



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

September 16, 2016

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 14/971,931

FILING DATE: December 16, 2015

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS US14/971,931

**By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office**



P. R. GRANT
Certifying Officer

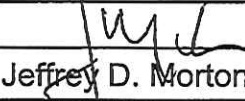
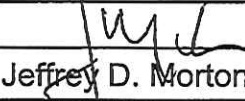
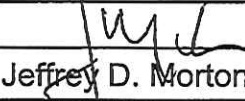
Electronic Acknowledgement Receipt	
EFS ID:	24387714
Application Number:	14971931
International Application Number:	
Confirmation Number:	7257
Title of Invention:	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF
First Named Inventor/Applicant Name:	Laura Deming
Customer Number:	21971
Filer:	Maya Skubatch/Simona Bandong (JM:sb)
Filer Authorized By:	Maya Skubatch
Attorney Docket Number:	47819-702.301
Receipt Date:	16-DEC-2015
Filing Date:	
Time Stamp:	19:59:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	\$6540
RAM confirmation Number	6880
Deposit Account	232415
Authorized User	BANDONG, SIMONA
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)					
Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)					
Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)					
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Sequence Listing (Text File)	47819702301SEQLIST.txt	129580	no	0
Warnings:					
Information:					
2		47819702301TRANSMITTALAN DTRACKONE.pdf	265490 cafcdfcda5063826631f356b46b09c16a74da7a0	yes	2
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Transmittal of New Application		1	1	
	TrackOne Request		2	2	
Warnings:					
Information:					
3	CRF Statement Paper and CRF are the same	47819702301SEQSTATEMENT. pdf	97205 d77c8def7a0811126951a2dc2816416ecd22a9aa	no	1
Warnings:					
Information:					
4		47819702301SPECIFICATION. pdf	1597249 a332b6cfca8aa6e9327b878edf0cbca69fbee5366	yes	87
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	83	
	Claims		84	86	
	Abstract		87	87	
Warnings:					
Information:					

5	Drawings-only black and white line drawings	47819701601_PCT_Figures.pdf	904429 a2de1522130290490a3a74225d3ba7b51a1deu9d	no	10
Warnings:					
Information:					
6	Application Data Sheet	47819702301ADS.pdf	310037 3d0a45b5fd9aa6a41d222abc07f145788f959986	no	9
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
7	Fee Worksheet (SB06)	fee-info.pdf	42315 f222b39252fa543f55f672c05fad03d13b02ae26	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			3346305		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

PTO/AIA/15 (03-13) Approved for use through 01/31/2014. OMB 0651-0032 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE											
Under the Paperwork Reduction Act of 1995 no persons are required to respond to a collection of information unless it displays a valid OMB control number											
UTILITY PATENT APPLICATION TRANSMITTAL (Only for new nonprovisional applications under 37 CFR 1.53(b))	<table border="1" style="width: 100%; border-collapse: collapse;"><tr><td style="width: 30%;">Attorney Docket No.</td><td>47819-702.301</td></tr><tr><td>First Named Inventor</td><td>Laura DEMING</td></tr><tr><td>Title</td><td>SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF</td></tr><tr><td>Express Mail Label No.</td><td></td></tr></table>	Attorney Docket No.	47819-702.301	First Named Inventor	Laura DEMING	Title	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF	Express Mail Label No.			
Attorney Docket No.	47819-702.301										
First Named Inventor	Laura DEMING										
Title	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF										
Express Mail Label No.											
APPLICATION ELEMENTS See MPEP chapter 600 concerning utility patent application contents.	ADDRESS TO: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450										
1. ☒ Fee Transmittal Form (PTO/SB/17 or equivalent) 2. ☐ Applicant asserts small entity status. See 37 CFR 1.27 3. ☐ Applicant certifies micro entity status. See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent. 4. ☒ Specification [Total Pages 87] Both the claims and abstract must start on a new page. (See MPEP § 608.01(a) for information on the preferred arrangement) 5. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets 10] 6. ☒ Inventor's Oath or Declaration [Total Pages 97] (Including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e)) a. ☐ Newly executed (original or copy) b. ☐ A copy from a prior application (37 CFR 1.63(d)) 7. ☒ Application Data Sheet * See note below. See 37 CFR 1.76 (PTO/AIA/14 or equivalent) 8. ☐ CD-ROM or CD-R in duplicate, large table, or Computer Program (Appendix) ☐ Landscape Table on CD 9. Nucleotide and/or Amino Acid Sequence Submission (If applicable, items a. – c. are required) a. ☒ Computer Readable Form (CRF) b. ☒ Specification Sequence Listing on: i. ☐ CD-ROM or CD-R (2 copies); or ii. ☐ Paper c. ☒ Statements verifying identity of above copies <td style="padding: 5px; vertical-align: top;">ACCOMPANYING APPLICATION PAPERS 10. ☐ Assignment Papers (cover sheet & document(s)) Name of Assignee _____ 11. ☐ 37 CFR 3.73(c) Statement ☐ Power of Attorney (when there is an assignee) 12. ☐ English Translation Document (if applicable) 13. ☐ Information Disclosure Statement (PTO/SB/08 or PTO-1449) ☐ Copies of citations attached 14. ☐ Preliminary Amendment 15. ☐ Return Receipt Postcard (MPEP § 503) (Should be specifically itemized) 16. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed) 17. ☐ Nonpublication Request Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent. 18. ☐ Other: _____ _____ _____ _____ </td>	ACCOMPANYING APPLICATION PAPERS 10. ☐ Assignment Papers (cover sheet & document(s)) Name of Assignee _____ 11. ☐ 37 CFR 3.73(c) Statement ☐ Power of Attorney (when there is an assignee) 12. ☐ English Translation Document (if applicable) 13. ☐ Information Disclosure Statement (PTO/SB/08 or PTO-1449) ☐ Copies of citations attached 14. ☐ Preliminary Amendment 15. ☐ Return Receipt Postcard (MPEP § 503) (Should be specifically itemized) 16. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed) 17. ☐ Nonpublication Request Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent. 18. ☐ Other: _____ _____ _____ _____										
*Note: (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 must be included in an Application Data Sheet (ADS). (2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).											
19. CORRESPONDENCE ADDRESS											
☒ The address associated with Customer Number: 021971 OR ☐ Correspondence address below											
<table border="1" style="width: 100%; border-collapse: collapse;"><tr><td colspan="2">Name</td></tr><tr><td colspan="2">Address</td></tr><tr><td>City</td><td>State</td></tr><tr><td>Country</td><td>Zip Code</td></tr><tr><td>Telephone</td><td>Email</td></tr></table>		Name		Address		City	State	Country	Zip Code	Telephone	Email
Name											
Address											
City	State										
Country	Zip Code										
Telephone	Email										
<table border="1" style="width: 100%; border-collapse: collapse;"><tr><td style="width: 60%;">Signature </td><td style="width: 40%;">Date December 16, 2015</td></tr><tr><td>Name (Print/Type) Jeffrey D. Morton</td><td>Registration No. (Attorney/Agent) L1070</td></tr></table>	Signature 	Date December 16, 2015	Name (Print/Type) Jeffrey D. Morton	Registration No. (Attorney/Agent) L1070							
Signature 	Date December 16, 2015										
Name (Print/Type) Jeffrey D. Morton	Registration No. (Attorney/Agent) L1070										
This collection of information is required by 37 CFR 1.53(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.											
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.											

PATENT APPLICATION
SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF

Inventors: Laura DEMING
Corey GOODMAN
Jaume PONS
Bang Janet SIM
Marija VRLJIC

Assignee: Alexo Therapeutics International



Wilson Sonsini Goodrich & Rosati
PROFESSIONAL CORPORATION

650 Page Mill Road
Palo Alto, CA 94304
(650) 493-9300 (Main)
(650) 493-6811 (Facsimile)

Filed Electronically on: December 16, 2015

SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF

CROSS-REFERENCE

This application claims the benefit of PCT Application No. PCT/US15/44528 which claims priority to U.S. Provisional Application No. 62/035,057, filed August 08, 2014, U.S. Provisional Application No. 62/138,257, filed March 25, 2015, U.S. Provisional Application No. 62/138,306 filed March 25, 2015 and U.S. Provisional Application No. 62/138,282, filed March 25, 2015 which are incorporated herein by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION

Signal-regulatory protein α (SIRP- α) is a protein widely expressed on the membrane of myeloid cells. SIRP- α interacts with CD47, a protein broadly expressed on many cell types in the body. The interaction of SIRP- α with CD47 prevents engulfment of "self" cells, which could otherwise be recognized by the immune system. SIRP- α was first discovered as a binder of SHP-2 (an SH-2 domain containing tyrosine phosphatase). CD47 was early characterized as an overexpressed antigen on ovarian carcinoma cells.

In 2000, Oldenborg et al. showed that administration of CD47-deficient red blood cells (RBCs) in a mouse model resulted in rapid clearance of the RBCs from the system, demonstrating CD47 to be a "protective" signal on some subset of "self" cells. Subsequently, the potential link between the SIRP- α and cancer was further explored. It was found that high CD47 expression on tumor cells acted, in acute myeloid leukemia (AML) and several solid tumor cancers, as a negative prognostic factor for survival. Strategies focused on disrupting the interaction between CD47 and SIRP- α , such as administration of agents that mask either CD47 or SIRP- α , have been explored as potential anticancer therapies.

However, in considering these therapeutic strategies, it is a concerning issue that SIRP- α could bind to CD47 on many different cell types in the human body. Thus, there exists a need to engineer SIRP- α to preferentially bind to CD47 only on diseased cells or on cells at a diseased site.

SUMMARY OF THE INVENTION

The invention features signal-regulatory protein α (SIRP- α) constructs, which refer to a polypeptide comprising a SIRP- α polypeptide attached to, e.g., a blocking peptide, an Fc domain monomer, an HSA, an albumin-binding peptide, a polymer, an

antibody-binding peptide, an antibody. The SIRP- α polypeptide can be either a wild-type SIRP- α or a SIRP- α variant. The SIRP- α variant constructs include SIRP- α variants. In some embodiments, the SIRP- α variant constructs have preferential activity at a diseased site (e.g., at the site of a tumor than at a non-diseased site). In
5 certain embodiments, the SIRP- α variant constructs have higher binding affinity to CD47 on diseased cells (e.g., tumor cells). In some embodiments, the SIRP- α variants bind with higher affinity to CD47 under acidic pH (e.g., less than around pH 7) and/or under hypoxic condition than under physiological conditions. In some
10 embodiments, the SIRP- α variants contain one or more substitutions of amino acids with histidine residues or with other amino acids that allow preferential binding of SIRP- α variant constructs at a diseased site. In some embodiments, the SIRP- α variant constructs are prevented from binding to CD47 in a non-diseased site by a
15 blocking peptide. In some embodiments, the SIRP- α variant constructs are targeted to the diseased site (e.g., the tumor) by a targeting moiety (e.g., an antibody directed to a tumor-associated antigen or an antibody-binding peptide). The invention also features methods and pharmaceutical compositions containing SIRP- α variant constructs to treat various diseases, such as cancer, preferably solid tumor cancer and hematological cancer.

In one aspect, the invention features a signal-regulatory protein α (SIRP- α)
20 variant construct, wherein the SIRP- α variant construct preferentially binds CD47 on diseased cells or at a diseased site than on non-diseased cells. In some embodiments, the SIRP- α variant construct binds to CD47 on diseased cells or at a diseased site with higher affinity than it binds CD47 on non-diseased cells.

In some embodiments, the SIRP- α variant construct includes a SIRP- α variant
25 attached to a blocking peptide. In some embodiments, the blocking peptide binds with higher affinity to a wild-type SIRP- α than to the SIRP- α variant. In some embodiments, the SIRP- α variant binds with higher affinity to a wild-type CD47 than to the blocking peptide.

In some embodiments, the blocking peptide is a CD47-based blocking
30 peptide. In some embodiments, the CD47-based blocking peptide includes a portion that has at least 80% amino acid sequence identity to the sequence of the wild-type, IgSF domain of CD47 (SEQ ID NO: 35) or a fragment thereof. In some embodiments, the CD47-based blocking peptide has the sequence of SEQ ID NO: 38 or 40.

Provided herein are SIRP- α variant constructs comprising a SIRP- α variant described herein, wherein said SIRP- α variant is attached to a blocking peptide described herein by use of at least one linker (e.g, a cleavable linker). In some embodiments, the SIRP- α variant may comprise the same CD47 binding site as a wild type SIRP- α . In some embodiments, the SIRP- α variant may comprise one or more mutations, or insertions as compared to a wild type SIRP- α . In some embodiments, the SIRP- α variant may be a truncated form of the wild type SIRP- α . In some embodiments, the blocking peptide may be a CD47 mimic, variant, or fragment described herein. In some embodiments, the blocking peptide may exhibit a higher affinity for a wild-type SIRP- α , as compared to the SIRP- α variant in the SIRP- α variant construct. In some embodiments, the blocking peptide may be a CD47 variant polypeptide that demonstrates a lower affinity for a SIRP- α variant as compared to the wild-type CD47. In some embodiments, the linker between the SIRP- α variant and the blocking peptide may be at least one linker that is optionally cleavable by one or more proteases. In some embodiments, the linker optionally also comprises one or more spacers.

In some embodiments, the SIRP- α variant is attached to a blocking peptide by way of a cleavable linker and optionally one or more spacers. In some embodiments, the cleavable linker is cleaved under acidic pH and/or hypoxic condition. In some embodiments, the cleavable linker is cleaved by a tumor-associated enzyme. In some embodiments, the tumor-associated enzyme is a protease. In some embodiments, the protease is selected from the group consisting of matriptase (MTSP1), urinary-type plasminogen activator (uPA), legumain, PSA (also called KLK3, kallikrein-related peptidase-3), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP9)human neutrophil elastase (HNE), and proteinase 3 (Pr3). In some embodiments, the protease is matriptase. In some embodiments, the cleavable linker has the sequence of LSGRSDNH (SEQ ID NO: 47) or any one of the sequences listed in Table 7. In some embodiments, the cleavable linker includes one or a combination of the following sequences (see, e.g., Table 7): PRFKIIGG, PRFRIIGG, SSRHRRALD, RKSSIIIRMRDVVL, SSSFDKGGKYKKGDDA, SSSFDKGGKYKRGDDA, IEGR, IDGR, GGSIDGR, PLGLWA, GPLGIAGI, GPEGLRVG, YGAGLGVV, AGLGVVER, AGLGISST, DVAQFVLT, VAQFVLTE, AQFVLTEG, PVQPIGPQ, L/S/G-/R-/S-/D/N/H, -/s/gs/Rk-/rv/-/-, SGR-SA, r/-/-/Rk-/v/-/-/g/-, RQAR-VV, r/-/-/Rk/v/-/-/g, /Kr/RKQ/gAS/RK/A, -/-/-

/N/-/-/, AAN-L, ATN-L, si/sq/-/yqrs/s/-/-, S/S/K/L/Q, -/p/-/- /li/-/-/-, g/pa/-/gl/-/gl/-, G/P/L/G/I/A/G/Q, P/V/G/L/I/G, H/P/V/G/L/L/A/R, -/-/-/viat/-/-/-, and -/y/y/vta -/-/-/-, where “-” means any amino acid (i.e., any naturally occurring amino acid), capital case indicates a strong preference for that amino acid, lower case indicates a minor preference for that amino acid, and “/” separates amino acid positions in cases where more than one amino acid at a position adjacent to the “/” is possible.

In some embodiments, the SIRP- α variant is attached to an antibody-binding peptide. In some embodiments, the antibody-binding peptide binds to a constant region of an antibody reversibly or irreversibly. In some embodiments, the antibody-binding peptide binds to the fragment antigen-binding (Fab) region of an antibody reversibly or irreversibly. In some embodiments, the antibody-binding peptide binds to a variable region of an antibody reversibly or irreversibly. In some embodiments, the antibody is Cetuximab. In some embodiments, the antibody-binding peptide has at least 75% amino acid sequence identity to the sequence of a disease localization peptide (DLP) (CQFDLSTRRLKC (SEQ ID NO: 64) or CQYNLSSRALKC (SEQ ID NO: 65)) or a fragment thereof. In some embodiments, the antibody-binding peptide has the sequence of SEQ ID NO: 64.

In some embodiments, the SIRP- α variant is attached to an Fc domain monomer. In some embodiments, the SIRP- α variant is attached to a human serum albumin (HSA). In some embodiments, the HSA includes amino acid substitution C34S and/or K573P, relative to SEQ ID NO: 67. In some embodiments, the HSA has the sequence of

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLVNEVTEFAKTCVAD
ESAENCCKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPN
LPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTFC
CQAADKAACLLPKLDEL RDEGKASSAKQRLKCASLQKFGERAFAKAWAVARLSQRF
PKAEFAEVSKLVTDLT KVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCE
KPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVLLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCE
LFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPC
AEDYLSVVLNQLCVLHEKTPVSDRVTKCTESLVNRRPCFSALEVDETYVPKEFNA
ETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCKKA
DDKETCFAEEGKKLVAASQAALGL (SEQ ID NO: 68).

In some embodiments, the SIRP- α variant is attached to an albumin-binding peptide. In some embodiments, the albumin-binding peptide has the sequence of SEQ ID NO: 2. In some embodiments, the SIRP- α variant is attached to a polymer, wherein the polymer is polyethylene glycol (PEG) chain or polysialic acid chain.

5 In some embodiments, the SIRP- α variant is attached to an antibody. In some embodiments, the antibody is a tumor-specific antibody. In some embodiments, the antibody (e.g., a tumor-specific antibody) is selected from the group consisting of cetuximab, pembrolizumab, nivolumab, pidilizumab, MEDI0680, MEDI6469, Ipilimumab, tremelimumab, urelumab, vantictumab, varlilumab, 10 mogamalizumab, anti-CD20 antibody, anti-CD19 antibody, anti-CS1 antibody, herceptin, trastuzumab, and pertuzumab. In some embodiments, the antibody (e.g., a tumor-specific antibody) may bind to one or more of the following: 5T4, AGS-16, ALK1, ANG-2, B7-H3, B7-H4, c-fms, c-Met, CA6, CD123, CD19, CD20, CD22, EpCAM, CD30, CD32b, CD33, CD37, CD38, CD40, CD52, CD70, CD74, CD79b, 15 CD98, CEA, CEACAM5, CLDN18.2, CLDN6, CS1, CXCR4, DLL-4, EGFR, EGP-1, ENPP3, EphA3, ETBR, FGFR2, fibronectin, FR-alpha, GCC, GD2, glypican-3, GPNMB, HER-2, HER3, HLA-DR, ICAM-1, IGF-1R, IL-3R, LIV-1, mesothelin, MUC16, MUC1, NaPi2b, Nectin-4, Notch 2, Notch 1, PD-L1, PD-L2, PDGFR- α , PS, PSMA, SLTRK6, STEAP1, TEM1, VEGFR, CD25, CD27L, DKK-1, CSF-1R, and/or 20 MSB0010718C.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to a sequence of any one of SEQ ID NOs: 3-12 and 24-34.

