlimited number of different binding domains to a BEAT antibody. Consequently, the cLc allows the engineering of a BEAT CD38/CD47 bispecific antibody that has two anti-CD38 binding units with different VH sequences that bind two distinct epitopes in CD38, in addition to the VH domain that binds CD47. Such a construct is referred herein as a “2+1” biparatopic BEAT bispecific antibody. A biparatopic BEAT CD38/CD47 bispecific antibody may induce a different geometry of binding on the target cell as compared to an antibody in the “1+1” format or an antibody in the “2+1” format that is monoparatopic and has two identical anti-CD38 binding units, and this in turn may modulate effector function.

“2+1” BEAT bispecific antibodies were engineered analogously to those in the “1+1” format with the anti-CD47 BEAT (A) arm designed as those described above. The anti-CD38 arm of the heterodimeric immunoglobulin consisted of a BEAT (B) Hc encompassing a first VH domain selected from Table 3 (anti-CD38-E2-RecA or anti-CD38-B6), a CH1 γ1 region, a glycine-serine linker (GGGGSGGGSGGGG), a second VH domain selected from Table 3 (anti-CD38-E2-RecA), a second CH1 γ1 region, a γ1 hinge region, a γ1 CH2 region, and a γ1 based BEAT (B) CH3 domain assembled with a cLc. “2+1” BEAT bispecific antibodies

resulting from the assembly of the BEAT (A) and BEAT (B) Hcs as well as the cLc are listed in Table 8. Importantly, biparatopic “2+1” BEAT bispecific antibodies consisted of VH domains that targeted non-overlapping epitopes in CD38 and that could bind simultaneously (Figure 2) (BEAT CD38/CD47-38, -39, -40 and -48). Contrariwise, monoparatopic “2+1” BEAT bispecific antibodies contained two copies of the same VH domain (BEAT CD38/CD47-32 and -34). “2+1” BEAT bispecific antibodies were expressed and purified as above. For all antibodies, monodispersity of >95% was observed by SE-HPLC with <1% homodimeric contaminants as determined by RP-HPLC-MS. Endotoxin levels were inferior to 5 EU/mg. BEAT CD38/CD47 bispecific antibodies were then further tested for their biological activity.

Table 8. List of CD38/CD47 “2+1” BEAT bispecific antibody constructs with and without effector function enhancing Fc mutations and their associated SEQ ID NOs.

Construct	Fc mutations	CD38 engagement	BEAT (A) Hc	BEAT (B) Hc	cLc
BEAT CD38/CD47-32	BEAT (A): E269D + S298A + S324N + E333A BEAT (B): E269D + S298A + S324N + E333A	Monoparatopic	SEQ ID NO: 281	SEQ ID NO: 287	SEQ ID NO: 10
BEAT CD38/CD47-34	BEAT (A): E269D + S298A + S324N + K334A BEAT (B): E269D + S298A + S324N + K334A	Monoparatopic	SEQ ID NO: 278	SEQ ID NO: 288	SEQ ID NO: 10
BEAT CD38/CD47-38	None	Biparatopic	SEQ ID NO: 259	SEQ ID NO: 289	SEQ ID NO: 10
BEAT CD38/CD47-39	BEAT (A): E269D + S298A + S324N + E333A BEAT (B): E269D + S298A + S324N + E333A	Biparatopic	SEQ ID NO: 281	SEQ ID NO: 290	SEQ ID NO: 10
BEAT CD38/CD47-40	BEAT (A): E269D + S298A + S324N + K334A BEAT (B): E269D + S298A + S324N + K334A	Biparatopic	SEQ ID NO: 278	SEQ ID NO: 291	SEQ ID NO: 10
BEAT CD38/CD47-48	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	Biparatopic	SEQ ID NO: 280	SEQ ID NO: 292	SEQ ID NO: 10

The capacity of “2+1” BEAT bispecific antibodies to mediate CDC compared to those in the “1+1” format was assessed in a cell-based assay on Daudi cells. Moving from the “1+1” format to the “2+1” format, in

the context of an Fc that was not further engineered for effector function, did not improve CDC potency (BEAT CD38/CD47-19 vs -38) (Figure 7). In the context of enhanced BEAT Fc however, the “2+1” architecture led to a gain in CDC activity at lower antibody concentrations over the equivalent antibody in the “1+1” format (BEAT CD38/CD47-40 and -48 vs -24 and -42). Next, “2+1” BEAT bispecific antibodies were compared for their capacity to induce ADCP in a cell based assay on Raji cells (Figure 8). Monoparatopic “2+1” BEAT bispecific antibodies showed a gain in ADCP potency over their “1+1” counterparts (BEAT CD38/CD47-32 and -34 vs -23 and -24), however the level of phagocytosis could not match that of the most favorable “1+1” construct BEAT CD38/CD47-42. Intriguingly, biparatopic “2+1” BEAT bispecific antibodies showed significant gain in ADCP potency, even with a non-enhanced Fc (BEAT CD38/CD47-38), to levels of the most enhanced “1+1” construct BEAT CD38/CD47-42, confirming the hypothesis that the geometry of antibody binding on the cell surface impacts effector function and that biparatopic engagement positively affects ADCP potency. Mild increase in FcγR affinities in the context of biparatopic “2+1” BEAT bispecific antibodies did not further improve ADCP activity (BEAT CD38/CD47-39 and -40). A biparatopic construct with mutations S239D + I332E, which mediate the strongest increases in FcγR binding, was not yet available at the time the assay above was performed but was later shown in a subsequent assay to have further potentiated ADCP activity over the equivalent “1+1” construct (BEAT CD38/CD47-48 vs -42) (Figure 9). FcγR affinities for favourable constructs were measured by SPR and compared to those of trastuzumab control IgG1 (Table 9).

Table 9. Modulation of FcγR affinities for “2+1” constructs as measured by SPR.

Construct	Fc mutations	Ia [fold gain in affinity vs control]	IIa [fold gain in affinity vs control]	IIb [fold gain in affinity vs control]	IIIa [fold gain in affinity vs control]
BEAT CD38/CD47-48	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	864	7	16	10
BEAT CD38/CD47-32	BEAT (A): S324N + S298A + E269D + E333A BEAT (B): S324N + S298A + E269D + E333A	12	0.5	1	3

BEAT CD38/CD47-40	BEAT (A): S324N + S298A + E269D + K334A BEAT (B): S324N + S298A + E269D + K334A	4513	0.4	1	4
BEAT CD38/CD47-34	BEAT (A): S324N + S298A + E269D + K334A BEAT (B): S324N + S298A + E269D + K334A	874	0.4	1	4

Taken together, mutation S324N favorably affected CDC potency and the biparatopic “2+1” format further potentiated the same (Figure 4 and Figure 7). S239D + I332E introduced asymmetrically in the BEAT (B) heavy chain resulted in the strongest gain in ADCP activity, which was further enhanced by the biparatopic “2+1” architecture.

Construct BEAT CD38/CD47-48 which encompassed all of the aforementioned, was engineered with an “Fc distal” anti-CD38 binder of low affinity (anti-CD38-B6, SPR KD = 225 nM). Affinity maturation of said binder (Table 3) allowed the engineering of an additional biparatopic “2+1” BEAT bispecific antibody with high affinity “Fc proximal” (anti-CD38-E2-RecA) and “Fc distal” (anti-CD38-B6-D9) anti-CD38 binding domains (BEAT CD38/CD47-60) (Table 10). BEAT CD38/CD47-60 as well as -42 and 48 were transiently expressed in CHO, purified and characterized as described above. Monodispersity of >95% was observed by SE-HPLC with <1% homodimeric contaminants as determined by RP-HPLC-MS. Endotoxin levels were inferior to 5 EU/mg.

Table 10. SEQ ID NOs for construct BEAT CD38/CD47-60.

Construct	Fc mutations	CD38 engagement	BEAT (A) Hc	BEAT (B) Hc	cLc
BEAT CD38/CD47-60	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	Biparatopic	SEQ ID NO: 280	SEQ ID NO: 293	SEQ ID NO: 10

Next, the capacity to induce CDC, ADCP as well as ADCC were assessed in cell based assays for construct BEAT CD38/CD47-60 compared to favorable candidates -42 and -48. Notably, BEAT CD38/CD47-60, which had a high affinity anti-CD38 binding domain in the “Fc distal” position, induced significantly more potent CDC than BEAT CD38/CD47-48 that had a low affinity anti-CD38 binding domain in the “Fc distal” position

(Figure 9). ADCC was similar for all constructs and ADCP of Daudi cells showed minimal gain for BEAT CD38/CD47-60 over -48.

Biophysical characterization of BEAT CD38/CD47 bispecific antibodies

BEAT CD38/CD47 bispecific antibodies were further characterized by DSC to investigate the impact of effector function engineering in the Fc on thermal stability (Table 11). Corresponding thermograms for candidates BEAT CD38/CD47-42, -48 and -60 are depicted in Figure 10. All BEAT Fcs with effector function enhancing mutations had reduced thermal stability compared to non-enhanced BEAT Fc. Mutations S239D + I332E had the strongest impact on thermal stability followed by K334E. Other combinations of mutations also reduced thermal stability but to a lesser extent.

Table 11. Summary of thermal transitions (T_m) observed by DSC for BEAT CD38/CD47 bispecific antibodies.

Construct	Fc mutations	T _m 1 [°C] Fc	T _m 2 [°C] Fab 1	T _m 3 [°C] Fab 2
BEAT CD38/CD47-3	None	69.5	83.5	-
BEAT CD38/CD47-21	BEAT (A): S324N + K334E BEAT (B): S324N + K334E	62.7	78.2	82.0
BEAT CD38/CD47-32	BEAT (A): E269D + S298A + S324N + E333A BEAT (B): E269D + S298A + S324N + E333A	64.2	77.6	81.9
BEAT CD38/CD47-34	BEAT (A): E269D + S298A + S324N + K334A BEAT (B): E269D + S298A + S324N + K334A	65.1/67.6	77.6	81.9
BEAT CD38/CD47-40	BEAT (A): S324N + S298A + E269D + K334A BEAT (B): S324N + S298A + E269D + K334A	64.7/67.5	79.1	82.4
BEAT CD38/CD47-42	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	61.3	78.3	82.8
BEAT CD38/CD47-48	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	61.9	79.7	83.0

BEAT CD38/CD47-60	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	62.3	79.0	82.8
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Given the reduced thermal stability of the enhanced BEAT Fcs, we further assessed the stability of the different variants in a 2 week forced degradation study at 40 °C. Percent monodispersity as determined by SE-HPLC was unchanged after 2 weeks for the original BEAT Fc as well as for all of the tested enhanced BEAT Fc variants while that of trastuzumab control IgG1 was severely reduced (Table 12).

