

CD47 ANTIBODIES, METHODS, AND USES

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted
5 electronically in ASCII format and is hereby incorporated by reference in its entirety.
Said ASCII copy, created on November 5, 2015, is named PRD3361WOPCT_SL.txt
and is 72,842 bytes in size.

FIELD

10 The subject matter herein relates generally to antibodies binding to CD47. The
described anti-CD47 antibodies are useful as therapeutic agents for hematological
disorders such as leukemias.

BACKGROUND

15 The protein cluster of differentiation 47 (CD47), also known as integrin
associated protein (IAP), ovarian cancer antigen OA3, and Rh-related antigen, is a
ubiquitously expressed cell surface pentaspan transmembrane Ig superfamily member.
CD47 interacts with SIRP alpha (signal-regulatory protein alpha) on macrophages and
thereby dampens phagocytosis. Cancer cells that co-opt this pathway evade
20 phagocytosis and thereby promote cancer cell survival (Jaiswal, S., et al., Trends in
Immunol 31: 212-219, 2010). This is a newly discovered mechanism of tumor immune
avoidance; thus therapeutically targeting CD47 has widespread application in numerous
cancers.

The expression of CD47 correlates with worse clinical outcomes in many
25 distinct malignancies including Non-Hodgkin lymphoma (NHL), acute lymphocytic
leukemia (ALL), acute myelogenous leukemia (AML), ovarian cancer, glioma,
glioblastoma, etc. In addition, CD47 has been identified as a cancer stem cell marker in
both leukemias and solid tumors (Jaiswal, et al., 2009 Cell, 138(2): 271-85; Chan, et
al., 2009 Proc Natl Acad Sci USA, 106(33): 14016-21; Chan et al., 2010 Curr Opin
30 Urol, 20(5): 393-7; Majeti R, et al., 2011 Oncogene, 30(9): 1009-19).

CD47-blocking antibodies have demonstrated anti-tumor activity for multiple *in vivo* tumor models. Blocking the interaction of CD47 with SIRP alpha is capable of promoting phagocytosis of CD47-expressing cells by macrophages (reviewed in Chao, et al., 2012 Curr Opin Immunol, 24(2): 225-32). Furthermore, these CD47-blocking
5 antibodies have been shown to synergize with other therapeutic antibodies including Rituxan® and Herceptin® in tumor models.

However, CD47 antibodies have been reported to cause platelet aggregation and hemagglutination of red blood cells. Platelet aggregation and hemagglutination are examples of homotypic interactions, wherein two CD47-expressing cells are caused to
10 aggregate or clump when treated with a bivalent CD47 binding entity. For example, the CD47 antibody, MABL, as a full IgG or F(ab')₂, has been reported to cause hemagglutination of erythrocytes and only when MABL was altered into an scFv or bivalent scFv was this effect mitigated. (See e.g., Uno S, Kinoshita Y, Azuma Y, et al., 2007 Oncol Rep, 17(5):1189-94). Similarly, the CD47 antibody, B6H12, has been
15 reported to induce direct aggregation of the platelets of certain subjects that have certain polymorphisms of the Fc receptor, FcγRII (Dorahy DJ, Thorne RF, Fecondo JV and Burns GF, 1997 Journal of Biol. Chem. 272:1323-1330). Thus, platelet aggregation and hemagglutination of RBCs represent a major limitation of therapeutically targeting CD47 with existing CD47 antibodies.

SUMMARY

Described herein are antibodies that specifically bind to CD47, inhibit CD47 from interacting with SIRP alpha, and do not have significant platelet aggregation activity.

25 In some embodiments, the antibody specifically binds to CD47 by interacting with CD47 (SEQ ID NO: 21 excluding the signal sequence) amino acid residues: Q1, L3, N27, E29, E97, L101, T102, R103, and E104. In some embodiments, the antibody interacts with CD47 (SEQ ID NO: 21 excluding the signal sequence) amino acid residues Y37, K39, R45, D46, T49, A53, L54, N55, K56, S57, T58, V59, P60, T61,
30 S64, A66, and K67. In some embodiments, the antibody interacts with CD47 (SEQ ID

NO: 21 excluding the signal sequence) amino acid residues E35, K39, Y37, D46, 49, D51, A53, L54, K56, T58, V59, T99, L101, and T102. In some embodiments, the antibody interact with CD47 (SEQ ID NO: 21 excluding the signal sequence) amino acid residues E29, Q31, N32, T34, E35, V36, Y37, K39, N41, D46, D51, A53, E97, 5 T99, E100, L101, T102, R103, and E104.

Another aspect of some described embodiments feature an antibody that specifically binds to human or cyno CD47 comprising a variable heavy chain selected from SEQ ID NOs: 4-6. The antibody optionally comprises a variable light (VL) chain region selected from SEQ ID NOs: 7 and 8. In some cases, the antibody comprises a 10 VH chain region selected from SEQ ID NOs: 4-6 and a VL chain region selected from SEQ ID NOs: 7 and 8. In other aspects, the antibody has a VH chain region comprising SEQ ID NO: 4 or SEQ ID NO: 5 paired with a VL chain region comprising SEQ ID NO: 7. In yet another aspect, the antibody has a VH chain region comprising SEQ ID NO: 6 paired with a VL chain region comprising SEQ ID NO: 8.

15 In some embodiments, the CD47 antibody comprises a VH complementarity determining region 1 (CDR1) sequence set forth in SEQ ID NO: 9 or SEQ ID NO: 10, a VH CDR2 sequence set forth in SEQ ID NO: 11 or SEQ ID NO: 12, a VH CDR3 sequence set forth in SEQ ID NO: 13 or SEQ ID NO: 14, a VL CDR1 sequence set forth in SEQ ID NO: 15 or SEQ ID NO: 16, a VL CDR2 sequence set forth in SEQ ID 20 NO: 17 or SEQ ID NO: 18 and a VL CDR3 sequence set forth in SEQ ID NO: 19 or SEQ ID NO: 20. For example, the CD47 antibody comprises a VH CDR1 sequence set forth in SEQ ID NO: 9, a VH CDR2 sequence set forth in SEQ ID NO: 11, a VH CDR3 sequence set forth in SEQ ID NO: 13, a VL CDR1 sequence set forth in SEQ ID NO: 15, a VL CDR2 sequence set forth in SEQ ID NO: 17, and a VL CDR3 sequence set 25 forth in SEQ ID NO: 19. In another example, the CD47 antibody comprises a VH CDR1 sequence set forth in SEQ ID NO: 10, a VH CDR2 sequence set forth in SEQ ID NO: 12, a VH CDR3 sequence set forth in SEQ ID NO: 14, a VL CDR1 set forth in SEQ ID NO: 16, a VL CDR2 sequence set forth in SEQ ID NO: 18, and a VL CDR3 sequence set forth in SEQ ID NO: 20.

30 In some embodiments, the antibodies bind to CD47 in a position wherein a VH

chain has more extensive contacts with CD47 than a VL chain, wherein the VH chain of the antibody is positioned near the membrane of a CD47 expressing cell, and wherein a VL chain of the antibody occludes a SIRP alpha binding site on CD47.

In some embodiments, the antibodies do not have significant hemagglutination activity. In some embodiments, the platelet-aggregation activity of the antibodies is no more than 10% greater than the degree of platelet-aggregation observed in the absence of the antibody. In some embodiments, the antibody is chimeric, humanized, or fully human. In some embodiments, the antibody binds to human CD47 or cynomolgus (cyno) CD47. In some embodiments, the antibody promotes macrophage-mediated phagocytosis of a CD47-expressing cell. In some embodiments, the antibody or antigen binding fragment thereof comprises an IgG isotype selected from IgG1 isotype and IgG2 isotype.

Described herein are also pharmaceutical compositions that include an antibody described herein and a pharmaceutical acceptable carrier. The described antibodies can be included in kits. Polynucleotides encoding the described antibodies as well as vectors and cells suitable for expressing the described polynucleotides are also described.

Another embodiment comprises methods of alleviating a symptom of a cancer or other neoplastic condition by administering to a subject in need thereof one or more antibodies described herein, wherein the antibody does not have significant platelet aggregation activity after administration. The antibody is administered in an amount sufficient to alleviate the symptom of the cancer or other neoplastic condition in the subject. In some embodiments, the subject is a human. In some embodiments, the antibody is chimeric, humanized, or fully human. In some embodiments, the antibody inhibits CD47 from interacting with SIRP alpha. In some embodiments, the antibody comprises an IgG isotype selected from the group consisting of IgG1 isotype and IgG2 isotype. In some embodiments, chemotherapy is administered along with the described antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B. Inhibition of SIRP alpha-Fc binding to Jurkat cells by phage-derived human IgG2 Fc-silent mAbs (Figure 1A). Binding to Jurkat cells by phage-derived human IgG2 Fc-silent mAbs. (Figure 1B). Shown are the SIRP alpha-blocking and CD47-binding activities of ten phage-derived mAbs in comparison to a positive control anti-CD47 mAb B6H12.2 and a human IgG2 Fc-silent (IgG2sigma) isotype control as determined by MSD (A) and FACS (B) assays respectively.

Figures 2A-2D. Dose dependent inhibition of SIRP alpha-Fc binding to CD47 expressing Jurkat cells by a subset of 23 human IgG2 Fc-silent mAbs. Shown are the dose response curves of 17 hybridoma mAbs and 3 phage mAbs in comparison to the positive control anti-CD47 mAb B6H12.2 in the cell-based SIRP alpha-blocking MSD assay.

Figures 3A and 3B. Hemagglutination of human red blood cells in response to varying doses of anti-human CD47 monoclonal antibodies IgG1/IgG2 Fc-silent B6H12.2 and commercially available BRIC126 (Figure 3A). Representative results of hemagglutination assays with 23 anti-CD47 mAbs. Shown are hemagglutination results in response to C47B91, C47B98, C47B116, C47B123, and C47B131 (Figure 3B).

Figure 4. Hemagglutination of human red blood cells in response to varying doses of anti-human IgG1/IgG2 Fc-silent C47B157, C47B161, and C47B222. IgG2 Fc silent C47B98 known to induce hemagglutination (see Figure 3B) was included as positive control.

Figures 5A-5D. Dose dependent inhibition of SIRP alpha-Fc binding to CD47 expressing Jurkat cells by HFA variants of C47B116. Shown are the dose response curves of 12 human frame work adapted variants in comparison to the parent hybridoma mAb C47B116 in the cell-based SIRP alpha-blocking MSD assay.

Figure 6. Epitope and paratope regions of C47B161 (SEQ ID NOS 56, 57 and 62, respectively, in order of appearance), C47B167 (SEQ ID NOS 56, 58 and 63, respectively, in order of appearance), C47B222 (SEQ ID NOS 56, 59 and 64, respectively, in order of appearance), C47B227 (SEQ ID NOS 56, 60 and 65, respectively, in order of appearance), and B6H12.2 (SEQ ID NOS 56, 61 and 66, respectively, in order of appearance). The epitope residues from the CD47 ECD -C15G mutant (SEQ ID NO 49) and paratope residues from the VH and VL of each C47B161 (SEQ ID NOS: 5 and 7), C47B167 (SEQ ID NOS: 50 and 51), C47B222 (SEQ ID NOS: 6 and 8), C47B227 (SEQ ID NOS: 41 and 46) and B6H12.2 (SEQ ID NOS: 52 and 53) are shaded, the CDR regions are underlined and labeled (Kabat definition), and SIRP alpha binding residues are marked with a dot above the CD47 sequence.

Figures 7A-7D. Epitope location and interactions between CD47 and C47B161. C47B161 binds to the epitope bin 2 shown in black (Figure 7A). 2D Interaction map between CD47 and C47B161. CDRs L1 and L2 are engaged in contacts with the antigen, while CDR L3 does not interact with CD47. Residues from all heavy chain CDRs contact CD47. Van der Waals interactions are shown as dashed lines, H bonds are solid lines with arrows indicating backbone H bonds and pointing to the backbone atoms. CD47, VL and VH residues are in gray boxes, white boxes and ovals, respectively. A distance cut-off of 4Å was used to define the contacting residues (Figure 7B). CD47 main interactions with the Fab light(Figure 7C) and heavy (Figure 7D) chains. The long CDR-L1 is represented by residues H31, N33, Y35 and Y37. H-bonds are shown as dashed lines. CD47 residues are underlined.

Figure 8A-8D. Epitope location and interactions between CD47 and C47B167. C47B167 recognizes the epitope bin 3 region in black. The antibody binds well to human CD47 (SEQ ID NO: 67) and weakly to cyno CD47 (SEQ ID NO: 68) due to sequence differences in the V59-T61 epitope region (Figure 8A). 2D Interaction map

between CD47 and C47B167. Van der Waals interactions are shown as dashed lines, H bonds are solid lines with arrows indicating backbone H bonds and pointing to the backbone atoms. CD47, VL and VH residues are in gray boxes, white boxes and ovals, respectively. A distance cut-off of 4Å was used to define the contacting residues (Figure 8B). CD47 main interactions with the Fab light (Figure 8C) and heavy (Figure 8D) chains. H-bonds are shown as dashed lines. CD47 residues are underlined.

Figures 9A and 9B. CD47 bound to both C47B161 and C47B167. The ternary complex was achieved by superposition of equivalent CD47 Cα atoms in both complexes (Figure 9A). There is no epitope overlap between the 2 antibodies (Figure 9B). The CD47 structure from the C47B167 complex is shown in Figure 9B. The C47B167 epitope is shown in black and the C47B161 epitope is in white.

Figures 10A-10D. Epitope location and interactions between CD47 and C47B222.

Epitope overall location: C47B161 binds to the epitope bin 1 region shown in black (Figure 10A). 2D Interaction map between CD47 and C47B222: there is segregation between the CD47 residues recognize by the LC and HC. Van der Waals interactions are shown as dashed lines, H bonds are solid lines with arrows indicating backbone H bonds and pointing to the backbone atoms. CD47, VL and VH residues are in gray boxes, white boxes and ovals, respectively (Figure 10B). A distance cut-off of 4Å was used to define the contacting residues. CD47 main interactions with the Fab light (Figure 10C) and heavy (Figure 10D) chains. CDR-L3 does not bind CD47. H-bonds are shown as dashed lines. CD47 residues are underlined.

Figure 11A-11D. Epitope location and interactions between CD47 and C47B227.

Epitope overall location: C47B227 binds to the epitope bin 1 region shown in black (Figure 11A). 2D Interaction map between CD47 and C47B227: there is epitope segregation with regions recognized either by the LC or HC. Van der Waals interactions are shown as dashed lines, H bonds are solid lines with arrows indicating backbone H bonds and pointing to the backbone atoms. CD47, VL and VH residues

are in gray boxes, white boxes and ovals, respectively. A distance cut-off of 4Å was used to define the contacting residues (Figure 11B). Main interactions of CD47 with the Fab light (Figure 11C) and heavy (Figure 11D) chains. H-bonds are shown as dashed lines. CD47 residues are underlined.

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Figures 12A and 12B. Epitope and paratope differences between C47B222 and C47B227. Differences in the light chain interactions: N30G, N31S and Y93S mutations in CDR-L1 and CDR-L3 result in repositioning of the FG loop of CD47 and changes in the H bond pattern (Figure 12A). Differences in the heavy chain interactions: A different conformation for the CDR-H3 loop of C47B222 increases the number of H bonds made by the H3 loop from none in C47B227 to 4 bonds in C47B222 (with CD47 residues Y37, K39, D46 and D51). The structural overlay was achieved by superposition of equivalent CD47 Ca atoms in both complexes. The CD47 residues are underlined (Figure 12B).

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Figures 13A-13D. Epitope location and interactions between CD47 and B6H12.2. Epitope overall location. B6H12.2 binds to the epitope bin 1 region shown in black (Figure 13A). 2D Interaction map between CD47 and B6H12.2: Van der Waals interactions are shown as dashed lines, H bonds are solid lines with arrows indicating backbone H bonds and pointing to the backbone atoms. CD47, VL and VH residues are in gray boxes, white boxes and ovals, respectively. A distance cut-off of 4Å was used to define the contacting residues (Figure 13B). CD47 main interactions with the Fab Light (Figure 13C) and Heavy (Figure 13D) chains. H-bonds are shown as dashed lines. CD47 residues are underlined.

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Figure 14. Phagocytosis of Jurkat target cells by human PBMC derived macrophages in response to 90 minute treatment with varying concentrations of anti-human CD47 IgG1/IgG2 Fc-silent C47B157, C47B161, C47B222, and B6H12.2.

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Figures 15A and 15B. Induction of apoptosis in (Figure 15A) Jurkat cells and (Figure

15B) HL60 cells in response to 24 hour treatment with varying concentrations of anti-human CD47 IgG1/IgG2 Fc-silent C47B157, C47B161, C47B222, and B6H12.2.

Figures 16A and 16B. Enhancement of complement dependent cytotoxicity against
5 Wil2-s target cells in response to varying concentrations of anti-human CD47 IgG1/IgG2 Fc-silent C47B157, C47B161, C47B222, B6H12.2. Rituximab was included as positive control.

Figures 17A-17D. Aggregation of human platelets in response to incubation of platelet
10 rich plasma with (Figure 17A) PBS, 200 µg/ml IgG1/IgG2 Fc silent B6H12.2 and 10 µM ADP; (Figure 17B) PBS, 200 µg/ml IgG1/IgG2 Fc-silent C47B157, and IgG1 B6H12.2; (Figure 17C) PBS, 200 µg/ml IgG1/IgG2sigma C47B161, and IgG1 B6H12.2; and (Figure 17D) PBS, 200 µg/ml IgG1/IgG2sigma C47B222, and IgG1 B6H12.2.

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Figures 18A-18D. *In vivo* activity of IgG1/IgG2 Fc-silent C47B157, C47B161, C47B222, and B6H12.2 in the HL60/NSG mice model. NSG mice were implanted with 10 million HL60 cells intravenously and antibody treatment was initiated on day 6 following tumor cell implant. Each group consisted of five mice. Animals received a
20 total of six doses, twice weekly (final dose day 23). Peripheral blood from the mice was collected weekly and analyzed via FACS to assess tumor cell outgrowth and treatment effects starting on day 14 (final collection on day 42).

Figures 19A and 19B. *In vivo* activity of IgG1/IgG2 Fc-silent C47B157, C47B161,
25 C47B222, and B6H12.2 in the MV4-11/NSG mice model. NSG mice were intravenously implanted with five million MV4-11 cells and antibody treatment was initiated on day 6 following tumor cell implant. Each group consisted of five mice. Animals received a total of six doses, twice weekly (final dose day 23). Peripheral blood from mice was collected on day 34 and was analyzed via FACS to assess effects
30 of treatment on tumor cell outgrowth.

Figure 20A-20D. *In vivo* activity of IgG1/IgG2 Fc-silent C47B157, C47B161, C47B222, and B6H12.2 in the Kasumi-3/NSG mice model. NSG mice were implanted with 10 million Kasumi-3 cells intravenously and antibody treatment was initiated on day 6 following tumor cell implant. Each treatment group consisted of five mice. Animals received a total of twelve doses, twice weekly (final dose day 44). Peripheral blood from the mice was collected weekly and analyzed via FACS to assess tumor cell outgrowth and treatment effects starting on day 14. Graph shows percentage of CD45% cells starting on day 34.