25 In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of
EEEX₁QX₂IQPDKSVLVAAGETX₃TLRCTX₄TSLX₅PVGPIQWFRGAGPGRX₆LIYNQX₇X₈GX₉FPRVTTVSDX₁₀TX₁₁RNNMDFSIRIGX₁₂ITX₁₃ADAGTYCYCX₁₄KX₁₅RKGSPDD
VEX₁₆KSGAGTELSVRAPKPS (SEQ ID NO: 13), wherein X₁ is L, I, or V; X₂ is V, L, or, 30 I; X₃ is A or V; X₄ is A, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or Q; X₉ is H, P, or R; X₁₀ is L, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEGX₁QX₂IQPDKSVSVAAGESX₃ILHCTX₄TSLX₅PVGPIQWFRGAGPGRX₆LIYNQX₇
X₈GX₉FPRVTTVSDX₁₀TX₁₁RNNMDFSIRIGX₁₂ITX₁₃ADAGTYYCX₁₄KX₁₅RKGSPDDV
5 EX₁₆KSGAGTELSVRAKPS (SEQ ID NO: 16), wherein X₁ is L, I, or V; X₂ is V, L, or I;
X₃ is A or V; X₄ is A, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or
Q; X₉ is H, P, or R; X₁₀ is L, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K,
L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T,
V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

10 In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDK FVLVAAGETX₃TLRCTX₄TSLX₅PVGPIQWFRGAGPGRX₆LIYNQX₇
X₈GX₉FPRVTTVSDX₁₀TX₁₁RNNMDFSIRIGX₁₂ITX₁₃ADAGTYYCX₁₄KX₁₅RKGSPDDV
EX₁₆KSGAGTELSVRAKPS (SEQ ID NO: 17), wherein X₁ is L, I, or V; X₂ is V, L, or I;
15 X₃ is A or V; X₄ is A, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or
Q; X₉ is H, P, or R; X₁₀ is L, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K,
L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T,
V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDKSVLVAAGETX₃TLRCTX₄TSLX₅PVGPIQWFRGAGPGRX₆LIYNQX
7X₈GX₉FPRVTTVSDX₁₀TX₁₁RNNMDFPIRIGX₁₂ITX₁₃ADAGTYYCX₁₄KX₁₅RKGSPDD
VEX₁₆KSGAGTELSVRAKPS (SEQ ID NO: 18), wherein X₁ is L, I, or V; X₂ is V, L, or
I; X₃ is A or V; X₄ is A, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E
25 or Q; X₉ is H, P, or R; X₁₀ is L, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I,
K, L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S,
T, V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDKSVLVAAGETX₃TLRCTX₄TSLX₅PVGPIQWFRGAGPGRX₆LIYNQX
30 7X₈GX₉FPRVTTVSDX₁₀TX₁₁RNNMDFSIRISX₁₂ITX₁₃ADAGTYYCX₁₄KX₁₅RKGSPDD
VEX₁₆KSGAGTELSVRAKPS (SEQ ID NO: 21), wherein X₁ is L, I, or V; X₂ is V, L, or
I; X₃ is A or V; X₄ is A, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E
or Q; X₉ is H, P, or R; X₁₀ is L, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I,

K, L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

5 EEEX₁QX₂IQPDKSVSVAAGESX₃ILHCTX₄TSLX₅PVGPIQWFRGAGPARX₆LIYNQX₇
X₈GX₉FPRVTTVSEX₁₀TX₁₁RENMDFSISISX₁₂ITX₁₃ADAGTYCYCX₁₄KX₁₅RKGSPDTE
X₁₆KSGAGTELSVRAPKS (SEQ ID NO: 14), wherein X₁ is L, I, or V; X₂ is V, L, or, I;
X₃ is A or V; X₄ is V, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or
Q; X₉ is H, P, or R; X₁₀ is S, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K,
10 L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T,
V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V;.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDKSVSVAAGESX₃ILLCTX₄TSLX₅PVGPIQWFRGAGPARX₆LIYNQX₇
15 X₈GX₉FPRVTTVSEX₁₀TX₁₁RENMDFSISISX₁₂ITX₁₃ADAGTYCYCX₁₄KX₁₅RKGSPDTE
X₁₆KSGAGTELSVRAPKS (SEQ ID NO: 15), wherein X₁ is L, I, or V; X₂ is V, L, or, I;
X₃ is A or V; X₄ is V, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or
Q; X₉ is H, P, or R; X₁₀ is S, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K,
L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T,
20 V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDKSVSVAAGESX₃ILHCTX₄TSLX₅PVGPIQWFRGAGPARX₆LIYNQX₇
X₈GX₉FPRVTTVSEX₁₀TX₁₁RENMDFSISISX₁₂ITX₁₃ADAGTYCYCX₁₄KX₁₅RKGSPDTE
25 X₁₆KSGAGTELSVRGKPS (SEQ ID NO: 19), wherein X₁ is L, I, or V; X₂ is V, L, or, I;
X₃ is A or V; X₄ is V, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or
Q; X₉ is H, P, or R; X₁₀ is S, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K,
L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T,
V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

30 In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDKSVSVAAGESX₃ILHCTX₄TSLX₅PVGPIQWFRGAGPARX₆LIYNQX₇
X₈GX₉FPRVTTVSEX₁₀TX₁₁RENMDFSISISX₁₂ITX₁₃ADAGTYCYCX₁₄KX₁₅RKGSPDTE
X₁₆KSGAGTELSVRAPKS (SEQ ID NO: 22), wherein X₁ is L, I, or V; X₂ is V, L, or, I;

X₃ is A or V; X₄ is V, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or Q; X₉ is H, P, or R; X₁₀ is S, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

5 In some embodiments, the SIRP- α variant in the SIRP- α variant construct has a sequence of

EEEX₁QX₂IQPDKSVLVAAGETX₃TLRCTX₄TSLX₅PVGPIQWFRGAGPARX₆LIYNQX₇X₈GX₉FPRVTTVSEX₁₀TX₁₁RENMDFSISIX₁₂ITX₁₃ADAGTYYCX₁₄KX₁₅RKGSPDTE
10 X₁₆KSGAGTELSVRAKPS (SEQ ID NO: 20), wherein X₁ is L, I, or V; X₂ is V, L, or, I; X₃ is A or V; X₄ is A, I, or L; X₅ is I, T, S, or F; X₆ is E, V, or L; X₇ is K or R; X₈ is E or Q; X₉ is H, P, or R; X₁₀ is S, T, or G; X₁₁ is K or R; X₁₂ is N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; X₁₃ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; X₁₄ is V or I; X₁₅ is F, L, or V; and X₁₆ is F or V.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has
15 a sequence of

EEX₁X₂QX₃IQPDKX₄VX₅VAAGEX₆X₇X₈LX₉CTX₁₀TSLX₁₁PVGPIQWFRGAGPX₁₂RX₁₃LIYNQX₁₄X₁₅GX₁₆FPRVTTVSX₁₇X₁₈TX₁₉RX₂₀NMDFX₂₁IX₂₂IX₂₃X₂₄ITX₂₅ADAGTYYC
20 X₂₆KX₂₇RKGSPDX₂₈X₂₉EX₃₀KSGAGTELSVRX₃₁KPS (SEQ ID NO: 23), wherein X₁ is E or G; X₂ is L, I, or V; X₃ is V, L, or, I; X₄ is S or F; X₅ is L or S; X₆ is S or T; X₇ is A or V; X₈ is I or T; X₉ is H or R; X₁₀ is A, V, I, or L; X₁₁ is I, T, S, or F; X₁₂ is A or G; X₁₃ is E, V, or L; X₁₄ is K or R; X₁₅ is E or Q; X₁₆ is H, P, or R; X₁₇ is D or E; X₁₈ is S, L, T, or G; X₁₉ is K or R; X₂₀ is E or N; X₂₁ is S or P; X₂₂ is S or R; X₂₃ is S or G; X₂₄ is N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, or Y; X₂₅ is P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, or Y; X₂₆ is V or I; X₂₇ is F, L, V; X₂₈ is D or absent; X₂₉
25 is T or V; X₃₀ is F or V; and X₃₁ is A or G.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to a sequence of any one of SEQ ID NOs: 13-23.

In some embodiments, a SIRP- α variant in the SIRP- α variant construct does
30 not include the sequence of any one of SEQ ID NOs: 3-12 and 24-34.

In some embodiments, the SIRP- α variant in the SIRP- α variant construct includes one or more substitutions of amino acid residues with histidine residues. In some embodiments, the one or more substitutions of amino acid residues with histidine residues are located at one or more of the following amino acid positions:

29, 30, 31, 32, 33, 34, 35, 52, 53, 54, 66, 67, 68, 69, 74, 93, 96, 97, 98, 100, 4, 6, 27, 36, 39, 47, 48, 49, 50, 57, 60, 72, 74, 76, 92, 94, 103, relative to a sequence of any one of SEQ ID NOs: 3-12.

5 In some embodiments, the SIRP- α variant construct binds with at least two, at least four, or at least six fold higher affinity to CD47 on diseased cells or at a diseased site than on non-diseased cells.

In some embodiments, the SIRP- α variant construct binds with at least two, at least four, or at least six fold higher affinity to CD47 under acidic pH than under neutral pH.

10 In some embodiments, the SIRP- α variant construct binds with at least two, at least four, or at least six fold higher affinity to CD47 under hypoxic condition than under physiological condition.

In some embodiments, the diseased cell is a cancer cell of a cancer disease.

In some embodiments, the acidic pH is a pH between about 4 to about 7.

15 In another aspect, the invention features a nucleic acid molecule encoding a SIRP- α variant construct described herein.

In another aspect, the invention features a vector including the nucleic acid molecule encoding a SIRP- α variant construct described herein.

20 In another aspect, the invention features a host cell that expresses a SIRP- α variant construct described herein, wherein the host cell includes a nucleic acid molecule encoding a SIRP- α variant construct described herein or a vector including the nucleic acid molecule, wherein the nucleic acid molecule or vector is expressed in the host cell.

25 In another aspect, the invention features a method of preparing a SIRP- α variant construct described herein, wherein the method includes: a) providing a host cell including a nucleic acid molecule of encoding a SIRP- α variant construct described herein or a vector including the nucleic acid molecule; b) expressing the nucleic acid molecule or vector in the host cell under conditions that allow for the formation of the SIRP- α variant construct; and c) recovering the SIRP- α variant
30 construct.

In another aspect, the invention features a pharmaceutical composition including a therapeutically effective amount of a SIRP- α variant construct described herein. In some embodiments, the pharmaceutical composition includes one or more pharmaceutically acceptable carriers or excipients.

In another aspect, the invention features a method of increasing phagocytosis of a target cell in a subject including administering to the subject a SIRP- α variant construct described herein or a pharmaceutical composition including a therapeutically effective amount of a SIRP- α variant construct described herein. In
5 some embodiments, the target cell is a cancer cell.

In another aspect, the invention features a method of eliminating regulatory T-cells in a subject including administering to the subject a SIRP- α variant construct described herein or a pharmaceutical composition including a therapeutically effective amount of a SIRP- α variant construct described herein.

10 In another aspect, the invention features a method for killing a cancer cell, the method includes contacting the cancer cell with a SIRP- α variant construct described herein or the pharmaceutical composition including a therapeutically effective amount of a SIRP- α variant construct described herein.

In another aspect, the invention features a method for treating a disease
15 associated with SIRP- α and/or CD47 activity in a subject, the method includes administering to the subject a therapeutically effective amount of the SIRP- α variant construct described herein or the pharmaceutical composition including a therapeutically effective amount of a SIRP- α variant construct described herein.

In another aspect, the invention features a method of treating a disease
20 associated with SIRP- α and/or CD47 activity in a subject, the method includes: (a) determining the amino acid sequences of SIRP- α of the subject; and (b) administering to the subject a therapeutically effective amount of a SIRP- α variant construct described herein; wherein the SIRP- α variant in the SIRP- α variant construct has the same amino acid sequence as that of a SIRP- α of the subject.

25 In another aspect, the invention features a method of treating a disease associated with SIRP- α and/or CD47 activity in a subject, the method includes: (a) determining the amino acid sequences of SIRP- α of the subject; and (b) administering to the subject a therapeutically effective amount of a SIRP- α variant construct described herein; wherein the SIRP- α variant in the SIRP- α variant
30 construct has minimal immunogenicity in the subject.

In another aspect, the invention features a method of treating a disease associated with SIRP- α and/or CD47 activity in a subject, the method includes: administering to the subject a SIRP- α variant construct described herein, wherein the

SIRP- α variant construct preferentially binds CD47 on diseased cells or at a diseased site over CD47 on non-diseased cells.

In some embodiments, the disease is cancer. In some embodiments, the cancer is selected from solid tumor cancer, hematological cancer, acute myeloid leukemia, chronic lymphocytic leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin lymphoma, Hodgkin lymphoma, multiple myeloma, bladder cancer, pancreatic cancer, cervical cancer, endometrial cancer, lung cancer, bronchus cancer, liver cancer, ovarian cancer, colon and rectal cancer, stomach cancer, gastric cancer, gallbladder cancer, gastrointestinal stromal tumor cancer, thyroid cancer, head and neck cancer, oropharyngeal cancer, esophageal cancer, melanoma, non-melanoma skin cancer, Merkel cell carcinoma, virally induced cancer, neuroblastoma, breast cancer, prostate cancer, renal cancer, renal cell cancer, renal pelvis cancer, leukemia, lymphoma, sarcoma, glioma, brain tumor, and carcinoma. In some embodiments, the cancer is a solid tumor cancer. In some
5
10
15

embodiments, the cancer is a hematological cancer.

In some embodiments, the disease is an immunological disease. In some embodiments, the immunological disease is an autoimmune disease or an inflammatory disease. In some embodiments, the autoimmune or inflammatory disease is multiple sclerosis, rheumatoid arthritis, a spondyloarthropathy, systemic lupus erythematosus, an antibody-mediated inflammatory or autoimmune disease, graft versus host disease, sepsis, diabetes, psoriasis, atherosclerosis, Sjogren's syndrome, progressive systemic sclerosis, scleroderma, acute coronary syndrome, ischemic reperfusion, Crohn's Disease, endometriosis, glomerulonephritis, myasthenia gravis, idiopathic pulmonary fibrosis, asthma, acute respiratory distress
20
25

syndrome (ARDS), vasculitis, or inflammatory autoimmune myositis.

In another aspect, the invention features a method of increasing hematopoietic stem cell engraftment in a subject including modulating the interaction between SIRP- α and CD47 in the subject by administering to the subject a SIRP- α variant described herein or a pharmaceutical composition including a therapeutically
30

effective amount of a SIRP- α variant described herein.

In another aspect, the invention features a method of altering an immune response in a subject including administering the subject a SIRP- α variant construct described herein or a pharmaceutical composition including a therapeutically

effective amount of a SIRP- α variant construct described herein, thereby altering the immune response in the subject.

In some embodiments, the subject is a mammal, preferably, the mammal is a human.

5

Definitions

As used herein, the term “diseased cells” and “diseased tissue” refer to, for example, cancer cells and tissue. In particular, the cancer may be a solid tumor cancer or a hematological cancer. For example, if the cancer is a solid tumor
10 cancer, the diseased cells are the cells of the solid tumor. Diseased cells are often living under conditions characteristic of a diseased site, such as acidic pH and hypoxia. “Diseased cells” and “diseased tissue” can also be associated with other diseases including, but not limited to, cancer. “Diseased cells” and “diseased tissue” can also be associated with an immunological disease or disorder, a cardiovascular
15 disease or disorder, a metabolic disease or disorder, or a proliferative disease or disorder. An immunological disorder includes an inflammatory disease or disorder and an autoimmune disease or disorder.

As used herein, the term “non-diseased cells” refers to normal, healthy cells of the body. Non-diseased cells often live under physiological conditions, such as
20 neutral pH and adequate oxygen concentration that maintain normal metabolism and regulatory functions of the cells.

As used herein, the term “diseased site” refers to the location or area proximal to the location of the disease in the body. For example, if the disease is solid tumor cancer located in the liver, then diseased site is the site of the tumor in the liver and
25 areas close to the tumor in the liver. Cells at a diseased site may include diseased cells as well as cells that support the disease at the diseased site. For example, if the diseased site is the site of a tumor, cells at the site of the tumor include both diseased cells (e.g., tumor cells) and cells supporting tumor growth at the site of the tumor. Similarly, the term “cancer site” refers to the location of the cancer in the
30 body.

As used herein, the term “SIRP- α D1 domain” or “D1 domain” refers to the membrane distal, extracellular domain of SIRP- α . The SIRP- α D1 domain is located at the N-terminus of a full-length, wild-type SIRP- α and mediates binding to CD47. Amino acid sequences of D1 domains are shown in Table 1.

As used herein, the term "SIRP- α D2 domain" or "D2 domain" refers to the second extracellular domain of SIRP- α . The SIRP- α D2 domain includes approximately amino acids 119 to 220 of a full-length, wild-type SIRP- α .

As used herein, the term "SIRP- α D3 domain" or "D3 domain" refers to the
5 third extracellular domain of SIRP- α . The SIRP- α D3 domain includes approximately amino acids 221 to 320 of a full-length, wild-type SIRP- α .

As used herein, the term "SIRP- α polypeptide" refers to a wild-type SIRP- α as well as a SIRP- α variant, as each term is defined and described herein.

As used herein, the term "SIRP- α variant" refers to a polypeptide containing a
10 SIRP- α D1 domain, or a CD47-binding portion of a full-length SIRP- α . In some embodiments, the SIRP- α variant has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.) sequence identity to a sequence of any one of SEQ ID NOs: 3-12 and 24-34. In some embodiments, a SIRP- α variant has higher affinity to CD47 than a wild-type SIRP- α . In some
15 embodiments, a SIRP- α variant contains a portion of wild-type human SIRP- α (preferably a CD47-binding portion of the wild-type SIRP- α) and/or has one or more amino acid substitutions. For example, a SIRP- α variant may contain substitutions of one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum of 20) amino acid residues relative to a wild-type SIRP- α . For example, a
20 SIRP- α variant may contain substitutions of one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum of 20) amino acid residues with histidine residues. In some embodiments, SIRP- α variants have a portion that has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) amino acid sequence identity to a sequence of wild-type human SIRP- α or to any of the SIRP- α variants described herein (e.g., to a sequence of a CD47-
25 binding portion of wild-type human SIRP- α). A CD47-binding portion of wild-type SIRP- α includes the D1 domain of the wild-type SIRP- α (a sequence of any one of SEQ ID NOs: 3-12).