Table 12. Results of force degradation study performed on CD38/CD47 BEAT bispecific antibodies.

Construct	t=0 [% monodispersity]	t=14, 4 °C [% monodispersity]	t=3d, 40 °C [% monodispersity]	t=7d, 40 °C [% monodispersity]	t=14d, 40 °C [% monodispersity]	Δ [% monodispersity] (t=0) – (t= 14)
BEAT CD38/CD47-3	95.8	96.5	96.4	97.2	96.9	1.2
BEAT CD38/CD47-21	98.8	99.5	98.8	99.0	99.2	0.3
BEAT CD38/CD47-40	99.0	99.5	98.9	98.6	97.9	-1.2
BEAT CD38/CD47-42	98.6	99.0	98.6	98.6	98.6	-0.0
BEAT CD38/CD47-48	97.8	98.0	97.4	97.0	95.9	-1.9
Control IgG1	94.9	95.2	73.7	36.9	0	-94.9

Next, we assessed whether engineering of enhanced BEAT Fc impacted binding of human neonatal Fc receptor (FcRn). FcRn binds at the interface between the CH2 and the CH3 domains and thus changes in the Fc could potentially modulate the binding affinity, which in turn could affect serum half-life (Martin *et al.*, (2001) Mol Cell 7(4): 867-877). Affinities of enhanced BEAT Fc variants for FcRn were determined by SPR (Table 13). Binding of FcRn was not negatively impacted by further engineering of the BEAT Fc and KDs were in the range of what was measured for trastuzumab control IgG1.

Table 13. SPR measurements for BEAT CD38/CD47 binding to FcRn.

Construct	Fc mutations	KD ratio [BEAT/Control IgG1]
BEAT CD38/CD47-21	BEAT (A): S324N + K334E BEAT (B): S324N + K334E	0.4
BEAT CD38/CD47-40	BEAT (A): S324N + S298A + E269D + K334A BEAT (B): S324N + S298A + E269D + K334A	0.3
BEAT CD38/CD47-42	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	0.5
BEAT CD38/CD47-48	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	0.2
BEAT CD38/CD47-60	BEAT (A): S239D + I332E + S324N BEAT (B): S324N	0.5

Example 6: *In vitro* biological characterization of BEAT CD38/47-48 and BEAT CD38/47-60 candidates

Example 6.1: *In vitro* characterization of BEAT CD38/47-48 and 60 binding activity of multiple myeloma and lymphoma cell lines

5 Material and methods

Hundred thousand cells per well were plated in U-bottom 96-well plate (TPP) and centrifuged at 350g for 5 minutes. Supernatants were discarded by flicking the plates and cell pellets were resuspended in 100 μ l of a dose-response of primary purified antibodies. Cells were incubated for 30 minutes at 4°C (dark). After two washing steps (350g for 5 minutes), cells were stained with monoclonal mouse anti-Human IgG-Fc PE
 10 diluted at 1/200 in FACS Buffer for 30 minutes at 4°C. After a last washing step, cells were suspended in FACS buffer containing Sytox (1/2000) or Dapi (1/50000).

Flow-cytometric analysis was then performed with a Cytotflex (Beckman Coulter).

Data were analyzed using FlowJo software (BD): PE Geometric Mean of Fluorescence Intensities (geoMFI) of viable single cells for each sample were extracted. Since antibodies can display some non-specific
 15 binding to cells, a normalization was performed using the geoMFI of the Isotype control antibody (ABC-IgG1). The values of geoMFI from the control staining were subtracted to the geoMFI values of each molecule to generate the relative fluorescence intensity (RFI). All the KD values were calculated using the

RFI. For some curves, geoMFI values at the highest concentration were excluded due to the observation of a hook effect.

For the KD calculation, a non-linear one site binding (hyperbola) regression was applied in Prism software (GraphPad) on the RFI values.

5 Results and conclusions

Binding of BEAT CD38/47-48 and -60 was evaluated by flow cytometry on a panel of multiple myeloma (KMS-12-PE, KMS-12-BM, NCI-H929), and lymphoma (Raji, Daudi) cell lines expressing different level of CD38 and CD47 (Figure 11). The rationale for this assessment was to evaluate the Kd of BEAT 38/47-48 and -60 as well as to understand their binding to tumor cell lines as compared to anti-CD38 (Darzalex) or anti-
10 CD47 (5F9-like, Magrolimab) monoclonal antibody binding.

BEAT CD38/47-48 and BEAT CD38/47-60 show higher binding to CD38 high tumor cell lines (Daudi, Raji, KMS-12-PE) as compared to benchmark Darzalex and 5F9. In addition, BEAT CD38/47-48 and BEAT CD38/47-60 show higher binding to CD38 low tumor cell lines (NCI-H929 and KMS-12-BM) as compared to Darzalex. Multiple myeloma cells in patients show several features of primary or acquired tumor escape
15 mechanisms, including the upregulation of CD47 “do not eat me” signal and the downregulation of cell surface CD38 (in patients receiving Darzalex as standard of care for example). BEATs 38/47 are designed to overcome both these tumor escape mechanisms and improve the targeting of CD38 expressing tumor cells as compared to standard of care Darzalex. The present cell binding data suggest that co-targeting of CD38 and CD47 could allow for a better therapeutic window as compared to current benchmark,
20 especially in CD38 low MM tumor cells.

Example 6.2: BEAT CD38/47-48 and BEAT CD38/47-60 inhibition CD47-SIRPα interactions

Materials and methods

10x10³ Daudi cells labelled with 8uM Hoescht (Thermofisher, cat# 33342) were plated in 20uL in a 384-well clear-bottom plates (Corning, cat# 3770) and co-incubated with 20uL of increasing concentration
25 (from 0.00015 nM to 640 nM) of test antibody for 30 minutes at 4 °C. In parallel, a mix of antibodies (detection mix) composed of 300 ng/mL SIRPα-mIgG1 Fc fusion protein (Accrobiosystem, cat# SIA-H52A8) and 2.5ug/mL of Alexa Fluor 647-conjugated anti-mouse IgG Fc detection reagent (Jackson ImmunoResearch, cat# 115-605-205) was prepared in assay buffer (PBS + 2.5% FBS + 0.1% NaN₃) and incubated for 30min at room temperature in the dark. The detection mix (20 uL) was added to the cells

and incubated for 3h at room temperature. Binding of recombinant SIRP α -Fc to tumor cells was quantified by image based analysis using CellInsight CX5 High Content Screening Platform (Thermo Fisher Scientific). CD47/SIRP α inhibition is shown as percentage of inhibition of the Detection Mix fluorescence signal.

Results and conclusions

- 5 The ubiquitous immune checkpoint CD47 works as a “Don’t eat me” signal inhibiting phagocytosis upon binding to its cognate receptor SIRP α expressed by macrophages. Tumor cells hijack this mechanism and overexpress CD47 as a mechanism of immune evasion to avoid phagocytosis. BEAT CD38/47-48 and BEAT CD38/47-60 are designed to selectively block CD47- SIRP α on CD38 expressing cells upon target-co-engagement and enhance antibody dependent cell phagocytosis. We therefore assessed using *in vitro*
- 10 models and image based analysis, the efficacy of BEAT CD38/47-48 and BEAT CD38/47-60 to inhibit CD47/SIRP α interaction on CD38 positive tumor cells (Figure 12).

- Despite low affinity for CD47, BEAT CD38/47-48 and BEAT CD38/47-60 show a potent inhibition of CD47/SIRP α interaction on Daudi cells. This is comparable to the ability of the high affinity anti-CD47 5F9 antibody to inhibit CD47/SIRP α interaction. Altogether the data suggest that combining a high affinity
- 15 anti-CD38 binder allow for inhibition of CD47/SIRP α interaction even in the presence of a low affinity CD47 binder.

Example 6.3. BEAT38/47-48 and -60 show superior killing of CD38^{low} expressing tumor cells through antibody dependent cell phagocytosis (ADCP) as compared to clinical benchmark Darzalex

Material and methods

- 20 1.5 x10⁴ tumor cells labelled with 1 μ g/mL pH-rodo Red SE were plated with 3x10³ monocytes-derived-macrophages stained with 2.5 μ M Cell trace violet (effector: target ratio = 5:1) in a 384-well clear-bottom plates. In some experiments, either 2.8 ng/ml sCD38 or 500 M/ml red blood cells were added to each well in order to assess the impact of CD38 and CD47 antigen-sink. Increasing concentration of test antibody was subsequently added to the well and incubated for 2h30 at 37°C, 5% CO₂. Phagocytosis was then
- 25 evaluated using CellInsightCX5 High Content Screening Platform and analyzed using HCS Studio Cell Analysis Software. Phagocytosis was quantified using image-based analysis as the average number of pHrodo-bright tumor cells for 100 cell trace violet-positive macrophages.

Results and conclusions

Tumor cells has evolved mechanisms to evade the recognition by the immune system, including hijacking CD47-SIRP α axis. Indeed, CD47 is overexpressed in MM patients where it is reported that nearly 100% of CD38 positive myeloma cells express CD47 at a high level. CD47 expression has been associated with disease progression and low response to therapies in both hematological malignancies and solid tumors.