Figures 21A and 21B. Mode of antibody neutralization. Structural overlay of CD47/Fab complexes onto the CD47/SIRP α complex showing regions of clash between Fab and SIRP alpha. The overlay was achieved by superposition of equivalent CD47 C α atoms in both complexes (Figure 21A). Overlap regions between each epitope and the SIRP alpha binding site (Figure 21B). The structure of CD47 from the C47B222 complex was used in Figure 21B.

DETAILED DESCRIPTION

Some characteristics of antibodies described herein include:

- specific binding to human CD47 and cyno CD47,
- blocking CD47 interaction with SIRP alpha,
- do not have significant human platelet aggregation activity,
- do not have significant hemagglutination activity,
- are capable of promoting phagocytosis of tumor cells by macrophages,
- display potent anti-tumor activity in a mouse model of human cancers.

Accordingly, the antibodies described herein stand to be of great importance in the treatment of a multitude of cancers.

Many existing CD47 antibodies block SIRP alpha. However, prior to the subject matter described herein, existing *full* IgG CD47 antibodies that blocked SIRP alpha caused the side effect of platelet aggregation and/or hemagglutination, which, as

described above, is an undesirable characteristic. Other existing antibodies, such as 2D3, do not cause hemagglutination, however, these antibodies also do not block SIRP alpha. Thus, there were previously no known CD47 antibodies in a full IgG format that blocked SIRP alpha without causing platelet and red blood cell clumping.

5 In some embodiments the IgG CD47 antibodies described herein do not exhibit significant platelet aggregation and hemagglutination, thereby increasing the efficacy of therapeutically targeting CD47, and maintain the ability to block the interaction of CD47 with SIRP alpha, which promotes phagocytosis of CD47-expressing cells. Specifically, the full IgG CD47 antibodies of the present disclosure (e.g., C47B157,
10 C47B161 and C47B222) do not have significant cell agglutination activity. For example, the CD47 antibodies described herein do not have significant platelet aggregation and hemagglutination activity. Thus, taken together, the antibodies described herein (e.g., C47B157, C47B161 and C47B222 and derivatives thereof) are unique among existing CD47 antibodies in their ability to block SIRP alpha, without
15 significant platelet aggregation and hemagglutination activity.

General

Those skilled in the art will appreciate that the disclosure herein is susceptible to variations and modifications other than those specifically described. It is to be
20 understood that the present disclosure encompasses all such variations and modifications. The present disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

25 The present disclosure is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions, and methods are clearly within the scope of the present disclosure.

30 The compositions of matter and methods described herein are produced or performed without undue experimentation using, unless otherwise indicated,

conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, peptide synthesis in solution, solid phase peptide synthesis, pharmaceutical chemistry, medicinal chemistry and immunology. Standard techniques are used for pharmaceutical preparation, formulation, and delivery, and treatment of patients.

Definitions

Unless otherwise defined, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art.

As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter. Thus, as used herein, the singular forms “a”, “an” and “the” include plural aspects unless the context clearly dictates otherwise. For example, reference to “a” includes a single as well as two or more; reference to “an” includes a single as well as two or more; reference to “the” includes a single as well as two or more and so forth.

As used herein, the terms “CD47”, “integrin-associated protein (IAP)”, “ovarian cancer antigen OA3”, and “Rh-related antigen” are synonymous and may be used interchangeably.

The terms “red blood cell(s)” and “erythrocyte(s)” are synonymous and used interchangeably herein.

As used herein, the term “platelet aggregation” refers to the adhesion of

activated platelets to one another that results in the formation of aggregates or clumps of activated platelets. Platelet aggregation is measured, as described in the Examples, using an aggregometer, which measures the increase in the transmittance of light as platelet aggregation occurs. “Significant platelet aggregation activity” occurs if there is an increase in light transmittance of at least 25% by 6 minutes after the addition of an antibody described herein relative to light transmittance prior to antibody addition.

The term “agglutination” refers to cellular clumping, while the term “hemagglutination” refers to clumping of a specific subset of cells, i.e., red blood cells. Thus, hemagglutination is a type of agglutination.

In the hemagglutination assay described herein, patterns in a given well are formed by erythrocytes which can be either “buttons,” “halos,” or intermediate between the two. The term “significant hemagglutination activity” refers to the presence of any halo pattern in a well upon the addition of an antibody described herein.

As used herein, the term “antibody” refers to immunoglobulin molecules and immunologically active portions of immunoglobulin (Ig) molecules, i.e., molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. By “specifically bind” or “immunoreacts with” or “directed against” is meant that the antibody reacts with one or more antigenic determinants of the desired antigen and does not react with, or binds at much lower affinity ($K_d > 10^{-6}$), to other polypeptides.

Antibodies include, but are not limited to monoclonal, chimeric, dAb (domain antibody), single chain, Fab, Fab- and F(ab')₂ fragments, F_v, scFvs, and an Fab expression library.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one “light” (about 25 kDa) and one “heavy” chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function. In general, antibody molecules obtained from humans relate to any of the classes IgG, IgM, IgA, IgE and IgD, which differ from one another by the nature of the heavy chain

present in the molecule. Certain classes have subclasses as well, such as IgG₁, IgG₂, and others. Furthermore, in humans, the light chain may be a kappa chain or a lambda chain.

The term “monoclonal antibody” (mAb) or “monoclonal antibody
5 composition”, as used herein, refers to a population of antibody molecules that contain only one molecular species of antibody molecule consisting of a unique light chain gene product and a unique heavy chain gene product. In particular, the complementarity determining regions (CDRs) of the monoclonal antibody are identical in all the molecules of the population. MAbs contain an antigen binding site capable of
10 immunoreacting with a particular epitope of the antigen.

In general, antibody molecules obtained from humans relate to any of the classes IgG, IgM, IgA, IgE and IgD, which differ from one another by the nature of the heavy chain present in the molecule. Certain classes have subclasses as well, such as IgG₁, IgG₂, and others. Furthermore, in humans, the light chain may be a kappa chain
15 or a lambda chain.

The term “antigen-binding site” or “binding portion” refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable (“V”) regions of the heavy (“H”) and light (“L”) chains. Three highly divergent stretches within the V regions of
20 the heavy and light chains, referred to as “hypervariable regions,” are interposed between more conserved flanking stretches known as “framework regions,” or “FRs”. Thus, the term “FR” refers to amino acid sequences which are naturally found between, and adjacent to, hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a
25 heavy chain are disposed relative to each other in three-dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as “complementarity-determining regions,” or “CDRs.” The assignment of amino acids to each domain is in accordance with the
30 definitions of Kabat Sequences of Proteins of Immunological Interest (National

Institutes of Health, Bethesda, Md. (1987 and 1991)), or Chothia & Lesk, J. Mol. Biol. 196:901-917 (1987), Chothia et al. Nature 342:878-883 (1989).

As used herein, the term “epitope” includes any protein determinant capable of specific binding to an immunoglobulin or fragment thereof, or a T-cell receptor. The
5 term “epitope” includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. An antibody is said to specifically bind an antigen when
10 the dissociation constant is $\leq 1 \mu\text{M}$.

As used herein, the terms “specifically binds,” refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation
15 constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction,
20 and geometric parameters that equally influence the rate in both directions. Thus, both the “on rate constant” (k_{on}) and the “off rate constant” (k_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. (See Nature 361:186-87 (1993)). The ratio of k_{off}/k_{on} enables the cancellation of all parameters not related to affinity, and is equal to the dissociation constant K_d . (See,
25 generally, Davies et al. (1990) Annual Rev Biochem 59:439-473). An antibody of the present disclosure is said to specifically bind to CD47, when the equilibrium binding constant (K_d) is $\leq 1 \mu\text{M}$, as measured by assays such as radioligand binding assays, surface plasmon resonance (SPR), flow cytometry binding assay, or similar assays known to those skilled in the art.

30 An “isolated” antibody is one which has been identified and separated and/or

recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will
5 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
10 antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "polypeptide" is used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein
15 fragments, and analogs are species of the polypeptide genus.

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

The term "oligonucleotide" referred to herein includes naturally occurring, and modified nucleotides linked together by naturally occurring, and non-naturally occurring oligonucleotide linkages. Oligonucleotides are a polynucleotide subset generally comprising a length of 200 bases or fewer. Preferably oligonucleotides are 10
25 to 60 bases in length and most preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length. Oligonucleotides are usually single stranded, e.g., for probes, although oligonucleotides may be double stranded, e.g., for use in the construction of a gene mutant. Oligonucleotides described herein are either sense or antisense oligonucleotides.

30 The term "sequence identity" means that two polynucleotide or amino acid

sequences are identical (i.e., on a nucleotide-by-nucleotide or residue-by-residue basis) over the comparison window. The term “percentage of sequence identity” is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U or I) or residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the comparison window (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms “substantial identity” as used herein denotes a characteristic of a polynucleotide or amino acid sequence, wherein the polynucleotide or amino acid comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 18 nucleotide (6 amino acid) positions, frequently over a window of at least 24-48 nucleotide (8-16 amino acid) positions, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the comparison window. The reference sequence may be a subset of a larger sequence.

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage. See Immunology—A Synthesis (2nd Edition, E. S. Golub and D. R. Gren, Eds., Sinauer Associates, Sunderland7 Mass. (1991)). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α -, α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present disclosure. 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-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance

with standard usage and convention.

Similarly, unless specified otherwise, the left-hand end of single-stranded polynucleotide sequences is the 5' end the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences", sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity, and most preferably at least 99 percent sequence identity.

Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

"Conservative" amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamic-aspartic, and asparagine-glutamine.

As discussed herein, minor variations in the amino acid sequences of antibodies or immunoglobulin molecules are contemplated as being encompassed by the present disclosure, providing that the variations in the amino acid sequence maintain at least

75%, more preferably at least 80%, 90%, 95%, and most preferably 99%. In particular, conservative amino acid replacements are contemplated. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into families: (1) acidic amino acids are aspartate, glutamate; (2) basic amino acids are lysine, arginine, histidine; (3) non-polar amino acids are alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, and (4) uncharged polar amino acids are glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. The hydrophilic amino acids include arginine, asparagine, aspartate, glutamine, glutamate, histidine, lysine, serine, and threonine. The hydrophobic amino acids include alanine, cysteine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine. Other families of amino acids include (i) serine and threonine, which are the aliphatic-hydroxy family; (ii) asparagine and glutamine, which are the amide containing family; (iii) alanine, valine, leucine and isoleucine, which are the aliphatic family; and (iv) phenylalanine, tryptophan, and tyrosine, which are the aromatic family. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the binding or properties of the resulting molecule, especially if the replacement does not involve an amino acid within a framework site. Whether an amino acid change results in a functional peptide can readily be determined by assaying the specific activity of the polypeptide derivative. Assays are described in detail herein. Fragments or analogs of antibodies or immunoglobulin molecules can be readily prepared by those of ordinary skill in the art. Preferred amino- and carboxy-termini of fragments or analogs occur near boundaries of functional domains. Structural and functional domains can be identified by comparison of the nucleotide and/or amino acid sequence data to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure and/or function. Methods to identify protein sequences that fold into a known three-dimensional structure are

known (Bowie et al. Science 253:164 (1991)). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in accordance with the present disclosure.

5 Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter binding affinity for forming protein complexes, (4) alter binding affinities, and (5) confer or modify other physicochemical or functional properties of such analogs. Analogs can include various mutants of a sequence other than the naturally-occurring peptide sequence. For
10 example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring sequence (preferably in the portion of the polypeptide outside the domain(s) forming intermolecular contacts. A conservative amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to
15 break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in Proteins, Structures and Molecular Principles (Creighton, Ed., W. H. Freeman and Company, New York (1984)); Introduction to Protein Structure (C. Branden and J. Tooze, eds., Garland
20 Publishing, New York, N.Y. (1991)); and Thornton et al. Nature 354:105 (1991).

The term “agent” is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials.

As used herein, the terms “label” or “labeled” refers to incorporation of a
25 detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods). In certain situations, the label or marker can also be therapeutic. Various methods of labeling polypeptides and glycoproteins are known
30 in the art and may be used. Examples of labels for polypeptides include, but are not

limited to, the following: radioisotopes or radionuclides

(e.g., ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I) fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, p-galactosidase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance. The term “pharmaceutical agent or drug” as used herein refers to a chemical compound or composition capable of inducing a desired therapeutic effect when properly administered to a patient.

The term “antineoplastic agent” is used herein to refer to agents that have the functional property of inhibiting a development or progression of a neoplasm in a human, particularly a malignant (cancerous) lesion, such as a carcinoma, sarcoma, lymphoma, or leukemia. Inhibition of metastasis is frequently a property of antineoplastic agents.

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (Parker, S., Ed., McGraw-Hill, San Francisco (1985)).

CD47 Antibodies

Monoclonal antibodies described herein have the ability to bind CD47, to inhibit the binding of SIRP alpha to CD47, decrease CD47-SIRP alpha-mediated signaling, promote phagocytosis, and to inhibit tumor growth and/or migration. Inhibition is determined, for example, using the cellular assay described herein in the Examples.

Exemplary antibodies described herein include an antibody having a variable heavy (VH) chain selected from SEQ ID NOs: 4-6, and having a variable light (VL) chain selected from SEQ ID NOs: 7 and 8. Specifically, exemplary antibodies include those provided in Table 1.

TABLE 1

Antibody Variable heavy (VH) chain Variable light (VL) chain

C47B157 SEQ ID NO: 4 SEQ ID NO: 7

C47B161 SEQ ID NO: 5 SEQ ID NO: 7

C47B222 SEQ ID NO: 6 SEQ ID NO: 8

The complementarity determining regions (CDRs) of the VH chain of the CD47 antibodies are highlighted below. In some embodiments, the amino acid sequence of VH CDR1 is DYNMH (SEQ ID NO: 9) or DYWIG (SEQ ID NO: 10). In some embodiments, the amino acid sequence of VH CDR2 is DIYPYNGGTGYNQKFKG (SEQ ID NO: 11) or IIYPGDS DTRYSPSFQG (SEQ ID NO: 12). In some
5 embodiments, the amino acid sequence of VH CDR3 is GGWHAMDS (SEQ ID NO: 13) or VGRFASHQLDY (SEQ ID NO: 14).

In some embodiments, the amino acid sequence of VL CDR1 is RSRQSIVHTNRYTYLA (SEQ ID NO: 15) or RASQSVNNRLA (SEQ ID NO: 16).

10 In some embodiments, the amino acid sequence of VL CDR2 is KVS NRFS (SEQ ID NO: 17) or WASTRAI (SEQ ID NO: 18). In some embodiments, the amino acid sequence of VL CDR3 is FQGSHVPYT (SEQ ID NO: 19) or QQGASWPFT (SEQ ID NO: 20).

Also included in this disclosure are antibodies that bind to the same epitope as
15 the CD47 antibodies described herein. For example, antibodies described in this application specifically bind to an epitope that includes one or more amino acid residues of an exemplary human CD47 that is provided below (GenBank Accession No. Q08722.1 (GI:1171879), incorporated herein by reference). The signal sequence (amino acids 1-18) is underlined.

20 SEQ ID NO: 21
MWPLVAALLLGSACCGSAQLLFNKTKSVEFTFCNDTVVIPCFVTNMEAQNTTE
VYVKWKFKGRDIYTFDGALNKSTVPTDFSSAKIEVSQLLKGDASLKMDKSDA
VSHTGNYTCEVTELTREGETIIE LKYRVVSWFSPNENILIVIFPIFAILLFWGQFGI
KTLKYRSGGMDEKTIALLVAGLVITVIVIVGAILFVPGEYSLKNATGLGLIVTST
25 GILILLHYVVFSTAIGLTSFVIAILVIQVIAYILAVVGLSLCIAACIPMHGPLLISGL

SILALAQLLGLVYMKFVASNQKTIQPPRKAVEEPLNAFKESKGMMNDE

In some embodiments the antibodies interact with Q1, L3, N27, E29, E97, L101, T102, R103, and E104 of SEQ ID NO: 21. In some embodiments the antibodies interact with Y37, K39, R45, D46, T49, A53, L54, N55, K56, S57, T58, V59, P60, T61, S64, A66, and K67 of SEQ ID NO:21. In some embodiments the antibodies interact with E35, K39, Y37, D46, 49, D51, A53, L54, K56, T58, V59, T99, L101, and T102 of SEQ ID NO: 21. In some embodiments the antibodies interact with E29, Q31, N32, T34, E35, V36, Y37, K39, N41, D46, D51, A53, E97, T99, E100, L101, T102, R103, and E104 of SEQ ID NO: 21.

Those skilled in the art will recognize that it is possible to determine, without undue experimentation, if an antibody has the same specificity as one of the antibodies described herein (e.g., C47B157, C47B161 and C47B222, or an antibody having a variable heavy chain selected from SEQ ID NOs: 4-6, and a variable light chain selected from SEQ ID NOs: 7 and 8) by ascertaining whether the former prevents the latter from binding to CD47. If the antibody being tested competes with the antibody of this disclosure, as shown by a decrease in binding by the antibody described herein, then the two antibodies likely bind to the same, or a nearby, epitope.

An alternative method for determining whether an antibody has the specificity of an antibody described herein is to pre-incubate the antibody described herein with soluble CD47 protein (with which it is normally reactive), and then add the antibody being tested to determine if the antibody being tested is inhibited in its ability to bind CD47. If binding of the antibody being tested is inhibited then, in all likelihood, it has the same, or functionally equivalent, epitope specificity as the antibody of this disclosure.

Antibodies of the Present Disclosure

The antibodies described herein, can be assessed for the ability to modulate, block, inhibit, reduce, antagonize, neutralize or otherwise interfere with CD47- and/or CD47/SIRP alpha-mediated signaling. For example, such assessments may include measuring CD47- and/or CD47/SIRP alpha-mediated signaling in the presence of one

or more of the antibodies described herein. These assays can include competitive binding assays or can measure a biologic readout, for example the ability to promote phagocytosis of a CD47 expressing cell by a macrophage, as is described in Example 7.

Various procedures known within the art may be used for the production of
5 monoclonal antibodies directed against CD47, or against derivatives, fragments, analogs homologs or orthologs thereof. (See, for example, Antibodies: A Laboratory Manual, Harlow E, and Lane D, 1988, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., incorporated herein by reference). Fully human antibodies are antibody molecules in which the entire sequence of both the light chain and the heavy
10 chain, including the CDRs, arise from human genes. Such antibodies are termed “human antibodies” or “fully human antibodies” herein. Human monoclonal antibodies are prepared, for example, using the procedures described in the Examples provided below. Human monoclonal antibodies can be also prepared by using the trioma technique; the human B-cell hybridoma technique (see Kozbor, et al., 1983 Immunol
15 Today 4: 72); and the EBV hybridoma technique to produce human monoclonal antibodies (see Cole, et al., 1985 In: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96). Human monoclonal antibodies may be utilized and may be produced by using human hybridomas (see Cote, et al., 1983. Proc Natl Acad Sci USA 80: 2026-2030) or by transforming human B-cells with Epstein
20 Barr Virus in vitro (see Cole, et al., 1985 In: MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc., pp. 77-96).

Antibodies are purified by well-known techniques, such as affinity chromatography using protein A or protein G, which provide primarily the IgG fraction of immune serum. Subsequently, or alternatively, the specific antigen which is the
25 target of the immunoglobulin sought, or an epitope thereof, may be immobilized on a column to purify the immune specific antibody by immunoaffinity chromatography. Purification of immunoglobulins is discussed, for example, by D. Wilkinson (The Scientist, published by The Scientist, Inc., Philadelphia Pa., Vol. 14, No. 8 (Apr. 17, 2000), pp. 25-28).