As used herein, the term "SIRP- α variant construct" refers to a polypeptide
30 comprising a SIRP- α polypeptide attached to, e.g., a blocking peptide, an Fc domain monomer, an HSA, an albumin-binding peptide, a polymer, an antibody-binding peptide, an antibody. The SIRP- α can be either a wild-type SIRP- α or a SIRP- α variant. In some embodiments, a SIRP- α variant construct has preferential activity at a diseased site. In some embodiments, SIRP- α variant constructs have preferential

activity at a diseased site and include a SIRP- α variant having a portion that has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) amino acid sequence identity to a sequence of wild-type human SIRP- α or to any of the SIRP- α variants described herein (e.g., to a sequence of a CD47-binding portion of wild-type human SIRP- α).

As used herein, the term "percent (%) identity" refers to the percentage of amino acid (or nucleic acid) residues of a candidate sequence, e.g., a SIRP- α variant, that are identical to the amino acid (or nucleic acid) residues of a reference sequence, e.g., a wild-type human SIRP- α or a CD47-binding portion thereof, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent identity (i.e., gaps can be introduced in one or both of the candidate and reference sequences for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). Alignment for purposes of determining percent identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. In some embodiments, the percent amino acid (or nucleic acid) sequence identity of a given candidate sequence to, with, or against a given reference sequence (which can alternatively be phrased as a given candidate sequence that has or includes a certain percent amino acid (or nucleic acid) sequence identity to, with, or against a given reference sequence) is calculated as follows:

$$100 \times (\text{fraction of } A/B)$$

where A is the number of amino acid (or nucleic acid) residues scored as identical in the alignment of the candidate sequence and the reference sequence, and where B is the total number of amino acid (or nucleic acid) residues in the reference sequence. In some embodiments where the length of the candidate sequence does not equal to the length of the reference sequence, the percent amino acid (or nucleic acid) sequence identity of the candidate sequence to the reference sequence would not equal to the percent amino acid (or nucleic acid) sequence identity of the reference sequence to the candidate sequence.

In particular embodiments, a reference sequence aligned for comparison with a candidate sequence may show that the candidate sequence exhibits from 50% to

100% identity across the full length of the candidate sequence or a selected portion of contiguous amino acid (or nucleic acid) residues of the candidate sequence. The length of the candidate sequence aligned for comparison purpose is at least 30%, e.g., at least 40%, e.g., at least 50%, 60%, 70%, 80%, 90%, or 100% of the length of the reference sequence. When a position in the candidate sequence is occupied by the same amino acid (or nucleic acid) residue as the corresponding position in the reference sequence, then the molecules are identical at that position.

As used herein, the term "tumor-associated protease" or "tumor enzyme" refers to an enzyme, e.g., a protease, that is present at an increased level in a cancer, e.g., a solid tumor cancer. In some embodiments, the tumor-associated protease may cleave a cleavable linker.

As used herein, the term "blocking peptide" refers to a peptide that can bind to a SIRP- α variant and block or "mask" the CD47-binding portion of the SIRP- α variant. In a SIRP- α variant construct, the blocking peptide may be attached to a SIRP- α variant by way of a linker that is optionally cleavable, and optionally one or more spacers. The blocking peptide may be coupled via non-covalent bonds to the SIRP- α variant and cleaved at a diseased site or diseased cell. In some embodiments, the blocking peptide may bind to a wild-type SIRP- α at the diseased site or diseased cell. A blocking peptide can be used to reduce or minimize binding of the SIRP- α variant with wild-type CD47 under normal physiological conditions or at a non-diseased site. In some embodiments, the blocking peptide has higher binding affinity to a wild-type SIRP- α than a SIRP- α variant. The blocking peptide may dissociate from the SIRP- α variant to bind to a wild-type SIRP- α at, for e.g., a diseased site or under non-physiological conditions. An example of a blocking peptide is a CD47-based blocking peptide, which is a peptide derived from CD47 or a fragment thereof. In some embodiments, a CD47-based blocking peptide is the extracellular, SIRP- α binding portion of CD47 (i.e., the IgSF domain of CD47). In some embodiments, a CD47-based blocking peptide includes one or more amino acid substitutions, additions, and/or deletions relative to the wild-type CD47.

As used herein, the term "cleavable linker" refers to a linker between two portions of a SIRP- α variant construct. In some embodiments, a cleavable linker may covalently attach a blocking peptide to a SIRP- α variant to block binding of the SIRP- α variant to CD47 under physiological conditions. In some embodiments, a cleavable linker may be installed within a blocking peptide, which may be non-

covalently associated with the SIRP- α variant to block binding of the SIRP- α variant to CD47 under physiological conditions. A cleavable linker may be cleaved under certain conditions. If the cleavable linker is within a blocking peptide, cleavage of the linker would inactivate the blocking peptide. The cleavable linker contains a moiety
5 that acts to cleave or induce cleavage of the linker under conditions characteristic of a diseased site, such as a cancer site, e.g., inside a solid tumor. The cleavable linker is stable under healthy physiological conditions (e.g., neutral pH and adequate oxygen concentration). The moiety may be a pH-sensitive chemical functional group (e.g., acetals, ketals, thiomaleamates, hydrazones, disulfide bonds) capable of being
10 hydrolyzed under acidic pH. The moiety may also be a hypoxia-sensitive chemical functional group (e.g., quinones, N-oxides, and heteroaromatic nitro groups) or amino acid capable of being reduced under hypoxic condition. The moiety in the cleavable linker may also be a protein substrate capable of being recognized and cleaved by a tumor-associated protease, enzyme, or peptidase.

15 As used herein, the term "spacer" refers to a covalent or non-covalent linkage between two portions of a SIRP- α variant construct, such as the linker (e.g., cleavable linker) and the SIRP- α variant, or the antibody-binding peptide and the SIRP- α variant. Preferably, the spacer is a covalent linkage. A spacer can be a simple chemical bond, e.g., an amide bond, or an amino acid sequence (e.g., a 3-
20 200 amino acid sequence). An amino acid spacer is part of the primary sequence of a polypeptide (e.g., joined to the spaced polypeptides or polypeptide domains via the polypeptide backbone). A spacer provides space and/or flexibility between the two portions. A spacer is stable under physiological conditions (e.g., neutral pH and adequate oxygen concentration) as well as under conditions characteristic of a
25 diseased site, e.g., acidic pH and hypoxia. A spacer is stable at a diseased site, such as a cancer site, e.g., inside a tumor. Descriptions of spacers are provided in detail further herein.

As used herein, the term "antibody" refers to intact antibodies, antibody fragments, provided that they exhibit the desired biological activity, monoclonal
30 antibodies, polyclonal antibodies, monospecific antibodies, and multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies. Preferably, the antibody is specific to a diseased cell, e.g., a tumor cell. For example, the antibody may specifically bind to a cell surface protein on a diseased cell, e.g., a tumor cell.

As used herein, the term "albumin-binding peptide" refers to an amino acid sequence of 12 to 16 amino acids that has affinity for and functions to bind serum albumin. An albumin-binding peptide can be of different origins, e.g., human, mouse, or rat. In some embodiments of the present invention, a SIRP- α variant
5 construct may include an albumin-binding peptide that is fused to the C-terminus of the SIRP- α variant to increase the serum half-life of the SIRP- α variant. An albumin-binding peptide can be fused, either directly or through a spacer, to the SIRP- α variant.

As used herein, the term "human serum albumin (HSA)" refers to the albumin
10 protein present in human blood plasma. Human serum albumin is the most abundant protein in the blood. It constitutes about half of the blood serum protein. In some embodiments, a human serum albumin has the sequence of amino acids 25-609 (SEQ ID NO: 67) of UniProt ID NO: P02768. In some embodiments, a human serum albumin further contains C34S relative to the sequence of SEQ ID NO:
15 67.

As used herein, the term "Fc domain monomer" refers to a polypeptide chain that includes second and third antibody constant domains (C_{H2} and C_{H3}). In some embodiment, the Fc domain monomer also includes a hinge domain. The Fc domain monomer can be any immunoglobulin antibody isotype, including IgG, IgE, IgM, IgA, or IgD. Additionally, the Fc domain monomer can be an IgG subtype (e.g., IgG1, IgG2a, IgG2b, IgG3, or IgG4). An Fc domain monomer does not include any portion
20 of an immunoglobulin that is capable of acting as an antigen-recognition region, e.g., a variable domain or a complementarity determining region (CDR). Fc domain monomers can include as many as ten changes from a wild-type Fc domain monomer sequence (e.g., 1-10, 1-8, 1-6, 1-4 amino acid substitutions, additions, or
25 deletions) that alter the interaction between an Fc domain and an Fc receptor. Examples of suitable changes are known in the art.

As used herein, the term "Fc domain" refers to a dimer of two Fc domain monomers. In the wild-type Fc domain, the two Fc domain monomers dimerize by
30 the interaction between the two C_{H3} antibody constant domains, as well as one or more disulfide bonds that form between the hinge domains of the two dimerizing Fc domain monomers. In some embodiments, an Fc domain may be mutated to lack effector functions, typical of a "dead Fc domain." In certain embodiments, each of the Fc domain monomers in an Fc domain includes amino acid substitutions in the

C_H2 antibody constant domain to reduce the interaction or binding between the Fc domain and an Fcγ receptor.

As used herein, the term “affinity” or “binding affinity” refers to the strength of the binding interaction between two molecules. Generally, binding affinity refers to the strength of the sum total of non-covalent interactions between a molecule and its binding partner, such as a SIRP-α variant and CD47. Unless indicated otherwise, binding affinity refers to intrinsic binding affinity, which reflects a 1:1 interaction between members of a binding pair. The binding affinity between two molecules is commonly described by the dissociation constant (K_D) or the affinity constant (K_A).

Two molecules that have low binding affinity for each other generally bind slowly, tend to dissociate easily, and exhibit a large K_D . Two molecules that have high affinity for each other generally bind readily, tend to remain bound longer, and exhibit a small K_D . The K_D of two interacting molecules may be determined using methods and techniques well known in the art, e.g., surface plasmon resonance. K_D is calculated as the ratio of k_{off}/k_{on} .

As used herein, the term “host cell” refers to a vehicle that includes the necessary cellular components, e.g., organelles, needed to express proteins from their corresponding nucleic acids. The nucleic acids are typically included in nucleic acid vectors that can be introduced into the host cell by conventional techniques known in the art (e.g., transformation, transfection, electroporation, calcium phosphate precipitation, direct microinjection, etc.). A host cell may be a prokaryotic cell, e.g., a bacterial cell, or a eukaryotic cell, e.g., a mammalian cell (e.g., a CHO cell). As described herein, a host cell is used to express one or more SIRP-α variant constructs.

As used herein, the term “pharmaceutical composition” refers to a medicinal or pharmaceutical formulation that contains an active ingredient as well as excipients and diluents to enable the active ingredient suitable for the method of administration. The pharmaceutical composition of the present invention includes pharmaceutically acceptable components that are compatible with the SIRP-α variant construct. The pharmaceutical composition may be in tablet or capsule form for oral administration or in aqueous form for intravenous or subcutaneous administration.

As used herein, the term “disease associated with SIRP-α and/or CD47 activity” refers to any disease or disorder that is caused by and/or related to SIRP-α and/or CD47 activity. For example, any disease or disorder that is caused by and/or

related to the increase and/or decrease of SIRP- α and/or CD47 activity. Examples of diseases associated with SIRP- α and/or CD47 activity include, but are not limited to, cancers and immunological diseases (e.g., autoimmune diseases and inflammatory diseases).

5 As used herein, the term “therapeutically effective amount” refers an amount of a SIRP- α variant construct of the invention or a pharmaceutical composition containing a SIRP- α variant construct of the invention effective in achieving the desired therapeutic effect in treating a patient having a disease, such as a cancer, e.g., solid tumor or hematological cancer. In particular, the therapeutic effective
10 amount of the SIRP- α variant construct avoids adverse side effects.

 As used herein, the term “optimized affinity” or “optimized binding affinity” refers to an optimized strength of the binding interaction between a SIRP- α variant and CD47. In some embodiments, the SIRP- α variant construct binds primarily or with higher affinity to CD47 on cells at a diseased site (i.e., cancer cells) and does
15 not substantially bind or binds with lower affinity to CD47 on cells at a non-diseased site (i.e., non-cancer cells). The binding affinity between the SIRP- α variant and CD47 is optimized such that the interaction does not cause clinically relevant toxicity. In some embodiments, in order to achieve an optimized binding affinity between the SIRP- α variant and CD47, the SIRP- α variant may be developed to have a lower
20 binding affinity to CD47 than which is maximally achievable.

 As used herein, the term “immunogenicity” refers to the property of a protein (e.g., a therapeutic protein) which causes an immune response in the host as though it is a foreign antigen. The immunogenicity of a protein can be assayed in vitro in a variety of different ways, in particular through in vitro T-cell proliferation assays (see,
25 e.g., Jawa et al., *Clinical Immunology* 149:534-555, 2013), some of which are commercially available (see, e.g., immunogenicity assay services offered by Proimmune).

 As used herein, the term “minimal immunogenicity” refers to an immunogenicity of a protein (e.g., a therapeutic protein) that has been modified, i.e.,
30 through amino acid substitutions, to be lower (e.g., at least 10%, 25%, 50%, or 100% lower) than what could have been before the amino acid substitutions are introduced. A protein (e.g., a therapeutic protein) is modified to have minimal immunogenicity means it causes no or very little host immune response even though it is a foreign antigen.

As used herein, the term “optimized pharmacokinetics” refers to that the parameters that are generally associated with the pharmacokinetics of a protein are improved and modified to produce an optimized protein for in vitro and/or in vivo use. Parameters that are associated with the pharmacokinetics of a protein are well-known to a skilled artisan, including, for examples, K_D , valency, and half-life. In the present invention, the pharmacokinetics of a SIRP- α variant construct of the invention are optimized for its interaction with CD47 for use in a therapeutic context.

DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a portion of the co-crystalized structure of CD47:SIRP- α (PDB: 4KJY, 4CMM), the N-terminus of CD47 exists as a pyro-glutamate and makes hydrogen bonding interactions with Thr66 of a SIRP- α variant or Leu66 of a wild-type SIRP- α .

FIG. 2 shows computation models of the interaction site between CD47 having T102Q and a wild-type SIRP- α having A27 (left) and the interaction site between CD47 having T102Q and a SIRP- α variant having I27.

FIG. 3 shows an SDS-PAGE of SIRP- α variant constructs (SEQ ID NOs: 48-56).

FIG. 4A shows an SDS-PAGE of SIRP- α variant construct (SEQ ID NO: 54) after in vitro cleavage with uPA and matriptase.

FIG. 4B shows an SDS-PAGE of SIRP- α variant construct (SEQ ID NO: 54) after in vitro cleavage with different amounts of matriptase.

FIG. 4C shows an SDS-PAGE of various SIRP- α variant constructs (SEQ ID NOs: 57-63) after in vitro cleavage with matriptase.

FIG. 5A shows a bar graph illustrating the different binding affinities of various SIRP- α variant constructs (SEQ ID NOs: 48-55) to CD47 before and after in vitro cleavage with matriptase.

FIG. 5B shows a bar graph illustrating the different binding affinities of various SIRP- α variant constructs (SEQ ID NOs: 52-63) and the SIRP- α variant (SEQ ID NO: 31) to CD47 before and after in vitro cleavage with matriptase.

FIG. 6 shows a sensorgram demonstrating that a SIRP- α variant construct (SEQ ID NO: 66) can bind Cetuximab and CD47 simultaneously.

FIG. 7A shows a scheme of the quaternary complex containing EGFR, Cetuximab, a SIRP- α variant construct (SEQ ID NO: 66), and CD47.

FIG. 7B shows a sensorgram demonstrating the formation of the quaternary complex shown in FIG. 7A.

FIG. 7C is an image of the sensorgram shown in FIG. 7B.

FIG. 8 is a scatter plot showing phagocytosis induced by the SIRP- α variant construct (SEQ ID NO: 66) and the SIRP- α variant (SEQ ID NO: 31).

DETAILED DESCRIPTION OF THE INVENTION

The invention features signal-regulatory protein α (SIRP- α) polypeptide constructs, including SIRP- α variant constructs, having preferential activity at a diseased site (e.g., at the site of a tumor than at a non-diseased site). In certain embodiments, the SIRP- α variant constructs have higher binding affinity to CD47 on diseased cells (e.g., tumor cells), cells. In some embodiments, the SIRP- α variants may contain one or more amino acid substitutions. In some embodiments, the amino acids may be substituted with histidine residues. In some embodiments, the amino acids may be substituted with other non-histidine amino acid residues. In some embodiments, the SIRP- α variant constructs bind with higher affinity to CD47 on diseased cells or at a diseased site than on non-diseased cells and under conditions characteristic of a diseased site, such as a cancer site, e.g., at the site of or inside a tumor. In some embodiments, the SIRP- α variant constructs bind with higher affinity to CD47 under acidic pH (e.g., less than around pH 7) and/or under hypoxic condition than under physiological conditions. In some embodiments, the SIRP- α variant constructs include a SIRP- α variant and a blocking peptide; the SIRP- α variant is prevented from binding to CD47 by the blocking peptide unless under conditions characteristic of a diseased site. In some embodiments, the SIRP- α variants are fused to an Fc domain monomer, a human serum albumin (HSA), an albumin-binding peptide, or a polymer (e.g., a polyethylene glycol (PEG) polymer). In some embodiments, the SIRP- α variant constructs have their immunogenicity, affinity, and/or pharmacokinetics optimized for use in a therapeutic context. In some embodiments, the SIRP- α variant constructs are preferentially targeted to diseased sites, e.g., a tumor, by way of a targeting moiety, e.g., a target-specific antibody. The invention features methods and pharmaceutical compositions containing SIRP- α variant constructs to treat various diseases, such as cancer, preferably solid tumor or hematological cancer, as well as methods of killing cancer cells and methods of

manufacturing SIRP- α variant constructs and pharmaceutical compositions containing such SIRP- α variant constructs.