CD47/SIRP α neutralizing molecules have shown anti-tumor activity against a wide range of tumor cell lines both *in vitro* and *in vivo*. *In vitro*, CD47/SIRP α blocking antibodies have been described to restore tumor cell phagocytosis and to potentiate antibody dependent cell phagocytosis initiated by tumor-targeting antibodies such as Rituximab or Trastuzumab. Several anti-CD47 targeting approaches have been developed both in preclinical and in clinical settings; the more prominent is the anti-CD47 antibody Hu5F9-G4, a monoclonal antibody with two high affinity anti-CD47 Fab arms with low/absent Fc function in an IgG4 format (herein referred to as 5F9). Owing to its low functionality IgG4 Fc portion, 5F9 primarily works in combination, as it is reported in clinical trials as combo partner synergizing with Rituximab in the treatments of Non-Hodgkin's Lymphomas. Differently for 5F9, BEAT CD38/47-48 and -60 enhances ADCP as a stand-alone molecule, by selectively blocking CD47-SIRP α interaction on CD38 expressing tumor cells while concomitantly activating phagocytosis through its Fc enhanced for effector functions, including enhanced ADCP. In addition, BEAT CD38/47-48 and -60 differentiates from MM standard of care Darzalex as it is predicted to potentiate ADCP activity against MM cells by targeting CD38 tumor cells and concomitantly CD47/SIRP α interactions. The ability of BEAT 38/47top candidates to inhibit CD47/SIRP α interaction was assessed *in vitro* in a competitive binding assay using a SIRP α -Fc-fusion protein. Both BEAT 38/47-48 and -60 efficiently inhibited CD47/SIRP α interactions on tumor cells *in vitro* to a comparable extent of that mediated by 5F9 (Example 6.2).

We assessed BEATs 38/47-48 and -60 ADCP potency against tumor cells as compared to clinical benchmarks 5F9 and Darzalex. Both BEAT 38/47-48 and -60 showed a comparable maximal level of phagocytosis induction of CD38^{high} expressing tumor cells as that mediated by Darzalex. Against CD38^{high} expressing tumor cells, Darzalex showed a slightly higher albeit statistical significant potency as that of BEAT 38/47-48 and -60 measured by its lower EC50 value. On the contrary, on CD38^{low} expressing tumor cells, both BEAT 38/47-48 and -60 induced a statistical significant higher phagocytosis of tumor cells, at a level that was twice the amount of that induced by Darzalex, as measured by their maximal phagocytosis index (Figure 13 and Table 14). Albeit blocking CD47/SIRP α interaction with only one Fab arm of low

affinity, Both BEAT 38/47-48 and -60 showed a comparable efficiency as that of 5F9 at inducing phagocytosis of both CD38^{high} or CD38^{low} expressing tumor cells. This latter point is of high relevance as it suggests that lowering anti-CD47 affinity do not impact on tumor cell phagocytosis in the BEAT CD38/47 format when targeted on tumor cells via concomitantly having two anti-CD38 high affinity Fab arms. When

5 comparing our internal clinical candidates BEAT 38/47-48 and -60, we found no differences in their potency as measured by both Maximal killing or EC50 in either CD38^{high} or CD38^{low} expressing tumor cells (Figure 13).

These data suggest that BEAT CD38/47 design allows for a comparable phagocytosis of tumor cells as that of 5F9, albeit drastically lowering the affinity to CD47 thereby potentially improving CD47 on target.

10 Indeed, targeting CD47 can be challenging due to its ubiquitous expression; RBCs and platelets are among the cell types better characterized for the involvement of their clearance being dependent on CD47 expression. Therefore, a critical aspect in designing anti-CD47 therapies is mitigating the crosslinking of RBCs (or platelets) that are the main antigen sinks. As a matter of fact, due to its on-target binding to RBCs, 5F9 caused a dose-dependent anemia in 27-41% of NHL patients treated. In addition, BEAT CD38/47

15 format showed to induce higher potency against CD38^{low} expressing tumor cells as that induced by standard of care Darzalex. Altogether our data suggest that both BEAT 38/47-48 and -60 have improved potency as compared to MM standard of care in the settings of low CD38 antigen availability while having a more favorable on target on RBCs as that reported by 5F9.

20 Table 14. Statistics values calculated in CD38^{high} (Daudi) or CD38^{low} (KMS12-BM) tumor cells using Tuckey's multiple comparison tests. NS = not significant. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Conditions	CD38 ^{high}		CD38 ^{low}	
	Max Phagocytosis	EC50	Max Phagocytosis	EC50
BEAT 38/47-48 vs. Darzalex	NS	> 0.001	> 0.001	NA
BEAT 38/47-60 vs. Darzalex	NS	> 0.05	> 0.01	NA
BEAT 38/47-48 vs. 5F9	NS	NS	NS	NS
BEAT 38/47-60 vs. 5F9	NS	NS	NS	NS
Darzalex vs 5F9	NS	> 0.05	> 0.01	NA
BEAT 38/47-60 vs. BEAT 38/47-48	NS	NS	NS	NS

Example 6.4: BEAT38/47-48 and -60 show superior killing of CD38^{low} expressing tumor cells as compared to mono arm anti-CD47

5 Material and methods

Experiments performed as described in paragraph "Material and methods" of Example 6.3.

Results and conclusions

To assess whether co-targeting CD38 and CD47 improved functions of BEATs 38/47 as compared to single targeting anti-CD38 or anti-CD47, we analyzed the capacity of BEAT 38/47-48 and -60 to induce ADCP of
 10 tumor cells relative to their CD38 or CD47 mono arms (Figure 14A). Both BEAT 38/47-48 and -60 showed enhanced ADCP of CD38^{low} expressing tumor cells as measured their higher maximal phagocytosis as compared to Monoarm CD38-59 and -79 respectively. In addition, albeit Monoarm CD47-54 reached a similar or higher induction of phagocytosis of tumor cells as compared to that of BEAT 38/47-48 and -60 (in 3 out of 7 experiments) its potency to induce phagocytosis was lower than that of both BEAT 38/47-48
 15 and -60 as measured by a higher EC50 (Figure 14B and Table 15).

Altogether BEAT 38/47-48 and -60 show improved potency over their relatives anti-CD38 and anti-CD47 mono arms suggesting that BEAT format it is luckily to induce a better potency in patients as that induced by targeting single CD38 or CD47.

Table 15. Statistic values calculated in CD38^{low} (KMS12-BM) tumor cells using Tuckey's multiple comparison
 20 tests.

Conditions	Max Phagocytosis	EC50
BEAT 38/47-48 vs. Monoarm CD47-54	> 0.05	> 0.05
BEAT 38/47-60 vs. Monoarm CD47-54	> 0.05	> 0.05
BEAT 38/47-48 vs. Monoarm CD38-59	> 0.05	> 0.05
BEAT 38/47-60 vs. Monoarm CD38-79	> 0.05	> 0.05

Example 6.5: BEAT38/47-48 and -60 show superior killing of tumor cells through complement dependent cytotoxicity (CDC) as compared to clinical benchmarks

Material and methods

5 Tumor cells were labelled with 5 μ M calcein AM and plated in 96-well plates with increasing concentration of test antibody and in presence of human serum for 4h30 at 37°C, 5% CO₂. In some experiments, either 2.8 ng/ml sCD38 or 500 M/ml red blood cells were added to each well in order to assess the impact of CD38 and CD47 antigen-sink.

10 Triton X-100 was used as a positive control for maximum tumor cell killing. After the completion of the assay, cells were centrifuged at 350g for 5 minutes to pellet the cells. 100 μ L of supernatant was then carefully transferred in a black flat bottom 96 wells plate, without transferring any cell. Fluorescence induced by the calcein release was read with a Synergy Plate reader using the following parameters:

- Excitation wavelength: 485 nm
- Emission wavelength: 515 nm

Specific killing was calculated according to the formula:

15 % Specific Killing= [(release in test condition – spontaneous release)/(maximum release TritonX100 – spontaneous release)].

Results and conclusions

20 Interaction of the Fc portion of a target bound antibody with C1q, the first subcomponent of the C1 complement system, activates the complement pathway and ultimately induces CDC. This is one of the mechanisms of action (MOA) of most clinically validated antibodies to kill tumor cells, including MM standard of care Darzalex. Tumor cells evolves and undergo several immune escape mechanisms, including escape to drug treatments (acquired escape mechanisms). Pertinent to MM, escape to CDC killing is one of the best-established mechanisms of resistance to Darzalex which eventually impact in the relapse of the disease. The acquired escape mechanisms to CDC is multifactorial and still need to be fully
25 understood. However, several factors influence it, including antigen density per tumor cells (CD38 downregulation) as well as the overexpression of complement regulatory proteins (including CD46, CD55 and CD59). For these reasons, BEATs 38/47-48 and -60 were designed with having dedicated engineering of their Fc portions to enhance Fc effector functions, including CDC (as reported WO2011104604). In addition, as previously reported in this patent, both BEATs 38/47-48 and -60 in their 2+1 architectures,

already enhanced CDC as compared to 1+1 architectures, most probably as a consequence of the enhancing CD38 clustering in the cell surface for optimal CDC killing (see Example 5, and Figure 7).

We assessed BEATs 38/47-48 and -60 potency against tumor cells to induce CDC as compared to clinical benchmarks. Both BEATs 38/47-48 and -60 showed a statistically significant higher tumor cells killing by CDC as compared to Darzalex. As expected, 5F9-like did not induce any detectable killing by CDC, in line with its low/absent IgG4 Fc effector format. When comparing our internal clinical candidates BEAT 38/47-48 and -60, we found that BEAT 38/47-60 showed a statistically significant difference in potency as measured by a lower CDC EC50. No differences in Maximal killing between the two BEATs CD38/47 were found (Figure 15 and Table 16).

These data suggest that, modulating Fc effector function with dedicated CDC enhancing mutations, improve potency as compared to using wild type (WT) IgG-1 class as in Darzalex. This improved potency against Darzalex is luckily to favor an enhanced activity in patients of BEATs 38/47 in context where CDC might be impaired.

Table 16. Statistics values calculated in CD38^{high} (Daudi) tumor cells using using Tuckey's multiple comparison test (for Max Killing) and T-test (for EC50). NS = not significant.

Conditions	Max Killing	EC50
BEAT 38/47-48 vs. Darzalex	> 0.0005	NS
BEAT 38/47-60 vs. Darzalex	> 0.0005	NS
BEAT 38/47-60 vs. BEAT 38/47-48	NS	> 0.005

Example 6.6: BEAT38/47-48 and -60 show superior killing of CD38^{low} expressing tumor cells through antibody dependent cell cytotoxicity (ADCC) as compared to clinical benchmarks

Material and methods

Tumor cells were first labelled with 1 μ M eFluor670 and then plated in a 96-well plate with increasing concentrations of BEAT 38/47 or control antibodies. Purified NK cells were then added to make a final Effector:Target (E:T) ratio of 5:1. The plates were incubated at 37°C, 5% CO₂. After 4h30 incubation, the plates were centrifugated. Cells were then resuspended in 100 μ l FACS buffer containing either Sytox (1/2000) or Dapi (1/50000).