30 The CD47 antibodies of this disclosure are monoclonal antibodies. Monoclonal

antibodies that modulate, block, inhibit, reduce, antagonize, neutralize or otherwise interfere with CD47- and/or CD47/SIRP alpha-mediated cell signaling are generated, e.g., by immunizing an animal with membrane bound and/or soluble CD47, such as, for example, human CD47 or an immunogenic fragment, derivative or variant thereof.

5 Alternatively, the animal is immunized with cells transfected with a vector containing a nucleic acid molecule encoding CD47 such that CD47 is expressed and associated with the surface of the transfected cells. Alternatively, the antibodies are obtained by screening a library that contains antibody or antigen binding domain sequences for binding to CD47. This library is prepared, e.g., in bacteriophage as protein or peptide
10 fusions to a bacteriophage coat protein that is expressed on the surface of assembled phage particles and the encoding DNA sequences contained within the phage particles (i.e., "phage displayed library"). Hybridomas resulting from myeloma/B cell fusions are then screened for reactivity to CD47.

Monoclonal antibodies are prepared, for example, using hybridoma methods,
15 such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent.

Alternatively, the lymphocytes can be immunized *in vitro*.

20 The immunizing agent will typically include the protein antigen, a fragment thereof, or a fusion protein thereof. Generally, either peripheral blood lymphocytes are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to
25 form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells can be cultured in a suitable culture medium that preferably contains one or more substances
30 that inhibit the growth or survival of the unfused, immortalized cells. For example, if

the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

5 Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection,
10 Manassas, Va. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of monoclonal antibodies. (See Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63)).

The culture medium in which the hybridoma cells are cultured can then be
15 assayed for the presence of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the
20 monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980). Moreover, in therapeutic applications of monoclonal antibodies, it is important to identify antibodies having a high degree of specificity and a high binding affinity for the target antigen.

After the desired hybridoma cells are identified, the clones can be subcloned by
25 limiting dilution procedures and grown by standard methods. (See Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells can be grown in vivo as ascites in a mammal.

30 The monoclonal antibodies secreted by the subclones can be isolated or purified

from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Monoclonal antibodies can also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567. DNA encoding the monoclonal antibodies described herein can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells described herein serve as a preferred source of such DNA. Once isolated, the DNA can be placed into expression vectors, which are then transfected into host cells such as Chinese hamster ovary (CHO) cells, Human Embryonic Kidney (HEK) 293 cells, simian COS cells, PER.C6®, NS0 cells, SP2/0, YB2/0, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also can be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (see U.S. Pat. No. 4,816,567; Morrison, Nature 368, 812-13 (1994)) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody described herein, or can be substituted for the variable domains of one antigen-combining site of an antibody described herein to create a chimeric bivalent antibody.

Human Antibodies and Humanization of Antibodies

The antibodies described herein include fully human antibodies or humanized antibodies. These antibodies are suitable for administration to humans without engendering an immune response by the human against the administered immunoglobulin.

A CD47 antibody is generated, for example, by phage-display methods using antibodies containing only human sequences. Such approaches are well-known in the

art, e.g., in WO92/01047 and U.S. Pat. No. 6,521,404, which are hereby incorporated by reference. In this approach, a combinatorial library of phage carrying random pairs of light and heavy chains are screened using natural or recombinant source of CD47 or fragments thereof. In another approach, a CD47 antibody can be produced by a process
5 wherein at least one step of the process includes immunizing a transgenic, non-human animal with human CD47 protein. In this approach, some of the endogenous heavy and/or kappa light chain loci of this xenogenic non-human animal have been disabled and are incapable of the rearrangement required to generate genes encoding immunoglobulins in response to an antigen. In addition, at least one human heavy
10 chain locus and at least one human light chain locus have been stably transfected into the animal. Thus, in response to an administered antigen, the human loci rearrange to provide genes encoding human variable regions immunospecific for the antigen. Upon immunization, therefore, the xenomouse produces B-cells that secrete fully human immunoglobulins.

15 A variety of techniques are well-known in the art for producing xenogenic non-human animals. For example, see U.S. Pat. No. 6,075,181 and No. 6,150,584, which is hereby incorporated by reference in its entirety. This general strategy was demonstrated in connection with generation of the first XenoMouse™ strains as published in 1994. See Green et al. Nature Genetics 7:13-21 (1994), which is hereby
20 incorporated by reference in its entirety. See also, U.S. Pat. Nos. 6,162,963; 6,150,584; 6,114,598; 6,075,181; and 5,939,598 and Japanese Patent Nos. 3 068 180 B2, 3 068 506 B2, and 3 068 507 B2 and European Patent No., EP 0 463 151 B1 and International Patent Applications No. WO 94/02602, WO 96/34096, WO 98/24893, WO 00/76310 and related family members.

25 The production of antibodies with reduced immunogenicity is also accomplished via humanization, chimerization and display techniques using appropriate libraries. It will be appreciated that murine antibodies or antibodies from other species can be humanized or primatized using techniques well known in the art. See e.g., Winter and Harris Immunol Today 14:43 46 (1993) and Wright et al. Crit.
30 Reviews in Immunol. 12:125-168 (1992). The antibody of interest may be engineered

by recombinant DNA techniques to substitute the CH1, CH2, CH3, hinge domains, and/or the framework domain with the corresponding human sequence (See WO 92102190 and U.S. Pat. Nos. 5,530,101; 5,585,089; 5,693,761; 5,693,792; 5,714,350; and 5,777,085). Also, the use of Ig cDNA for construction of chimeric immunoglobulin

5 genes is known in the art (Liu et al. P.N.A.S. 84:3439 (1987) and J. Immunol. 139:3521 (1987)). mRNA is isolated from a hybridoma or other cell producing the antibody and used to produce cDNA. The cDNA of interest may be amplified by the polymerase chain reaction using specific primers (U.S. Pat. Nos. 4,683,195 and 4,683,202).

Alternatively, a library is made and screened to isolate the sequence of interest. The

10 DNA sequence encoding the variable region of the antibody is then fused to human constant region sequences. The sequences of human constant regions genes may be found in Kabat et al. (1991) Sequences of Proteins of immunological Interest, N.I.H. publication no. 91-3242. Human C region genes are readily available from known clones. The choice of isotype will be guided by the desired effector functions, such as

15 complement fixation, or activity in antibody-dependent cellular cytotoxicity. Preferred isotypes are IgG1 and IgG2. Either of the human light chain constant regions, kappa or lambda, may be used. The chimeric, humanized antibody is then expressed by conventional methods.

Antibody fragments, such as Fv, F(ab')₂ and Fab may be prepared by cleavage

20 of the intact protein, e.g., by protease or chemical cleavage. Alternatively, a truncated gene is designed. For example, a chimeric gene encoding a portion of the F(ab')₂ fragment would include DNA sequences encoding the CH1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Consensus sequences of H and L J regions may be used to design

25 oligonucleotides for use as primers to introduce useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments. C region cDNA can be modified by site directed mutagenesis to place a restriction site at the analogous position in the human sequence.

30 Expression vectors include plasmids, retroviruses, YACs, EBV derived

episomes, and the like. A convenient vector is one that encodes a functionally complete human CH or CL immunoglobulin sequence, with appropriate restriction sites engineered so that any VH or VL sequence can be easily inserted and expressed. In such vectors, splicing usually occurs between the splice donor site in the inserted J
5 region and the splice acceptor site preceding the human C region, and also at the splice regions that occur within the human CH exons. Polyadenylation and transcription termination occur at native chromosomal sites downstream of the coding regions. The resulting chimeric antibody may be joined to any strong promoter, including retroviral LTRs, e.g., SV-40 early promoter, (Okayama et al. Mol. Cell. Bio. 3:280 (1983)), Rous
10 sarcoma virus LTR (Gorman et al. P.N.A.S. 79:6777 (1982)), and moloney murine leukemia virus LTR (Grosschedl et al. Cell 41:885 (1985)). Also, as will be appreciated, native Ig promoters and the like may be used.

Further, human antibodies or antibodies from other species can be generated through display type technologies, including, without limitation, phage display,
15 retroviral display, ribosomal display, and other techniques, using techniques well known in the art and the resulting molecules can be subjected to additional maturation, such as affinity maturation, as such techniques are well known in the art. Wright *et al.* Crit, Reviews in Immunol. 12:125-168 (1992), Hanes and Plückthun PNAS USA
94:4937-4942 (1997) (ribosomal display), Parmley and Smith Gene 73:305-318 (1988)
20 (phage display), Scott, TIBS, vol. 17:241-245 (1992), Cwirla et al. PNAS USA 87:6378-6382 (1990), Russel et al. Nucl. Acids Research 21:1081-1085 (1993), Hoganboom et al. Immunol. Reviews 130:43-68 (1992), Chiswell and McCafferty TIBTECH; 10:80-8A (1992), and U.S. Pat. No. 5,733,743. If display technologies are utilized to produce antibodies that are not human, such antibodies can be humanized as
25 described above.

Using these techniques, antibodies can be generated to CD47 expressing cells, soluble forms of CD47, epitopes or peptides thereof, and expression libraries thereto (See e.g., U.S. Pat. No. 5,703,057), which can thereafter be assessed as described previously for desired characteristics.

30 The CD47 antibodies described herein can be expressed by a vector containing

a DNA segment encoding the single chain antibody described above. These can include vectors, liposomes, naked DNA, adjuvant-assisted DNA, gene gun, catheters, etc. Vectors include chemical conjugates such as described in WO 93/64701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as *orthopox* or *avipox* vectors, herpesvirus vectors such as a herpes simplex I virus (HSV) vector (see Geller, A. I. et al., J. Neurochem, 64:487 (1995); Lim, F., et al., in DNA Cloning: Mammalian Systems, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); Geller, A. I. et al., Proc Natl. Acad. Sci.: U.S.A. 90:7603 (1993); Geller, A. I., et al., Proc Natl. Acad. Sci. USA 87:1149 (1990), Adenovirus Vectors (see LeGal LaSalle et al., Science, 259:988 (1993); Davidson, et al., Nat. Genet. 3:219 (1993); Yang, et al., J. Virol. 69:2004 (1995) and Adeno-associated Virus Vectors (see Kaplitt, M. G., et al., Nat. Genet. 8:148 (1994)).

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, CaPO₄ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofection, cell microinjection, and

viral vectors.

The vector can be employed to target essentially any desired target cell. For example, stereotaxic injection can be used to direct the vectors (e.g. adenovirus, HSV) to a desired location. Additionally, the particles can be delivered by

5 intracerebroventricular (icy) infusion using a minipump infusion system, such as a SynchroMed Infusion System. A method based on bulk flow, termed convection, has also proven effective at delivering large molecules to extended areas of the brain and may be useful in delivering the vector to the target cell. (See Bobo et al., Proc. Natl. Acad. Sci. USA 91:2076-2080 (1994); Morrison et al., Am. J. Physiol. 266:292-305

10 (1994)). Other methods that can be used include catheters, intravenous, parenteral, intraperitoneal and subcutaneous injection, and oral or other known routes of administration.

These vectors can be used to express large quantities of antibodies that can be used in a variety of ways. For example, to detect the presence of CD47 in a sample.

15 The antibody can also be used to try to bind to and disrupt CD47- and/or the CD47/SIRP alpha interaction and CD47/SIRP alpha-mediated signaling.

Techniques can be adapted for the production of single-chain antibodies specific to an antigenic protein described herein (see e.g., U.S. Pat. No. 4,946,778). In addition, methods can be adapted for the construction of Fab expression libraries (see e.g., Huse,

20 et al., 1989 Science 246: 1275-1281) to allow rapid and effective identification of monoclonal Fab fragments with the desired specificity for a protein or derivatives, fragments, analogs or homologs thereof. Antibody fragments that contain the idiotypes to a protein antigen may be produced by techniques known in the art including, but not limited to: (i) an F(ab')₂ fragment produced by pepsin digestion of an antibody

25 molecule; (ii) an Fab fragment generated by reducing the disulfide bridges of an F(ab')₂ fragment; (iii) an Fab fragment generated by the treatment of the antibody molecule with papain and a reducing agent and (iv) F_v fragments. Thus variations of the described embodiments are contemplated which include F_v, Fab, Fab' and F(ab')₂ CD47 fragments, single chain CD47 antibodies, single domain antibodies (e.g.,

30 nanobodies or VHHs), bispecific CD47 antibodies, and heteroconjugate CD47

antibodies.

Fc modifications

It can be desirable to modify the antibody described herein with respect to effector function, so as to enhance, e.g., the effectiveness of the antibody in treating diseases and disorders associated with aberrant CD47 signaling. For example, because CD47 is ubiquitously expressed, mutation(s) can be introduced into the Fc region of the antibody in order to silence effector function, thereby decreasing the likelihood of normal cell-killing.

In some embodiments, the antibody described herein is an IgG isotype. In some embodiments, the constant region of the antibody is of human IgG1 isotype, having the amino acid sequence of SEQ ID NO: 1.

In some embodiments, the constant region of the antibody is of human IgG2 isotype, having the amino acid sequence of SEQ ID NO: 2.

In some embodiments, the human IgG2 constant region is modified at amino acids Val234, Gly237, Pro238, His268, Val309, Ala329, and Pro330 (Kabat Numbering) to alter Fc receptor interactions (see WO11/066501 A1). For example, Val234Ala, Gly237Ala (G237A), Pro238Ser (P238S), His268Ala (H268A), Val309Leu (V309L), Ala329Ser (A329S), and Pro330Ser (P330S). (EU index of Kabat et al 1991 *Sequences of Proteins of Immunological Interest*). In some embodiments, the constant region of the antibody of the modified human IgG2 has the amino acid sequence of SEQ ID NO: 3.

Use of Antibodies Against CD47

It will be appreciated that therapeutics in accordance with the described embodiments will be administered with suitable carriers, excipients, and other agents that are incorporated into formulations to provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, Pa. (1975)), particularly Chapter 87 by

Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as Lipofectin™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate for use in treatments and therapies in accordance with the present disclosure, provided that the active ingredient in the formulation is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration.

In one embodiment, the described antibodies may be used as therapeutic agents. Such agents will generally be employed to treat, alleviate, and/or prevent a disease or pathology associated with aberrant CD47 expression, activity and/or signaling in a subject. A therapeutic regimen is carried out by identifying a subject, e.g., a human patient suffering from (or at risk of developing) a disease or disorder associated with aberrant CD47 expression, activity and/or signaling, e.g., a cancer or other neoplastic disorder, using standard methods. An antibody preparation, preferably one having high specificity and high affinity for its target antigen, is administered to the subject and will generally have an effect due to its binding with the target. Administration of the antibody may abrogate or inhibit or interfere with the expression, activity and/or signaling function of the target (e.g., CD47). Administration of the antibody may abrogate or inhibit or interfere with the binding of the target (e.g., CD47) with an endogenous ligand (e.g., SIRP alpha) to which it naturally binds. For example, the antibody binds to the target and modulates, blocks, inhibits, reduces, antagonizes, neutralizes, or otherwise interferes with CD47 expression, activity and/or signaling. In some embodiments an antibody having heavy and light chain CDRs with the amino acid sequences described in Table 2 may be administered to a subject in order to treat a disease or disorder associated with aberrant CD47 expression. In one embodiment the disease or disorder associated with aberrant CD47 expression may be cancer.

Table 2

Selected Antibody Sequences		
CDR	C47B157 and C47B161	C47B222
HC CDR 1	DYNMH (SEQ ID NO: 9)	DYWIG (SEQ ID NO: 10)
HC CDR 2	DIYPYNGGTGYNQKFKG (SEQ ID NO: 11)	IIYPGDS DTRYSPSFQG (SEQ ID NO: 12)
HC CDR 3	GGWHAMDS (SEQ ID NO: 13)	VGRFASHQLDY (SEQ ID NO: 14)
LC CDR 1	RSRQSIVHTNRYTYLA (SEQ ID NO: 15)	RASQSVNNRLA (SEQ ID NO: 16)
LC CDR 2	KVSNRFS (SEQ ID NO: 17)	WASTRAI (SEQ ID NO: 18)
LC CDR 3	FQGSHVPYT (SEQ ID NO: 19)	QQGASWPFT (SEQ ID NO: 20)

Diseases or disorders related to aberrant CD47 expression, activity and/or signaling include, by way of non-limiting example, hematological cancer and/or solid tumors. Hematological cancers include, e.g., leukemia, lymphoma and myeloma.

- 5 Certain forms of leukemia include, by way of non-limiting example, acute lymphocytic leukemia (ALL); acute myeloid leukemia (AML); chronic lymphocytic leukemia (CLL); chronic myelogenous leukemia (CML); Myeloproliferative disorder/neoplasm (MPDS); and myelodysplasia syndrome. Certain forms of lymphoma include, by way of non-limiting example, Hodgkin's lymphoma, both indolent and aggressive non-
- 10 Hodgkin's lymphoma, Burkitt's lymphoma, and follicular lymphoma (small cell and large cell). Certain forms of myeloma include, by way of non-limiting example, multiple myeloma (MM), giant cell myeloma, heavy-chain myeloma, and light chain or Bence-Jones myeloma. Solid tumors include, e.g., breast tumors, ovarian tumors, lung tumors, pancreatic tumors, prostate tumors, melanoma tumors, colorectal tumors, lung
- 15 tumors, head and neck tumors, bladder tumors, esophageal tumors, liver tumors, and

kidney tumors.

Symptoms associated with cancers and other neoplastic disorders include, for example, inflammation, fever, general malaise, fever, pain, often localized to the inflamed area, loss of appetite, weight loss, edema, headache, fatigue, rash, anemia, muscle weakness, muscle fatigue and abdominal symptoms such as, for example, abdominal pain, diarrhea or constipation.

A therapeutically effective amount of an antibody described herein relates generally to the amount needed to achieve a therapeutic objective. As noted above, this may be a binding interaction between the antibody and its target antigen that, in certain cases, interferes with the functioning of the target. The amount required to be administered will furthermore depend on the binding affinity of the antibody for its specific antigen, and will also depend on the rate at which an administered antibody cleared from the body. Common ranges for therapeutically effective dosing of an antibody or antibody fragment described herein may be, by way of nonlimiting example, from about 0.1 mg/kg body weight to about 100 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 0.1 mg/kg body weight to about 0.3 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 0.4 mg/kg body weight to about 0.6 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 0.7 mg/kg body weight to about 0.9 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 1.0 mg/kg body weight to about 2.0 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 2.0 mg/kg body weight to about 3.0 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 3.0 mg/kg body weight to about 4.0 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 4.0 mg/kg body weight to about 5.0 mg/kg body weight. In one embodiment the therapeutically effective dose of an antibody described herein is from about 5.0 mg/kg body weight to about 6.0 mg/kg body weight. In one embodiment the therapeutically

effective does of an antibody described herein is from about 6.0 mg/kg body weight to about 7.0 mg/kg body weight. In one embodiment the therapeutically effective does of an antibody described herein is from about 7.0 mg/kg body weight to about 8.0 mg/kg body weight. In one embodiment the therapeutically effective does of an antibody described herein is from about 8.0 mg/kg body weight to about 9.0 mg/kg body weight. In one embodiment the therapeutically effective does of an antibody described herein is from about 9.0 mg/kg body weight to about 10.0 mg/kg body weight. In one embodiment the therapeutically effective does of an antibody described herein is from about 10.0 mg/kg body weight to about 15.0 mg/kg body weight. In one embodiment the therapeutically effective does of an antibody described herein is from about 15.0 mg/kg body weight to about 20.0 mg/kg body weight. Common dosing frequencies may range, for example, from once a day to twice daily to once every other day to once a week.

Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular inflammatory-related disorder. Alleviation of one or more symptoms of the inflammatory-related disorder indicates that the antibody confers a clinical benefit.

In another embodiment, antibodies directed against CD47 may be used in methods known within the art relating to the localization and/or quantitation of CD47 (e.g., for use in measuring levels of CD47 and/or both CD47 and SIRP alpha within appropriate physiological samples, for use in diagnostic methods, for use in imaging the protein, and the like). In a given embodiment, antibodies specific to CD47, or derivative, fragment, analog or homolog thereof, that contain the antibody derived antigen binding domain, are utilized as pharmacologically active compounds (referred to hereinafter as “therapeutics”).

In another embodiment, an antibody specific for CD47 can be used to isolate a CD47 polypeptide, by standard techniques, such as immunoaffinity, chromatography or immunoprecipitation. Antibodies directed against the CD47 protein (or a fragment thereof) can be used to detect the protein in a biological sample. In some embodiments CD47 may be detected in a biological sample as part of a clinical testing procedure,

e.g., to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and
5 radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or
10 phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

In yet another embodiment, an antibody according to this disclosure can be used as an agent for detecting the presence of CD47 and/or both CD47 and SIRP alpha
15 protein (or a protein fragment thereof) in a sample. In some embodiments, the antibody contains a detectable label. Antibodies are polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab, scFv, or F(ab')_2) is used. The term “labeled”, with regard to an antibody, is intended to encompass direct labeling of the antibody by coupling (i.e., physically linking) a detectable substance to the antibody, as
20 well as indirect labeling of the antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently-labeled secondary antibody and end-labeling of an antibody with biotin such that it can be detected with fluorescently-labeled streptavidin. The term “biological sample” is intended to include tissues, cells and biological fluids isolated
25 from a subject, as well as tissues, cells and fluids present within a subject. Included within the usage of the term “biological sample”, therefore, is blood and a fraction or component of blood including blood serum, blood plasma, or lymph. That is, the detection method of a described embodiment can be used to detect an analyte mRNA, protein, or genomic DNA in a biological sample in vitro as well as in vivo. For
30 example, in vitro techniques for detection of an analyte mRNA include Northern

hybridizations and in situ hybridizations. *In vitro* techniques for detection of an analyte protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, and immunofluorescence. *In vitro* techniques for detection of an analyte genomic DNA include Southern hybridizations. Procedures for conducting immunoassays are described, for example in “ELISA: Theory and Practice: Methods in Molecular Biology”, Vol. 42, J. R. Crowther (Ed.) Human Press, Totowa, N.J., 1995; “Immunoassay”, E. Diamandis and T. Christopoulos, Academic Press, Inc., San Diego, Calif., 1996; and “Practice and Theory of Enzyme Immunoassays”, P. Tijssen, Elsevier Science Publishers, Amsterdam, 1985. Furthermore, in vivo techniques for detection of an analyte protein include introducing into a subject a labeled anti-analyte protein antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

Therapeutic Administration and Formulations of CD47 Antibodies

The antibodies described herein and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions suitable for administration. Principles and considerations involved in preparing such compositions, as well as guidance in the choice of components are well known in the art, for example, see Remington's Pharmaceutical Sciences: The Science And Practice Of Pharmacy 19th ed. (Alfonso R. Gennaro, et al., editors) Mack Pub. Co., Easton, Pa.: 1995; Drug Absorption Enhancement: Concepts, Possibilities, Limitations, And Trends, Harwood Academic Publishers, Langhorne, Pa., 1994; and Peptide And Protein Drug Delivery (Advances In Parenteral Sciences, Vol. 4), 1991, M. Dekker, New York.

Such compositions typically comprise the antibody and a pharmaceutically acceptable carrier. Where antibody fragments are used, the smallest inhibitory fragment that specifically binds to the binding domain of the target protein may be preferred. For example, based upon the variable-region sequences of an antibody, peptide molecules can be designed that retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology. (See, e.g., Marasco et al., Proc. Natl. Acad. Sci. USA,

90: 7889-7893 (1993)).

As used herein, the term “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with
5 pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, ringer's solutions, dextrose solution, and
10 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the antibody, use thereof in the compositions is contemplated.

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

15 A pharmaceutical composition of a described embodiment is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (i.e., topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application
20 can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or
25 phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous
30 solutions (where water soluble) or dispersions and sterile powders for the

extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the antibody in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the antibody into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, one or more of the described antibodies may be formulated into an ointment, salve, gel, or cream as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the described antibodies may be prepared with carriers that will protect against rapid elimination from the body, such as sustained/controlled release formulations, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

For example, the active ingredients can be encapsulated in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions.

Sustained-release preparations can be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate,

non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable
5 release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods.

Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) and can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled
10 in the art, for example, as described in U.S. Pat. No. 4,522,811.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of one or more of the
15 described antibodies calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the described embodiments are dictated by and directly dependent on the unique characteristics of the antibody and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an antibody for the treatment of
20 individuals.

The described pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

The formulation described herein can also contain more than one of the described antibodies as necessary for the particular indication being treated, preferably
25 those with complementary activities that do not adversely affect each other.

Alternatively, or in addition, the composition can comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

30 In one embodiment, one or more of the described antibodies may be

administered as a combination therapy, i.e., combined with other agents, e.g., therapeutic agents, that are useful for treating pathological conditions or disorders, such as various forms of cancer, autoimmune disorders and inflammatory diseases. The term “in combination” in this context means that the agents are given substantially
5 contemporaneously, either simultaneously or sequentially. If given sequentially, at the onset of administration of the second compound, the first of the two compounds is preferably still detectable at effective concentrations at the site of treatment.

For example, the combination therapy can include one or more antibodies described herein coformulated with, and/or coadministered with, one or more additional
10 therapeutic agents, e.g., one or more cytokine and growth factor inhibitors, immunosuppressants, anti-inflammatory agents, metabolic inhibitors, enzyme inhibitors, and/or cytotoxic or cytostatic agents, as described in more detail below. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications
15 associated with the various monotherapies.

Preferred therapeutic agents used in combination with an antibody described herein are those agents that interfere at different stages in an inflammatory response. In one embodiment, one or more antibodies described herein may be coformulated with, and/or coadministered with, one or more additional agents such as other cytokine or
20 growth factor antagonists (e.g., soluble receptors, peptide inhibitors, small molecules, ligand fusions); or antibodies or antigen binding fragments that bind to other targets (e.g., antibodies that bind to other cytokines or growth factors, their receptors, or other cell surface molecules); and anti-inflammatory cytokines or agonists thereof.

In other embodiments, the antibodies described herein are used as vaccine
25 adjuvants against autoimmune disorders, inflammatory diseases, etc. The combination of adjuvants for treatment of these types of disorders are suitable for use in combination with a wide variety of antigens from targeted self-antigens, i.e., autoantigens, involved in autoimmunity, e.g., myelin basic protein; inflammatory self-antigens, e.g., amyloid peptide protein, or transplant antigens, e.g., alloantigens. The antigen may comprise
30 peptides or polypeptides derived from proteins, as well as fragments of any of the

following: saccharides, proteins, polynucleotides or oligonucleotides, autoantigens, amyloid peptide protein, transplant antigens, allergens, or other macromolecular components. In some instances, more than one antigen is included in the antigenic composition.

5

Additional Screening Methods

The present disclosure provides methods (also referred to herein as “screening assays”) for identifying modulators, i.e., candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other drugs) that modulate or otherwise
10 interfere with the binding of CD47 to SIRP alpha, or candidate or test compounds or agents that modulate or otherwise interfere with the signaling function of CD47 and/or CD47-SIRP alpha. Also provided are methods of identifying compounds useful to treat disorders associated with aberrant CD47 and/or CD47-SIRP alpha expression, activity and/or signaling. The screening methods can include those known or used in the art or
15 those described herein. For example, CD47 can be immobilized on a microtiter plate and incubated with a candidate or test compound, e.g., a CD47 antibody, in the presence of SIRP alpha. Subsequently, bound SIRP alpha can be detected using a secondary antibody, and absorbance can be detected on a plate reader.

Methods of identifying compounds capable of promoting phagocytosis of tumor
20 cells by macrophages are also provided. These methods can include those known or used in the art or those described herein. For example, macrophages are incubated with labeled tumor cells in the presence of a candidate compound, e.g., a CD47 antibody. After a period of time, the macrophages can be observed for internalization of the tumor label to identify phagocytosis. Additional details regarding these methods, e.g.,
25 SIRP alpha blocking assays and phagocytosis assays, are provided in the Examples. This disclosure also includes compounds identified in the screening assays described herein.

In one embodiment, assays are provided for screening candidate or test compounds which modulate the signaling function of CD47. The test compounds can
30 be obtained using any of the numerous approaches in combinatorial library methods

known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the “one-bead one-compound” library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide
5 libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds. (See, e.g., Lam, 1997. *Anticancer Drug Design* 12: 145).

A “small molecule” as used herein, is meant to refer to a composition that has a molecular weight of less than about 5 kD and most preferably less than about 4 kD.

10 Small molecules can be, e.g., nucleic acids, peptides, polypeptides, peptidomimetics, carbohydrates, lipids or other organic or inorganic molecules. Libraries of chemical and/or biological mixtures, such as fungal, bacterial, or algal extracts, are known in the art and can be screened with any of the assays of described in this disclosure.

Examples of methods for the synthesis of molecular libraries can be found in the art, for
15 example in: DeWitt, et al., 1993. *Proc. Natl. Acad. Sci. U.S.A.* 90: 6909; Erb, et al., 1994. *Proc. Natl. Acad. Sci. U.S.A.* 91: 11422; Zuckermann, et al., 1994. *J. Med. Chem.* 37: 2678; Cho, et al., 1993. *Science* 261: 1303; Carrell, et al., 1994. *Angew. Chem. Int. Ed. Engl.* 33: 2059; Carell, et al., 1994. *Angew. Chem. Int. Ed. Engl.* 33: 2061; and Gallop, et al., 1994. *J. Med. Chem.* 37: 1233.

20 Libraries of compounds may be presented in solution (see e.g., Houghten, 1992. *Biotechniques* 13: 412-421), or on beads (see Lam, 1991. *Nature* 354: 82-84), on chips (see Fodor, 1993. *Nature* 364: 555-556), bacteria (see U.S. Pat. No. 5,223,409), spores (see U.S. Pat. No. 5,233,409), plasmids (see Cull, et al., 1992. *Proc. Natl. Acad. Sci. USA* 89: 1865-1869) or on phage (see Scott and Smith, 1990. *Science* 249: 386-390;
25 Devlin, 1990. *Science* 249: 404-406; Cwirla, et al., 1990. *Proc. Natl. Acad. Sci. U.S.A.* 87: 6378-6382; Felici, 1991. *J. Mol. Biol.* 222: 301-310; and U.S. Pat. No. 5,233,409.). In one embodiment, a candidate compound is introduced to an antibody-antigen complex and determining whether the candidate compound disrupts the antibody-antigen complex, wherein a disruption of this complex indicates that the candidate
30 compound modulates the signaling function of CD47 and/or the interaction between

CD47 and SIRP alpha. In another embodiment, a soluble CD47 and/or both CD47 and SIRP alpha protein described herein is provided and exposed to at least one neutralizing monoclonal antibody. Formation of an antibody-antigen complex is detected, and one or more candidate compounds are introduced to the complex. If the antibody-antigen complex is disrupted following introduction of the one or more candidate compounds, the candidate compounds is useful to treat disorders associated with aberrant CD47 and/or CD47-SIRP alpha signaling.

Determining the ability of the test compound to interfere with or disrupt the antibody-antigen complex can be accomplished, for example, by coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the antigen or biologically-active portion thereof can be determined by detecting the labeled compound in a complex. For example, test compounds can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, test compounds can be enzymatically-labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

In one embodiment, the assay comprises contacting an antibody-antigen complex with a test compound, and determining the ability of the test compound to interact with the antigen or otherwise disrupt the existing antibody-antigen complex. In this embodiment, determining the ability of the test compound to interact with the antigen and/or disrupt the antibody-antigen complex comprises determining the ability of the test compound to preferentially bind to the antigen or a biologically-active portion thereof, as compared to the antibody.

In another embodiment, the assay comprises contacting an antibody-antigen complex with a test compound and determining the ability of the test compound to modulate the antibody-antigen complex. Determining the ability of the test compound to modulate the antibody-antigen complex can be accomplished, for example, by determining the ability of the antigen to bind to or interact with the antibody, in the presence of the test compound.

Those skilled in the art will recognize that, in any of the screening methods disclosed herein, the antibody may be a neutralizing antibody, which modulates or otherwise interferes with CD47 activity and/or signaling.

The screening methods disclosed herein may be performed as a cell-based assay or as a cell-free assay. The cell-free assays of the described embodiments are amenable to use of either the soluble form or the membrane-bound form of CD47 and fragments thereof. In the case of cell-free assays comprising the membrane-bound form of CD47, it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the proteins are maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)_n, N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate, 3-(3-cholamidopropyl)dimethylamminiol-1-propane sulfonate (CHAPS), or 3-(3-cholamidopropyl)dimethylamminiol-2-hydroxy-1-propane sulfonate (CHAPSO).

In more than one embodiment, it may be desirable to immobilize either the antibody or the antigen to facilitate separation of complexed from uncomplexed forms of one or both following introduction of the candidate compound, as well as to accommodate automation of the assay. Observation of the antibody-antigen complex in the presence and absence of a candidate compound can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein that adds a domain that allows one or both of the proteins to be bound to a matrix can be provided. For example, GST-antibody fusion proteins or GST-antigen fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtiter plates, that are then combined with the test compound, and the mixture is incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or

indirectly. Alternatively, the complexes can be dissociated from the matrix, and the level of antibody-antigen complex formation can be determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of a described embodiment. For example, either the antibody (e.g., C47B157, C47B161 and C47B222, or an antibody having a variable heavy chain selected from SEQ ID NOs: 4-6 and a variable light chain selected from SEQ ID NOs: 7 and 8) or the antigen (e.g. CD47 protein) can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated antibody or antigen molecules can be prepared from biotin-NHS(N-hydroxy-succinimide) using techniques well-known within the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, other antibodies reactive with the antibody or antigen of interest, but which do not interfere with the formation of the antibody-antigen complex of interest, can be derivatized to the wells of the plate, and unbound antibody or antigen trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using such other antibodies reactive with the antibody or antigen.

The present disclosure further pertains to novel agents identified by any of the aforementioned screening assays and uses thereof for treatments as described herein.

Diagnostic and Prophylactic Formulations

The antibodies described herein may be used in diagnostic and prophylactic formulations. In one embodiment, one or more of the described antibodies may be administered to a subject that are at risk of developing one or more of the aforementioned diseases, such as for example, without limitation, cancer or other neoplastic condition. A subject's or organ's predisposition to one or more of the aforementioned cancers or other neoplastic conditions can be determined using genotypic, serological or biochemical markers.

Antibodies described herein are also useful in the detection of CD47 and/or

SIRP alpha in patient samples and accordingly are useful as diagnostics. For example, the CD47 antibodies described herein are used in in vitro assays, e.g., ELISA, to detect CD47 and/or SIRP alpha levels in a patient sample.

In one embodiment, an antibody described herein is immobilized on a solid support (e.g., the well(s) of a microtiter plate). The immobilized antibody serves as a capture antibody for any CD47 and/or SIRP alpha that may be present in a test sample. Prior to contacting the immobilized antibody with a patient sample, the solid support is rinsed and treated with a blocking agent such as milk protein or albumin to prevent nonspecific adsorption of the analyte.

Subsequently the wells are treated with a test sample suspected of containing the antigen, or with a solution containing a standard amount of the antigen. Such a sample is, e.g., a serum sample from a subject suspected of having levels of circulating antigen considered to be diagnostic of a pathology. After rinsing away the test sample or standard, the solid support is treated with a second antibody that is detectably labeled. The labeled second antibody serves as a detecting antibody. The level of detectable label is measured, and the concentration of CD47 and/or SIRP α in the test sample is determined by comparison with a standard curve developed from the standard samples.

It will be appreciated that based on the results obtained using the described antibodies in an *in vitro* diagnostic assay, it is possible to determine the stage of disease (e.g., a clinical indication associated with ischemia, an autoimmune or inflammatory disorder) in a subject based on expression levels of CD47 and/or SIRP alpha. For a given disease, samples of blood are taken from subjects diagnosed as being at various stages in the progression of the disease, and/or at various points in the therapeutic treatment of the disease. Using a population of samples that provides statistically significant results for each stage of progression or therapy, a range of concentrations of the antigen that may be considered characteristic of each stage is designated.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general

knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

EXAMPLES

5

EXAMPLE 1: GENERATION OF CD47 ANTIBODIES USING HYBRIDOMA TECHNOLOGY

Balb/c mice were immunized with recombinant human CD47-Fc Chimera (R&D systems) to initiate anti-CD47 antibody development utilizing Freund's adjuvant (Sigma), InterFAD (mouse Interferon alpha, PBL InterferonSource), or 2-dose adjuvant (Creative Diagnostics) using standard immunization protocols. To develop hybridomas expressing CD47 antibodies, spleens from immunized mice were harvested and B-cells were isolated for fusion with SP20-Bcl2 myeloma cells. Hybridoma clones from 4 fusions (C47Y1, C47Y2, C47Y3, and C47Y4) were assayed by ELISA for antibody binding to CD47 but not to the Fc-Tag. The hybridoma supernatants showing specific binding to CD47 were further screened for binding to CD47-expressing Jurkat cells (TIB-152, ATCC) and for blocking SIRP alpha binding to Jurkat cells by meso scale discovery (MSD)-based assays.

Briefly, Jurkat cells were washed and resuspended in phosphate buffered saline and then captured onto MSD 96-well high bind plates at 30,000 cells per well by incubating at 37°C for 1.5 hours. The plates were blocked with 15% fetal bovine serum (FBS) (or heat-inactivated FBS) for 30 min at room temperature with gentle agitation. For cell-binding activity, hybridoma supernatants were incubated with cells captured on MSD high binding plates at 4°C for 1 hour, and the bound antibodies were detected with a MSD Sulfo-Tag labeled goat anti-mouse antibody.

For SIRP alpha-blocking activity, recombinant SIRP alpha-Fc (R&D systems) at 2 µg/ml was incubated with Jurkat cells in the presence of hybridoma supernatants for 1-1.5 hours, and the bound SIRP alpha was detected with a MSD Sulfo-Tag labeled mouse anti-SIRP alpha antibody (R&D systems). The plates were read on a Sector 6000 imager and electrochemiluminescence (ECL) signals were recorded. The percent

inhibition was normalized to no antibody and no SIRP alpha controls included in the assay. Hybridoma hits showing neutralization of SIRP alpha binding were selected for molecular cloning of antibody V-regions. Briefly, the cDNAs from hybridoma hits of interest were isolated by reverse transcriptase reaction with Invitrogen's SuperScript III
 5 cells Direct cDNA System, and then V regions were amplified through PCR with pre-mixed forwards primers and reverse primers prior to infusion cloning onto murine IgG1/K constant regions. After recombinant expression in HEK293 cells (Life Technologies), the transient transfection supernatants containing mIgG1 mAbs were re-screened in the Jurkat cell-binding and SIRP alpha-blocking assays. A total of 20
 10 sequence unique mIgG1 mAbs with confirmed SIRP alpha-blocking activity (>40% inhibition, Table 3) were selected for conversion into chimeric human IgG2 Fc-silent /human kappa mAbs.