5 In some embodiments, a SIRP- α variant construct includes a SIRP- α variant attached to a blocking peptide. In some embodiments, the preferential binding of the SIRP- α variant in the SIRP- α variant construct to CD47 on diseased cells or diseased sites may be obtained by attaching the block peptide to the SIRP- α variant by use of a cleavable linker, which is cleaved at the diseased cells or diseased sites. In some embodiments, the preferential binding of the SIRP- α variant in the SIRP- α variant construct to CD47 on diseased cells or diseased sites may be obtained by
10 attaching the block peptide to the SIRP- α variant, wherein the blocking peptide can be detached or simply dissociated from the SIRP- α variant at the diseased cells or diseased sites.

I. SIRP- α variants

15 There exist at least ten natural variants of wild-type human SIRP- α . The amino acid sequences of the D1 domains of the ten wild-type human SIRP- α variants are shown in SEQ ID NOs: 3-12 (see Table 1). In some embodiments, the SIRP- α variant has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.) sequence identity to a sequence of any one of
20 SEQ ID NOs: 3-12. Table 2 lists possible amino acid substitutions in each D1 domain variant (SEQ ID NOs: 13-23). In some embodiments, the SIRP- α variant binds with an optimized binding affinity to CD47. In some embodiments, the SIRP- α variant construct including a SIRP- α variant binds primarily or with higher affinity to CD47 on cancer cells and does not substantially bind or binds with lower affinity to
25 CD47 on non-cancer cells. In some embodiments, the binding affinity between the SIRP- α variant construct and CD47 is optimized such that the interaction does not cause clinically relevant toxicity. In some embodiments, the SIRP- α variant construct has minimal immunogenicity. In some embodiments, the SIRP- α variant has the same amino acids as that of the SIRP- α polypeptide in a biological sample of the
30 subject, except for the amino acids changes introduced to increase affinity of the SIRP- α variant. Techniques and methods for generating SIRP- α variants and determining their binding affinities to CD47 are described in detail further herein.

Table 2 lists specific amino acid substitutions in a SIRP- α variant, relative to each D1 domain variant sequence. A SIRP- α variant may include one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten) of the substitutions listed in Table 2. In some embodiments, a SIRP- α variant includes at most ten amino acid substitutions relative to a wild-type D1 domain. In some embodiments, a SIRP- α variant includes at most seven amino acid substitutions relative to a wild-type D1 domain.

In some embodiments, a SIRP- α variant is a chimeric SIRP- α variant that includes a portion of two or more wild-type D1 domain variants (e.g., a portion of one wild-type D1 domain variant and a portion of another wild-type D1 domain variant). In some embodiments, a chimeric SIRP- α variant includes at least two portions (e.g., three, four, five, etc.) of wild-type D1 domain variants, wherein each of the portions is from a different wild-type D1 domain variant. In some embodiments, a chimeric SIRP- α variant further includes one or more amino acid substitutions listed in Table 2. In some embodiments, the SIRP- α variant has at least 80% (e.g., at least 85%, 87%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.) sequence identity to a sequence of any one of SEQ ID NOs: 24-34 in Table 3.

Table 1. Sequences of wild-type SIRP- α D1 domains

Wild-type D1 domain variant 1 (SEQ ID NO: 3)	EEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTKRNNMDFSIRIGNITPADAGTYICVKFRKGSPD DVEFKSGAGTELSVRAKPS
Wild-type D1 domain variant 2 (SEQ ID NO: 4)	EEELQVIQPDKSVSVAAGESAILHCTVTSLIPVGPIQWFRGAGPARELIYNQKEGHFPRVTTVSESTKRENMDFSISISNITPADAGTYICVKFRKGSPD TEFKSGAGTELSVRAKPS
Wild-type D1 domain variant 3 (SEQ ID NO: 5)	EEELQVIQPDKSVSVAAGESAILLCTVTSLIPVGPIQWFRGAGPARELIYNQKEGHFPRVTTVSESTKRENMDFSISISNITPADAGTYICVKFRKGSPD TEFKSGAGTELSVRAKPS
Wild-type D1 domain variant 4 (SEQ ID NO: 6)	EEGLQVIQPDKSVSVAAGESAILHCTATSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTKRNNMDFSIRIGNITPADAGTYICVKFRKGSPD DVEFKSGAGTELSVRAKPS
Wild-type D1 domain variant 5 (SEQ ID NO: 7)	EEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTKRNNMDFSIRIGNITPADAGTYICVKFRKGSPD DVEFKSGAGTELSVRAKPS
Wild-type D1 domain variant 6 (SEQ ID NO: 8)	EEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTKRNNMDFPIRIGNITPADAGTYICVKFRKGSPD DVEFKSGAGTELSVRAKPS

Wild-type D1 domain variant 7 (SEQ ID NO: 9)	EEELQVIQPDKSVSVAAGESAILHCTVTSVIPVGPIQWFRGAGPARELIYNQKEGHFPRVTTVSESTKRENMDFSISISNITPADAGTYICVKFRKGSPDTEFKSGAGTELSVRGKPS
Wild-type D1 domain variant 8 (SEQ ID NO: 10)	EEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPARELIYNQKEGHFPRVTTVSESTKRENMDFSISISNITPADAGTYICVKFRKGSPDTEFKSGAGTELSVRAPKS
Wild-type D1 domain variant 9 (SEQ ID NO: 11)	EEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPGRELIYNQKEGHFPRVTTVSDLTNRNMDFSIRISNITPADAGTYICVKFRKGSPDVVEFKSGAGTELSVRAPKS
Wild-type D1 domain variant 10 (SEQ ID NO: 12)	EEELQVIQPDKSVSVAAGESAILHCTVTSVIPVGPIQWFRGAGPARELIYNQKEGHFPRVTTVSESTKRENMDFSISISNITPADAGTYICVKFRKGSPDTEFKSGAGTELSVRAPKS

Table 2. Amino acid substitutions in a SIRP-α variant, relative to each D1 domain variant

D1 domain v1 (SEQ ID NO: 13)	EEEX ₁ QX ₂ IQPDKSVLVAAGETX ₃ TLRCTX ₄ TSIX ₅ PVGPIQWFRGAGPGRX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSDX ₁₀ TX ₁₁ RNNMDFSIRIGX ₁₂ ITX ₁₃ ADAGTYICX ₁₄ KX ₁₅ RKGSPDDVEX ₁₆ KSGAGTELSVRAPKS
Amino acid substitutions relative to SEQ ID NO: 13	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =A, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =L, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v2 (SEQ ID NO: 14)	EEEX ₁ QX ₂ IQPDKSVSVAAGESX ₃ ILHCTX ₄ TSIX ₅ PVGPIQWFRGAGPARX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSEX ₁₀ TX ₁₁ RENMDFSISISX ₁₂ ITX ₁₃ ADAGTYICX ₁₄ KX ₁₅ RKGSPDTEX ₁₆ KSGAGTELSVRAPKS
Amino acid substitutions relative to SEQ ID NO: 14	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =V, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =S, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v3 (SEQ ID NO: 15)	EEEX ₁ QX ₂ IQPDKSVSVAAGESX ₃ ILLCTX ₄ TSIX ₅ PVGPIQWFRGAGPARX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSEX ₁₀ TX ₁₁ RENMDFSISISX ₁₂ ITX ₁₃ ADAGTYICX ₁₄ KX ₁₅ RKGSPDTEX ₁₆ KSGAGTELSVRAPKS
Amino acid substitutions relative to SEQ ID NO: 15	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =V, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =S, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V

D1 domain v4 (SEQ ID NO: 16)	EEGX ₁ QX ₂ IQPDKSVSVAAGESX ₃ ILHCTX ₄ TSLX ₅ PVGPIQWFRGAGPG RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSDX ₁₀ TX ₁₁ RNNMDFSIRIGX ₁₂ ITX ₁₃ ADAGTYCYX ₁₄ KX ₁₅ RKGSPDDVEX ₁₆ KSGAGTELSVRAKPS
Amino acid substitutions relative to SEQ ID NO: 16	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =A, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =L, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v5 (SEQ ID NO: 17)	EEEX ₁ QX ₂ IQPDKFVLVAAGETX ₃ TLRCTX ₄ TSLX ₅ PVGPIQWFRGAGPG RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSDX ₁₀ TX ₁₁ RNNMDFSIRIGX ₁₂ ITX ₁₃ ADAGTYCYX ₁₄ KX ₁₅ RKGSPDDVEX ₁₆ KSGAGTELSVRAKPS
Amino acid substitutions relative to SEQ ID NO: 17	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =A, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =L, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v6 (SEQ ID NO: 18)	EEEX ₁ QX ₂ IQPDKSVLVAAGETX ₃ TLRCTX ₄ TSLX ₅ PVGPIQWFRGAGPG RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSDX ₁₀ TX ₁₁ RNNMDFPIRIGX ₁₂ ITX ₁₃ ADAGTYCYX ₁₄ KX ₁₅ RKGSPDDVEX ₁₆ KSGAGTELSVRAKPS
Amino acid substitutions relative to SEQ ID NO: 18	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =A, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =L, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v7 (SEQ ID NO: 19)	EEEX ₁ QX ₂ IQPDKSVSVAAGESX ₃ ILHCTX ₄ TSLX ₅ PVGPIQWFRGAGPA RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSEX ₁₀ TX ₁₁ RENMDFSISISX ₁₂ ITX ₁₃ ADAGTYCYX ₁₄ KX ₁₅ RKGSPDTEX ₁₆ KSGAGTELSVRGKPS
Amino acid substitutions relative to SEQ ID NO: 19	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =V, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =S, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v8 (SEQ ID NO: 20)	EEEX ₁ QX ₂ IQPDKSVLVAAGETX ₃ TLRCTX ₄ TSLX ₅ PVGPIQWFRGAGPA RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSEX ₁₀ TX ₁₁ RENMDFSISISX ₁₂ ITX ₁₃ ADAGTYCYX ₁₄ KX ₁₅ RKGSPDTEX ₁₆ KSGAGTELSVRAKPS
Amino acid substitutions relative to SEQ ID NO: 20	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =A, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =S, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V

D1 domain v9 (SEQ ID NO: 21)	EEEX ₁ QX ₂ IQPDKSVLVAAGETX ₃ TLRCTX ₄ TS LX ₅ PVGPIQWFRGAGPG RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSDX ₁₀ TX ₁₁ RNNMDFSIRISX ₁₂ ITX ₁₃ ADAGTY YCX ₁₄ KX ₁₅ RKGSPDDVEX ₁₆ KSGAGTELSVRAKPS
Amino acid substitutions relative to SEQ ID NO: 21	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =A, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =L, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
D1 domain v10 (SEQ ID NO: 22)	EEEX ₁ QX ₂ IQPDKSVSVAAGESX ₃ ILHCTX ₄ TS LX ₅ PVGPIQWFRGAGPA RX ₆ LIYNQX ₇ X ₈ GX ₉ FPRVTTVSEX ₁₀ TX ₁₁ RENMDFSISISX ₁₂ ITX ₁₃ ADAGTY YCX ₁₄ KX ₁₅ RKGSPDTEX ₁₆ KSGAGTELSVRAKPS
Amino acid substitutions relative to SEQ ID NO: 22	X ₁ =L, I, V; X ₂ =V, L, I; X ₃ =A, V; X ₄ =V, I, L; X ₅ =I, T, S, F; X ₆ =E, V, L; X ₇ =K, R; X ₈ =E, Q; X ₉ =H, P, R; X ₁₀ =S, T, G; X ₁₁ =K, R; X ₁₂ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₁₃ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₁₄ =V, I; X ₁₅ =F, L, V; X ₁₆ =F, V
Pan D1 domain (SEQ ID NO: 23)	EEX ₁ X ₂ QX ₃ IQPDKX ₄ VX ₅ VAAGEX ₆ X ₇ X ₈ LX ₉ CTX ₁₀ TS LX ₁₁ PVGPIQWFR GAGPX ₁₂ RX ₁₃ LIYNQX ₁₄ X ₁₅ GX ₁₆ FPRVTTVSX ₁₇ X ₁₈ TX ₁₉ RX ₂₀ NMDFX ₂₁ IX ₂₂ IX ₂₃ X ₂₄ ITX ₂₅ ADAGTY YCX ₂₆ KX ₂₇ RKGSPDX ₂₈ X ₂₉ EX ₃₀ KSGAGTEL SVRX ₃₁ KPS
Amino acid substitutions relative to SEQ ID NO: 23	X ₁ =E, G; X ₂ =L, I, V; X ₃ =V, L, I; X ₄ =S, F; X ₅ =L, S; X ₆ =S, T; X ₇ =A, V; X ₈ =I, T; X ₉ =H, R; X ₁₀ =A, V, I, L; X ₁₁ =I, T, S, F; X ₁₂ =A, G; X ₁₃ =E, V, L; X ₁₄ =K, R; X ₁₅ =E, Q; X ₁₆ =H, P, R; X ₁₇ =D, E; X ₁₈ =S, L, T, G; X ₁₉ =K, R; X ₂₀ =E, N; X ₂₁ =S, P; X ₂₂ =S, R; X ₂₃ =S, G; X ₂₄ =N, A, C, D, E, F, G, H, I, K, L, M, P, Q, R, S, T, V, W, Y; X ₂₅ =P, A, C, D, E, F, G, H, I, K, L, M, N, Q, R, S, T, V, W, Y; X ₂₆ =V, I; X ₂₇ =F, L, V; X ₂₈ =D or absent; X ₂₉ =T, V; X ₃₀ =F, V; and X ₃₁ =A, G

Table 3. SEQ ID NOs: 24-34

SEQ ID NO	Sequence
24	EEELQVIQPDKSVSVAAGESAILHCTITSLIPVGPIQWFRGAGPARELIYNQREG HFPRVTTVSETTRRENMDFSISISNITPADAGTYYCVKFRKGSPDTEVKSGAGTE LSVRAKPS
25	EEEVQVIQPDKSVSVAAGESAILHCTLTSLIPVGPIQWFRGAGPARVLIYNQRQG HFPRVTTVSEGTRRENMDFSISISNITPADAGTYYCIKFRKGSPDTEFKSGAGTE LSVRAKPS
26	EEEVQIIQPDKSVSVAAGESVILHCTITSLTPVGPIQWFRGAGPARLLIYNQREG PFPRVTTVSETTRRENMDFSISISNITPADAGTYYCVKLRKGSPDTEFKSGAGTE LSVRAKPS
27	EEELQIIQPDKSVSVAAGESAILHCTITSLSPVGPIQWFRGAGPARVLIYNQRQG PFPRVTTVSEGTKRENMDFSISISNITPADAGTYYCIKLRKGSPDTEFKSGAGTE LSVRAKPS
28	EEEIQVIQPDKSVSVAAGESVIIHCTVTSLFPVGPIQWFRGAGPARVLIYNQRQG RFPRVTTVSEGTKRENMDFSISISNITPADAGTYYCVKVRKGSPDTEVKSGAGTE LSVRAKPS
29	EEEVQIIQPDKSVSVAAGESIILHCTVTSLFPVGPIQWFRGAGPARVLIYNQREG RFPRVTTVSEGTRRENMDFSISISNITPADAGTYYCIKLRKGSPDTEFKSGAGTE LSVRAKPS
30	EEEVQLIQPDKSVSVAAGESAILHCTVTSLFPVGPIQWFRGAGPARVLIYNQREG PFPRVTTVSEGTKRENMDFSISISNITPADAGTYYCIKFRKGSPDTEVKSGAGTE LSVRAKPS
31	EEELQIIQPDKSVLVAAGETATLRCTITSLFPVGPIQWFRGAGPGRVLIYNQRQG PFPRVTTVSDTTKRNNMDFSIRIGNITPADAGTYYCIKFRKGSPDDVEFKSGAGT ELSVRAKPS
32	EEELQIIQPDKSVSVAAGESAILHCTITSLFPVGPIQWFRGAGPARLLIYNQRQG PFPRVTTVSETTKRENMDFSISISNITPADAGTYYCVKFRKGSPDTEFKSGAGTE LSVRAKPS
33	EEEVQIIQPDKSVSVAAGESAILHCTITSLFPVGPIQWFRGAGPARVLIYNQKQG PFPRVTTISETTRRENMDFSISISNITPADAGTYYCIKFRKGSPDTEFKSGAGTE LSVRAKPS
34	EEELQIIQPDKSVSVAAGESAILHCTITSLTPVGPIQWFRGAGPARVLIYNQRQG PFPRVTTVSEGTRRENMDFSISISNITPADAGTYYCIKFRKGSPDTEVKSGAGTE LSVRAKPS

Desirably, the SIRP- α variant constructs of the invention bind with higher affinity to CD47 under conditions characteristic of a diseased site, such as a cancer site, e.g., inside a tumor, than under physiological conditions (e.g., neutral pH and adequate oxygen concentration). Conditions characteristic of a diseased site, such as a cancer site, e.g., inside a tumor, are, e.g., acidic pH and hypoxia. In some embodiments, SIRP- α variant constructs of the invention may be engineered to preferentially bind to diseased cells over non-diseased cells. In particular, the

disease cells may be cancer cells of a cancer disease, e.g., solid tumor or hematological cancer. Preferably, the SIRP- α variant constructs bind with higher affinity to CD47 under acidic pH (e.g., less than around pH 7) than under neutral pH, e.g., pH 7.4. Preferably, the SIRP- α variant constructs bind with higher affinity to

5 CD47 under hypoxic condition than under a condition with adequate oxygen concentration. In some embodiments, a SIRP- α variant construct includes a SIRP- α variant attached to a blocking peptide. In some embodiments, the preferential binding of the SIRP- α variant in the SIRP- α variant construct to CD47 on diseased cells or diseased sites may be obtained by attaching the block peptide to the SIRP- α

10 variant by use of a cleavable linker, which is cleaved at the diseased cells or diseased sites. In some embodiments, the preferential binding of the SIRP- α variant in the SIRP- α variant construct to CD47 on diseased cells or diseased sites may be obtained by attaching the block peptide to the SIRP- α variant, wherein the blocking peptide can be detached or simply dissociated from the SIRP- α variant at the

15 diseased cells or diseased sites.