Flow-cytometric analysis was then performed with a Cytoflex (Beckman Coulter). Viable tumor cells were identified as positive for eFluor670 and negative for Sytox dead cell stain.

Absolute number of live tumor cells were then reported and % of Killing and %Specific ADCC were calculated with the following formulas:

5

$$\% \text{Specific Killing} = (1 - (\text{Target Abs.count/well of } \textit{sample} / \text{Average of Target Abs.count/well of } \textit{Target only})) * 100$$

$$\% \text{Specific ADCC} = \% \text{Specific Killing of } \textit{sample} - \% \text{Specific Killing of } \textit{no Ab}$$

Results and conclusions

10 Interaction of the Fc portion of a target bound antibody Fcγ-Receptor III (CD16) activates NK cells and induces potent degranulation leading to ADCC of tumor cells. Together with CDC, ADCC is one of best characterized MOA of Fc competent antibodies to kill tumor cells and is considered one of the more prominent mechanism induced by Darzalex to kill MM cells.

We assessed BEAT 38/47-48 and -60 ADCC potency against tumor cells as compared to clinical benchmarks (Figure 16 and Table 17). Both BEATs 38/47-48 and -60 showed a comparable killing of CD38^{high} expressing tumor cells as that induced by Darzalex. On the contrary, on CD38^{low} expressing tumor cells, albeit reaching a similar maximal level of tumor cell killing as that of Darzalex, both BEATs 38/47-48 and -60 showed a more prominent potency as measured by a statistical significant lower EC50 of killing. Similarly to what already shown with CDC, 5F9-like did not induce any detectable killing by ADCC, in line with its low/absent IgG4 Fc effector format. These data suggest that by having two bi-paratopic CD38 Fab arms in BEAT CD38/47-48 and -60, can improve the efficacy of ADCC when CD38 level are scarce on cell surface. When comparing our internal clinical candidates BEAT 38/47-48 and -60, we found no differences in their potency as measured by both Maximal killing or EC50 in either CD38^{high} or CD38^{low} expressing tumor cells.

The improved ADCC potency against Darzalex on CD38^{low} expressing tumor cells is luckily to favor an enhanced activity in patients that show low expression of CD38 as a consequence of antigen down modulation due to Darzalex treatment as well as on MM clones having low CD38 due to acquired immune escape mechanisms.

25

Table 17. Statistics values calculated using Tuckey's multiple comparison tests in CD38^{high} (Raji) or CD38^{low-int} (NCI-H929) tumor cells. NS = not significant.

Conditions	CD38 ^{high} tumor cells		CD38 ^{low/int} tumor cells	
	Max Phagocytosis	EC50	Max Phagocytosis	EC50
BEAT 38/47-48 vs. Darzalex	NS	NS	NS	> 0.05
BEAT 38/47-60 vs. Darzalex	NS	NS	NS	> 0.05
BEAT 38/47-60 vs. BEAT 38/47-48	NS	NS	NS	NS

Example 6.7: BEAT CD38/47-48 and BEAT CD38/47-60 induce killing of NCI-H929 MM tumor cells measure by Multiple mode of action of killing (MMoAK) assay

Material and methods

Peripheral Blood Mononuclear Cells (PBMCs) from Healthy Volunteers were harvested from buffy coats obtained from the La Chaux-de-Fonds Transfusion Center. For blood filter processing, buffy coats were diluted in 50 ml of PBS (1/2 dilution) and then transferred in 3 SepMate tubes previously loaded with Ficoll. Isolation was performed following manufacturer's instructions (centrifugation at 1200g for 10 minutes with brake). The mononuclear layer was transferred into 50 ml falcon tubes, the PBMCs were washed 3 times in PBS (300 g for 10 min), counted and frozen at -80°C. The day before the assay, autologous PBMCs were thawed and incubated overnight at 37°C in complete medium. Human monocytes were purified from frozen PBMCs and differentiated into macrophages by culturing them during 7 days in complete medium containing 50ng/mL M-CSF.

MMoAK Assay

On the day of the experiment, 1x10⁵ PBMCs + 5000 autologous Monocyte-Derived-Macrophages were plated with tumor cells labelled with 5μM eFluor 670 in presence of 50% human serum in ultra-low attachment 96-well plates. In some experiments (Figure 18), either 2.8 ng/ml sCD38 or 500 M/ml red blood cells were added to each well in order to assess the impact of CD38 and CD47 antigen-sink. Increasing concentration of test antibody and control antibodies was then added to the well and incubated for further 48h at 37°C, 5% CO₂. After completion of the assay, cells were centrifuged at 350g for 10 minutes and resuspended in 100 μl FACS buffer containing either Sytox (1/2000) or Dapi (1/50000). Samples were acquired using Cytoflex flow cytometer and analyzed using Flowjo software (tree star).

Viable tumor cells were identified as positive for eFluor-670 and negative for Sytox dead cell stain. Absolute number of live tumor cells were then reported and % of Killing and %Specific Killing were calculated with the following formulas:

- Killing capacity of BEAT 38/47 was quantified using the formula:
- 5 - %Specific Killing = $(1 - (\text{Target Abs.count/well of sample} / \text{Average of Target Abs.count/well of Target only})) * 100$

Results and conclusions

Physiologically, antibody dependent effector functions do not occur as separate entities but rather concomitantly. To accurately compare the potency of BEATs 38/47-48 and -60 and benchmarks, an optimized multiple mode of killing assay was established *in vitro* (Figure 17A). It consists of a co-culture of human PBMCs stimulated with 100 U/ml of IL-2 with tumor cells in the presence of human serum and autologous monocyte-derived macrophages. With this approach, in the presence of test antibodies, tumor cells are killed through multiple mechanisms such as ADCC by IL-2 activated NK cells, Re-Directed Lysis (RDL) by T cells, CDC by complement and ADCP (mediated by the presence of phagocytes).
10
15 Importantly, in MMoAK assays, human serum acts also as a source of human immunoglobulins (Igs), a key element to assess the potency of antibody-dependent effector functions in the presence of physiological levels of Igs. Hence, BEAT 38/47-48 and -60 killing potency can be quantified and accurately compared to benchmarks through this assay.

Under this condition, 5F9 failed to induce detectable level of tumor cells killing, most probably as a consequence of having an IgG4 Fc with minimal effector function thereby outcompeting for Fc receptors with Igs present in the serum. In marked contrast, BEAT 38/47-48 and -60 exhibited a prominent tumor cell killing which was twice as high as that induced by Darzalex (Figure 17B). When comparing our internal clinical candidates, no differences in Maximal killing between the two BEATs CD38/47 were found, however, BEAT 38/47-60 showed a statistically significant difference in potency as measured by a lower EC50 in MMoAK (Figure 17C and D).
20
25

CD38 can be released in serum and the fraction of circulating soluble CD38 (sCD38) increases with disease stage in MM thereby potentially functioning as a sink for CD38 and thus limiting the efficacy of CD38 targeting therapies. Together with this, high number of circulating RBCs could be a second major antigen sink for BEATs 38/47-48 and -60 based on high level CD47 expression on RBCs. Thus, the impact of sCD38

and a high concentration of RBCs were evaluated in MMoAK to test whether antigen sink impact BEATs 38/47-48 and -60 potency *in vitro*. Presence or absence of sCD38 at 2.8 ng/ml (a concentration that is detected in patient bone marrow) did not impact BEATs 38/47-48 and -60 killing of tumor cells through MMoAK as measured by no difference in maximal killing or EC50 (Figure 18 and Table 18). Likewise, this mode of killing was not impacted by a high concentration of RBCs (500 million/ml) (Figure 18).

Altogether, by using an *in vitro* assay that aim at recapitulating the complexity of the MOAs of Fc competent antibody *in vivo*, we found that BEAT 38/47-48 and -60 show important differentiations in terms of potency as compared to MM standard of care Darzalex. In addition, this assay remarks how critical is the correct activity of Fc functions, as in the presence of serum, 5F9 is completely abrogated in its capacity to phagocytose CD38^{low} expressing tumor cells. Importantly, CD38 or CD47 antigen sink did not impact BEAT 38/47-48 and -60 potency *in vitro*. These findings and their translational relevance strongly support that BEAT 38/47-48 and -60 will have prominent activity in MM patients which is luckily to be superior as that of standard of care Darzalex. Based on all the parameters tested, both BEATs show no major differentiation in terms of potency *in vitro*. However, BEAT 38/47-60 showed enhanced potency compared to BEAT 38/47-48 in CDC and MMoAK and therefore it was prioritized over BEAT 38/47-48 as our clinical candidate.

Table 18. Statistics values calculated using One way Anova multiple comparison tests in CD38^{low}-int (NCI-H929) tumor cells. NS = not significant. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Conditions	Max Killing	EC50
BEAT 38/47-48 vs. Darzalex	> 0.01	NS
BEAT 38/47-60 vs. Darzalex	> 0.05	NS
BEAT 38/47-48 vs. 5F9	> 0.0001	NA
BEAT 38/47-60 vs. 5F9	> 0.0001	NA
Darzalex vs 5F9	NS	NA
BEAT 38/47-60 vs. BEAT 38/47-48	NS	> 0.05

Example 6.8: BEAT38/47-60 show higher potency as compared to combination of benchmark Darzalex and 5F9 *in vitro*

Material and methods

MMoAK performed as described in Example 6.7. For combination condition (Darzalex + 5F9), Darzalex was tested in dose dependent fashion like BEAT 38/47-60 while 5F9 was used at saturating concentration (160nM).