15 Table 3. List of hybridoma-derived mIgG1 mAbs that demonstrated >40% inhibition of SIRP alpha blocking.

Hybridoma	mIgG1 Ab	% Inhibition
C47Y3_22G02	C47M27	98.9
C47Y2_4E11	C47M87	98.8
C47Y2_33D06	C47M38	98.6
C47Y3_5F06	C47M26	98.4
C47Y2_11E03	C47M69	96.9
C47Y2_9A04	C47M71	96.6
C47Y2_30E07	C47M41	96.5
C47Y3_16C06	C47M32	96.4
C47Y2_28E03	C47M47	93.4
C47Y3_11H10	C47M36	91.0

C47Y4_3A12	C47M22	85.9
C47Y3_13A03	C47M34	85.4
C47Y1_01D03	C47M6	79.0
C47Y4_2E06	C47M23	77.5
C47Y2_5D02	C47M80	70.0
C47Y2_19F06	C47M57	52.3
C47Y4_2C02	C47M24	49.4
C47Y2_13A02	C47M66	43.1
C47Y2_29H05	C47M45	41.0
C47Y3_11B09	C47M37	40.4

EXAMPLE 2: GENERATION OF CD47 ANTIBODIES USING PHAGE DISPLAY TECHNOLOGY

5 **CD47 Reagents and Methods:** A recombinant human CD47 extracellular domain (ECD) protein (SEQ ID NO. 22) with the addition of a C-terminal 6xHIS tag (SEQ ID NO: 55) was generated in-house for phage panning. The cDNA clone for human CD47 was obtained from Origene, and the ECD region was amplified by PCR and subcloned into a mammalian expression vector. After transient transfection of

10 HEK 293F cells, the secreted His-tagged human CD47-ECD proteins were purified via Immobilized-metal affinity chromatography (IMAC) using HisTrap columns (GE Healthcare). Peak fractions were pooled and concentrated before being chromatographed over a 26/60 Superdex 200 column (GE Healthcare) for a final polishing step to obtain a monomeric form and a dimeric form of the CD47-ECD

15 proteins. The CD47-ECD proteins were biotinylated using a 10-fold molar excess of sulfo-NHS-LC-Biotin (Pierce) for phage panning experiments.

Phage Display: In-house de novo phage libraries have been described in detail (Shi et al (2010) J. Mol. Biol. 397:385-396; Int. Pat. Publ. No. WO09/085462). These libraries were built on three human VH germline genes (IGHV1-69, 3-23, 5-51) and four human VL germline genes (A27, B3, L6, O12) designed to have high diversity in CDR-H3. Three de novo phage libraries (DNP00004 -169HC /LC mix, DNP00005 -323HC /LC mix and DNP00006 -551HC / LC mix) displaying Fab variants on phage coat protein pIX were panned against biotinylated human CD47-ECD in the dimer form according to standard protocols. The panning conditions used include: panning with biotinylated human CD47 ECD dimer at room temperature for 1 hour, with Round 1 antigen concentration at 100 nM and using Streptavidin beads in capturing phage; Round 2 antigen concentration at 100 nM and using Neutravidin beads in capturing phage; and Round 3 antigen concentration at 10 nM and using with Streptavidin beads in capturing phage. Fab proteins were produced and captured onto ELISA plates by a polyclonal anti-Fd (CH1) antibody. Biotinylated CD47 ECD was added at the desired nM concentration, and the bound biotinylated CD47 was detected by HRP-conjugated streptavidin and chemiluminescence read in a plate reader. Fab clones were also sequenced for their VH and VL identity. The VHs from selected Fabs were amplified with framework-specific primers from *E. coli* clone expressing the Fab of interest using PCR and subcloned into mammalian expression vector containing signal peptide for mammalian expression and the human IgG2 effector function silent (IgG2 Fc silent) heavy chain constant region. Similarly, the VLs were amplified and were subcloned into a mammalian expression vector containing the kappa light chain constant region. The subcloning was done by Infusion cloning (Clontech) with the primers used summarized in Table 4. Infusion cloning is also known as ligation-independent cloning (LIC), where DNA fragments were connected to each other based on sequence homology without the use of restriction endonucleases or DNA ligase.

Table 4. Primers used to convert Fab hits to mAb expressing constructs

Primer Name	SEQ ID NO:	Primer Sequences
HuG1_DNVH_F169	23	CAAAGTATACAGGCCCGAGGTGCAGCT GGTGCAGAG
HuG1_DNVH_F323	24	CAAAGTATACAGGCCCGAAGTGCAGCT GCTGGAAAG
HuG1_DNVH_F551	25	CAAAGTATACAGGCCCGAAGTGCAGCT GGTGCAGAGC
HuG1_DNVH_R	26	GCCCTTGGTGGAGGCGCTGCTCACGG TCACCAG
HuK_DNVL_FA27L6muSP	27	CAAAGTATCCAAGCAGAAATTGTGCT GACCCAGAG
HuK_DNVL_FO12muSP	28	CAAAGTATCCAAGCAGATATTCAGAT GACCCAGAGC
HuK_DNVL_R	29	TGCAGCCACCGTACGTTTAATTTCCAC TTTGGTGCC

The newly generated VH and VL mammalian expression DNA constructs were paired together to generate 10 mAbs for further characterization. They were transiently transfected in HEK293F cells, and the resulting supernatants containing mAbs were characterized for their ability to bind to CD47 on Jurkat cells by FACS and to block SIRP alpha binding to Jurkat cells as previously described. A commercially available anti-CD47 B6H12.2 was subcloned into the human IgG2sigma constant region and used as a positive control in the assays. Figure 1 shows the activity of ten phage-derived mAbs, among which four displayed strong SIRP alpha-blocking activity. Three of them C47B91 (=C47M91), C47B96 (=C47M96) and C47B98 (=C47M98) were

confirmed as CD47 specific cell-binders and therefore selected for further characterization.

EXAMPLE 3: BIOACTIVITY OF CD47 ANTIBODIES

5 The ability of the 23 total CD47 antibodies generated (20 from hybridoma methods and 3 from phage display methods) to bind CD47 and to block certain bioactivities of CD47 was analyzed using various *in vitro* assays as described below.

10 **CD47 binding assay:** Human CD47 and Cyno CD47 ECD proteins were generated in-house as His-tagged proteins as described in Example 2. Kinetic binding affinities to human CD47 and Cyno CD47 ECD proteins were determined by a Protein Interaction Array System (ProteOn). Briefly, mAbs were captured on the sensor chip via anti-IgG-Fc to reach surface density of 200-350 RU. The CD47 ECD monomeric proteins were serially titrated from 300 nM down to 3.7 nM and injected for 5 min. The dissociation was monitored for 30 min. Data were fitted to 1:1 binding model. The K_D values of the 23 mAbs as well as the ratios between affinities to human and Cyno CD47 proteins are listed in Table 3. Most of the mAbs showed cyno CD47 cross-reactivity (within 5 fold affinity of human CD47) with the exception of C47B121, C47B120, and C47B131.

20 **Binning assay:** This assay permits assessment of the panel of antibodies individually as both capture and detection reagents with the rest of the antibodies in the panel. Antibodies forming effective capture/detection reagents with each other theoretically recognize spatially-separated epitopes on a monomeric protein, thus allowing both antibodies to bind to the target protein at the same time. Groups of clones exhibiting similar patterns of activity across the entire panel are hypothesized to bind to similar epitopes. Selecting clones from different groups should therefore provide antibodies recognizing different epitopes. The CD47 antibodies were directly immobilized on GLC sensors (BioRad). Competing samples were pre-incubated with 200 nM of CD47-ECD for 4 hrs before injection over the chip surface for 4 min to allow association. Dissociation was then monitored for 4 min. The results suggest there

might be 4 distinct epitope groups, with group 1 and 2 overlapping and competing with each other, while group 3 only overlaps with group 1 but not with group 2.

SIRP alpha Blocking Activity: The potency of SIRP alpha blocking by the 23 CD47 antibodies was measured by serially titrating the antibodies and incubating with Jurkat cells captured on MSD high-binding plates for 1 hour, then removed before incubation of recombinant SIRP alpha-Fc to Jurkat cells for another 1.5 hrs. The bound SIRP alpha was detected with a MSD Sulfo-Tag labeled mouse anti-SIRP alpha antibody. The ECL signal was plotted as a function of antibody concentrations, and EC₅₀ values were obtained from fitting the dose-response curves using non-linear regression model in GraphPad Prism. All antibodies demonstrated dose-dependent inhibition of SIRP alpha binding to CD47-expressing cells. Figure 2 shows the dose-response curves of a subset of the anti-CD47 mAbs in the assay.

Hemagglutination Activity: The 23 mAbs were also evaluated for activity in hemagglutination assays. Briefly, blood was collected from healthy donor volunteers into Vacutainer blood collection tubes (BD Biosciences), buffered with sodium citrate. Blood was washed with PBS three times and a 2% erythrocyte suspension was prepared in PBS. 50 µl of the serially diluted mAbs (2-fold) were incubated with 50 µl of the 2% erythrocyte suspension for 2 hours at room temperature in clear 96-well round bottom plates and subsequently were scored for hemagglutination when RBCs did not appear as tight pellets in the well. The starting concentration from which 2-fold serial dilutions were prepared was 200 µg/ml for most mAbs tested. In instances where antibody stock concentrations were less concentrated (C47B118 and C47B119) the starting concentration prepared for antibodies was 85µg/ml. Most of the tested mAbs did induce hemagglutination (representative results shown in Figure 3 and Figure 4), however no association of hemagglutination with any other properties of these mAbs was found.

The characteristics of the 23 mAbs in these assays were summarized in Table 5. Among them, C47B116 and C47B91 were selected for further optimization, as they did not induce hemagglutination, showed potent SIRP alpha-blocking activity as well as

good affinity to both human CD47 and Cyno CD47.

Table 5: Characteristics of the 23 anti-CD47 mAbs including affinity to human and cyno CD47, ability to block SIRP alpha, epitope bins and hemagglutination (Hg)

5 activities. Lowest concentrations at which hemagglutination was visibly observed are indicated where appropriate. Note: the K_D numbers for B6H12.2 represent averages of 5 readings in the same experiment. NA: not tested in the same experiment.

mAb ID	SIRPa- blocking EC ₅₀ (µg/ml)	Human CD47 K _D (M)	Cyno CD47 K _D (M)	K _D Ratio Cyno/ Human	Epitope Bin	Hg
B6H12.2	0.21	5.39E-10	5.72E-10	1.1	1	N
C47B115	NA	1.41E-09	7.12E-09	5.0	2	Y ≥0.195 µg/ml
C47B116	0.10	1.41E-09	9.67E-10	0.7	2	N
C47B117	2.96	8.72E-08	1.52E-07	1.7	4	N
C47B118	0.09	3.96E-09	2.64E-09	0.7	2	Y ≥0.332 µg/ml
C47B119	0.32	2.46E-09	2.35E-09	1.0	2	N
C47B120	0.26	5.56E-09	7.85E-08	14.1	1	N
C47B121	0.20	7.16E-09	NB		3	N

C47B122	0.08	1.64E-09	2.91E-09	1.8	2	Y ≥0.098 μg/ml
C47B123	0.07	2.07E-09	1.71E-09	0.8	2	Y ≥0.098 μg/ml
C47B124	0.11	3.65E-09	2.90E-09	0.8	2	Y ≥0.781 μg/ml
C47B125	~	4.05E-08	3.32E-08	0.8	2	N
C47B126	0.04	3.44E-10	2.60E-10	0.8	1	Y ≥0.098 μg/ml
C47B127	0.19	3.28E-09	4.14E-09	1.3	2	Y ≥0.781 μg/ml
C47B128	0.05	2.23E-09	2.22E-09	1.0	1	Y ≥0.098 μg/ml
C47B129	0.12	2.78E-09	4.94E-09	1.8	2	Y ≥0.195 μg/ml

C47B130	0.11	4.84E-09	4.28E-09	0.9	1	Y ≥0.064 µg/ml
C47B131	0.33	1.45E-09	1.62E-07	111.7	3	N
C47B132	NA	2.65E-09	9.62E-09	3.6	2	Y ≥0.083 µg/ml
C47B133	0.13	1.06E-09	3.80E-09	3.6	2	Y ≥0.098 µg/ml
C47B134	NA	2.54E-09	2.71E-09	1.1	2	Y ≥0.391 µg/ml
C47B91	0.06	1.56E-08	2.45E-08	1.6	1	N
C47B96	0.46	6.08E-08	3.58E-08	0.6	1	Y ≥3.125 µg/ml
C47B98	0.95	3.24E-09	3.24E-09	1.0	2	Y ≥0.098 µg/ml

EXAMPLE 4: HUMAN FRAMEWORK ADAPTATION OF C47B116

Initial analysis of the amino acid sequence of the C47B116 VH domain (SEQ ID NO: 30) suggests that the closest mouse germline genes are IGHV1S29*02 for HV
5 and IGHJ4*01 for HJ. Initial analysis of the amino acid sequence of the C47B116 VL

domain (SEQ ID NO: 54) suggests that the closest mouse germline genes are IGKV117*01 and IGKJ2*01. For human framework adaption (HFA) of C47B116 to reduce immunogenicity in humans, four human VH (IGHV1-3, IGHV1-46, IGHV1-69, and IGHV5-51) and 3 human VL (IGKV2-28, IGKV3-1, and IGKV4-1) variants chains
5 have been designed to replace the mouse framework in C47B116 VL and VH sequences with human frameworks while keeping the CDRs intact, based on sequence homology analysis and consideration for future affinity maturation using in-house de novo libraries. Constructs for each VH (SEQ ID NO: 4, 5, 31, and 32) and VL (SEQ ID NO: 7, 33, and 34) chains were generated by gene assembly and cloned into vectors
10 for human IgG2sigma expression. Combinations of VH and VL variants were co-transfected into HEK 293Expi expression system and the resulting 12 HFA variants were purified from culture supernatants by Protein-A chromatography. The purified C47B116 HFA variants were again characterized for SIRP alpha – blocking, binding affinities to human and Cyno CD47, and hemagglutination as previously described. For
15 the hemagglutination assays, the starting concentration from which 2-fold serial dilutions were prepared was 200 µg/ml for most mAbs tested. In instances where antibody stock concentrations were less concentrated, starting concentrations for the serial dilutions were prepared at 125 µg/ml (C47B160), 170 µg/ml (C47B156), and 195 µg/ml (C47B159). All variants demonstrated potent SIRP alpha-blocking activity
20 (Figure 5). C47B148, C47B151, C47B152, C47B156, and C47B160 induced hemagglutination, suggesting frameworks may contribute to this activity. The selected C47B116 HFA variants investigated for kinetic binding studies showed similar affinity to human CD47 and cyno CD47. Some HFA variants (C47B155, C47B157, C47B159 and C47B161) retained good binding affinity (within 5-fold of the parent mAb
25 C47B116) to CD47, whereas others (C47B147, C47B149, C47B151 and C47B153) showed more significant loss in affinity (more than 6 fold decrease compared to C47B116). The data are summarized in Table 6. C47B157 and C47B161 were chosen for further characterization because they maintained potent SIRP alpha –blocking activity, good binding affinity to both human and cyno CD47, but exhibited no
30 hemagglutination activity or biophysical property concerns.

Table 6. Characterization of 12 C47B116 HFA variants for SIRP alpha-blocking, affinity to human CD47 and cyno CD47, and hemagglutination activities. The lowest concentrations at which hemagglutination was visibly observed are indicated where appropriate.

C47BB116 HFA Ab	SIRPa- blocking EC₅₀ (μg/ml)	Human CD47 K_D (M)	Cyno CD47 K_D (M)	KD Ratio Cyno/ Human	Hemagglutination
C47B147	0.24	9.55E-09	1.35E-08	1.4	Y ≥ 0.781 μg/ml
C47B148	0.09	NA	NA		Y ≥ 0.098 μg/ml
C47B149	0.09	7.95E-09	1.16E-08	1.5	N
C47B151	0.29	6.89E-09	9.34E-09	1.4	Y ≥ 0.781 μg/ml
C47B152	0.26	NA	NA		Y ≥ 0.195 μg/ml
C47B153	0.21	6.39E-09	7.92E-09	1.2	N
C47B155	0.13	4.27E-09	4.65E-09	1.1	N
C47B156	0.04	NA	NA		Y ≥ 0.083 μg/ml
C47B157	0.13	3.53E-09	3.71E-09	1.1	N

C47B159	0.03	2.60E-09	2.29E-09	0.9	N
C47B160	0.02	NA	NA		Y ≥ 0.061 µg/ml
C47B161	0.06	2.87E-09	2.96E-09	1.0	N
C47B116	0.12	1.02E-09	9.50E-10	0.9	N

EXAMPLE 5: AFFINITY MATURATION OF C47B91

The heavy chain of C47B91 (SEQ ID NO: 35) is coming from 5-51 germline, and the light chain (SEQ ID NO: 36) is coming from L6 germline. Two Fab libraries were designed for the affinity maturation of C47B91. One of the Fab libraries is heavy chain library (C47F8L1), adding diversity from de novo V5.0 to the CDR1 and CDR2 in VH of C47B91 (Table 7); and another Fab library is light chain library (C47F18L1), adding VL diversity from de novo V5.0 based on the VL germline (L6) of C47B91 (Table 8). The Fab libraries were constructed in a pIX phage display system as described in U.S. Pat. No. 6,472,147 and International application No. WO09/085462 with minor modifications to restriction enzyme sites for cloning purposes.

Table 7. Affinity maturation design for the light chain library

Loop	Position	Parent (L6)	VL library diversification scheme
L1	30	S	D, N, R, S
	31	S	N, S, T
	32	Y	D, N, R, S, Y
L2	49	Y	E, H, K, Y

	50	D	D, G, S, W, Y
	53	N	D, N, S, T, Y
L3	91	R	A, D, E, G, H, N, R, S, W, Y
	92	S	A, D, E, G, H, N, R, S, W, Y
	93	N	A, D, E, G, H, N, R, S, W, Y
	94	W	A, D, E, G, H, N, R, S, W, Y
	96	L	F, I, L, N, R, W, Y

Table 8. Affinity maturation design for the heavy chain library

Loop	Position (Kabat)	Parent (5-51)	VH library diversification Scheme
H1	30	T	D, K, T
	31	S	D, N, S, T
	32	Y	A, D, S, Y
	33	W	A, D, G, S, W, Y
	35	G	G, H, N, S
H2	50	I	A, E, I, N, R, T, W, Y
	52	Y	A, D, L, N, R, Y
	55	D	D, E, N, S, Y

	57	D	D, N, R, S, T, Y
	59	R	E, G, Q, R, Y
H3	93	M	V

Affinity maturation Fab libraries displayed on phage coat protein IX was panned against CHO-S mammalian cells expressing full length of human CD47. The phage library was pre-cleared with 1×10^8 CHO-S parental cells by incubating together
5 in 10% FBS/DMEM at 4°C overnight. Pre-cleared libraries were recovered through centrifugation to remove the CHO-S parental cells, and concentrated by PEG/NaCl precipitation. About 1×10^7 CHO-S CD47 expressing cells were used for each round of panning, panning was about 2 hours at 4°C. Cells binding with the phage were washed with 40% Ficoll/2%BSA in PBS once, and 3 times with ice-cold
10 PBS/0.2%FBS. Round 1 and 2 used the CHO-S CD47 higher expressing clone, while round 3 used either the CHO-S CD47 higher expressing clone or low expressing clone in order to enrich tighter binders. Same libraries were also used to pan with biotinylated CD47 human CD47 ECD monomer. The panning was performed for one hour at 25°C, and the antigen concentrations were at 1 nM for round 1, 0.1 nM for
15 round 2 and 0.1 nM or 0.01 nM for round 3. Binders were retrieved by addition of Streptavidin-beads to form a bead/antigen/phage complex, which was washed in TBST. Alternatively overnight wash was used in attempt to enrich binders with slower off-rate.