In some embodiments, a SIRP- α variant construct includes a SIRP- α variant and a blocking peptide. In some embodiments, SIRP- α variant may be attached to a blocking peptide through a linker (e.g., a cleavable linker). The blocking peptide serves to block the CD47 binding site of the SIRP- α variant to prevent binding of

20 SIRP- α variant to CD47 under physiological conditions (e.g., neutral pH and adequate oxygen concentration). The cleavable linker is a linker capable of being cleaved only under conditions characteristic of a diseased site (such as a cancer site, e.g., inside a tumor), such as acidic pH and hypoxia. In some embodiments, the cleavable linker is cleaved by a tumor-associated protease at a diseased site. In

25 some embodiments, the linker is not cleaved and the blocking peptide simply dissociates from the SIRP- α variant at a diseased site such that the SIRP- α variant is free to bind to nearby CD47 on diseased cells, e.g., tumor cells. Therefore, only when the SIRP- α variant is at a diseased site would it be released from the blocking peptide and be free to bind to nearby CD47 on diseased cells, e.g., tumor cells.

30 Blocking peptides and linkers (e.g., cleavable linkers) are described in detail further herein.

In some embodiments, a SIRP- α variant construct includes a SIRP- α variant and a targeting moiety. In some embodiments, a SIRP- α variant may be attached to

a targeting moiety, such as an antibody, e.g., a tumor-specific antibody, or another protein or peptide, e.g., an antibody-binding peptide, that exhibit binding affinity to a diseased cell. After administration, the tumor-specific antibody or antibody-binding peptide serves as a targeting moiety to bring the SIRP- α variant to the diseased site, such as a cancer site, e.g., inside a solid tumor, where the SIRP- α can interact specifically with CD47 on diseased cells. In some embodiments, a SIRP- α variant may be fused to a protein or peptide, e.g., an antibody-binding peptide, capable of binding to an antibody (e.g., tumor-specific antibody), i.e., binding to a constant or variable region of the antibody. SIRP- α variants capable of binding to one or more antibodies are described in detail further herein. In other embodiments, other SIRP- α variants, such as the ones described in International Publication No. WO2013109752 (hereby incorporated by reference), may be attached to a tumor-specific antibody or to a protein or peptide, e.g., an antibody-binding peptide, capable of binding to a tumor-specific antibody. In some embodiments, the SIRP- α variant may be attached to the antibody either *in vitro* (prior to administration to a human) or *in vivo* (after administration).

In some embodiments, a SIRP- α variant may further include a D2 and/or D3 domain of a wild-type human SIRP- α . In some embodiments, a SIRP- α variant may be attached to an Fc domain monomer, a human serum albumin (HSA), a serum-binding protein or peptide, or an organic molecule, e.g., a polymer (e.g., a polyethylene glycol (PEG)), in order to improve the pharmacokinetic properties of the SIRP- α variant, e.g., increase serum half-life. Fc domain monomers, HSA proteins, serum-binding proteins or peptides, and organic molecules such as a PEG that serve to increase the serum half-life of the SIRP- α variants of the invention are described in detail further herein. In some embodiments, a SIRP- α variant does not include the sequence of any one of SEQ ID NOs:3-12 and 24-34.

II. Amino acid substitutions with histidine residues in SIRP- α variants

In some embodiments, in addition to the amino acid substitutions in a SIRP- α variant listed in Table 2, the SIRP- α variant may include one or more amino acid substitutions with histidine residues. The SIRP- α variant constructs including a SIRP- α variant bind with higher affinity to CD47 on diseased cells or at a diseased site than on non-diseased cells and under conditions characteristic of a diseased site (e.g., acidic pH, hypoxia) than under physiological conditions. Amino acid residues

to be substituted with histidine residues may be identified using histidine scanning mutagenesis, protein crystal structures, and computational design and modeling methods. Techniques and methods that may be used to generate SIRP- α variants and ways to determine their binding affinities to CD47 on diseased and non-diseased cells are described in detail further herein. The histidine residue substitutions may be located at the interface of a SIRP- α variant and CD47 or may be at internal regions of a SIRP- α variant. Preferentially, histidine residue substitutions are located at the interface of a SIRP- α variant and CD47. Table 4 lists specific SIRP- α amino acids that may be substituted with histidine residues. The amino acid numbering in Table 4 is relative to the sequence of SEQ ID NO: 3; one or more amino acids at the corresponding positions in any one of the sequences of SEQ ID NOs: 4-12 may also be substituted with histidine residues. Contact residues are the amino acids located at the interface of a SIRP- α variant and CD47. Core residues are the internal amino acids not directly involved in the binding between a SIRP- α variant and CD47. The SIRP- α variants may include one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, or all) of the substitutions listed in Table 4. The SIRP- α variants may contain a maximum of 20 histidine substitutions.

Table 4. SIRP- α amino acid substitutions (amino acid numbering is relative to the sequence of SEQ ID NO: 3)

Contact residues	S29H, L30H, I31H, P32H, V33H, G34H, P35H, Q52H, K53H, E54H, L66H, T67H, K68H, R69H, F74H, K93H, K96H, G97H, S98H, D100H
Core residues	L4H, V6H, A27H, I36H, F39H, E47H, L48H, I49H, Y50H, F57H, V60H, M72H, F74H, I76H, V92H, F94H, E103H

III. pH-dependent binding

Studies have shown that tumor cell mediated oncogenic metabolism generates a large amount of lactic acid and protons, leading to the reduction in the extracellular pH values to as low as 6 in tumor tissue (Icard et al., *Biochim. Biophys. Acta*. 1826:423-433, 2012). In some embodiments, the SIRP- α variant constructs including a SIRP- α variant are engineered to bind with high affinity to CD47 under acidic pH than under neutral pH (e.g., around pH 7.4). Thus, the SIRP- α variant constructs of the invention are engineered to selectively bind to CD47 on diseased cells (e.g., tumor cells) or on cells at a diseased site (e.g., cells in the tumor micro-environment supporting tumor growth), over CD47 on non-diseased cells.

In one embodiment, to engineer pH-dependent binding of a SIRP- α variant construct of the invention, histidine mutagenesis may be performed on the SIRP- α variant, especially on the region of SIRP- α that interacts with CD47. Crystal structures of a SIRP- α and CD47 complex (see, e.g., PDB ID No. 2JJS) and computer modeling may be used to visualize the three-dimensional binding site of SIRP- α and CD47. Computational design and modeling methods useful in designing a protein with pH-sensitive binding properties are known in the literature and described in, e.g., Strauch et al., *Proc Natl Acad Sci* 111:675-80, 2014, which is incorporated by reference herein in its entirety. In some embodiments, computer modeling may be used to identify key contact residues at the interface of SIRP- α and CD47. Identified key contact residues may be substituted with histidine residues using available protein design software (e.g., RosettaDesign), which can generate various protein designs that can be optimized, filtered, and ranked based on computed binding energy and shape complementarity. Therefore, energetically favorable histidine substitutions at certain amino acid positions may be identified using computational design methods. Computer modeling may be also be used to predict the change in the three-dimensional structure of SIRP- α . Histidine substitutions that generate a significant change in the three-dimensional structure of SIRP- α may be avoided.

Once energetically and structurally optimal amino acid substitutions are identified, the amino acids may be systematically substituted with histidine residues. In some embodiments, one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum of 20) amino acids of SIRP- α may be substituted with histidine residues. In particular, amino acids located at the interface of SIRP- α and CD47, preferably, amino acids directly involved in the binding of SIRP- α to CD47, may be substituted with histidine residues. The SIRP- α variant may include one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum of 20) histidine residue substitutions. In other embodiments, naturally occurring histidine residues of SIRP- α may be substituted with other amino acid residues. In yet other embodiments, one or more amino acids of SIRP- α may be substituted with non-histidine residues in order to affect the binding of naturally occurring or substituted histidine residues with CD47. For example, substituting amino acids surrounding a naturally occurring histidine residue with other amino acids may "bury" the naturally occurring histidine residue. In some embodiments,

amino acids not directly involved in binding with CD47, i.e., internal amino acids (e.g., amino acids located at the core of SIRP- α) may also be substituted with histidine residues. Table 4 lists specific SIRP- α amino acids that may be substituted with histidine or non-histidine residues. Contact residues are the amino acids located at the interface of SIRP- α and CD47. Core residues are the internal amino acids not directly involved in the binding between SIRP- α and CD47. The SIRP- α variants may include one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, or all) of the substitutions listed in Table 4.

SIRP- α variants containing one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum number of 20) histidine residue substitutions may be tested for their binding to CD47 under different pH conditions (e.g., at pH 5, 5.5, 6, 6.5, 7, 7.4, 8). In some embodiments, purified CD47 protein may be used to test binding. Various techniques known to those skilled in the art may be used to measure the affinity constant (K_A) or dissociation constant (K_D) of a SIRP- α variant/CD47 complex under different pH conditions (e.g., at pH 5, 5.5, 6, 6.5, 7, 7.4, 8). In a preferred embodiment, the binding affinity of a SIRP- α variant to a CD47 may be determined using surface plasmon resonance (e.g., Biacore3000™ surface plasmon resonance (SPR) system, Biacore, INC, Piscataway N.J.). In an exemplary embodiment, a SIRP- α variant with pH-dependent binding, which specifically binds a CD47 with higher affinity at pH 6 than at pH 7.4, exhibits a lower K_D at pH 6 than at pH 7.4.

IV. Hypoxia-dependent binding

Tumor hypoxia is the condition in which tumor cells have been deprived of oxygen. As a tumor grows, its blood supply is constantly redirect to the most fast growing parts of the tumor, leaving portions of the tumor with oxygen concentration significantly lower than in healthy tissues.

In some embodiments, a SIRP- α variant may be attached to a hypoxia-activated prodrug, which may act to increase the efficacy of a SIRP-alpha variant against the relevant diseased cells under specifically hypoxic conditions. Hypoxia-activated prodrugs are known in the literature, such as those described by Kling et al. (*Nature Biotechnology*, 30:381, 2012), herein incorporated by reference.

V. Antibody binding

Another strategy to provide selective SIRP- α activity at a diseased site than at a non-diseased site is to attach the SIRP- α protein to a protein or peptide that can bind to a region of an antibody. Preferably, the antibody is specific to a diseased cell, e.g., a tumor cell. For example, the antibody may specifically bind to a cell surface protein on a diseased cell, e.g., a tumor cell. The SIRP- α protein may bind to the antibody reversibly or irreversibly.

General antibody binding

In some embodiments, to engineer a SIRP- α protein that can bind to different antibodies regardless of antibody specificity, the SIRP- α protein may be fused to a protein or peptide that recognizes the constant region of an antibody, e.g., the C_H2 or C_H3 constant domain of the Fc domain of an antibody. A SIRP- α protein is capable of binding CD47 and has at least 50% amino acid sequence identity to a sequence of a wild-type SIRP- α (e.g., variant 1 (SEQ ID NO: 1, shown below)) or to a sequence of a CD47-binding portion of a wild-type SIRP- α (e.g., a sequence of any one of SEQ ID NOs: 3-12 listed in Table 1).

SEQ ID NO: 1

1 MEPAGPAPGR LGPLLCLLLA ASCAWSGVAG EEELQVIQPD KSVLVAAGET
ATLRCTATSL
61 IPVGPIQWFR GAGPGRELIY NQKEGHFPRV TTVSDLTKRN
NMDFSIRIGN ITPADAGTTY
121 CVKFRKGSPD DVEFKSGAGT ELSVRAKPSA PVVSGPAARA
TPQHTVSFTC ESHGFSPRDI
181 TLKWFKNONE LSDFQTNVDP VGESVSYSIH STAKVVLTRE
DVHSQVICEV AHVTLQGDPL
241 RGTANLSETI RVPPTLEVTQ QPVRAENQVN VTCQVRKFYP
QRLQLTWLEN GNVSRTEAS
301 TVTENKDGTY NWMSWLLNV SAHRDDVKLT CQVEHDGQPA
VSKSHDLKVS AHPKEQGSNT
361 AAENTGSNER NIYIVGVVVC TLLVALLMAA LYLVRIRQKK
AQGSTSSTRL HEPEKNAREI
421 TQDTNDITYA DLNLPKGKKP APQAAEPNNH TEYASIQTSP
QPASEDTLTY ADLDMVHLNR
481 TPKQPAPKPE PSFSEYASVQ VPRK

A CD47-binding portion of a wild-type SIRP- α includes the D1 domain of a wild-type SIRP- α (e.g., a sequence of any one of SEQ ID NOs: 3-12 listed in Table 1). Proteins and peptides exhibiting general binding to the constant region of an

antibody are known in the art. For example, the bacterial antibody-binding proteins, e.g., Proteins A, G, and L, bind to the constant regions of an antibody. Proteins A and G bind to the Fc domains, while Protein L binds to the constant region of the light chain. In an exemplary embodiment, Protein A, G, or L may be fused to the N- or C-terminus of a SIRP- α protein. Preferentially, in this embodiment, the fusion protein of Protein A, G, or L and the SIRP- α protein may be attached, i.e., through chemical conjugation, to an antibody prior to administration to prevent the fusion protein from binding to various other antibodies in serum. Protein A, G, or L may also be evolved and screened using conventional techniques in the field (i.e., directed evolution and display libraries) for higher binding affinity to the constant regions of an antibody. In some embodiments, a SIRP- α protein may be directly attached to an antibody using conventional genetic or chemical conjugation techniques in the art. In other embodiments, a SIRP- α protein may also be attached to an antibody by way of a spacer, which allows for additional structural and spatial flexibility of the protein. Various spacers are described in detail further herein. In some embodiments, the SIRP- α protein may bind, either directly or through an antibody-binding protein or peptide, to the antibody reversibly or irreversibly. Further, screening of modified antibodies which can be utilized in accordance with the embodiments of the invention described herein can be carried out as described in, e.g., US Patent Publication No. US 20100189651.

Other proteins or peptides capable of binding to a constant region of an antibody and methods of screening for such proteins or peptides are described in US Patent Publication No. US20120283408, which is incorporated by reference herein in its entirety.

Specific antibody binding

In some embodiments, to provide selective targeting of SIRP- α variants at a diseased site and to engineer a SIRP- α variant capable of binding to a specific antibody, e.g., a tumor-specific antibody, the SIRP- α variant construct may include a SIRP- α variant and an antibody-specific protein or peptide. The SIRP- α variant may be fused to an antibody-specific protein or peptide (e.g., an antibody-binding peptide). Preferably, the protein or peptide specifically binds to a tumor-specific antibody. In some embodiments, the fusion protein of the SIRP- α variant and the antibody-binding protein or peptide may be co-administered with the tumor-specific

antibody in a combination therapy. In other embodiments, the fusion protein and the tumor-specific antibody may be administered separately, i.e., within hours of each other, preferably, the antibody is administered first. In yet other embodiments, prior to administration, the fusion protein may be covalently attached to the tumor-specific antibody using genetic or chemical methods commonly known in the art.

Examples of antibody-binding peptides include a disease localization peptide (DLP) (SEQ ID NO: 64 or 65), a small peptide that can bind to the center of the fragment antigen-binding (Fab) region of Cetuximab (see, e.g., Donaldson et al., *Proc Natl Acad Sci U S A.* 110: 17456-17461, 2013). Cetuximab is an epidermal growth factor receptor (EGFR) IgG1 antibody. Antibody-binding peptides that can be fused to a SIRP- α variant also include, but are not limited to, peptides having at least 75% amino acid sequence identity to the sequence of the DLP (SEQ ID NO: 64 or 65) or a fragment thereof. In some embodiments, the antibody-binding peptide has the sequence of SEQ ID NO: 64.

In a recent study, SIRP- α has been shown to enhance in vitro phagocytosis of DLD-1 cells in combination with the antibody Cetuximab (Weiskopf et al., *Science* 341: 88-91, 2013). In some embodiments, a SIRP- α variant may be fused to a specific antibody-binding peptide, e.g., a DLP having the sequence of SEQ ID NO: 64. In these embodiments, the SIRP- α variant construct including a SIRP- α variant and a DLP may target its activity in Cetuximab-bound, EGFR expressing tumors. This in turn may further improve the delivery of the SIRP- α variant construct including a SIRP- α variant and DLP and Cetuximab to anti-EGFR responsive patients. An example of a SIRP- α variant construct including a SIRP- α variant and DLP is shown in SEQ ID NO: 66, in which single-underlined portion indicates the DLP and bold portion indicates the SIRP- α variant. Two DLPs are genetically linked to both N- and C-terminus of SIRP- α variant for avidity and to improve affinity for Cetuximab. The sequence of the SIRP- α variant (bold portion) in SEQ ID NO: 66 may be replaced by a sequence of any SIRP- α variant described herein. Other antibody-binding peptides may also be fused to a SIRP- α variant. Such antibody-binding peptides include, but are not limited to, peptides that can specifically bind to antibodies such as cetuximab, pembrolizumab, nivolumab, pidilizumab, MEDI0680, MEDI6469, Ipilimumab, tremelimumab, urelumab, vantictumab, varlilumab,

mogamalizumab, anti-CD20 antibody, anti-CD19 antibody, anti-CS1 antibody, herceptin, trastuzumab, and/or pertuzumab.

SEQ ID NO: 66

5 CQFDLSTRRLKCGGGGSGGGGSGGGGSGGGGSEELQIIQPDKSVLVAAGETATLRC
 TITSLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRTTVSDTTKRNNMDFSIRIGNIT
 PADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPSGGGGSGGGGSGGGGSGGGGS
 CQFDLSTRRLKC

10 In some embodiments, a SIRP- α variant construct including a SIRP- α variant
 and a DLP may be further combined with a CD47-based blocking peptide described
 herein to block the binding of the SIRP- α variant in the construct before the construct
 reaches the diseased site where the cleavable linker may be cleaved. In these
 embodiments, the therapeutic window can be expanded as the SIRP- α variant
15 construct containing a SIRP- α variant, a CD47-based blocking peptide, and a DLP
 accumulates at the diseased site and is only active at the diseased site after linker
 cleavage induced by a protease (e.g., a tumor-specific protease) or other
 characteristics of the diseased site (e.g., acidic pH, hypoxia).