Results and conclusions

Darzalex is the standard of care in MM treatment and Magrolimab has successfully been used in combination with tumor targeting antibody (i.e. Rituximab) (MARK P. CHAO ET AL. Anti-CD47 Antibody Synergizes with Rituximab to Promote Phagocytosis and Eradicate Non-Hodgkin Lymphoma. September 3, 2010. Cell 142, 699–713) to enhance phagocytosis of tumor cells. Therefore, a critical question to address was to understand whether the BEAT 38/47 concept would show a better efficacy against tumor cells as compared to a potential combination of Darzalex with a CD47 blocker. Indeed, such a combination has already been presented in the context of acute lymphoblastic leukemia where it was found to show improved efficacy as compared to monotherapy CD38 (FOTINI VOGIATZI, ET AL. Co-Targeting of CD38 and CD47 in T Cell Acute Lymphoblastic Leukemia 614. Acute lymphoblastic leukemia: therapy, excluding transplantation. November 5, 2020. Blood (2020) 136 (Supplement 1) : 39). We therefore assessed the potency of BEAT 38/47-60 against the combination of Darzalex with Magrolimab-like (5F9) in MMoAK assays in order to recapitulate all the effector functions as well as the competition of Fc Receptor with circulating Igs. To have a stringent comparison, Magrolimab-like was used at saturating doses in combination with Darzalex tested at different concentration as BEAT 38/47-60. In these settings, BEAT 38/47-60 showed a statistical significant higher killing of tumor cells as compared to that induced by combination of Darzalex with 5F9 (Figure 19 and Table 19). These data suggest that, albeit having a lower affinity for CD47 in a single Fab arm, by blocking CD47 at the effector to target interface of the using a BEAT format by avidity-dependent crosslinking using high affinity anti-CD38 Fab arms, induce a higher potency of tumor cells as compared to the combination of high affinity anti-CD38 and anti-CD47 monoclonal antibodies.

Table 19. Statistics values calculated using Tuckey's multiple comparison tests in CD38^{high} (Raji) or CD38^{low-int} (NCI-H929) tumor cells. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

Conditions	Max Phagocytosis	EC50
BEAT 38/47-60 vs. (Darzalex + 5F9)	<0.01	NA

Example 6.9: BEAT38/47-60 do not compete for binding to CD38 with Darzalex on cells and its potency is not impacted by concomitant Darzalex treatment *in vitro*

Materials and methods

- 5 Raji-CD47KO cells were incubated with saturating concentration of Darzalex-AF647, BEAT 38/47-AF488 or mix of Darzalex-AF647 + BEAT 38/47-AF488 (Figure 20A-C). After washing steps, fluorescence was analysed with a Cytoflex cytometer. Figure 20A-C shows a representative Flow Cytometry Dot Plots.

- With regard to Figure 20D, Raji-CD47KO cells were pre-incubated with saturating concentration of IgG1 control, Darzalex or BEAT 38/47 purified antibodies for 30min at 4°C before the addition of saturating
10 concentration of BEAT 38/47-AF488. After washing steps, fluorescence was analysed with a Cytoflex cytometer and % of competition with BEAT 38/47-AF488 was then calculated. Mean of duplicates is represented here +/- Standard Deviations (SD).

MMoAK performed as described in Example 6.7. BEAT38/47 + Darzalex combination was performed with increasing doses of both BEAT 38/47 and Darzalex at the concentrations indicated in X axis of the figure.

15 Results and conclusions

- Darzalex is a standard of care for the treatment of multiple myeloma and BEAT 38/47-60 is envisioned to be used in relapsed refractory MM patients post Darzalex treatment. Therefore, we assessed if BEAT 38/47-60 is impacted for cell binding in the presence of Darzalex as well as whether Darzalex impact BEAT 38/47-60 potency against tumor cells *in vitro*. To precisely address competition for CD38, we used CD47-
20 KO Raji tumor cells to avoid CD47 binding of BEAT 38/47-60. By concomitantly incubating CD47-KO Raji cells with BEAT 38/47-60 and Darzalex at 4°C, we found that Darzalex did not impact binding of BEAT 38/47-60 to CD47-KO Raji cells (Figure 20A-C). In addition, we found only a low (20%) inhibition of BEAT 38/47-60 binding when Darzalex was incubated with to CD47-KO Raji tumor cells prior to BEAT 38/47-60 staining (Figure 20D). To address whether the low competition mediated by Darzalex on cell binding was
25 affecting BEAT 38/47-60 potency *in vitro*, we tested ADCP, ADCC and CDC activity of BEAT 38/47-60 in the presence of Darzalex. Overall, we found no impact in any of the effector function of BEAT 38/47-60 by concomitant treatment with Darzalex *in vitro* (Figure 21, and Table 20).

Altogether these data suggest that BEAT 38/47-60 can maintain its efficacy even in presence of bound Darzalex.

Table 20. Statistics values calculated using Tuckey's multiple comparison tests in indicated tumor cell lines. NS = Not significant. When killing curves do not reach comparable maximal level, comparing EC50 is not applicable (NA).

	Conditions	Max Phagocytosis	EC50
ADCP (CD38 ^{high})	BEAT 38/47-60 vs. Darzalex	NS	NS
	BEAT 38/47-60 vs. (Darzalex + BEAT 38/47-60)	NS	NS
ADCP (CD38 ^{low})	BEAT 38/47-60 vs. Darzalex	NA	NA
	BEAT 38/47-60 vs. (Darzalex + BEAT 38/47-60)	NS	NS
ADCC (CD38 ^{high})	BEAT 38/47-60 vs. Darzalex	<0.001	NS
	BEAT 38/47-60 vs. (Darzalex + BEAT 38/47-60)	NS	NS
ADCC (CD38 ^{low})	BEAT 38/47-60 vs. Darzalex	<0.01	NS
	BEAT 38/47-60 vs. (Darzalex + BEAT 38/47-60)	NS	NS
CDC (CD38 ^{high})	BEAT 38/47-60 vs. Darzalex	<0.05	NA
	BEAT 38/47-60 vs. (Darzalex + BEAT 38/47-60)	NS	<0.001

5

Example 7: *Ex vivo* evaluation of BEAT CD38/47-60 on-target profiles

CD47 is expressed ubiquitously and is upregulated in several hematological cancers. To test the on-target specificity of BEAT CD38/47-60 on non-tumor cells, we assessed their binding in RBCs and platelets that are known to expressed high level of CD47.

- 10 For the assessment of BEAT CD38/47-60 a different batch of molecule was used as clinical development batch. Albeit showing improved purity as compared to BEAT CD38/47-60 used for the examples before, both BEAT CD38/47-60 early and late development batches showed comparable functionality and potency against tumor cell lines.

Example 7.1: BEAT38/47-60 show lower on target/off tumor activity in RBCs and platelets as compared to 5F9 *in vitro*

Materials and methods

Binding of BEAT 38/47 and 5F9 to human RBCs (Figure 22A)

- 5 Representative example of binding of BEAT 38/47 or 5F9 human red blood cells. The graphs in Figure 22A show the overlay of nonlinear sigmoidal regression binding curves (Relative Geometric Mean Fluorescence Intensity) for a given donor. Binding was evaluated on six different human red blood cell donors. Staining of cells were performed as described in Example 6.1.

Ability of BEAT 38/47 to induce erythrocyte agglutination (Figure 22B) was evaluated using an indirect

10 *Coombs assay*

Preparation of a 0.8% blood solution

Fresh peripheral blood from healthy volunteers collected on EDTA K3 tubes were obtained from Bern Transfusion Center. 2 ml of fresh blood was added to 48 ml of sterile PBS in a 50 ml Falcon tube. The tubes were centrifuged for 10 min at 3500 G. Supernatant was gently removed with a 25 ml pipette. A 0.8% red
15 blood cell suspension solution was prepared by adding 100 μ l of packed red cells into 10 ml of ID-Diluent 2 in a new clean tube.

Indirect Coombs test

- Test and control antibodies were then prepared 3X concentrated in sterile PBS. 50 μ l of blood from the 0.8% blood solution and 25 μ l of each antibody dilution was then added in each column of the ID-card
20 Coombs anti-IgG. The cards were incubated at 37°C for 15 minutes and then were centrifuged in the ID-centrifuge. Each microtube of the ID-card contains anti-human globulin anti-IgG (rabbit) within a gel matrix. The gel column acts as a filter that traps agglutinated red blood cells as they pass through the gel column during the centrifugation of the card. The gel column separates agglutinated red blood cells from non-agglutinated red blood cells based on size. Any agglutinated red blood cells are captured at the top
25 of or along the gel column, and non- agglutinated red blood cells descend to the bottom of the microtube forming a pellet. Extent of agglutination was scored from 0 (no agglutination) to 4 (complete agglutination).

Red Blood cell depletion assay (Figure 22C)

Ability of BEAT 38/47 to induce red-blood cell loss was evaluated in-house using a hematology analyzer (Sigma 5H with human blood) (Figure 22C). Fresh peripheral blood from healthy human volunteers collected on EDTA K3 tubes were obtained from Bern Transfusion Center.

- 5 Test and control antibodies were incubated 13X concentrated in 500ul of complete peripheral blood for 1h30 at 37°C under gentle agitation (100 rpm). After incubation, blood cell counting was performed using Sigma 5H hematology Analyzer (SwissAvans). Red Blood cell concentration in fresh blood were then reported and analyzed in GraphPad Prism software.

Hemolysis of RBCs (Figure 22D)

- 10 Fresh peripheral blood from healthy volunteers collected on EDTA K3 tubes were obtained from Bern Transfusion Center. BEAT 38/47-induction of hemolysis (Figure 22D) was tested *in vitro* by incubating test and control antibodies in fresh peripheral blood at 37°C for 3h30 and analysis the hemoglobin absorbance in plasma at 414nm. Triton X-100 was used as a positive control for hemolysis. Samples were centrifuged and plasma was transferred into a clear bottom 96 well plate. Hemolysis was quantified by measuring the
- 15 absorbance of plasma at 414nm. Results were normalized in GraphPad Prism software to no antibody treatment condition (negative control) and triton X-100 (positive control).

*Platelet aggregation (Figure 22E)***Blood Collection and Preparation**

- Following written informed consent, blood was obtained by careful venepuncture, using a 19-gauge
- 20 needle and 60 mL syringe, from six healthy volunteers who denied recent ingestion of non-steroidal anti-inflammatory agents or other drugs known to affect platelet function. Blood (total volume 50 mL) was dispensed into 10 mL graduated plastic tubes, each containing 3.13% (w/v) tri-sodium citrate dihydrate (1 part citrate to 9 parts blood), and inverted carefully 3 times to mix with the anticoagulant. After careful mixing, the blood was left to rest on the bench for 10 minutes before being centrifuged at 180 x g for 10
- 25 min to prepare platelet rich plasma (PRP). The PRP was carefully aspirated using a plastic pipette, pooled into a sterile plastic 30 ml Universal container. Some of the residual blood was centrifuged further (3,000 x g for 10 min) to obtain platelet poor plasma (PPP) that was required to set 100% light transmission aggregometry (LTA) on the aggregometer, AggRAM.