Fab production was induced from phage plasmid DNA enriched after panning.
20 The supernatants containing secreted Fabs were used directly to test for inhibition of recombinant human SIRP alpha binding to human CD47 on Jurkat cells and to check their binding to human CD47 ECD monomer and dimer by ELISA as previously described. The Fab clones were also sequenced to check for their VH and VL identity. Fab hits were selected based on unique sequences, binding activity to ECD proteins,
25 and SIRP alpha-blocking activity.

The V_H from selected Fabs was amplified with framework-specific primers from *E. coli* clone expressing the Fab of interest using PCR. The amplified fragments were subcloned into mammalian expression vector containing signal peptide for mammalian expression and the human IgG2 Sigma heavy chain constant region.

5 Similarly, the V_L fragments were amplified and were subcloned into a mammalian expression vector containing the kappa light chain constant region. The subcloning was done by Infusion cloning (Clontech) with the following primers: HuG1_DNVH_F551, HuG1_DNVH_R, HuK_DNVL_FA27L6muSP, and HuK_DNVL_R (sequences listed in Table 3). The newly generated V_H and V_L
10 mammalian expression DNA constructs were paired together to generate mAbs for further characterization.

A total of 32 C47B91 affinity matured mAbs were generated through pairing of 10 affinity matured V_H chains (SEQ ID NO: 6, and 37-45) with the parent V_L chain, 4 affinity matured V_L chains (SEQ ID NO: 8, and 46-48) with the parent V_H chain or
15 from best chains crosses. They were expressed in HEK 293 Expi cells, purified via Protein-A chromatography, and characterized for SIRP alpha-blocking activity, binding affinity to human and Cyno CD47, and hemagglutination activity. A biochemical assay was conducted to investigate the ability of the variants to block SIRP alpha-binding to CD47. Here, His-tagged CD47-ECD dimer protein was pre-incubated with the mAbs
20 before addition to the SIRP alpha-Fc captured on MSD standard plates and then the amount of CD47 bound was detected by a sheep polyclonal anti-CD47 antibody (R&D systems) combined with an MSD SulfoTag-labeled anti-sheep antibody (Meso Scale Discovery). The SIRP alpha-blocking activity of the antibodies was normalized to the percentage of inhibition relative to the signal of no Ab (maximum binding) and no
25 CD47 (background). The inhibitory activities of these mAbs at 10 µg/ml were shown in Table 9, and 25 out of 32 mAbs demonstrated greater inhibition of SIRP alpha binding than the parent C47B91 at 10 µg/ml. Binding affinities to human and Cyno CD47 were measured by ProteOn as described previously. Most of the variants showed improvement in affinity over the parent C47B91 mAb, with C47B222, C47B223,
30 C27B226 and C47B227 showing the most, 5-7 fold tighter affinity compared to

C47B91. Importantly, the off-rates were significantly improved with some of these variants. Some of the variants did induce hemagglutination, suggesting that variations in the CDR sequences may also contribute to hemagglutination activity. The data for SIRP alpha-blocking, binding affinities to human and Cyno CD47, and

5 hemagglutination are summarized in Table 8.

Table 9. Characterization of 32 C47B91 AM variants for SIRP alpha-blocking, affinity to human CD47 and cyno CD47, and hemagglutination activities. The lowest concentrations at which hemagglutination was visibly observed are indicated where appropriate. For C47B214, C47B218, C47B241, C47B242, C47B243, and C47B244, two donors were tested, and one donor demonstrated hemagglutination in response to these mAbs.

C47B91 AM Ab	% SIRP alpha Inhibition	Human CD47 KD (M)	Cyno CD47 KD (M)	KD Ratio Cyno/ Human	Hemagglutination
C47B213	71.6	2.33E-09	4.25E-09	1.8	Y ≥ 3.125 µg/ml
C47B214	99.7	2.30E-09	1.40E-09	0.6	Y/N
C47B215	99.6	2.05E-09	1.44E-09	0.7	N
C47B216	99.8	3.35E-09	2.74E-09	0.8	N
C47B217	83.1	7.67E-09	5.56E-09	0.7	N
C47B218	98.9	2.56E-09	1.58E-09	0.6	Y/N
C47B219	99.4	2.58E-09	1.66E-09	0.6	N
C47B220	98.6	4.30E-09	3.74E-09	0.9	N
C47B221	95.0	5.21E-09	4.50E-09	0.9	N

C47B222	99.9	1.12E-09	8.42E-10	0.8	N
C47B223	100.0	1.14E-09	8.07E-10	0.7	N
C47B224	99.7	2.46E-09	2.19E-09	0.9	N
C47B225	96.3	3.31E-09	3.76E-09	1.1	Y ≥ 3.125 µg/ml
C47B226	100.0	8.72E-10	5.43E-10	0.6	N
C47B227	100.0	9.02E-10	5.74E-10	0.6	N
C47B228	99.3	2.69E-09	2.53E-09	0.9	N
C47B229	16.1	2.15E-09	8.32E-09	3.9	Y ≥ 1.563 µg/ml
C47B230	28.4	1.99E-09	7.28E-09	3.7	Y ≥ 1.563 µg/ml
C47B231	83.7	1.13E-08	1.23E-08	1.1	N
C47B232	99.6	2.80E-09	2.42E-09	0.9	N
C47B233	98.4	3.90E-09	3.02E-09	0.8	N
C47B234	86.6	4.49E-09	7.93E-09	1.8	N
C47B235	99.9	2.35E-09	3.06E-09	1.3	Y ≥ 0.781 µg/ml
C47B236	9.8	2.92E-09	1.46E-08	5.0	N
C47B237	97.9	2.57E-09	3.51E-09	1.4	N
C47B238	99.1	3.53E-09	3.73E-09	1.1	N
C47B239	96.7	6.69E-09	9.70E-09	1.4	N
C47B240	93.0	8.59E-09	9.23E-09	1.1	N

C47B241	99.8	2.62E-09	2.03E-09	0.8	Y/N
C47B242	99.1	3.47E-09	3.46E-09	1.0	Y/N
C47B243	99.8	2.02E-09	2.08E-09	1.0	Y/N
C47B244	99.8	2.12E-09	1.55E-09	0.7	Y/N
C47B91	82.6	5.95E-09	1.78E-08	3.0	N

EXAMPLE 6: EPITOPE AND PARATOPE IDENTIFICATION BY X-RAY CRYSTALLOGRAPHY

- 5 The detailed epitopes and paratopes of antibodies C47B161, C47B167, C47B222, C47B227 and B6H12.2 were determined by co-crystallization of their corresponding Fabs with the CD47 ECD -C15G mutant [(SEQ ID NO: 49) hereafter simply CD47] and structure determination by X-ray crystallography. C47B167 was derived from human framework adaption of C47B131 of epitope bin 3 using a similar approach as
- 10 described for C47B116 in example 4. It blocks SIRPalpha binding ($EC_{50} = 0.79 \mu\text{g/ml}$), binds to human CD47 with moderate affinity ($K_D = 5.2 \text{ nM}$) and does not induce hemagglutination.

- The His-tagged C47B161, C47B167, C47B222, C47B227 Fabs were expressed in HEK293 cells and purified using affinity and size exclusion chromatography. The His-
- 15 tagged CD47 was deglycosylated and further purified by affinity chromatography. The complex CD47:Fab was prepared by mixing Fabs with excess CD47 at a molar ratio ranging from 1.2:1.0 to 1.5:1.0. The complex was incubated overnight at 4°C, separated from the uncomplexed species using size exclusion chromatography, and concentrated to 7.5-20 mg/mL in 20 mM HEPES pH 7.4, 100 mM NaCl, 5% glycerol.
- 20 The concentrated complex was then used to set up crystallization trials using the sitting drop method at 20 °C and crystals were optimized by varying the protein to reservoir ratio. The optimized crystallization conditions for each of the Fab-CD47 complexes are listed in Table 10.

Table 10. Crystallization Conditions for the CD47/Fab complexes.

CD47/C47B161 Complex
CD47 was treated with 200 mU endoH/mg of CD47 (endoH from Sigma E7642) and the fully deglycosylated protein was purified in Concanavalin A Sepharose 4B (GE 17-0440-03) and buffer exchanged to 50mM Tris pH 8, 50mM NaCl (ELN CD47-2013-196). The CD47/C47B161 complex was then prepared by mixing deglycosylated CD47 with C47B161 at a molar ratio of 1.2:1.0 (excess CD47), incubated at 4°C overnight, purified by size-exclusion chromatography, and concentrated to 7.5 mg/mL in 20mM Hepes pH 7.4, 0.1 M NaCl, 5% glycerol. Crystals suitable for X-diffraction were obtained from 34% PEG 8kDa, 0.1M Hepes pH 7.5 using the sitting drop vapor-diffusion method at 20°C.
CD47/C47B167 Complex
The complex was prepared by mixing CD47 with C47B167 at a molar ratio of 1.2:1.0 (excess CD47), incubated at 4°C overnight, buffer exchanged to 20mM Tris pH 7.5, and then eluted from monoS with a gradient of 43-53 mM NaCl in 20mM Tris pH 7.5 and concentrated to 15 mg/mL. Crystals suitable for X-diffraction were obtained from 18% PEG 3kDa, 0.2M (NH ₄) ₂ SO ₄ , 0.1M Mes pH 6.5 using the sitting drop vapor-diffusion method at 20°C.
CD47/C47B222 Complex
The complex was prepared by mixing CD47 with C47B222 at a molar ratio of 1.5:1.0 (excess CD47), incubated at 4°C overnight, buffer exchanged to 20mM Tris pH 7.5, and then eluted from monoS with a gradient of 43-61 mM NaCl in 20mM Tris pH 7.5 and concentrated to 17 mg/mL. Crystals suitable for X-diffraction were obtained from 25% PEG 3k, 1M LiCl, 0.1M Mes pH 6.5 using the sitting drop vapor-diffusion with microseed matrix screening method ¹⁰ at 20°C.
CD47/C47B227 Complex
The CD47/ C47B227 complex was prepared similarly to the C47B167 complex. Crystals suitable for X-diffraction were obtained from 4.5M Na Formate, 0.1M Tris pH 8.5 using the sitting drop vapor-diffusion with microseed matrix screening method ¹⁰ at 20°C and the complex at 11 mg/mL.
CD47/B6H12.2 Complex
The complex was prepared similarly to the C47B167 complex, with the exception of B6H12.2 complex being eluted from monoS with a gradient of 119-137mM NaCl in 20mM Hepes pH 7.0 and concentrated to 3 mg/mL. Crystals suitable for X-diffraction were obtained from 2.4M (NH ₄) ₂ SO ₄ , 0.1M Na Acetate pH 5.5 using the sitting drop vapor-diffusion with microseed matrix screening method ¹⁰ at 20°C.
C47B161, C47B167, C47B222, C47B227 Fabs

C47B161, C47B167 and C47B227 were respectively, concentrated to 8, 17 and 20 mg/mL without further purification. Crystals suitable for X-diffraction were obtained from the conditions listed in Table 2 using the sitting drop vapor-diffusion method at 20°C (ELN CD47-2013-219). In the case of C47B222, the best quality dataset (described in Table 2) was derived from a crystal that was set up to be of a CD47/ C47B222 complex but, instead contained only the free Fab. This CD47/ C47B222 complex was prepared similarly to the C47B222 complex described previously, with the difference of the complex being eluted from monoS with a gradient of 119-145mM NaCl in 20mM Hepes pH 7.0 and concentrated to 23 mg/mL.

For X-ray data collection, one crystal was soaked for a few seconds in a cryo-protectant solution containing crystallization solution supplemented with 20% glycerol, and flash frozen in the stream of nitrogen at 100 K. Diffraction data were collected at the Dectris Pilatus 6M Pixel Array detector at the beamline 17-ID of the Advanced Photon Source (APS) at Argonne National Laboratory over a 240° crystal rotation with 2-min exposures per 0.25°-image and were processed with the program HKL2000. X-ray data statistics are given in Tables 11 and 12.

Table 11. Crystal data, X-ray data and refinement statistics for the CD47/Fab complexes.

Complex	CD47/ C47B161	CD47/ C47B167	CD47/ C47B222	CD47/ C47B227	CD47/ B6H12.2
<i>Crystal data</i>					
Crystallization solution					
0.1M Buffer	Hepes pH	Mes pH 6.5	Mes pH 6.5	Tris pH 8.5	Acetate pH
Precipitant	34% PEG 8K	18% PEG 3K	25% PEG 3K	4.5M NaFormate	2.4M (NH ₄) ₂ SO ₄
Additive		0.2M (NH ₄) ₂ SO ₄	1M LiCl		
Space group	P2 ₁	P2 ₁ 2 ₁ 2	P2 ₁	P2 ₁	C2
Complex/asym.unit	2	4	1	2	1
Unit cell					
<i>a</i> (Å)	74.56	173.61	60.57	94.95	161.76

<i>b</i> (Å)	60.97	210.84	72.93	63.55	54.53
<i>c</i> (Å)	124.13	89.37	72.70	142.00	83.49
β (°)	90.05	90.00	108.96	102.50	95.89
V_m (Å ³ /Da)	2.0	3.0	2.2	3.0	2.7
Solvent content (%)	39	59	45	59	54
<i>X-ray data*</i>					
Resolution (Å)	50.00-2.90	50.00-2.90	50.00-2.30	50.00-3.00	40.00-2.10
High Resolution Shell (Å)	(2.95-2.90)	(2.95-2.90)	(2.34-2.30)	(3.05-3.00)	(2.14-2.10)
Measured reflections	77,044	476,433	82,617	94,048	130,229
Unique reflections	24,865	72,505	26,271	32,700	42,025
Completeness (%)	98.6 (90.9)	100 (100)	98.8 (97.0)	98.6 (98.6)	98.7 (99.1)
Redundancy	3.1 (2.9)	6.6 (6.3)	3.1 (2.8)	2.9 (2.3)	3.1 (3.0)
Rsym (%)	11.9 (46.5)	18.0 (72.9)	8.3 (43.2)	16.5 (53.6)	10.5 (36.8)
$\langle I/\sigma \rangle$	9.1 (2.0)	12.0 (3.1)	12.8 (2.4)	7.2 (2.1)	10.8 (4.2)
<i>Refinement</i>					
Resolution (Å)	41.4-2.9	45.0-2.9	32.2-2.3	41.3-3.0	38.6-2.1
Number of reflections	24,832	72,349	26,217	32,646	41,995
Number of all atoms	8,243	17,321	4,291	7,358	4,441
Number of waters	57	195	99	10	208
Rfactor (%)	24.9	18.7	18.8	20.9	18.2
Rfree (%)	29.7	24.1	24.4	26.6	22.9
RMSD					
bond lengths (Å)	0.003	0.003	0.003	0.009	0.007
bond angles (°)	0.768	0.697	0.712	1.516	1.119
Wilson B-factor (Å ²)	53.18	38.90	49.94	66.83	25.4
MolProbity					
Ramachandran favored (%)	93.93	96.06	95.87	94.22	97.76
Ramachandran allowed (%)	5.70	3.62	4.13	5.57	2.24
Ramachandran outliers (%)	0.37	0.32	0.00	0.21	0.00

Rotamer outliers (%)	0.00	0.38	0.89	0.38	0.64
Clash score	6.69	2.74	3.16	6.62	3.47

*Values for high resolution shell are in parenthesis.

Table 12. Crystal data, X-ray data and refinement statistics for the free Fabs.

Fab	C47B161	C47B167	C47B222	C47B227
<i>Crystal data</i>				
Crystallization solution				
0.1M Buffer	Mes pH 6.5	Acetate pH 4.5	Tris pH 8.5	Tris pH 8.5
Precipitant	24% PEG 3K	25% PEG 3K	2.4M (NH ₄) ₂ SO ₄	2.4M (NH ₄) ₂ SO ₄
Additive	1M Na Acetate	0.2M (NH ₄) ₂ Ac	5% MPD	5% MPD
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Fab/asym.unit	1	2	1	1
Unit cell				
<i>a</i> (Å)	56.63	73.96	63.74	62.60
<i>b</i> (Å)	60.59	79.90	74.47	73.98
<i>c</i> (Å)	122.97	144.40	103.98	114.04
V _m (Å ³ /Da)	2.1	2.1	2.6	2.6
Solvent content (%)	42	42	53	53
<i>X-ray data*</i>				
Resolution (Å)	40.00-2.00	50.00-2.00	50.00-1.60	50.00-2.60
High Resolution Shell (Å)	(2.03-2.00)	(2.03-2.00)	(1.63-1.60)	(2.64-2.60)
Measured reflections	176,113	370,967	388,172	104,467
Unique reflections	28,937	58,215	65,229	16,591
Completeness (%)	99.2 (96.5)	99.1 (97.3)	97.5 (88.5)	98.8 (97.3)
Redundancy	6.1 (5.1)	6.4 (5.7)	6.0 (5.1)	6.3 (6.1)
Rsym (%)	5.4 (14.9)	5.7 (24.6)	8.6 (29.2)	11.8 (60.4)
<I/σ>	26.9 (10.6)	27.7 (8.3)	16.4 (5.2)	14.8 (3.8)
<i>Refinement</i>				
Resolution (Å)	34.3-2.0	35.0-2.0	35.0-1.6	33.8-2.6
Number of reflections	28,865	58,140	65,163	16,518

Number of all atoms	3,427	6,840	3,581	3,268
Number of waters	105	173	325	75
Rfactor (%)	19.4	19.8	17.05	18.18
Rfree (%)	24.0	24.8	19.79	27.24
RMSD				
bond lengths (Å)	0.007	0.007	0.014	0.007
bond angles (°)	1.209	1.148	1.625	1.128
Wilson B-factor (Å ²)	26.86	28.03	18.68	38.57
MolProbity				
Ramachandran favored (%)	98.38	97.33	98.09	95.93
Ramachandran allowed (%)	1.39	2.67	1.91	3.83
Ramachandran outliers (%)	0.23	0.00	0.00	0.24
Rotamer outliers (%)	1.07	0.80	0.28	0.29
Clash score	2.59	1.60	2.03	5.76

*Values for high resolution shell are in parenthesis.

The overall structures

The structural models for the CD47 molecules include residues from 1 to at least 114, corresponding to the IgV domain, and glycans in positions 5, 16, 32, 55, and 93, except for CD47 in the C47B161 Fab complex with no visible glycans. The CD47 C-terminal 6xHis (SEQ ID NO: 55) is disordered. The Fab structural models contain residues from 1 to at least 211 for the light chain and from 1 to at least 217 for the heavy chain. The Fab C-terminal 6xHis tag (SEQ ID NO: 55) and inter-chain disulfide bond are disordered. In the case of the C47B222/C47B227 heavy chains, residues 135-140 are also disordered. The antibody/antigen combining site is well defined by electron density in all 5 complexes, which allows reliable positioning of the binding residues. The Fabs are numbered sequentially in all figures and CD47 numbering starts at the N-terminus of the mature protein.