 In some embodiments, proteins or peptides capable of binding to tumor-
20 specific antibodies may be identified using techniques commonly used in the art,
 such as directed evolution and display libraries, e.g., a phage display library.
 Methods and techniques directed to identifying proteins and peptides capable of
 binding to tumor-specific antibodies are known in the art, such as those described by
 Donaldson et al. (*Proc Natl Acad Sci* 110:17456-61, 2013), which is incorporated by
25 reference herein in its entirety. In a phage display library, a potential antibody-
 specific protein or peptide is typically covalently linked to a bacteriophage coat
 protein. The linkage results from translation of a nucleic acid encoding the protein or
 peptide fused to the coat protein. Bacteriophage displaying the peptide can be
 grown and harvested using standard phage preparatory methods, e.g. PEG
30 precipitation from growth media. These displaying phages can then be screened
 against other proteins, e.g., tumor-specific antibodies, in order to detect interaction
 between the displayed protein and the tumor-specific antibodies. Once the tumor-
 specific protein or peptide is identified, the nucleic acid encoding the selected tumor-
 specific protein or peptide can be isolated from cells infected with the selected

VI. Blocking peptides

A blocking peptide may be attached a SIRP- α variant by way of a cleavable linker. In some embodiments, a blocking peptide may also be non-covalently attached to a SIRP- α variant. The blocking peptide acts to block the CD47 binding site of the SIRP- α variant such that the SIRP- α variant cannot bind to CD47 on the cell surface of non-diseased cells under physiological conditions (e.g., neutral pH and adequate oxygen concentration). Under abnormal conditions (i.e., an acidic and/or hypoxic environment or an environment with increased protease expression) at a diseased site, such as a cancer site, e.g., inside a tumor, the cleavable linker may be cleaved to release the SIRP- α variant from the blocking peptide. The SIRP- α variant would then be free to bind to CD47 on nearby tumor cells. Examples of cleavable linkers are described in detail further herein.

In some embodiments, the blocking peptide has higher affinity towards wild-type SIRP- α than engineered SIRP- α variant. Once the linker is cleaved, the blocking peptide dissociates from the SIRP- α variant and may bind to a wild-type SIRP- α . A blocking peptide with different binding affinities to wild-type SIRP- α and SIRP- α variant may be identified using methods and techniques commonly known in the art, e.g., directed evolution and display libraries (e.g., phage or yeast display). In one exemplary embodiment, a nucleotide encoding the SIRP- α binding region of CD47 or a nucleotide encoding the variable region of an anti-SIRP- α antibody may be mutated and/or recombined at random to create a large library of gene variants using techniques such as, e.g., error-prone PCR and DNA shuffling. Once a genetic library is created, the mutant peptides encoded by the nucleotides may be screened for their ability to bind to wild-type SIRP- α and SIRP- α variant using, e.g., phage or yeast display. Identified peptides that can bind to both wild-type SIRP- α and SIRP- α variant may undergo a second screening process such that the proteins that bind with higher affinity to wild-type SIRP- α than to SIRP- α variant may be isolated. The identified peptides, once bound to wild-type SIRP- α or SIRP- α variant should prevent the binding of CD47 to wild-type SIRP- α or SIRP- α variant. Various techniques known to those skilled in the art may be used to measure the affinity constant (K_A) or dissociation constant (K_D) of a SIRP- α variant/blocking peptide complex or a wild-type SIRP- α /blocking peptide complex. A blocking peptide may bind with at least three fold higher affinity to a wild-type SIRP- α than a SIRP- α variant.

CD47-based blocking peptides

A blocking peptide may be a CD47 mimic polypeptide, or a CD47 fragment that can bind a SIRP- α variant described herein. Some blocking peptides may bind a SIRP- α variant at a site that is different from the CD47 binding site. Some blocking
5 peptides may bind a SIRP- α variant in a manner that is different from CD47. In some cases, the blocking peptide may comprise at least one stabilizing disulfide bond. A blocking peptide may comprise a polypeptide sequence of CERVIGTGWRC, or a fragment or variant thereof. A variant blocking peptide may contain one or more conservative or non-conservative modification. In some cases a
10 variant blocking peptide may contain modifications of a cysteine to a serine and/or one or more modifications of an asparagine to a glutamine. A blocking peptide may bind to the SIRP- α variant at the same site as a peptide that comprises a polypeptide sequence of CERVIGTGWRC, or a variant or fragment thereof. A blocking peptide may comprise a polypeptide sequence of GNYTCEVTELTREGETIIEELK, or a
15 fragment or variant thereof. A blocking peptide may bind to the SIRP- α variant at the same site as a peptide that comprises a polypeptide sequence of GNYTCEVTELTREGETIIEELK, or a variant or fragment thereof. In some cases a blocking peptide may comprise a polypeptide sequence of EVTELTREGE, or a fragment or variant thereof. A blocking peptide may bind to the SIRP- α variant at the
20 same site as a peptide that comprises a polypeptide sequence of EVTELTREGE, or a variant or fragment thereof. In some cases a blocking peptide may comprise a polypeptide sequence of CEVTELTREGE, or a fragment or variant thereof. A blocking peptide may bind to the SIRP- α variant at the same site as a peptide that comprises a polypeptide sequence of CEVTELTREGE, or a variant or fragment
25 thereof.

Provided herein are SIRP- α variant constructs comprising a SIRP- α variant and a blocking peptide, wherein the blocking peptide may comprise a polypeptide sequence of SEVTELTREGET, or a fragment or variant thereof. A blocking peptide may bind to the SIRP- α variant at the same site as a peptide that comprises a
30 polypeptide sequence of SEVTELTREGET, or a variant or fragment thereof. In some cases, the blocking peptide may comprise a polypeptide sequence of GQYTSEVTELTREGETIIEELK, or a fragment or variant thereof. A blocking peptide may bind to the SIRP- α variant at the same site as a peptide that comprises a

polypeptide sequence of GQYTSEVTELTREGETIIEELK, or a variant or fragment thereof.

In some cases, the blocking peptide may be a CD47 variant polypeptide, that exhibits a higher affinity for wild-type SIRP- α , as compared to the SIRP- α variant. As compared to wild-type CD47, the blocking polypeptide may comprise at least one of the following mutations: T102Q, T102H, L101Q, L101H, and L101Y. As compared to wild-type CD47, the blocking polypeptide may comprise an introduction of an additional glycine or any other amino acid residue at or near the N-terminus. The additional amino acid may be introduced adjacent to a glutamine and/or a leucine at or near the N-terminus of CD47. In some cases, a blocking peptide may be a CD47 variant polypeptide that demonstrates a lower affinity for a SIRP- α variant as compared to a wild-type CD47. Such CD47 variant polypeptides are easily identified and tested using methods described herein.

Provided herein are SIRP- α variant constructs comprising a SIRP- α variant described herein, wherein said SIRP- α variant is connected to a blocking peptide described herein by use of at least one linker. The SIRP- α variant may comprise the same CD47 binding site as a wild type SIRP- α . The SIRP- α variant may comprise one or more mutations, or insertions as compared to a wild type SIRP- α . The SIRP- α variant may be a truncated form of the wild type SIRP- α . The blocking peptide maybe a CD47 mimic, variant, or fragment described herein. The blocking peptide may exhibit a higher affinity for wild-type SIRP- α , as compared to the SIRP- α variant in the SIRP- α variant construct. The blocking peptide may be a CD47 variant polypeptide that demonstrates a lower affinity for a SIRP- α variant as compared to wild-type CD47. The linker may be at least one linker that is optionally cleavable by one or more proteases, and optionally also comprises one or more spacers. The cleavable linker may comprise the sequence LSGRSDNH. The spacers may comprise one or more units of glycine-serine spacers, each unit of which may comprise the sequence GGGGS.

In some embodiments, the blocking peptide that is attached to a SIRP- α variant by way of a cleavable linker is a SIRP- α -binding peptide derived from CD47 (i.e., a CD47-based blocking peptide). In some embodiments, the CD47-based blocking peptide is derived from the SIRP- α binding portion of CD47. The SIRP- α binding portion of CD47 is often referred to as the immunoglobulin superfamily

(IgSF) domain of CD47, the sequence of which is shown below (SEQ ID NO: 35; Ref. NP_0017681).

SEQ ID NO: 35: wild-type, IgSF domain of human CD47

5 1-50 QLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW
 KFKGRDIYTF
 51-100 DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH
 TGNYTCEVTE
 101-123 LTREGETIIE LKYRVVSWFS PNE

10

 In some embodiments, the CD47-based blocking peptide contains the full-length, IgSF domain of CD47 (SEQ ID NO: 35) or a fragment thereof. In some
embodiments, the CD47-based blocking peptide contains one or more amino acid
substitutions, deletions, and/or additions relative to the wild-type, IgSF domain of
15 CD47 (SEQ ID NO: 35) or a fragment thereof. In some embodiments, a CD47-
based blocking peptide has at least 80% (e.g., 83%, 86%, 90%, 93%, 96%, etc)
amino acid sequence identity to the sequence of the wild-type, IgSF domain of CD47
(SEQ ID NO: 35) or a fragment thereof.

 In some embodiments, the amino acid substitutions, deletions, and/or
20 additions in the CD47-based blocking peptide results in the CD47-based blocking
peptide having low binding affinity for a SIRP- α variant and relatively higher binding
affinity for the wild-type SIRP- α . In some embodiments, the amino acid substitutions
in the CD47-based blocking peptide are located at the interface of CD47 and SIRP-
 α . For example, amino acid substitution T102Q in the CD47 IgSF domain sterically
25 clashes with amino acid substitution A27I in a SIRP- α variant, while a wild-type
SIRP- α having A27 would not sterically clash with the amino acid substitution T102Q
(see FIG. 2). Thus, a CD47-based blocking peptide having T102Q would bind with
higher affinity to a wild-type SIRP- α having A27 than to a SIRP- α variant having
A27I. Examples of amino acid substitutions in a CD47-based blocking peptide that
30 may create steric clashes with specific amino acids in a SIRP- α variant are listed in
Table 5. Each of these amino acid substitutions in a CD47-based blocking peptide
may reduce the binding affinity of the CD47-based blocking peptide to a SIRP- α
variant, depending on the specific amino acid in the SIRP- α variant at the SIRP- α -
CD47 interaction site.

Table 5: Examples of amino acid substitutions in a CD47-based blocking peptide that may create steric clashes with specific amino acids in a SIRP- α variant

Amino acid substitution in a CD47-based blocking peptide (amino acid numbering is relative to SEQ ID NO: 35)	Amino acid in a SIRP- α variant (amino acid numbering is relative to any one of SEQ ID NOs: 13-23)
T102Q	27I
T102H	27I
L101Q	31F
L101H	31F
L101Y	31F

In addition to creating steric clashes between a CD47-based blocking peptide and a SIRP- α variant, amino acid substitutions, additions, and/or deletions can also be used to break specific non-covalent interactions between a CD47-based blocking peptide and a SIRP- α variant, thus, reducing the binding affinity of the CD47-based blocking peptide to the SIRP- α variant. In some embodiments, extending the N-terminus of the CD47-based blocking peptide by one or more amino acids (e.g., one amino acid), either by adding the one or more amino acids directly to the N-terminus and/or by inserting the one or more amino acids between other amino acids at the N-terminus, breaks non-covalent interactions (e.g., hydrogen bonding interactions) between the N-terminus of the CD47-based blocking peptide and a SIRP- α variant. For example, an amino acid addition, e.g., a glycine addition, at the N-terminus of the CD47-based blocking peptide will prevent cyclization of glutamine to pyroglutamate at the N-terminus and also create unwanted contacts and interactions that will likely will disrupt the hydrogen bonding interactions between the N-terminal pyroglutamate of the CD47-based blocking peptide and the amino acid L66 in a wild-type SIRP- α or amino acid substitution L66T in a SIRP- α variant (see also Example 5). In some embodiments, an amino acid residue, e.g., glycine, is added at the N-terminus of the CD47-based blocking peptide such that the N-terminus of CD47 is changed from QLLFNK to GQLLFNK or QQLLFNK. The choice of the amino acid substitutions, deletions, and/or additions in a CD47-based blocking peptide would depend on the specific amino acid substitutions in a SIRP- α variant.

Furthermore, fusing the N-terminus of the CD47-based blocking peptide to the C-terminus of a SIRP- α variant through a cleavable linker and optionally one or more spacers also affects the binding interactions between the CD47-based blocking

peptide and the SIRP- α variant and reduces the binding affinity of the CD47-based blocking peptide to the SIRP- α variant. In some embodiments, in a SIRP- α variant construct, the N-terminus of a CD47-based blocking peptide is fused to the C-terminus of a SIRP- α variant by way of a cleavable linker and optionally one or more spacers. In some embodiments, in a SIRP- α variant construct, the C-terminus of a CD47-based blocking peptide is fused to the N-terminus of a SIRP- α variant by way of a cleavable linker and optionally one or more spacers. Examples of cleavable linkers and spacers are described in detail further herein.

Exemplary CD47-based blocking peptides are shown in Table 6. In some embodiments, the CD47-based blocking peptide has or includes the sequence SEVTELTREGET (SEQ ID NO: 38). In some embodiments, the CD47-based blocking peptide has or includes the sequence GQYTSEVTELTREGETIIE LK (SEQ ID NO: 40).

Table 6

SEQ ID NO	CD47-based blocking peptides	Portion of the CD47 IgSF domain
36	EVTELTREGE	Amino acids 97-106 of SEQ ID NO: 35
37	CEVTELTREGE C	Amino acids 96-107 of SEQ ID NO: 35 with T125C
38	SEVTELTREGET	Amino acids 96-107 of SEQ ID NO: 35 with C96S
39	GNYTCEVTELTREGETIIE LK	Amino acids 92-112 of SEQ ID NO: 35
40	GQYTSEVTELTREGETIIE LK	Amino acids 92-112 of SEQ ID NO: 35 with N93Q and C96S
41	QLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW KFKGRDIYTF DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH TGNYTCEVTE QTREGETIIE LKYRVVSWFS PNE	CD47 IgSF domain with L101Q

SEQ ID NO	CD47-based blocking peptides	Portion of the CD47 IgSF domain
42	QLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW KFKGRDIYTF DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH TGNYTCEVTE YTREGETIIE LKYRVVSWFS PNE	CD47 IgSF domain with L101Y
43	QLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW KFKGRDIYTF DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH TGNYTCEVTE HTREGETIIE LKYRVVSWFS PNE	CD47 IgSF domain with L101H
44	QLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW KFKGRDIYTF DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH TGNYTCEVTE LQREGETIIE LKYRVVSWFS PNE	CD47 IgSF domain with T102Q
45	QLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW KFKGRDIYTF DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH TGNYTCEVTE LHREGETIIE LKYRVVSWFS PNE	CD47 IgSF domain with T102H
46	GQLLFNKTKSV EFTFCNDTVV IPCFVTNMEA QNTTEVYVKW KFKGRDIYTF DGALNKSTVP TDFSSAKIEV SQLLKGDASL KMDKSDAVSH TGNYTCEVTE LTREGETIIE LKYRVVSWFS PNE	CD47 IgSF domain with N-terminal glycine addition

VII. Cleavable linkers

In some embodiments, a SIRP- α variant construct includes a SIRP- α variant attached to a blocking peptide. In some embodiments, a SIRP- α variant construct includes a wild-type SIRP- α attached to a blocking peptide. A linker used to fuse a SIRP- α variant or a wild-type SIRP- α and a blocking peptide can be a cleavable linker or a non-cleavable linker. In some embodiments, the preferential binding of the SIRP- α variant in the SIRP- α variant construct to CD47 on diseased cells or diseased sites may be obtained by attaching the block peptide to the SIRP- α variant by use of a cleavable linker, which is cleaved at the diseased cells or diseased sites.

In some embodiments, a cleavable linker is used between a SIRP- α variant and a blocking peptide. In some embodiments, a cleavable linker may be installed within a blocking peptide, which may be non-covalently associated with the SIRP- α variant to block binding of the SIRP- α variant to CD47 under physiological conditions. A cleavable linker may be cleaved under certain conditions. If the cleavable linker is within a blocking peptide, cleavage of the linker would inactivate

the blocking peptide. Under conditions characteristic of a diseased site, such as a cancer site, e.g., inside a tumor, the linker is cleaved to release the SIRP- α variant from the blocking peptide such that the SIRP- α variant can bind to nearby CD47 on the cell surface of diseased cells, e.g., tumor cells. In this manner, in a SIRP- α construct that includes a SIRP- α variant and a blocking peptide, the SIRP- α variant can only bind to CD47 on diseased cells (e.g., tumor cells) or cells at a diseased site (e.g., cells in the tumor micro-environment supporting tumor growth), and is unable to bind to CD47 on non-diseased cells under physiological conditions, since the cleavable linker remains stable under physiological conditions and the CD47-binding site of the SIRP- α variant would be blocked by the blocking peptide. A cleavable linker may include amino acids, organic small molecules, or a combination of amino acids and organic small molecules that cleave or induce cleavage of the linker under conditions characteristic of a diseased site, such as acidic pH, hypoxia, and increased protease expression. Cleavable linkers are stable at physiological conditions (e.g., neutral pH and adequate oxygen concentration). In some embodiments, a cleavable linker may not be cleaved and the blocking peptide may simply dissociate from the SIRP- α variant at a diseased site such that the SIRP- α variant is free to bind to nearby CD47 on diseased cells, e.g., tumor cells. In these embodiments, the SIRP- α variants may be engineered to have pH-dependent binding to CD47, the details of which are described previously. The SIRP- α variants may be engineered to bind with high affinity to CD47 under acidic pH of a diseased site than under neutral pH (e.g., around pH 7.4) of a non-diseased site. Thus, the blocking peptide (e.g., a CD-47 based blocking peptide or a CD47 IgSF domain blocking protein) may dissociate away from the SIRP- α variant under the acidic pH of a diseased site. In some embodiments, to engineer pH-dependent binding of a SIRP- α variant to CD47 at a diseased site, histidine mutagenesis may be performed on the SIRP- α , especially on the region of SIRP- α that interacts with CD47.

pH-dependent cleavable linkers

One of the characteristics of a cancer site, e.g., inside a tumor, is acidic pH. In some embodiments, a linker may be cleaved under acidic pH (e.g., less than around pH 7). An acid-sensitive linker is stable at physiological pH (e.g., around pH 7.4). The cleavage at acidic pH may be through acid hydrolysis or by proteins present and active at acidic pH of a diseased site, such as a cancer site, e.g., inside

a tumor. Acid-sensitive linkers may include a moiety, such as a chemical functional group or compound, capable of being hydrolyzed under acidic pH. Acid-sensitive chemical functional groups and compounds include, but are not limited to, e.g., acetals, ketals, thiomaleamates, hydrazones, and disulfide bonds. Acid-sensitive linkers, as well as acid-sensitive chemical groups and compounds, which may be used in the construction of acid-sensitive linkers, are well known in the art and described in US Patent Nos. 8,748,399, 5,306,809, and 5,505,931, Laurent et al., (*Bioconjugate Chem.* 21:5–13, 2010), Castaneda et al. (*Chem. Commun.* 49:8187-8189, 2013), and Ducry et al. (*Bioconjug. Chem.* 21:5-13, 2010), each of which is incorporated by reference herein in its entirety. In one embodiment, a disulfide bond may be installed in a cleavable linker using a peptide synthesizer and/or conventional chemical synthesis techniques. In another embodiment, a thiomaleamic acid linker (Castaneda et al. *Chem. Commun.* 49:8187-8189, 2013) may be used as the cleavable linker. In this embodiment, to insert a thiomaleamic acid linker between a SIRP- α variant and a blocking peptide, one of the two thiol groups of the thiomaleamic acid linker (see, e.g., Scheme 2, Castaneda et al.) may be attached to the C-terminus of a SIRP- α variant, while the ester group of the thiomaleamic linker may be attached to the N-terminus of the blocking peptide. The contents of the referenced publications are incorporated herein by reference in their entireties.