LTA procedure

The AggRAM aggregometer was used according to the manufacturers' instructions. The instrument was pre-warmed to 37°C prior to the start of the experiment. For each individual sample (aggregation curve) 100% optical transmission was set using a 250 µL sample of autologous PPP. Following this, 215 µL of PRP was added to the cuvette containing a stirrer bar and 25 µL of the test compounds or LeoA1 was added to the sample, which was then placed into the warming well of the AggRAM for 2 minutes without stirring. After 1 minute, the cuvette was moved to the optical well of the AggRAM with stirring, where light transmission was set to record for a further minute to establish the 0% transmission baseline prior to the addition of 10 µL of collagen, ADP or PBS. The aggregation response was recorded for a further 6 minutes. Aggregation traces, showing the changes in light transmission, and reports for each run were printed directly from the aggregometer. Hard copies of raw data for all individual volunteers were stored in a designated secure location within Platelet Services and can be provided upon request. The numerical output (AUC and % Max) was taken directly from the machine and is presented in tabulated form in Platelet Services' report and were displayed using GraphPad Prism software.

Results and conclusions

CD38 and CD47, albeit upregulated in multiple myeloma cells, can be expressed at low level in other tissues and cellular compartments, including RBCs, platelets and several white blood cells. Specifically, Human RBCs are especially prone to be targeted by anti-CD47 based therapies due their high basal expression of CD47. Indeed, transient anemia in about 60% of the patients in phase I clinical trial was a major side effect of Magrolimab (SIKIC, B. I. ET AL. First-in-Human, First-in-Class Phase I Trial of the Anti-CD47 Antibody Hu5F9-G4 in Patients With Advanced Cancers. J Clin Oncol 37, 946-953, 2019). Based on this, BEAT 38/47-60 was designed to have a low affinity anti-CD47 Fab arm to risk mitigate RBCs on target induced by the anti-CD47 monotherapies like the high affinity anti-CD47 Magrolimab. We therefore addressed several on target/off tumor parameters of BEAT 38/47-60 *in vitro*, as compared to Magrolimab-like monoclonal antibody. We analysed binding to RBCs and whole blood cell subsets, induction of hemagglutination, RBCs depletion and hemolysis as well as platelet aggregation (Figure 22).

BEAT 38/47-60 showed a remarkably lower binding to RBCs as compared to Magrolimab (5F9) in whole blood incubated with increasing concentration of antibodies (Figure 22A) suggesting a more favorable on target on RBCs. This is in line with its lower affinity against CD47 as compared to Magrolimab. To assess whether the lower binding RBCs resulted in a lower on target effect on RBCs, we assessed

- hemagglutination, hemolysis and RBCs depletion potentials of BEAT 38/47-60. To have a robust and reproducible measurement of RBCs agglutination *in vitro*, we set up the Coombs assay, a method that indirectly induces agglutination upon crosslinking of antibodies bound on RBCs. Using this method, Magrolimab induced prominent hemagglutination of RBCs *in vitro* while BEAT 38/47-60 induced a lower Coombs score as compared to it (Figure 22B). In addition, using a hematoanalyser machine to count RBCs, we found that incubation of whole blood with BEAT 38/47-60 induce no detectable depletion of RBCs in whole blood (Figure 22C). On the contrary, Magrolimab-like antibodies induced a dose-dependent depletion of RBCs in whole blood (Figure 22C). Both BEAT 38/47-60 and Magrolimab-like antibodies did not induce a detectable of RBC lysis *in vitro* (Figure 22D). Next, the impact of BEAT 38/47-60 on platelet aggregation *in vitro* was evaluated. Leao-1, a well-established antibody that induce platelet aggregation (JUDITH L. SCOTT at al. Characterization of a novel membrane glycoprotein involved in platelet activation. J Biol Chem. 1989 Aug 15;264(23):13475-82) was used as positive control. Accordingly, it induced high level of platelet aggregation (Figure 22E). On the contrary, BEAT 38/47-60 or Magrolimab-like antibody failed to induce platelet aggregation (Figure 22E).
- Altogether these data suggest that BEAT 38/47-60 have a more favorable on target profile on RBCs as compared to anti-CD47 Magrolimab-like antibody.

Table 21: Statistics values calculated using Tuckey's multiple comparison tests in indicated conditions of Coombs score.

	Conditions	EC50
Coombs Score	BEAT 38/47-60 vs. 5F9	<0.0001

- Example 7.2: BEAT38/47-60 show lower on target/off tumor activity in RBCs and platelets as compared to 5F9 *in vitro***

Materials and methods

Binding was evaluated on six different human red blood cell donors. Staining of cells were performed as described in Figure 11.

- Results and conclusions**

Next, we analyzed the binding level of BEAT 38/47-60 in whole blood as CD47 and CD38 can be expressed on multiple white blood cells subsets. The binding of BEAT 38/47-60 and Magrolimab-like antibody to

immune cells was compared by flow cytometry following staining of whole blood from healthy donors. BEAT 38/47-60 showed a trend of lower binding to CD4 and CD8 T cell subsets, B cells and NK cells relative to 5F9. Binding to granulocytes was the only exception where BEAT 38/47-60 was showing a trend of higher binding at the highest concentrations tested (Figure 23).

- 5 Altogether these data suggest that BEAT 38/47-60 show no major liability as measured by its on target profile on whole blood as compared to anti-CD47 Magrolimab-like antibody.

Example 8: Efficacy of BEAT CD38/CD47-48 and BEAT CD38/CD47-60 *in vivo* models

Example 8.1: Efficacy of BEAT CD38/CD47-48 and BEAT CD38/CD47-60 in an *in vivo* Raji tumor mouse model expressing high level of CD38

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Material and Method

Animal husbandry

In vivo experiments were performed in female 6-7-week-old immune-competent SCID mice from JANVIER LABS. All mice were maintained under standardized environmental conditions in rodent cages ($20 \pm 1^\circ\text{C}$ room temperature, $50 \pm 10\%$ relative humidity, 12 hours light dark cycle). Mice received irradiated food and bedding and $0.22 \mu\text{m}$ -filtered drinking water. Studies were performed in collaboration with Transcure company (FRANCE).

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Raji tumor mouse model CD38 high expressing

Two experiments were conducted and were named respectively Raj_9 and Raj_10 studies.

20 Raj_9 study. Dose escalation of BEAT CD38/CD47-48 and BEAT CD38/CD47-60 in Raji tumors. SCID mice were xenograft subcutaneously with Raji tumor cells only at day 0 (5 mice per group; 8 groups; 40 mice total). 12 days after the xenograft, mice were randomized based on the tumor volume and were injected intravenously with PBS1X; BEAT CD38/CD47-48 molecules at 10, 1 and 0.1mg/kg and BEAT CD38/CD47-60 molecules at 10, 1 and 0.1mg/kg once per week for 3 weeks. No *ex vivo* analysis was performed on these animals. This experiment was done only once.

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Raj_10 study. BEAT CD38/CD47-48 and BEAT CD38/CD47-60 efficacy study in Raji tumors. SCID mice were xenograft subcutaneously with Raji tumor cells only at day 0 (10 mice per group; 8 groups; 80 mice total).

12 days after the xenograft, mice were randomized based on the tumor volume and were injected intravenously with PBS1X; BEAT CD38/CD47-48 molecules; BEAT CD38/CD47-60 molecules; BEAT CD38/CD47-59 molecules; BEAT CD38/CD47-79 molecules once per week for 3 weeks. Darzalex was injected intravenously at 16mg/kg twice per week for 3 weeks. *Ex vivo* analysis was performed on 5 animals per group at day 16. Mice serum and tumors were harvested for *ex vivo* analysis. Luminex was performed on mice serum and tumor supernatant samples. FACS analysis was performed on mice tumors. This experiment was done only once.

Tumor growth inhibition is calculated using the $1-\Delta t/\Delta c$ method (change in test tumor size divided by change in control tumor size) and expressed as a percentage. Averages for each group are used for this calculation):

$$\text{TGI} = (1 - (\text{Treatment } V_{\text{current}} - \text{Treatment } V_{\text{start}}) / (\text{Control } V_{\text{current}} - \text{Control } V_{\text{start}})) \times 100 \text{ as a \%}$$

Tumor regression was defined as achieving a negative Δt .

Mice samples preparation for flow cytometry

For tumor samples, tumors were harvested and dissociated with GentleMACS. Cell suspensions were filtered and centrifuged. Cells were then counted and stained for immune cell profiling. Staining with a complete antibody panel and the relative controls were prepared in FACS buffer. Samples were analyzed on the Northern lights instrument (CYTEK). Data were analyzed using FlowJo v10.7.1 and GraphPad Prism 8.

Mice samples preparation for Luminex assay

Serum samples and tumor supernatant samples were assessed by Multiplex Luminex quantification according to the manufacturer's instructions. Beads, in-vivo samples and/or supernatants and diluted standards provided by the kit, were added to the plates, incubated overnight. The detection antibody was added to the plates and incubated for 30 minutes at room temperature. The plates were washed, and streptavidin-PE was added and incubated for 30 minutes at room temperature. The plates were washed, and the reading buffer was added and incubated at room temperature before reading with the Luminex 200 instrument. Luminex data were analyzed using ProcartaPlex 1.0 Analyst software. Cytokines concentration was normalized to the upper (ULOQ) and lower (LLOQ) limit of quantification. All data

below LLOQ were set to the lowest point of the standard curves and considered like unanalyzable (for GraphPad Prism analysis no zero allowed). Data were analyzed using Excel and GraphPad Prism 8.

Statistical analysis

Data were analyzed using GraphPad Prism 9 software. Statistical analysis performed: one-way analysis of variance (ANOVA) and if significant followed by Dunnett's with PBS control and Tukey's multiple comparison $P < 0.05$ was considered as statistically significant. Level of significance is represented by asterisks. (**** for < 0.0001 ; *** for 0.0001 ; ** for 0.001 and * for 0.05).