The CD47 molecules in the Fab complexes superimposed among themselves

and with CD47 bound to SIRP alpha (Hatherley, 2008) with RMSD of 0.45-0.98Å, indicating a high degree of structural similarity of CD47 in the various complexes and absence of large conformational changes induced by Fab or receptor binding.

5 **The epitopes, paratopes and antibody/antigen interactions**

C47B161, C47B167, and C47B222/ C47B227/B6H12.2 bind to 3 distinct epitope bins as revealed by antibody competition binning assays. C47B222 and C47B227 bind to the same overall location, while C47B167 binds closer to the cell membrane and C47B161 and B6H12.2 bind to a more apical region of CD47. Binding
 10 to an epitope closer to the membrane can cause additional constraints to the Fab that might propagate to the 2nd Fab arm in the antibody and make binding of this arm to another CD47 molecule more challenging. We speculate that constraints to the 2nd Fab arm due to epitope location could translate into less cell aggregation from CD47/antibody/CD47 cross-linkings. In the case of the C47B222 Fab, the HC has
 15 more extensive contacts with CD47 than the LC and it is the chain closer to the membrane.

The epitope and paratope sequences are shown in Figure 6. The details of the interaction made between CD47 and each of the Fabs is discussed in detail below.

20 **CD47/C47B161 Complex**

C47B161 recognizes a conformational epitope composed of residues in the CD47 N-terminal (Q1 and L3), BC (N27 and E29) and FG (residues 101-103) loop regions and the F (residue E97) and G (E104) β-strands as seen in Figure 7. M259 binds to epitope bin 2 region and covers an area of about 690Å² on CD47.

25 The paratope is composed of residues from five CDRs except CDR-L3. The LC is positioned mostly on the CD47 β-sheet, while the HC covers the apical loop regions of the antigen. In comparison to the other anti-CD47 Fabs under evaluation, C47B161 has a long CDR-L1 with an insertion of six residues at position 30. The Y35 and Y37 residues near the tip of the CDR-L1 loop enhance the affinity of the antibody for its
 30 antigen by H bonding the β-sheet of CD47 (Figure 7C). The other residues at the L1

loop tip (H31 and N33 residues), are not involved in direct contact with CD47 but, they play an important role in orienting Y35 and, specially, Y37 for effective interaction with CD47. On the VH side, all CDRs are involved in interactions with the antigen, particularly with the N-terminus Q1 residue, which is cyclized as pyroglutamate, and the 27-29 and 102-103 loop segments (Figure 7B and 7D).

CD47/C47B167 Complex

C47B167 recognizes a conformational epitope composed of residues of the C (Y37 and K39), C' (R45, D46 and T49) and C'' (N55-T58) β -strands and C'C'' (A53, L54) and C''E (V59-T61, S64, A66, K67) loop regions as shown in Figure 8. C47B167 binds to the epitope bin 3 region and covers an area of about $930\text{\AA}^2$ on CD47.

The C47B167 paratope is made of residues from all six CDRs. The C and C' β -strands (Y37, K39, R45, D46 and T49) interact only with the LC CDRs, while the loop region 64-67 is contacted only by the long CDR-H3 (9 residue insertion).

C47B167 has reduced cross-reactivity to cyno monkey CD47 due to sequence divergence in the epitope region V59-T61 (see stretch of sequence alignment in Figure 8A). The T61A human to cynoCD47 change kills the H bonds between residue 61 and LC residues D49 and R52 in cyno (Figure 8C). Additionally, a potential N-linked glycosylation site in position 62 of cyno CD47 (and not in human) could create steric hindrance for antibody binding to the V59-T61 region. The V59A change should have a minor impact (Figure 8D).

There is no common residue between the epitope bins 2 and 3. C47B161 (bin 2) and C47B167 (bin 3) can bind simultaneously to CD47 without any clash regions as shown in Figure 9. This result has also been demonstrated using antibody competition binning assays (see Example 3). C47B167 recognizes a larger area of the CD47 β -sheet than C47B161 and binds considerably closer to the cell membrane.

CD47/C47B222 and CD47/C47B227 Complexes

C47B222 recognizes a conformational epitope composed of residues of the C (Y37 and K39), C' (D46, T49, D51), C'' (K56, T58) and F (T99) β -strands and the BC

(E35), C'C'' (A53 and L54), C''E (V59), and FG (L101, T102) loop regions as shown in Figure 10. The epitope of C47B222 is located in the bin 1 region and covers a CD47 area of about $730\text{\AA}^2$. C47B222 competes for CD47 binding with C47B161, C47B167, C47B227 and B6H12.2. The regions of C47B222 epitope overlap with the other
 5 epitopes are displayed in Figure 6.

The C47B222 paratope is composed of residues from all CDRs except CDR-L3. The majority of the C47B222 interactions with CD47 are made by the heavy chain using a combination of H bonds and van der Waals contacts (Figure 10). There is clear segregation between the epitope regions recognized by the HC and LC paratopes. The
 10 heavy chain interacts exclusively with the C'C'' loop and all β -strand epitope regions except for the F strand. The light chain binds, also with exclusivity, the BC and FG loops and the F strand, occupying a more apical position on CD47 that serves to block SIRP alpha binding to CD47 very effectively. Leucine 54 in the C'C'' loop is in a central location of the combining site and contacts all three heavy chain CDRs (Figure
 15 10B). The C'' strand hydrogen bonds CDR-H2 and the C and C' β -strands contact CDR-H3.

The C47B227 conformational epitope is also located in bin 1. The epitope covers a CD47 area of about $800\text{\AA}^2$ composed of residues of all strands of the β -sheet (strand C: Y37; strand C': T49 and D51; strand C'': N55, K56 and T58; strand F: T99;
 20 strand G: E104) and segments of the BC (E35), C'C'' (residue range 53-55) and FG (L101, T102) loop regions as shown in Figure 11.

The C47B227 paratope is composed of residues from all CDRs. Similar to C47B222, the heavy chain of C47B227 interacts exclusively with the C, C', and C'' β -strands and most of the C'C'' loop region – the exception is A53, which is in the C'C''
 25 loop and has a van der Waals contact with CDR-L3. The light chain of M263 binds to the F and G strands and the BC and FG loops. C47B222 and C47B227 bind to CD47 with a similar orientation and share many epitope and paratope residues (Figure 6).

C47B222 has 97% sequence identity to C47B227 with 6 amino acid changes in the regions near or at the CDRs (HC: T30D and LC: N30G, N31S, H49Y, I56T and
 30 S93Y changes from C47B222 to C47B227. See Figure 6). Nevertheless, C47B222 and

C47B227 bind to CD47 with the same overall orientation and recognize roughly the same epitope region with similar affinity (about 1nM). This epitope tolerance to different light chains is a direct consequence of the C47B222 / C47B227 recognition of CD47 being heavily based on the HC contacts and the separation between the epitope regions recognized by the HC and LC paratopes (epitope segregation). Furthermore, the effective number of residues changes between C47B222 and C47B227 is only three (N30G, N31S and S93Y) since the heavy chain T30D and light chain H49Y and I56T changes are too far away (greater than 6Å) from the combining site to have an effect in CD47 recognition. Replacement of G30 with Asn in C47B222 only induces accommodation of the CD47 FG loop in a slightly different position to avoid steric clashes between L101 and N30 as seen in Figure 11A. The N31S and S93Y changes create new H bonds with the T102 main chain and E35 in M263, strengthening the interaction of the C47B227 LC with CD47 and contra-balancing the fewer H bonds made by the CDR-H3 of C47B227 in comparison to the C47B222, which adopts a different conformation and uniquely H bonds CD47 residues Y37, K39, D46 and D51 (Figures 10-12).

CD47/B6H12.2 Complex

B6H12.2 binds to the epitope bin 1 region and it was used as a control antibody. B6H12.2 recognizes a conformational epitope composed of residues of the C (V36, Y37, K39 and K41), C' (D46, D51), F (E97, T99) and G (E104) β-strands and the BC (E29, Q31, N32, T34 and E35), C'C'' (A53) and FG (E100-R103) loop regions as shown in Figure 10. Unlike C47B222 and C47B227, which were also clustered in bin 1, B6H12.2 does not recognize any residues in neither the C'' β-strand nor the C''D loop region. Nevertheless, B6H12.2 has a larger epitope area than C47B161, C47B167, C47B222 and C47B227 (about 1055Å² versus 690Å² for C47B161, 930Å² for C47B167, 730Å² for C47B222, and 800Å² for C47B227).

The B6H12.2 paratope is composed of residues from all CDRs (Figure 13). The antibody and antigen recognition appears to be mostly driven by H bonds. The light chain makes 6 H bonds with CD47 side chains and 3 with backbone atoms. The heavy

chain, on the other hand, is engaged in 13 H bonds with CD47, most of them involving backbone atoms. B6H12.2 has a short CDR-H3 that goes deep into the combining site, stabilizes the FG loop and gives space for CDR-H2 to spread on top of the BC loop. The heavy chain contacts with exclusivity the BC (mostly CDR-H2), C'C'' (CDR-H3) and most of the FG (CDRs-H2 and H3) loop regions, while the LC contacts exclusively the C' strand and the adjacent C-terminal area of the C strand. However, there is less epitope segregation for B6H12.2 than for C47B222 and C47B227. There are multiple epitope residues in the F strand and FG loop that interact with both HC and LC.

10 **Epitope Summary**

Unlike C47B161, B6H12.2 does not recognize the N-terminal region of CD47. Unlike C47B167, C47B222, and C47B227, B6H12.2 does not recognize any residues in either the C'' β -strand or the C''D loop regions of CD47 (see Figure 6). Specifically, the epitope residues of each Fab that do not coincide with the epitope identified for B6H12.2 are:

C47B161: residues in the N-terminal (Q1 and L3) and the BC loop (N27) regions.

C47B167: residues in the β -strand C' (R45 and T49), C'C'' loop (L54), β -strand C'' (K56, S57, and T58) and C''D loop (V59, P60, T61, S64 and K67) regions.

C47B222: residues in the β -strand C' (T49), C'C'' loop (L54), β -strand C'' (K56 and T58) and C''D loop (V59) regions.

C47B227: residues in the C'C'' loop (L54) and β -strand C'' (K56 and T58) regions.

EXAMPLE 7: CHARACTERIZATION OF CD47 ANTIBODIES

Several human CD47 IgG2sigma antibodies with desired characteristics were converted to an IgG1 Fc format for further evaluation in bioassays. Their IgG1 variants were confirmed to bind to human CD47, and block SIRP alpha-binding.

IgG1/IgG2sigma Fc variants that did not induce hemagglutination (C47B222, C47B157, and C47B161) were further evaluated in vitro for enhancement of phagocytosis, apoptosis, complement mediated cytotoxicity (CDC), and platelet

aggregation activity. In addition, the anti-tumor activity of these three mAbs was tested *in vivo*.

Phagocytosis:

5 *In vitro* phagocytosis assays were performed to assess whether C47B157, C47B161, and C47B222 enhance phagocytosis of CD47 expressing target cells by human macrophages. Briefly, CD14 positive monocytes were isolated from leukopak purified PBMCs by negative depletion using the Stemcell EasySep human monocyte enrichment kit without CD16 depletion. Purified monocytes were plated at 0.1×10^6 cells/cm² in X-VIVO-10 medium (Lonza) supplemented with 10% FBS (Invitrogen) and 25 ng/ml M-CSF (R&D Systems) and were differentiated to macrophages for seven days. IFN- γ (R&D Systems) was added at 50 ng/ml during the final 24 hours of differentiation. Subsequently, adherent macrophages were detached from tissue culture dishes by Accutase (Sigma) treatment and macrophages (1×10^5) were plated in 96-well U-bottom plates at a 1:1 ratio with GFP expressing Jurkat cells (1×10^5) in the presence of varying concentrations of anti-CD47 mAbs. Cells were incubated at 37°C for 90 minutes. Upon completion of incubation, cells were washed once with PBS and cells were detached with Accutase (20 minute incubation). Cells were pelleted, washed and macrophages were stained with an APC conjugated anti-human CD11b antibody (eBiosciences) for 30 minutes followed by two washes with stain Buffer (BD Biosciences). Cells were acquired on the MacsQuant flow cytometer. Data was analyzed with the FloJo software. Percent phagocytosis was determined by the following equation $[(\text{GFP}^{\text{pos}}, \text{CD11b}^{\text{pos}} \text{ cells}) / (\text{GFP}^{\text{pos}}, \text{CD11b}^{\text{pos}} + \text{GFP}^{\text{pos}} \text{ cells})] \times 100\%$.

25 These experiments demonstrated that IgG1 B157, B161, B222 and B6H12.2 demonstrated robust phagocytosis enhancing ability, while IgG2sigma B157, B161, B222, and B6H12.2 (Figure 14) enhanced phagocytosis minimally.

Apoptosis:

30 Several anti-CD47 mAbs (e.g., AD-22, 1F7 and MABL-1) have been described

to mediate apoptosis of target cells in soluble form upon ligation of CD47. To test whether C47B157, C47B161, and C47B222 mediate apoptosis upon ligation of CD47, Jurkat cells or HL60 cells (1×10^6 cells/ml in 100 μ l) were incubated for 24 hours at 37°C in RPMI1640 supplemented with 10% FBS in the presence or absence of antibodies (0.05, 0.5, and 5 μ g/ml). Apoptosis was detected with the FITC Annexin-V Apoptosis Detection Kit (BD Pharmingen). Apoptosis was expressed as the sum of early apoptosis (Annexin-V positive/PI negative) and late apoptosis (Annexin-V positive/ PI negative).

These experiments revealed that the control mAb B6H12.2 induced robust apoptosis as IgG2sigma in Jurkat and HL60 cells, but not as IgG1 (see Figure 15). Similarly, C47B157 and C47B161 induced low levels of apoptosis as IgG2sigma in Jurkat cells, but not as IgG1. IgG1/IgG2sigma C47B157 and C47B161 had no proapoptotic effects on HL60 cells. 24 hour treatment of Jurkat and HL60 cells with IgG1 or IgG2sigma C47B222 did not result in apoptosis induction.

Complement Mediated Cytotoxicity:

C47B157, C47B161, and C47B222 were tested for complement mediated cytotoxicity (CDC). CDC assays were performed with Wil2-S target cells. 50,000 Wil2-S cells were plated in opaque white 96-well plates in 25 μ l RPMI-1640 supplemented with 10% heat inactivated FBS and 0.1 mM NEAA (all reagents from Invitrogen). Upon addition of 25 μ l of medium supplemented with or without antibodies cells were incubated for 30 minutes at 37°C. Subsequently, 50 μ l of human serum (Bioreclamation, complement preserved) was added and cells were incubated at 37°C for 2 hours. To assess maximal lysis 20 μ l of 2% Triton-X-100 were added to control wells and cells were incubated at 37°C for 2 hours. Once the incubation was completed, 100 μ l of CellTiter-Glo Reagent (Promega; pre-mixture of buffer and substrate) was added to each well, contents were gently mixed to induce cell lysis and luminescence was recorded on the 2104 EnVision Multilabel reader (Perkin Elmer). Specific lysis was calculated by the following equation: [specific lysis = ((Experimental release-spontaneous release)/(maximum release-Spontaneous release))*100].

As shown in Figure 16, IgG1/IgG2sigma C47B157, C47B161, and C47B222 did not enhance CDC as IgG1 or IgG2sigma. In contrast, the tool mAb B6H12.2 IgG1mediated robust CDC similar to the positive control rituximab, and did not enhance CDC in the effector function silent IgG2sigma backbone.

5

Platelet aggregation:

Based on the observation that the commercially available anti-CD47 mAb B6H12 (mIgG1) induced platelet aggregation (Fujimoto TT, Katsutani S, Shimomura T, Fujimura K. (2003) J Biol Chem 278: 26655-26665), assays were established to determine the platelet aggregation activity of C47B157, C47B161, and C47B222. Briefly, blood was collected from healthy donor volunteers into Vacutainer blood collection tubes (BD Biosciences) buffered with sodium citrate. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared with the PDQ platelet function centrifuge (Biodata Corporation) according to the manufacturer's protocol. Platelet aggregation was measured with the PAP-8E aggregometer (Biodata Corporation) as recommended by the manufacturer. Specifically, 25 µl of ADP (positive control; Biodata Corporation) or antibodies were added to 225 µl of PRP for a final concentration of 10 µM ADP or 100, 140, 150, or 200 µg/ml test antibodies (depending on availability of antibody tested). Aggregation was determined by measuring the transmission of light through the 250 µl sample at 37 °C with continuous stirring. The transmission of PPP was set as 100%. Aggregation was recorded for a total of 6 minutes.

To assess platelet aggregation activity of the herein described mAbs, eight assays with independent donors were evaluated: 1.) five experiments were conducted where the activity of IgG1/IgG2sigma B6H12.2 was compared to the positive control 10 µM ADP and negative control PBS; and 2.) three experiments were conducted where the activity of IgG1/IgG2sigma C47B157, C47B161, and C47B222 was compared to IgG1 B6H12.2 and negative PBS control. The platelet aggregation assays demonstrated that C47B157, C47B161, and C47B222 did not induce platelet aggregation in either the IgG1 or IgG2sigma backbone with three independent donors

when tested at concentrations ranging from 100 to 200 µg/ml (see Figure 17 b-d depicting results for an experiment run at 200 µg/ml). Maximal aggregation observed for IgG1/IgG2sigma C47B157, C47B161, and C47B222 ranged from 0 to 7% similar to what was observed for the PBS control (eight independent donors; maximal aggregation ranged from 2-11%). In contrast, B6H12.2 tested at 100 and 200 µg/ml induced aggregation as IgG1, ranging from 40 to 93% (eight donors) similar to the positive control 10 µM ADP (five donors; aggregation ranging from 55 to 120%), but not as IgG2sigma ranging from 1 to 6% (five independent donors).

10 ***In vivo* anti-tumor efficacy of Fc variants of CD47 antibodies:**

The *in vivo* anti-tumor activity of C47B157, C47B161, and C47B222 was tested in three human tumor cell models. Briefly, for the first two models 10x10⁶ HL-60 or 5x10⁶ MV4-11 cells were intravenously implanted into NSG mice. For both of these models, antibody treatment was initiated on day 6 following tumor cell implant, and animals were dosed twice weekly at 0.2 and 10 mg/kg via intraperitoneal injection. A total of six doses were administered (final dose on day 23) and each dosing group consisted of five mice. For the HL-60/NSG mice model peripheral blood from the mice was collected weekly and analyzed via FACS to assess tumor cell outgrowth and treatment effects starting on day 14 (final collection on day 42). For the MV4-11/NSG model, peripheral blood from mice was collected on day 34 and was analyzed via FACS to assess effects of treatment on tumor cell outgrowth in the peripheral blood. In a third *in vivo* model 10x10⁶ Kasumi-3 cells were intravenously implanted into NSG mice, and treatment was initiated on day 6 following tumor cell implant. Animals were dosed twice weekly at 0.2 and 10 mg/kg via intraperitoneal injection for a total of 12 doses. Final dose was administered on day 44 and each dosing group consisted of five mice. Peripheral blood was collected from mice weekly and analyzed via FACS to assess tumor cell outgrowth in the peripheral blood starting on day 14.