Hypoxia-dependent cleavable linkers

In some embodiments, a linker may be cleaved under hypoxic condition, which is another characteristic of a cancer site, e.g., inside a tumor. A SIRP- α variant attached to a blocking peptide by way of a hypoxia-sensitive linker is prevented from binding to CD47 on non-diseased cells while the linker remains stable under physiological conditions (e.g., neutral pH and adequate oxygen concentration). Once the fusion protein is at the site of cancer, e.g., inside a tumor, where oxygen concentration is significantly lower than in healthy tissues, the linker is cleaved to release the SIRP- α variant from the blocking peptide, which can then bind to cell surface CD47 on tumor cells. The hypoxia-sensitive linker may include a moiety, e.g., an amino acid or a chemical functional group, capable of being cleaved under hypoxic condition. Some examples of chemical moieties that may be cleaved, i.e., cleaved through reduction, under hypoxic condition include, but are not limited

to, quinones, N-oxides, and heteroaromatic nitro groups. These chemical moieties may be installed in the cleavable linker using conventional chemical and peptide synthesis techniques. Examples of hypoxia-sensitive amino acids are also known in the art, such as those described by Shigenaga et al. (*European Journal of Chemical*
5 *Biology* 13:968–971, 2012), which is incorporated herein by reference in its entirety.

In a preferred embodiment, the hypoxia-sensitive amino acid described by Shigenaga et al. (*European J. Chem, Biol.* 13:968–971, 2012) may be inserted between a SIRP- α variant and a blocking peptide. For example, the amino group of the hypoxia-sensitive amino acid may be attached to the C-terminus of the SIRP- α
10 variant through a peptide bond, and similarly, the carboxylic acid group of the hypoxia-sensitive amino acid may be attached to the N-terminus of the blocking peptide through a peptide bond. Under hypoxic condition, the reduction of the nitro group induces the cleavage of the peptide bond between the hypoxia-sensitive amino acid and the N-terminus of the blocking peptide, thus, successfully releasing
15 the SIRP- α variant from the blocking peptide. The SIRP- α variant can then bind to CD47 on tumor cells.

In another embodiment, the hypoxia-sensitive 2-nitroimidazole group described by Duan et al. (*J. Med. Chem.* 51:2412–2420, 2008) may be inserted between a SIRP- α variant and a blocking peptide or installed in a cleavable linker
20 inserted between a SIRP- α variant and a blocking peptide. Under hypoxic condition, the reduction of the nitro group induces further reduction, which eventually leads to elimination of the 2-nitroimidazole group from its attachment, e.g., the SIRP-g variant, the blocking peptide, or the cleavable linker.

25 *Tumor-associated enzyme-dependent cleavable linkers*

In other embodiments, a SIRP- α variant construct may include a SIRP- α variant attached to a blocking peptide by way of a linker (e.g., a cleavable linker) and optionally one or more spacers (examples of spacers are described in detail further herein). In some embodiments, the linker (e.g., a cleavable linker) may be cleaved
30 by a tumor-associated enzyme. In some embodiments, a linker, which can be cleaved by a tumor-associated enzyme, may be contained within a blocking peptide, which may be non-covalently attached to a SIRP- α variant. Once the fusion protein is at the site of cancer, e.g., inside a tumor, the linker is cleaved by a tumor-associated enzyme to release the SIRP- α variant from the blocking peptide, which

can then bind to cell surface CD47 on tumor cells. A linker sensitive to a tumor-associated enzyme may contain a moiety, e.g., a protein substrate, capable of being specifically cleaved by an enzyme, e.g., a protease, that is only present at the cancer site, e.g., inside a tumor. The moiety may be selected based on the type of enzyme, e.g., a protease, present at the cancer site, e.g., inside a tumor. An exemplary cleavable linker that can be cleaved by a tumor-associated enzyme is LSGRSDNH (SEQ ID NO: 47), which can be cleaved by multiple proteases, e.g., matriptase (MTSP1), urinary-type plasminogen activator (uPA), legumain, PSA (also called KLK3, kallikrein-related peptidase-3), matrix metalloproteinase-2 (MMP-2), MMP9, human neutrophil elastase (HNE), and proteinase 3 (Pr3).. Other cleavable linkers that are susceptible to cleavage by enzymes (e.g., proteases) are also available. In addition to the aforementioned proteases, other enzymes (e.g., proteases) that can cleave a cleavable linker include, but are not limited to, urokinase, tissue plasminogen activator, trypsin, plasmin, the cathepsin protease family and another enzyme having proteolytic activities. According to some embodiments of the present invention, a SIRP- α variant or a wild-type SIRP- α is attached to a blocking peptide by way of a linker (e.g., a cleavable linker) susceptible to cleavage by enzymes having proteolytic activities, such as a urokinase, a tissue plasminogen activator, plasmin, or trypsin.

In some embodiments, sequences of cleavable linkers can be derived and selected by putting together several sequences based on different enzyme preferences. Non-limited examples of several potential proteases and their corresponding protease sites are shown in Table 7. In Table 7, “-” means any amino acid (i.e., any naturally occurring amino acid), capital case indicates an strong preference for that amino acid, lower case indicates a minor preference for that amino acid, and “/” separates amino acid positions in cases where more than one amino acid at a position adjacent to the “/” is possible. Other cleavable sequences include, but are not limited to, a sequence from a human liver collagen (α 1(III) chain (e.g., GPLGIAGI (SEQ ID NO: 100))), a sequence from a human PZP (e.g., YGAGLGVV (SEQ ID NO: 101), AGLGVVER (SEQ ID NO: 102), or AGLGISST (SEQ ID NO: 103)), and other sequences that are autolytic (e.g., VAQFVLTE (SEQ ID NO: 104), AQFVLTEG (SEQ ID NO: 105), or PVQPIGPQ (SEQ ID NO: 106)).

Table 7

Protease	Potential protease sites
uPA:	L/S/G-/R-/S-/D/N/H (SEQ ID NO: 69); -/s/gs/Rk-/rv/-/-/- (SEQ ID NO: 70); SGR-SA (SEQ ID NO: 71)
Matriptase:	L/S/G-/R-/S-/D/N/H (SEQ ID NO: 72); r/-/-/Rk-/v/-/-/g/- (SEQ ID NO: 73); RQAR-VV (SEQ ID NO: 74); r/-/-/Rk/v/-/-/g (SEQ ID NO: 75); /Kr/RKQ/gAS/RK/A (SEQ ID NO: 76)
Legumain:	L/S/G-/R-/S-/D/N/H (SEQ ID NO: 77); ---/-/-/N/-/-/- (SEQ ID NO: 78); AAN-L (SEQ ID NO: 79); ATN-L (SEQ ID NO: 80)
PSA	si/sq/-/yqr s/s/-/- (SEQ ID NO: 81); S/S/K/L/Q (SEQ ID NO: 82)
MMP2	-/p/-/- /li/-/-/- (SEQ ID NO: 83)
MMP9	g/pa/-/gl/-/g/- (SEQ ID NO: 84); G/P/L/G/I/A/G/Q (SEQ ID NO: 85); P/V/G/L/I/G (SEQ ID NO: 86); H/P/V/G/L/L/A/R (SEQ ID NO: 87)
HNE	-/-/-/viat/-/-/- (SEQ ID NO: 88)
Pr3	-/y/y/vta -/-/-/- (SEQ ID NO: 89)
Pro-urokinase	PRFKIIGG (SEQ ID NO: 90); PRFRIIGG (SEQ ID NO: 91)
TGFβ	SSRHRRALD (SEQ ID NO: 92)
Plasminogen	RKSSIIIRMRDVVL (SEQ ID NO: 93)
Staphylokinase	SSSFDKGKYKKGDDA (SEQ ID NO: 94); SSSFDKGKYKRGDDA (SEQ ID NO: 95)
Factor Xa	IEGR; IDGR (SEQ ID NO: 96); GGSIDGR (SEQ ID NO: 97)
Gelatinase	PLGLWA (SEQ ID NO: 98)
Human fibroblast collagenase	DVAQFVLT (SEQ ID NO: 99)

There are reports in the literature of increased levels of enzymes having known substrates in various types of cancers, e.g., solid tumors. See, e.g., La
5 Rocca et al., *Brit. J. Cancer* 90:1414-1421 and Ducry et al., *Bioconjug. Chem.* 21:5-13, 2010, each of which is incorporated by reference herein in its entirety. Tumor-associated enzymes may also be identified using conventional techniques known in the art, e.g., immunohistochemistry of tumor cells. In one exemplary embodiment, the enzyme-sensitive moiety in a linker may be a matrix metalloproteinase (MMP)

substrate, which may be cleaved by an MMP present at the cancer site, e.g., inside a tumor. In another exemplary embodiment, the enzyme-sensitive moiety in a linker may be a maleimido-containing dipeptide linker (see, e.g., Table 1 in Ducry et al.), which may be cleaved through proteolysis by proteases (e.g., cathepsin or plasmin) present at elevated levels in certain tumors (Koblinski et al., *Chim. Acta* 291:113–135, 2000). In this embodiment, the maleimide group of the maleimido-containing dipeptide linker may be conjugated to a cysteine residue of the SIRP- α variant and the carboxylic acid group at the C-terminus of the maleimido-containing dipeptide linker may be conjugated to the amino group at the N-terminus of the blocking peptide. Similarly, the maleimide group of the maleimido-containing dipeptide linker may be conjugated to a cysteine residue of the blocking peptide and the carboxylic acid group at the C-terminus of the maleimido-containing dipeptide linker may be conjugated to the amino group at the N-terminus of the SIRP- α variant. Mass-spectrometry and other available techniques in the field of proteomics may be used to confirm the cleavage of the tumor-associated enzyme-dependent cleavable linkers. Other enzyme-sensitive moieties are described in US Patent No. 8,399,219, which is incorporated by reference herein in its entirety. In some embodiments, the moiety sensitive to a tumor-associated enzyme, e.g., a protein substrate, may be inserted between a SIRP- α variant and a blocking peptide using conventional molecule cell biology and chemical conjugation techniques well known in the art.

Peptide linkers which are susceptible to cleavage by enzymes of the complement system, such as but not limited to urokinase, tissue plasminogen activator, trypsin, plasmin, or another enzyme having proteolytic activity may be used herein. According to one method of the present invention, a polypeptide is attached to a masking peptide via a linker susceptible to cleavage by enzymes having a proteolytic activity such as a urokinase, a tissue plasminogen activator, plasmin, or trypsin.

VIII. Serum albumin

Fusion to serum albumins can improve the pharmacokinetics of protein pharmaceuticals, and in particular, a SIRP- α variant described here may be joined with a serum albumin. Serum albumin is a globular protein that is the most abundant blood protein in mammals. Serum albumin is produced in the liver and constitutes about half of the blood serum proteins. It is monomeric and soluble in the blood.

Some of the most crucial functions of serum albumin include transporting hormones, fatty acids, and other proteins in the body, buffering pH, and maintaining osmotic pressure needed for proper distribution of bodily fluids between blood vessels and body tissues. In some embodiments, a SIRP- α variant may be fused to a serum

5 albumin. In preferred embodiments, serum albumin is human serum albumin (HSA). In some embodiments of the present invention, the N-terminus of an HSA is joined to the C-terminus of the SIRP- α variant to increase the serum half-life of the SIRP- α variant. An HSA can be joined, either directly or through a linker, to the C-terminus of the SIRP- α variant. Joining the N-terminus of an HSA to the C-terminus of the

10 SIRP- α variant keeps the N-terminus of the SIRP- α variant free to interact with CD47 and the proximal end of the C-terminus of the HSA to interact with FcRn. An HSA that can be used in the methods and compositions described here are generally known in the art. In some embodiments, the HSA includes amino acids 25-609 (SEQ ID NO: 67) of the sequence of UniProt ID NO: P02768. In some

15 embodiments, the HSA includes one or more amino acid substitutions (e.g., C34S and/or K573P), relative to SEQ ID NO: 67. In some embodiments, the HSA has the sequence of SEQ ID NO: 68.

IX. Albumin-binding peptides

Binding to serum proteins can improve the pharmacokinetics of protein

20 pharmaceuticals, and in particular the SIRP- α variants described here may be fused with serum protein-binding peptides or proteins. In some embodiments, a SIRP- α variant may be fused to an albumin-binding peptide that displays binding activity to serum albumin to increase the half-life of the SIRP- α variant. Albumin-binding peptides that can be used in the methods and compositions described here are

25 generally known in the art. See, e.g., Dennis et al., *J. Biol. Chem.* 277:35035-35043, 2002 and Miyakawa et al., *J. Pharm. Sci.* 102:3110-3118, 2013. In one embodiment, the albumin binding peptide includes the sequence DICLPRWGCLW (SEQ ID NO: 2). An albumin-binding peptide can be fused genetically to a SIRP- α variant or attached to a SIRP- α variant through chemical means, e.g., chemical conjugation. If

30 desired, a spacer can be inserted between the SIRP- α variant and the albumin-binding peptide to allow for additional structural and spatial flexibility of the fusion protein. Specific spacers and their amino acid sequences are described in detail further herein. In some embodiments, an albumin-binding peptide may be fused to the N- or C-terminus of a SIRP- α variant. In one example, the C-terminus of the

albumin-binding peptide may be directly fused to the N-terminus of the SIRP- α variant through a peptide bond. In another example, the N-terminus of the albumin-binding peptide may be directly fused to the C-terminus of the SIRP- α variant through a peptide bond. In yet another example, the carboxylic acid at the C-terminus of the albumin-binding peptide may be fused to an internal amino acid residue, i.e., the side-chain amino group of a lysine residue of the SIRP- α variant using conventional chemical conjugation techniques. Without being bound to a theory, it is expected that fusion of an albumin-binding peptide to a SIRP- α variant may lead to prolonged retention of the therapeutic protein through its binding to serum albumin.

X. Fc domains

In some embodiments, a SIRP- α variant construct may include a SIRP- α variant and an Fc domain monomer. In some embodiments, a SIRP- α variant may be fused to an Fc domain monomer of an immunoglobulin or a fragment of an Fc domain monomer. As conventionally known in the art, an Fc domain is the protein structure that is found at the C-terminus of an immunoglobulin. An Fc domain includes two Fc domain monomers that are dimerized by the interaction between the C_H3 antibody constant domains. A wild-type Fc domain forms the minimum structure that binds to an Fc receptor, e.g., Fc γ RI, Fc γ RIIa, Fc γ RIIb, Fc γ RIIIa, Fc γ RIIIb, Fc γ RIV. In the present invention, an Fc domain monomer or a fragment of an Fc domain fused to a SIRP- α variant to increase serum half-life of the SIRP- α variant may include a dimer of two Fc domain monomers or an Fc domain monomer, provided that the Fc domain monomer can bind to the Fc receptor (e.g., an FcRn receptor). Furthermore, an Fc domain or a fragment of the Fc domain fused to a SIRP- α variant to increase serum half-life of the SIRP- α variant does not induce any immune system-related response. In some embodiments, an Fc domain may be mutated to lack effector functions, typical of a “dead” Fc domain. For example, an Fc domain may include specific amino acid substitutions that are known to minimize the interaction between the Fc domain and an Fc γ receptor. In some embodiments, an Fc domain monomer or a fragment of the Fc domain may be fused to the N- or C-terminus of a SIRP- α variant through conventional genetic or chemical means, e.g., chemical conjugation. If desired, a linker (e.g., a spacer) can be inserted between the SIRP- α variant and the Fc domain monomer.

Heterodimerization of Fc domain monomers

In some embodiments, each of the two Fc domain monomers in an Fc domain includes amino acid substitutions that promote the heterodimerization of the two monomers. Heterodimerization of Fc domain monomers can be promoted by introducing different, but compatible, substitutions in the two Fc domain monomers, such as “knob-into-hole” residue pairs and charge residue pairs. The use of “knob-into-hole” residue pairs is described by Carter and co-workers (Ridgway et al., *Protein Eng.* 9:617-612, 1996; Atwell et al., *J Mol Biol.* 270:26-35, 1997; Merchant et al., *Nat Biotechnol.* 16:677-681, 1998). The knob and hole interaction favors heterodimer formation, whereas the knob-knob and the hole-hole interaction hinder homodimer formation due to steric clash and deletion of favorable interactions. The “knob-into-hole” technique is also disclosed in U.S. Patent Application Publication No. 8,216,805, Merchant et al., *Nature Biotechnology* 16:677-681, 1998, and Merchant et al., *Proc Natl Acad Sci U S A.* 110:E2987–E2996, 2013, each of which is incorporated herein by reference in its entirety. A hole is a void that is created when an original amino acid in a protein is replaced with a different amino acid having a smaller side-chain volume. A knob is a bump that is created when an original amino acid in a protein is replaced with a different amino acid having a larger side-chain volume. Specifically, the amino acid being replaced is in the C_H3 antibody constant domain of an Fc domain monomer and is involved in the dimerization of two Fc domain monomers. In some embodiments, a hole in one C_H3 antibody constant domain is created to accommodate a knob in another C_H3 antibody constant domain, such that the knob and hole amino acids act to promote or favor the heterodimerization of the two Fc domain monomers. In some embodiments, a hole in one C_H3 antibody constant domain is created to better accommodate an original amino acid in another C_H3 antibody constant domain. In some embodiments, a knob in one C_H3 antibody constant domain is created to form additional interactions with original amino acids in another C_H3 antibody constant domain.

A hole can be constructed by replacing amino acids having larger side chains such as tyrosine or tryptophan with amino acids having smaller side chains such as alanine, valine, or threonine, such as Y407V mutation in the C_H3 antibody constant domain. Similarly, a knob can be constructed by replacing amino acids having

smaller side chains with amino acids having larger side chains, such as T366W mutation in the C_H3 antibody constant domain. In a preferred embodiment, one Fc domain monomer includes the knob mutation T366W and the other Fc domain monomer includes hole mutations T366S, L358A, and Y407V. A SIRP- α D1 variant of the invention may be fused to an Fc domain monomer including the knob mutation T366W to limit unwanted knob-knob homodimer formation. Examples of knob-into-hole amino acid pairs are included, without limitation, in Table 8.