Results and conclusions

BEAT CD38/CD47-48 and BEAT CD38/CD47-60 were evaluated in a dose response experiment *in vivo* in a Raji tumor cell line derived xenograft (CDX) model, this tumor cell line expresses high levels of CD38 and has been reported to respond to Darzalex (YU et al, Novel anti-CD38 humanized mAb SG003 possessed enhanced cytotoxicity in lymphoma than Daratumumab via antibody-dependent cell-mediated cytotoxicity. BMC Biotechnology (2019) 19:28). SCID mice were xenograft subcutaneously with Raji tumor cells only at day 0 (5 mice per group; 8 groups; 40 mice total). 12 days after the xenograft, mice were randomized based on the tumor volume and were injected intravenously with PBS, BEAT CD38/CD47-48 molecules at 10, 1 and 0.1mg/kg and BEAT CD38/CD47-60 molecules at 10, 1 and 0.1mg/kg once per week for 3 weeks. Tumor volumes at enrollment varied from 40-270 mm³. 3 mice with no measurable tumor on D12 were excluded from further evaluation.

15 days after the start of treatment (27 days post xenograft) control tumors were reaching the ethical endpoint of 1000 mm³ and the study was halted. Tumor growth inhibition was evaluated for both molecules at all 3 doses. Tumor regression during treatment was observed for 3/5 mice treated with BEAT CD38/CD47-48 and 4/5 mice treated with BEAT CD38/CD47-60. Tumor growth is shown in Figure 24 and Figure 25. Based on number of regressions 10 mg/kg was identified as the efficacious dose. One way ANOVA analysis on D27 was not significant due to high variability in tumor volumes and no post hoc test was conducted.

The efficacy of BEAT CD38/CD47-48 and BEAT CD38/CD47-60 at 10 mg/kg was tested in one further experiment *in vivo* in the Raji tumor model. SCID mice were xenograft subcutaneously with Raji tumor cells at day 0 (10 mice per group; 8 groups; 80 mice total). 12 days after the xenograft, mice were

randomized based on the tumor volume and were injected intravenously with PBS1X; BEAT CD38/CD47-48 molecules; BEAT CD38/CD47-60 molecules; BEAT CD38/CD47-59 molecules (Single arm CD38 control for BEAT CD38/CD47-48) and BEAT CD38/CD47-79 molecules (Single arm CD38 control for BEAT CD38/CD47-60) once per week for 3 weeks. All BEAT molecules were injected at 10mg/kg. Darzalex was injected intravenously at 16mg/kg twice per week for 3 weeks. *Ex vivo* analysis were performed on 5 animals per group at day 16. Mice serum and tumors were harvested for *ex vivo* analysis. Luminex was performed on mice serum and tumor supernatant samples. FACS analysis was performed on mice tumors.

36 days after the xenograft control mice had tumors exceeding 1000 mm³ and the study was terminated. Tumor growth inhibition (TGI) was calculated, all BEATs and Darzalex showed significant TGI ranging from 47% to 110% (regression) (Table 22). Both BEAT CD38/CD47-48 and BEAT CD38/CD47-60 showed a significant efficacy on tumor reduction (Figure 26, Figure 27 and Figure 28, and Table 22).

Table 22. Statistical analysis at day 36 of Figure 26, Figure 27 and Figure 28 (one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison). TGI (tumor growth inhibition) is shown.

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value	TGI
PBS treated mice vs. BEAT CD38/47-48	1124	775.1 to 1472	Yes	****	<0.0001	110%
PBS treated mice vs. BEAT CD38/47-59	716.6	368.1 to 1065	Yes	****	<0.0001	70%
PBS treated mice vs. BEAT CD38/47-60	982.8	634.3 to 1331	Yes	****	<0.0001	96%
PBS treated mice vs. BEAT CD38/47-79	922.2	573.7 to 1271	Yes	****	<0.0001	91%
PBS treated mice vs. Darzalex	479	130.5 to 827.5	Yes	**	0.0038	47%

BEAT CD38/CD47-48 achieved regression in 5/5 mice; BEAT CD38/CD47-60 achieved regression in 3/5 mice while Darzalex showed regression in none and as shown in Table 23 Tukey's post hoc testing showed no difference between BEAT CD38/CD47-48 BEAT and CD38/CD47-60. At day 36, in this experiment design, BEAT CD38/CD47-48 and BEAT CD38/CD47-60 showed a better efficacy than Darzalex (respectively P=0.0003 and P=0.0076) (Table 23).

Table 23. Statistical analysis at day 36 of Figure 26, Figure 27 and Figure 28 (one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison).

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
PBS treated mice vs. BEAT CD38/47-48	1124	714.9 to 1532	Yes	****	<0.0001
PBS treated mice vs. BEAT CD38/47-59	716.6	307.9 to 1125	Yes	****	<0.0001
PBS treated mice vs. BEAT CD38/47-60	982.8	574.1 to 1392	Yes	****	<0.0001
PBS treated mice vs. BEAT CD38/47-79	922.2	513.5 to 1331	Yes	****	<0.0001
PBS treated mice vs. Darzalex	479	70.29 to 887.7	Yes	*	0.0127
BEAT CD38/47-48 vs. BEAT CD38/47-59	-407	-815.7 to 1.713	No	ns	0.0516
BEAT CD38/47-48 vs. BEAT CD38/47-60	-140.8	-549.5 to 267.9	No	ns	0.9485
BEAT CD38/47-48 vs. BEAT CD38/47-79	-201.4	-610.1 to 207.3	No	ns	0.749
BEAT CD38/47-48 vs. Darzalex	-644.6	-1053 to -235.9	Yes	***	0.0003
BEAT CD38/47-59 vs. BEAT CD38/47-60	266.2	-142.5 to 674.9	No	ns	0.4304
BEAT CD38/47-59 vs. BEAT CD38/47-79	205.6	-203.1 to 614.3	No	ns	0.7296
BEAT CD38/47-59 vs. Darzalex	-237.6	-646.3 to 171.1	No	ns	0.5715
BEAT CD38/47-60 vs. BEAT CD38/47-79	-60.6	-469.3 to 348.1	No	ns	0.9997
BEAT CD38/47-60 vs. Darzalex	-503.8	-912.5 to -95.09	Yes	**	0.0076
BEAT CD38/47-79 vs. Darzalex	-443.2	-851.9 to -34.49	Yes	*	0.026

15 days after the xenograft (3 days after first treatment), 5 animals per group were sacrificed. Mice serum and tumors were harvested and analyzed *ex vivo* by Luminex and FACS. BEAT CD38/CD47-48 and BEAT CD38/CD47-60 showed a trend of IL-1b decrease level in the tumors (Figure 29A). BEAT CD38/CD47-60 showed a significant decrease of MHCII+ macrophages in the tumors (Figure 29B, and Table 24).

- 5 Table 24. Statistical analysis of Figure 29B (one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison).

Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
PBS treated mice vs. BEAT CD38/47-48	91691	-79161 to 262544	No	ns	0.532
PBS treated mice vs. BEAT CD38/47-59	210665	39812 to 381518	Yes	*	0.0106
PBS treated mice vs. BEAT CD38/47-60	199452	28599 to 370305	Yes	*	0.0166
PBS treated mice vs. BEAT CD38/47-79	126510	-54707 to 307727	No	ns	0.2696
PBS treated mice vs. Darzalex	62652	-108200 to 233505	No	ns	0.8491

- 10 In conclusion, BEAT CD38/CD47-48 and BEAT CD38/CD47-60 at 10mg/kg showed a significant efficacy of tumor reduction in Raji xenografted mouse model. BEAT CD38/CD47-60 showed a corresponding activation of macrophages consistent with ADCP.

Example 8.2: Efficacy of BEAT CD38/CD47-48 and BEAT CD38/CD47-60 in an *in vivo* KMS-12-BM tumor mouse model expressing low level of CD38

Material and Method

Animal husbandry

- 15 *In vivo* experiments were performed in female 6-7-week-old immune-competent SCID mice from JANVIER LABS. All mice were maintained under standardized environmental conditions in rodent cages (20 ± 1°C room temperature, 50 ± 10% relative humidity, 12 hours light dark cycle). Mice received irradiated food and bedding and 0.22 µm-filtered drinking water. Studies were performed in collaboration with Transcure company (FRANCE).

KMS-12-BM tumor mouse model Darzalex resistant

Once experiment was conducted and was named KMS_7 study.

KMS_7 study. Dose escalation of BEAT CD38/CD47-48 and BEAT CD38/CD47-60 in KMS-12-BM tumors. SCID mice were xenograft subcutaneously with KMS-12-BM tumor cells only at day 0 (5 mice per group; 9 groups; 45 mice total). 15 days after the xenograft, mice were randomized based on the tumor volume and were injected intravenously with PBS1X; BEAT CD38/CD47-48 molecules at 10, 1 and 0.1 mg/kg and BEAT CD38/CD47-60 molecules at 10, 1 and 0.1mg/kg once per week for 3 weeks. Darzalex was injected intravenously at 16mg/kg twice per week for 3 weeks. No *ex vivo* analysis was performed on these animals. This experiment was done only once.

- 10 Tumor growth inhibition is calculated using the $1-\Delta t/\Delta c$ method (change in test tumor size divided by change in control tumor size) and expressed as a percentage. Averages for each group are used for this calculation):

$$\text{TGI} = (1 - (\text{Treatment } V_{\text{current}} - \text{Treatment } V_{\text{start}}) / (\text{Control } V_{\text{current}} - \text{Control } V_{\text{start}})) \times 100 \text{ as a \%}$$

Tumor regression was defined as achieving a negative Δt (tumor smaller at end of study than beginning).

15 *Statistical analysis*

Data were analyzed using GraphPad Prism 9 software. Statistical analysis performed: one-way analysis of variance (ANOVA) on the tumor growth and if significant followed by Dunnett's with PBS control and Tukey's multiple comparison $P < 0.05$ was considered as statistically significant. Level of significance is represented by asterisks. (**** for < 0.0001 ; *** for 0.0001 ; ** for 0.001 and * for 0.05).