In all three models tested, C47B157, C47B161, C47B222, and B6H12.2 demonstrated anti-tumor activity. When tested in the HL60 model (see Figure 18), the efficacy of each mAb was dependent on the dose tested and the Fc effector function of

each mAb. The effector function competent IgG1 and effector function silent IgG2 sigma versions of all mAbs demonstrated dose dependent activity. IgG1 robustly suppressed tumor cell outgrowth at 10 mg/kg and delayed tumor cell outgrowth at 0.2 mg/kg. IgG2sigma delayed tumor cell growth at 10 mg/kg, while at 0.2 mg/kg the anti-tumor effects were less pronounced. Similar to the HL60 model, when tested within the MV4-11 models (Figure 19), the mAbs effectively suppress tumor cell growth as IgG1 at 0.2 and 10 mg/kg. Tumor cell outgrowth suppression was less when the mAbs were tested in the IgG2 sigma backbone. While effector function provided an enhancement in efficacy in the HL60 and MV4-11 models, IgG1 and IgG2sigma C47B157, C47B161, C47B222, and B6H12.2 demonstrate robust anti-tumor activity when tested in the IgG1 or IgG2sigma backbone at both 0.2 and 10 mg/kg (Figure 20).

EXAMPLE 8: PROPOSED MODE OF ANTIBODY NEUTRALIZATION

As noted in the previous examples, antibodies have been generated that can promote cell phagocytosis likely by blocking the binding of CD47 in the target cell to the SIRP alpha receptor in macrophages and, consequently, disrupting the “eat-me-not” signal that otherwise the target cell would send to the macrophage. The overlay of CD47/Fab structures with the CD47/SIRP alpha structure in Figure 21 shows regions of clash between all Fv domains and SIRP alpha D1 domain, making impossible for both antibody and SIRP alpha to bind simultaneously to CD47.

5

10

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO:	Type	Species	Description	Sequence
1	PRT	human	IgG1-Fc Wild-type	ASTKGPSVFPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKKVEPKSCDKTHTCP PCPAPELLGGPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFNWYVDGV EVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQV SLTCLVKGFYPSDIAVEWESNGQPENN YKTTTPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLS PGK

2	PRT	human	IgG2-Fc- Wild-type	ASTKGPSVFPLAPCSRSTSESTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVPSSNFGTQTYTC NVDHKPSNTKVDKTKVERKCCVECPCP APPVAGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSHEDPEVQFNWYVDGVEVHN AKTKPREEQFNSTFRVVSVLTVVHQDW LNGKEYKCKVSNKGLPAPIEKTISKTKG QPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPP MLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK
3	PRT	human	IgG2 sigma-Fc	ASTKGPSVFPLAPCSRSTSESTAALGCL VKDYFPEPVTVSWNSGALTSGVHTFPA VLQSSGLYSLSSVVTVTSSNFGTQTYTC NVDHKPSNTKVDKTKVERKCCVECPCP APPAAASSVFLFPPKPKDTLMISRTPEVT CVVVDVSAEDPEVQFNWYVDGVEVHN AKTKPREEQFNSTFRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSSIEKTISKTKG QPREPQVYTLPPSREEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPP MLDSDGSFFLYSKLTVDKSRWQQGNVF SCSVMHEALHNHYTQKSLSLSPGK
4	PRT	artificial	C47B157- VH	QVQLVQSGAEVKKPGASVKVSCKASG YTFTDYNMHWVRQAPGQGLEWMGDI YPYNGGTGYNQKFKGRVTMTRDTSTST VYMELSSLRSEDTAVYYCARGGWHAM DSWGQGTLLTVSS

5	PRT	artificial	C47B161-VH	QVQLVQSGAEVKKPGASVKVSCKASG YTFTDYNMHWVRQAPGQRLEWMGDI YPYNGGTGYNQKFKGRVTITRDTSAST AYMELSSLRSEDNAVYYCARGGWHAM DSWGQGTLVTVSS
6	PRT	artificial	C47B222-VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FTDYWIGWVRQMPGKGLEWMGIHYPG DSDTRYSPSFQGGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLVTVSS
7	PRT	artificial	C47B157 and C47B161-VL	DIVMTQSPLSLPVTPGEPASISCRSRQSI VHTNRYTYLAWYLQKPGQSPQLLIYKV SNRFSGV PDRFSGSGSGTDFTLKISRVE AEDVGVYYCFQGSHVPYTFGGGTKLEIK
8	PRT	artificial	C47B222-VL	EIVLTQSPATLSLSPGERATLSCRASQSV NNRLAWYQQKPGQAPRLLIHWASTRAI GIPARFSGSGSGTDFTLTISSELPEDFAV YYCQQGASWPFTFGQGTKVEIK
9	PRT	mouse	C47B157 and C47B161-HCDR1	DYNMH
10	PRT	mouse	C47B222-HCDR1	DYWIG
11	PRT	mouse	C47B157 and C47B161-HCDR2	DIYPYNGGTGYNQKFKG

12	PRT	mouse	C47B222-HCDR2	IIYPGDS DTRYSPSFQG
13	PRT	mouse	C47B157 and C47B161-HCDR3	GGWHAMDS
14	PRT	mouse	C47B222-HCDR3	VGRFASHQLDY
15	PRT	mouse	C47B157 and C47B161-LCDR1	RSRQSIVHTNRYTYLA
16	PRT	mouse	C47B222-LCDR1	RASQSVNNRLA
17	PRT	mouse	C47B157 and C47B161-LCDR2	KVSNRFS
18	PRT	mouse	C47B222-LCDR2	WASTRAI
19	PRT	mouse	C47B157 and C47B161-LCDR3	FQGSHVPYT
20	PRT	mouse	C47B222-LCDR3	QQGASWPFT

21	PRT	human	CD47	MWPLVAALLLGSACCGSAQLLFNKT KSVEFTFCNDTVVIPCFVTNMEAQNTTEV YVKWKFKGRDIYTFDGALNKSTVPTDF SSAKIEVSQLLKGDASLKMDKSDAVSH TGNYTCEVTELTREGETIIE LKYRVVSWFSPNE NILIVIFPIFAILLFWGQFGIKTLK YRSGGMDEKTIALLVAGLVITVIVIVGA ILFVPGEYSLKNATGLGLIVTSTGILILLH YYVFSTAIGLTSFVIAILVIQVIAYILAVV GLSLCIAACIPMHGPLLISGLSILALAQL LGLVYMKFVASNQKTIQPPRKAVEEPL NAFKESKGMMNDE
22	PRT	human	CD47-ECD	QLLFNKT KSVEFTFCNDTVVIPCFVTNMEAQNTTEV YVKWKFKGRDIYTFDGALNKSTVPTDF SSAKIEVSQLLKGDASLKMDKSDAVSHT TGNYTCEVTELTREGETIIE LKYRVVSWFSPNE
23	DNA	artificial	HuG1_DN VH_F169	CAAAGTATACAGGCCCGAGGTGCAGCT GGTGCAGAG
24	DNA	artificial	HuG1_DN VH_F323	CAAAGTATACAGGCCCGAAGTGCAGCT GCTGGAAAG
25	DNA	artificial	HuG1_DN VH_F551	CAAAGTATACAGGCCCGAAGTGCAGCT GGTGCAGAGC
26	DNA	artificial	HuG1_DN VH_R	GCCCTTGGTGGAGGCGCTGCTCACGG TCACCAG

27	DNA	artificial	HuK_DNV L_FA27L6 muSP	CAAAGTATCCAAGCAGAAATTGTGCT GACCCAGAG
28	DNA	artificial	HuK_DNV L_FO12mu SP	CAAAGTATCCAAGCAGATATTCAGAT GACCCAGAGC
29	DNA	artificial	HuK_DNV L_R	TGCAGCCACCGTACGTTTAATTCCAC TTTGGTGCC
30	PRT	mouse	C47B116- VH	EVQLQQSGPELVKPGASVKISCKASGYT FTDYNMHWVKQSHGKSLEWIGDIYPY NGGTGYNQKFKSKATLTVDNSSSTAY MELRSLTSEDSAVYYCARGGWHAMDS WGQGTSVTVSS
31	PRT	artificial	C47B151, C47B152, and C47B153- VH	QVQLVQSGAEVKKPGSSVKVSCKASGY TFTDYNMHWVRQAPGQGLEWMGDIYP YNGGTGYNQKFKGRVTITADESTSTAY MELSSLRSED TAVYYCARGGWHAMDS WGQGTLVTVSS
32	PRT	artificial	C47B147, C47B148 and C47B149- VH	EVQLVQSGAEVKKPGESLKISCKGSGY TFTDYNMHWVRQMPGKGLEWMGDIY PYNGGTGYNQKFKGQVTISADKSISTA YLQWSSLKASDTAMYYCARGGWHAM DSWGQGT LVT VSS
33	PRT	artificial	C47B159, C47B155, C47B151, C47B147- VL	DIVMTQSPDSLAVSLGERATINCRSRQSI VHTNRYTYLAWYQQKPGQPPKLLIYKV SNRFSGVPDRFSGSGSGTDFTLTISLQA EDVAVYYCFQGSHVPYTFGGGTKLEIK

34	PRT	artificial	C47B160, C47B156, C47B152, C47B148- VL	EIVLTQSPATLSLSPGERATLSCRSRQSI VHTNRYTYLAWYQQKPGQAPRLLIYK VSNRFSGIPARFSGSGSGTDFTLTISSLEP EDFAVYYCFQGSHVPYTFGGGTKLEIK
35	PRT	artificial	C47B237, C47B238, C47B239, C47B240, C47B91 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FTSYWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGGVTISADKSISTAYLQ WSSLKASDTAMYYCARVGRFASHQLD YWGQGTLLTVSS
36	PRT	artificial	C47B91, C47B213, C47B217, C47B221, C47B229, C47B230, C47B225, C47B231, C47B234, C47B235, C47B236 VL	EIVLTQSPATLSLSPGERATLSCRASQSV NKALAWYQQKPGQAPRLLIYGASNRA TGIPARFSGSGSGTDFTLTISSLEPEDFA VYYCQQGKGWPFTFGQGTKVEIK
37	PRT	artificial	C47B213, C47B214, C47B215, C47B216, C47B241 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FDDSWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGGVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS

38	PRT	artificial	C47B217, C47B218, C47B219, C47B220, C47B242 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FTDSWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
39	PRT	artificial	C47B229 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FDDAWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
40	PRT	artificial	C47B230 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FTDDWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
41	PRT	artificial	C47B225, C47B226, C47B227, C47B228, C47B244 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FDDYWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
42	PRT	artificial	C47B231, C47B232, C47B233 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FTNYWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS

43	PRT	artificial	C47B234 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FDNYWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
44	PRT	artificial	C47B235 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FDDYWISWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
45	PRT	artificial	C47B236 VH	EVQLVQSGAEVKKPGESLKISCKGSGYS FKDDWIGWVRQMPGKGLEWMGIIYPG DSDTRYSPSFQGQVTISADKSISTAYLQ WSSLKASDTAVYYCARVGRFASHQLD YWGQGTLLTVSS
46	PRT	artificial	C47B238, C47B215, C47B219, C47B223, C47B227, C47B233 VL	EIVLTQSPATLSLSPGERATLSCRASQSV GSRLAWYQQKPGQAPRLLIYWASTRAT GIPARFSGSGSGTDFTLTISSELEPEDFAV YYCQQGAYWPFTFGQGTKVEIK
47	PRT	artificial	C47B239, C47B241, C47B242, C47B243, C47B244 VL	EIVLTQSPATLSLSPGERATLSCRASQSV SNRLAWYQQKPGQAPRLLIYGASNRAT GIPARFSGSGSGTDFTLTISSELEPEDFAV YYCQQGRSWPFTFGQGTKVEIK

48	PRT	artificial	C47B240, C47B216, C47B220, C47B224, C47B228 VL	EIVLTQSPATLSLSPGERATLSCRASQSV SNRQAWYQQKPGQAPRLLIHSASNRAT GIPARFSGSGSGTDFTLTISSELEPEDFAV YYCQQGRSWPFTFGQGTKVEIK
49	PRT	human	CD47- ECD-C15G	QLLFNKTKSVEFTFGNDTVVIPCFVTNM EAQNTTEVYVKWKFKGRDIYTFDGAL NKSTVPTDFSSAKIEVSQLLKGDASLKM DKSDAVSHTGNYTCEVTELTREGETIIE LK YRVVSWFSPNE
50	PRT	artificial	C47B167- VH	EVQLVQSGAEVKKPGESLKISCKGSGY TFTSYWMQWVRQMPGKGLEWMGEIN PSNGRTDYNEKFRGQVTISADKSISTAY LQWSSLKASDTAMY YCARQGSGSYGN SYGFFDVWGQGT TTVTVSS
51	PRT	artificial	C47B167- VL	EIVLTQSPATLSLSPGERATLSCRASSSV SYMHWYQQKPGQAPRLLIYDTSRLASG IPARFSGSGSGTDFTLTISSELEPEDFAVY YCQQWRSNPYTFGGGGTKVEIK
52	PRT	mouse	B6H12.2- VH	EVKLVESGGDLVKPGGSLKLSCAASGF TFSGYGMSWVRQTPDKRLEWVATITSG GT YTYYPDSVKGRFTISRDN AKNTLYL QIDSLKSEDTAIYFCARSLAGNAMDYW GQGTSVTVSS
53	PRT	mouse	B6H12.2- VL	DIVMTQSPATLSVTPGDRVSLSCRASQT ISDYLHWYQQKSHESPRLLIKFASQSISG IPSRFSGSGSGSDFTLSINSVEPEDVGVY YCQNGHGFPRTFGGGGTKLEIK

54	PRT	mouse	C47B116 VL	DVVMTQTPLSLPVSLGDQASISCRSRQS IVHTNRYTYLAWYLQKPGQSPKLLIYK VSNRFSGVPDRFSGSGSGTDFTLKISRV EAEDLGVYYCFQGSHVPYTFGGGTKLE IK
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WHAT IS CLAIMED:

1. An isolated antibody comprising the following properties:
 - a. the antibody specifically binds to CD47;
 - b. the antibody prevents CD47 from interacting with signal-regulatory protein alpha (SIRP alpha);
 - c. the antibody does not have significant platelet aggregation activity.
2. The antibody of claim 1, wherein the antibody specifically binds to CD47 by interacting with CD47 (SEQ ID NO: 21 excluding the signal sequence) amino acid residues:
 - a. Q1, L3, N27, E29, E97, L101, T102, R103, and E104;
 - b. Y37, K39, R45, D46, T49, A53, L54, N55, K56, S57, T58, V59, P60, T61, S64, A66, and K67;
 - c. E35, K39, Y37, D46, 49, D51, A53, L54, K56, T58, V59, T99, L101, and T102; or
 - d. E29, Q31, N32, T34, E35, V36, Y37, K39, N41, D46, D51, A53, E97, T99, E100, L101, T102, R103, and E104.
3. An isolated antibody that specifically binds to human or cyno CD47, and comprises a variable heavy (VH) chain region selected from the group consisting of SEQ ID NOs: 4-6.
4. The antibody of claim 3, wherein the antibody or antigen binding fragment thereof comprises a variable light (VL) chain region selected from the group consisting of SEQ ID NOs: 7 and 8.
5. The antibody of claim 3, wherein the antibody or antigen binding fragment thereof comprises a VH region selected from the group consisting of SEQ ID NOs: 4-6 and a VL region selected from the group consisting of SEQ ID NOs: 7 and 8.
6. The antibody of claim 5, wherein the VH chain region comprises SEQ ID NO: 4 or SEQ ID NO: 5 paired with a VL chain region comprising SEQ ID NO: 7.
7. The antibody of claim 5, wherein the VH chain region comprises SEQ ID

- NO: 6 paired with a VL chain region comprising SEQ ID NO: 8.
8. The antibody of claim 3, wherein the antibody or antigen binding fragment thereof comprises a VH complementarity determining region 1 (CDR1) sequence set forth in SEQ ID NO: 9 or SEQ ID NO: 10, a VH CDR2 sequence set forth in SEQ ID NO: 11 or SEQ ID NO: 12, a VH CDR3 sequence set forth in SEQ ID NO: 13 or SEQ ID NO: 14, a VL CDR1 sequence set forth in SEQ ID NO: 15 or SEQ ID NO: 16, a VL CDR2 sequence set forth in SEQ ID NO: 17 or SEQ ID NO: 18 and a VL CDR3 sequence set forth in SEQ ID NO: 19 or SEQ ID NO: 20.
 9. The antibody of claim 8, wherein the antibody or antigen binding fragment thereof comprises a VH CDR1 sequence set forth in SEQ ID NO: 9, a VH CDR2 sequence set forth in SEQ ID NO: 11, a VH CDR3 sequence set forth in SEQ ID NO: 13, a VL CDR1 sequence set forth in SEQ ID NO: 15, a VL CDR2 sequence set forth in SEQ ID NO: 17, and a VL CDR3 sequence set forth in SEQ ID NO: 19.
 10. The antibody of claim 8, wherein the antibody or antigen binding fragment thereof comprises a VH CDR1 sequence set forth in SEQ ID NO: 10, a VH CDR2 sequence set forth in SEQ ID NO: 12, a VH CDR3 sequence set forth in SEQ ID NO: 14, a VL CDR1 set forth in SEQ ID NO: 16, a VL CDR2 sequence set forth in SEQ ID NO: 18, and a VL CDR3 sequence set forth in SEQ ID NO: 20.
 11. The antibody of any one previous claim, wherein the VH chain of the antibody has more extensive contacts with CD47 than the VL chain of the antibody.
 12. The antibody of any one of claims 2-10, wherein the epitope bound by the VH chain of the antibody is positioned near the membrane of a CD47 expressing cell, and wherein the VL chain of the antibody occludes a SIRP alpha binding site on CD47.
 13. The antibody of any one previous claim, wherein the antibody does not have significant hemagglutination activity.

14. The antibody of any one previous claim, wherein the platelet-aggregation activity of the antibody is no more than 10% greater than the degree of platelet-aggregation observed in the absence of the antibody.
15. The antibody of any one previous claim, wherein the antibody is chimeric, humanized, or fully human.
16. The antibody of any one previous claim, wherein the CD47 is human CD47 or cyno CD47.
17. The antibody of any one previous claim, wherein the antibody or antigen binding fragment thereof promotes macrophage-mediated phagocytosis of a CD47-expressing cell.
18. The antibody of any one previous claim, wherein the antibody or antigen binding fragment thereof comprises an IgG isotype selected from the group consisting of IgG1 isotype and IgG2 isotype.
19. A pharmaceutical composition comprising the antibody of anyone of claims 1 to 17 and a pharmaceutical acceptable carrier.
20. A method of alleviating a symptom of a cancer or other neoplastic condition, the method comprising administering the antibody of anyone of claims 1 to 17 to a subject in need thereof in an amount sufficient to alleviate the symptom of the cancer or other neoplastic condition in the subject.
21. The method of claim 20, wherein the subject is a human.
22. The method of claim 20 or 21, wherein the antibody is chimeric, humanized, or fully human.
23. The method of any one of claims 20 to 22, wherein the antibody or antigen binding fragment thereof prevents CD47 from interacting with SIRP alpha
24. The method of any one of claims 20 to 23, wherein the antibody comprises an IgG isotype selected from the group consisting of IgG1 isotype and IgG2 isotype.
25. The method of any one of claims 20 to 24, further comprising administering chemotherapy.