20 Results and conclusions

BEAT CD38/CD47-48 and BEAT CD38/CD47-60 were tested in one experiment *in vivo* in KMS-12-BM tumor model, which expresses low levels of CD38. SCID mice were xenografted subcutaneously with KMS-12-BM tumor cells at day 0 (5 mice per group; 9 groups; 45 mice total). 15 days after the xenograft, mice were randomized based on the tumor volume and were injected intravenously with PBS, BEAT CD38/CD47-48 molecules at 10, 1 and 0.1mg/kg and BEAT CD38/CD47-60 molecules at 10, 1 and 0.1mg/kg once per week for 3 weeks. Darzalex was injected intravenously at 16 mg/kg twice per week for 3 weeks.

Tumor growth was monitored, and tumor growth inhibition assessed at the end of the planned 3 week treatment on Day 37 (Figure 30 and Figure 31). Control tumors had grown an average of 300 mm³ and Tumor growth inhibition ranged from -37% (progression faster than control, though this was not significant) for Darzalex through to 86% tumor growth inhibition for BEAT CD38/CD47-48 at 10 mg/kg (Table 25). At the highest dose (10 mg/kg) both BEAT CD38/CD47-48 and BEAT CD38/CD47-60 were statistically superior to PBS and Darzalex treatment (Table 25 and Table 26). At Day 37 there was no statistical difference between BEAT CD38/CD47-48 and BEAT CD38/CD47-60 (Table 25 and Table 26).

Table 25. Statistical analysis at day 37 of Figure 30 and Figure 31.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value	TGI
PBS treated mice vs. BEAT CD38/47-48 at 10mg/kg	258	56.97 to 459.0	Yes	**	0.0073	86%
PBS treated mice vs. BEAT CD38/47-48 at 1mg/kg	72.6	-128.4 to 273.6	No	ns	0.856	24%
PBS treated mice vs. BEAT CD38/47-48 at 0.1mg/kg	63.4	-137.6 to 264.4	No	ns	0.9169	21%
PBS treated mice vs. BEAT CD38/47-60 at 10mg/kg	223.2	22.17 to 424.2	Yes	*	0.0244	74%
PBS treated mice vs. BEAT CD38/47-60 at 1mg/kg	9.2	-191.8 to 210.2	No	ns	0.9998	3%
PBS treated mice vs. BEAT CD38/47-60 at 0.1mg/kg	55.2	-145.8 to 256.2	No	ns	0.9559	18%
PBS treated mice vs. Darzalex at 16mg/kg	-109.6	-310.6 to 91.43	No	ns	0.5143	-37%

At day 55, in this experiment design, BEAT CD38/CD47-48 showed a better efficacy than Darzalex (P=0.0478) (Table 26).

Table 26. Statistical analysis at day 55 of Figure 30 and Figure 31.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
PBS treated mice vs. BEAT CD38/47-48 at 10mg/kg	258	22.21 to 493.8	Yes	*	0.024
PBS treated mice vs. BEAT CD38/47-48 at 1mg/kg	72.6	-163.2 to 308.4	No	ns	0.9715

PBS treated mice vs. BEAT CD38/47-48 at 0.1mg/kg	63.4	-172.4 to 299.2	No	ns	0.9867
PBS treated mice vs. BEAT CD38/47-60 at 10mg/kg	223.2	-12.59 to 459.0	No	ns	0.0741
PBS treated mice vs. BEAT CD38/47-60 at 1mg/kg	9.2	-226.6 to 245.0	No	ns	>0.9999
PBS treated mice vs. BEAT CD38/47-60 at 0.1mg/kg	55.2	-180.6 to 291.0	No	ns	0.9941
PBS treated mice vs. Darzalex at 16mg/kg	-109.6	-345.4 to 126.2	No	ns	0.7988
BEAT CD38/47-48 at 10mg/kg vs. BEAT CD38/47-48 at 1mg/kg	-185.4	-421.2 to 50.39	No	ns	0.2128
BEAT CD38/47-48 at 10mg/kg vs. BEAT CD38/47-48 at 0.1mg/kg	-194.6	-430.4 to 41.19	No	ns	0.1678
BEAT CD38/47-48 at 10mg/kg vs. BEAT CD38/47-60 at 10mg/kg	-34.8	-270.6 to 201.0	No	ns	0.9997
BEAT CD38/47-48 at 10mg/kg vs. BEAT CD38/47-60 at 1mg/kg	-248.8	-484.6 to -13.01	Yes	*	0.0327
BEAT CD38/47-48 at 10mg/kg vs. BEAT CD38/47-60 at 0.1mg/kg	-202.8	-438.6 to 32.99	No	ns	0.1343
BEAT CD38/47-48 at 10mg/kg vs. Darzalex at 16mg/kg	-367.6	-603.4 to -131.8	Yes	***	0.0004
BEAT CD38/47-48 at 1mg/kg vs. BEAT CD38/47-48 at 0.1mg/kg	-9.2	-245.0 to 226.6	No	ns	>0.9999
BEAT CD38/47-48 at 1mg/kg vs. BEAT CD38/47-60 at 10mg/kg	150.6	-85.19 to 386.4	No	ns	0.455
BEAT CD38/47-48 at 1mg/kg vs. BEAT CD38/47-60 at 1mg/kg	-63.4	-299.2 to 172.4	No	ns	0.9867
BEAT CD38/47-48 at 1mg/kg vs. BEAT CD38/47-60 at 0.1mg/kg	-17.4	-253.2 to 218.4	No	ns	>0.9999
BEAT CD38/47-48 at 1mg/kg vs. Darzalex at 16mg/kg	-182.2	-418.0 to 53.59	No	ns	0.2304
BEAT CD38/47-48 at 0.1mg/kg vs. BEAT CD38/47-60 at 10mg/kg	159.8	-75.99 to 395.6	No	ns	0.3809
BEAT CD38/47-48 at 0.1mg/kg vs. BEAT CD38/47-60 at 1mg/kg	-54.2	-290.0 to 181.6	No	ns	0.9947
BEAT CD38/47-48 at 0.1mg/kg vs. BEAT CD38/47-60 at 0.1mg/kg	-8.2	-244.0 to 227.6	No	ns	>0.9999
BEAT CD38/47-48 at 0.1mg/kg vs. Darzalex at 16mg/kg	-173	-408.8 to 62.79	No	ns	0.2865

BEAT CD38/47-60 at 10mg/kg vs. BEAT CD38/47-60 at 1mg/kg	-214	-449.8 to 21.79	No	ns	0.0975
BEAT CD38/47-60 at 10mg/kg vs. BEAT CD38/47-60 at 0.1mg/kg	-168	-403.8 to 67.79	No	ns	0.3204
BEAT CD38/47-60 at 10mg/kg vs. Darzalex at 16mg/kg	-332.8	-568.6 to -97.01	Yes	**	0.0016
BEAT CD38/47-60 at 1mg/kg vs. BEAT CD38/47-60 at 0.1mg/kg	46	-189.8 to 281.8	No	ns	0.9981
BEAT CD38/47-60 at 1mg/kg vs. Darzalex at 16mg/kg	-118.8	-354.6 to 117.0	No	ns	0.7281
BEAT CD38/47-60 at 0.1mg/kg vs. Darzalex at 16mg/kg	-164.8	-400.6 to 70.99	No	ns	0.3433

In conclusion BEAT CD38/CD47-48 and BEAT CD38/CD47-60 at 10 mg/kg showed efficacy and tumor growth inhibition of 86% and 74% respectively vs. PBS control.

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Claims

1. A bispecific antibody comprising at least two binding portions, at least one of which binds to human CD38 and at least one of which binds to human CD47.
- 5 2. The bispecific antibody according to claim 1, comprising at least one binding portion which binds to human CD47 and at least two binding portions which bind to human CD38.
3. The bispecific antibody according to claim 2, wherein said at least two CD38 binding portions are monoparatopic.
4. The bispecific antibody according to claim 2, wherein said at least two CD38 binding portions are
10 biparatopic.
5. The bispecific antibody according to any one of claims 1 to 4, wherein at least one of said binding portions which binds to human CD47 can also bind to cynomolgus CD47.
6. The bispecific antibody according to any one of claims 1 to 5, wherein at least one of said binding portions which binds to human CD38 can also bind to cynomolgus CD38.
- 15 7. The bispecific antibody according to any one of claims 1 to 6, for use as a medicament.
8. The bispecific antibody according to claim 7, for use in treating multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myeloid leukemia, lymphoma, breast cancer such as Her2+ breast cancer, prostate cancer, cervical cancer, germinal center B-cell lymphoma or B-cell acute lymphoblastic leukemia, Chronic lymphocytic leukemia (CLL),
20 Myelodysplastic syndrome (MDS), Non-Hodgkin lymphoma, diffuse large B-cell lymphoma, non small cell lung cancer (NSCLC), Hepatocellular carcinoma (HCC), High-grade serous ovarian carcinoma, peritoneal cancer.
9. The bispecific antibody according to any one of claims 1 to 8, wherein said at least one binding portion which binds to human CD38 comprises a CDR set selected from the group comprising: SEQ
25 ID NO: 103, SEQ ID NO: 163, SEQ ID NO: 223; SEQ ID NO: 117, SEQ ID NO: 177, SEQ ID NO: 237; SEQ ID NO: 121; SEQ ID NO: 181, SEQ ID NO: 241.
10. The bispecific antibody according to any one of claims 1 to 7, wherein said at least one binding portion which binds to human CD47 comprises a CDR set comprising: SEQ ID NO: 75, SEQ ID NO: 135, SEQ ID NO: 195.
- 30 11. The bispecific antibody according to any one of the preceding claims, wherein said bispecific antibody comprises a Fc region.

12. The bispecific antibody according to claim 11, wherein said Fc region is a variant which comprises at least one amino acid modification relative to the Fc region of the parent antibody, whereas the antibody comprising the variant Fc region exhibits altered effector function compared to the parent antibody.
- 5 13. The bispecific antibody of any one of the preceding claims wherein said at least one binding portion which binds to human CD47 has an affinity to human CD47 lower than the affinity that said at least one binding portion which binds to human CD38 has to human CD38.
14. An epitope on the human CD38 extracellular domain which is bound by the bispecific antibody of any one of claims 1 to 13.
- 10 15. An epitope on the human CD47 extracellular domain which is bound by the bispecific antibody of any one of claims 1 to 13.
16. An isolated nucleic acid encoding the bispecific antibody of any one of claims 1 to 13.
17. A host cell comprising the isolated nucleic acid of claim 16.

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