Table 8

Fc domain monomer 1	Y407T	Y407A	F405A	T394S	T366S L358A Y407V	T394W Y407T	T394S Y407A	T366W T394S
Fc domain monomer 2	T366Y	T366W	T394W	F405W	T366W	T366Y F405A	T366W F405W	F405W Y407A

In addition to the knob-into-hole strategy, electrostatic steering strategy may also be used to control the dimerization of Fc domain monomers. Electrostatic steering is the utilization of favorable electrostatic interactions between oppositely charged amino acids in peptides, protein domains, and proteins to control the formation of higher ordered protein molecules. In particular, to control the dimerization of Fc domain monomers using electrostatic steering, one or more amino acid residues that make up the C_H3-C_H3 interface are replaced with positively- or negatively-charged amino acid residues such that the interaction becomes electrostatically favorable or unfavorable depending on the specific charged amino acids introduced. In some embodiments, a positively-charged amino acid in the interface, such as lysine, arginine, or histidine, is replaced with a negatively-charged amino acid such as aspartic acid or glutamic acid. In some embodiments, a negatively-charged amino acid in the interface is replaced with a positively-charged amino acid. The charged amino acids may be introduced to one of the interacting C_H3 antibody constant domains, or both. Introducing charged amino acids to the interacting C_H3 antibody constant domains of the two Fc domain monomers can promote the selective formation of heterodimers of Fc domain monomers as controlled by the electrostatic steering effects resulting from the interaction between

charged amino acids. The electrostatic steering technique is also disclosed in U.S. Patent Application Publication No. 20140024111, Gunasekaran et al., *J Biol Chem.* 285:19637-46, 2010, and Martens et al., *Clin Cancer Res.* 12:6144-52, 2006, each of which is incorporated herein by reference in its entirety. Examples of electrostatic steering amino acid pairs are included, without limitation, in Table 9.

Table 9

Fc domain monomer 1	K409 D	K409 D	K409 E	K409 E	K392 D	K392 D	K392 E	K392 E	K409 D K392 D	K370 E K409 D K439 E
Fc domain monomer 2	D399 K	D399 R	D399 K	D399 R	D399 K	D399 R	D399 K	D399 R	D399 K D356 K	D356 K E357 K D399 K

XI. Polyethylene glycol (PEG) polymer

In some embodiments, a SIRP- α variant may also be fused to a polymer, e.g., polyethylene glycol (PEG). The attachment of a polymer to a protein pharmaceutical can “mask” the protein pharmaceutical from the host’s immune system (Milla et al., *Curr Drug Metab.* 13:105-119, 2012). In addition, certain polymers, e.g., hydrophilic polymers, can also provide water solubility to hydrophobic proteins and drugs (Gregoriadis et al., *Cell Mol. Life Sci.* 57:1964-1969, 2000; Constantinou et al., *Bioconjug. Chem.* 19:643-650, 2008). Various polymers, such as PEG, polysialic acid chain (Constantinou et al., *Bioconjug. Chem.* 19:643-650, 2008), and PAS chain (Schlapschy et al., *Protein Eng. Des. Sel.* 26:489-501, 2013), are known in the art and can be used in the present invention. In some embodiments, a polymer, e.g., PEG, may be covalently attached to a SIRP- α variant, either at the N- or C-terminus or at an internal location, using conventional chemical methods, e.g., chemical conjugation. In some embodiments, a polymer, e.g., PEG, may be covalently attached to a cysteine substitution or addition in the SIRP- α variant. The cysteine substitution in the SIRP- α variant may be I7C, A16C, S20C, T20C, A45C, G45C, G79C, S79C, or A84C, relative to the sequence of any one of SEQ ID NOs: 13-23. The addition of a cysteine residue in the SIRP- α variant may be introduced using

conventional techniques in the art, e.g., peptide synthesis, genetic modification, and/or molecular cloning. The polymer, e.g., PEG, may be attached to the cysteine residue using cysteine-maleimide conjugation well-known to one of skill in the art. The contents of the referenced publications are incorporated herein by reference in
5 their entireties.

In addition to the embodiments described above, other half-life extension technologies are also available and may be used in the present invention to increase the serum half-life of SIRP- α variants. Half-life extension technologies include, but are not limited to, and EXTEN (Schellenberger et al., *Nat. Biotechnol.* 27:1186-1192,
10 2009) and Albu tag (Trussel et al., *Bioconjug Chem.* 20:2286-2292, 2009). The contents of the referenced publications are incorporated herein by reference in their entireties.

XII. Spacers

15 In some embodiments, spacers may be used in the SIRP- α variant construct. For examples, a SIRP- α variant construct may include a SIRP- α variant attached to a blocking peptide by way of a linker (e.g., a cleavable linker). In such SIRP- α constructs, a spacer may be inserted between the SIRP- α variant and the linker (e.g., a cleavable linker), and/or between the linker (e.g., a cleavable linker) and the
20 blocking peptide. To optimized the spacing between the SIRP- α variant and the linker, and/or the spacing between the linker and the blocking peptide, any one or more of the spacers described below may be used.

In some embodiments of a SIRP- α variant construct including a SIRP- α variant attached to a blocking peptide by way of a linker (e.g., a cleavable linker), the
25 spacer serves to position the cleavable linker away from the core of the SIRP- α variant and the blocking peptide such that the cleavable linker is more accessible to the enzyme responsible for cleavage. It should be understood that the attachment of two elements in a SIRP- α variant construct, for example, a SIRP- α variant and a linker (e.g., a cleavable linker) in a SIRP- α variant construct including (e.g., in this
30 order) a SIRP- α variant, a linker, and a blocking peptide, need not be of particular mode of attachment or through a particular reaction. Any reaction providing a SIRP- α variant construct of suitable stability and biological compatibility is acceptable.

A spacer refers to a linkage between two elements in a SIRP- α variant construct, e.g., a SIRP- α variant and a linker (e.g., a cleavable linker) in a SIRP- α variant construct including (e.g., in this order) a SIRP- α variant, a linker, and a blocking peptide, a linker (e.g., a cleavable linker) and a blocking peptide in a SIRP- α variant construct including (e.g., in this order) a SIRP- α variant, a linker, and a blocking peptide, a SIRP- α variant and a serum protein-binding peptide or protein, e.g., an albumin-binding peptide. A spacer may also refer to a linkage that can be inserted between a SIRP- α variant or a wild-type SIRP- α and an antibody, e.g., a tumor-specific antibody, or an antibody-binding peptide. A spacer can provide additional structural and/or spatial flexibility of the SIRP- α variant construct. A spacer can be a simple chemical bond, e.g., an amide bond, a small, organic molecule (e.g., a hydrocarbon chain), an amino acid sequence (e.g., a 3-200 amino acid sequence), or a combination of a small, organic molecule (e.g., a hydrocarbon chain) and an amino acid sequence (e.g., a 3-200 amino acid sequence). A spacer is stable under physiological conditions (e.g., neutral pH and adequate oxygen concentration) as well as under conditions characteristic of a diseased site, e.g., acidic pH and hypoxia. A spacer is stable at a diseased site, such as a cancer site, e.g., inside a tumor.

A spacer may include 3-200 amino acids. Suitable peptide spacers are known in the art, and include, for example, peptide linkers containing flexible amino acid residues, such as glycine and serine. In certain embodiments, a spacer can contain motifs, e.g., multiple or repeating motifs, of GS, GGS, GGGS, GGSG, or SGGG. In certain embodiments, a spacer can contain 2 to 12 amino acids including motifs of GS, e.g., GS, GSGS, GSGSGS, GSGSGSGS, GSGSGSGSGS, or GSGSGSGSGSGS. In certain other embodiments, a spacer can contain 3 to 12 amino acids including motifs of GGS, e.g., GGS, GGSGGS, GGSGGSGGS, and GGSGGSGGSGGS. In yet other embodiments, a spacer can contain 4 to 12 amino acids including motifs of GGSG, e.g., GGSG, GGSGGGSG, or GGSGGGSGGGSG. In other embodiments, a spacer can contain motifs of (GGGS)_n, wherein n is an integer from 1 to 10. In other embodiments, a spacer can also contain amino acids other than glycine and serine, e.g., GENLYFQSGG, SACYCELS, RSIAT, RPACKIPNDLKQKVMNH, GGSAGGSGSGSSGGSSGASGTGTAGGTGSGSGTGSG, AAANSSIDLISVPVDSR, or GGSGGGSEGGGSEGGGSEGGGSEGGGSEGGGSGGGS. In some

embodiments in the present invention, one or more 12- or 20-amino acid peptide spacers may be used in a SIRP- α variant construct. The 12- and 20-amino acid peptide spacers may contain sequences GGGSGGGSGGGS and SGGGSGGGSGGGSGGGSGGG, respectively. In some embodiments, one or more 18-amino acid peptide spacers containing sequence GGS GGSGGGSGGGSGGGSGGS may be used in a SIRP- α variant construct.

In some embodiments, a spacer may also have the general structure:

$\xi\text{-W-(CH}_2\text{)}_n\text{-Q-}\xi$, wherein W is NH or CH₂, Q is an amino acid or a peptide, and n is an integer from 0 to 20.

XIII. Fusion of a blocking peptide to a SIRP- α variant

A blocking peptide (e.g., a CD47-based blocking peptide having a sequence of any one of SEQ ID NOs: 36-46 in Table 6) may be fused to the N- or C-terminus of a SIRP- α variant by way of a linker, e.g., a cleavable linker (e.g., LSGRSDNH (SEQ ID NO: 47)), and optionally one or more spacers (e.g., (GGGGS)_n, fused genetically to either N- or C-terminus of the linker, wherein n is an integer from 1 to 10). Exemplary sequences of SIRP- α variant constructs including a CD47-based blocking peptide fused to a SIRP- α variant by way of a cleavable linker and one or more spacers are shown in sequences of SEQ ID NOs: 48-63. The length of the spacers may be changed to achieve the most optimized binding between the CD47-based blocking peptide and the SIRP- α variant.

XIV. Methods of producing SIRP- α variant constructs

The SIRP- α variant constructs of the invention can be produced from a host cell. A host cell refers to a vehicle that includes the necessary cellular components, e.g., organelles, needed to express the polypeptides and constructs described herein from their corresponding nucleic acids. The nucleic acids may be included in nucleic acid vectors that can be introduced into the host cell by conventional techniques known in the art (e.g., transformation, transfection, electroporation, calcium phosphate precipitation, direct microinjection, infection, etc). The choice of nucleic acid vectors depends in part on the host cells to be used. Generally,

preferred host cells are of either prokaryotic (e.g., bacterial) or eukaryotic (e.g., mammalian) origin.

Nucleic Acid Vector Construction and Host Cells

5 A polynucleotide sequence encoding the amino acid sequence of a SIRP- α variant construct may be prepared by a variety of methods known in the art. These methods include, but are not limited to, oligonucleotide-mediated (or site-directed) mutagenesis and PCR mutagenesis. A polynucleotide molecule encoding a SIRP- α variant construct of the invention may be obtained using standard techniques, e.g.,
10 gene synthesis. Alternatively, a polynucleotide molecule encoding a wild-type SIRP- α may be mutated to contain specific histidine substitutions using standard techniques in the art, e.g., QuikChangeTM mutagenesis. Polynucleotides can be synthesized using nucleotide synthesizer or PCR techniques.

Polynucleotide sequences encoding SIRP- α variant constructs may be
15 inserted into a vector capable of replicating and expressing the polynucleotides in prokaryotic or eukaryotic host cells. Many vectors are available in the art and can be used for the purpose of the invention. Each vector may contain various components that may be adjusted and optimized for compatibility with the particular host cell. For example, the vector components may include, but are not limited to, an origin of
20 replication, a selection marker gene, a promoter, a ribosome binding site, a signal sequence, a polynucleotide sequence encoding a SIRP- α variant construct of the invention, and a transcription termination sequence. In some embodiments, a vector can include internal ribosome entry site (IRES) that allows the expression of multiple SIRP- α variant constructs. Some examples of bacterial expression vectors include,
25 but are not limited to, pGEX series of vectors (e.g., pGEX-2T, pGEX-3X, pGEX-4T, pGEX-5X, pGEX-6P), pET series of vectors (e.g., pET-21, pET-21a, pET-21b, pET-23, pET-24), pACYC series of vectors (e.g., pACYCDuet-1), pDEST series of vectors (e.g., pDEST14, pDEST15, pDEST24, pDEST42), and pBR322 and its derivatives (see, e.g., U.S. Patent No. 5,648,237). Some examples of mammalian expression
30 vectors include, but are not limited to, pCDNA3, pCDNA4, pNICE, pSELECT, and pFLAG-CMV. Other types of nucleic acid vectors include viral vectors for expressing a protein in a cell (e.g., a cell of a subject). Such viral vectors include, but are not limited to, retroviral vectors, adenoviral vectors, poxviral vectors (e.g., vaccinia viral

vectors, such as Modified Vaccinia Ankara (MVA)), adeno-associated viral vectors, and alphaviral vectors.

In some embodiments, *E. coli* cells are used as host cells for the invention. Examples of *E. coli* strains include, but are not limited to, *E. coli* 294 (ATCC® 31,446), *E. coli* λ 1776 (ATCC® 31,537, *E. coli* BL21 (DE3) (ATCC® BAA-1025), and *E. coli* RV308 (ATCC® 31,608). In other embodiments, mammalian cells are used as host cells for the invention. Examples of mammalian cell types include, but are not limited to, human embryonic kidney (HEK) cells, Chinese hamster ovary (CHO) cells, HeLa cells, PC3 cells, Vero cells, and MC3T3 cells. Different host cells have characteristic and specific mechanisms for the posttranslational processing and modification of protein products. Appropriate cell lines or host systems may be chosen to ensure the correct modification and processing of the protein expressed. The above-described expression vectors may be introduced into appropriate host cells using conventional techniques in the art, e.g., transformation, transfection, electroporation, calcium phosphate precipitation, and direct microinjection. Once the vectors are introduced into host cells for protein production, host cells are cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Protein Production, Recovery, and Purification

Host cells used to produce the SIRP-α variant constructs of the invention may be grown in media known in the art and suitable for culturing of the selected host cells. Examples of suitable media for bacterial host cells include Luria broth (LB) plus necessary supplements, such as a selection agent, e.g., ampicillin. Examples of suitable media for mammalian host cells include Minimal Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), DMEM with supplemented fetal bovine serum (FBS), and RPMI-1640.

Host cells are cultured at suitable temperatures, such as from about 20 °C to about 39 °C, e.g., from 25 °C to about 37 °C. The pH of the medium is generally from about 6.8 to 7.4, e.g., 7.0, depending mainly on the host organism. If an inducible promoter is used in the expression vector of the invention, protein expression is induced under conditions suitable for the activation of the promoter.

Protein recovery typically involves disrupting the host cell, generally by such means as osmotic shock, sonication, or lysis. Once the cells are disrupted, cell

debris may be removed by centrifugation or filtration. The proteins may be further purified, for example, by affinity resin chromatography. Standard protein purification methods known in the art can be employed. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, chromatography on silica or on a cation-exchange resin, SDS-PAGE, and gel filtration.

Alternatively, SIRP- α variant constructs can be produced by the cells of a subject (e.g., a human), e.g., in the context of therapy, by administering a vector (e.g., a retroviral vector, adenoviral vector, poxviral vector (e.g., vaccinia viral vector, such as Modified Vaccinia Ankara (MVA)), adeno-associated viral vector, and alphaviral vector) containing a nucleic acid molecule encoding the SIRP- α variant construct. The vector, once inside a cell of the subject (e.g., by transformation, transfection, electroporation, calcium phosphate precipitation, direct microinjection, infection, etc) will promote expression of the SIRP- α variant construct, which is then secreted from the cell.

XV. Pharmaceutical compositions and preparations

In some embodiments, pharmaceutical compositions of the invention may contain one or more SIRP- α variant constructs of the invention as the therapeutic proteins. In addition to a therapeutic amount of the protein, the pharmaceutical compositions may contain a pharmaceutically acceptable carrier or excipient, which can be formulated by methods known to those skilled in the art. In other embodiments, pharmaceutical compositions of the invention may contain nucleic acid molecules encoding one or more SIRP- α variant constructs of the invention (e.g., in a vector, such as a viral vector). The nucleic acid molecule encoding a SIRP- α variant construct may be cloned into an appropriate expression vector, which may be delivered via well-known methods in gene therapy.

Acceptable carriers and excipients in the pharmaceutical compositions are nontoxic to recipients at the dosages and concentrations employed. Acceptable carriers and excipients may include buffers such as phosphate, citrate, HEPES, and TAE, antioxidants such as ascorbic acid and methionine, preservatives such as hexamethonium chloride, octadecyldimethylbenzyl ammonium chloride, resorcinol, and benzalkonium chloride, proteins such as human serum albumin, gelatin, dextran, and immunoglobulins, hydrophilic polymers such as polyvinylpyrrolidone, amino

The pharmaceutical composition for gene therapy can be in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Vectors that may be used as *in vivo* gene delivery vehicle include, but are not limited to, retroviral vectors, adenoviral vectors, poxviral vectors (e.g.,
5 vaccinia viral vectors, such as Modified Vaccinia Ankara (MVA)), adeno-associated viral vectors, and alphaviral vectors. In some embodiments, a vector can include internal ribosome entry site (IRES) that allows the expression of multiple SIRP- α variant constructs. Other vehicles and methods for gene delivery are described in U.S. Patent Nos. 5,972,707, 5,697,901, and 6,261,554, each of which is
10 incorporated by reference herein in its entirety.

Other methods of producing pharmaceutical compositions are described in, e.g., U.S. Patent Nos. 5,478,925, 8,603,778, 7,662,367, and 7,892,558, all of which are incorporated by reference herein in their entireties.

15 **XVI. Routes, dosage, and timing of administration**

Pharmaceutical compositions of the invention that contain one or more SIRP- α variant constructs as the therapeutic proteins may be formulated for parenteral administration, subcutaneous administration, intravenous administration, intramuscular administration, intra-arterial administration, intrathecal administration,
20 or intraperitoneal administration. The pharmaceutical composition may also be formulated for, or administered via, nasal, spray, oral, aerosol, rectal, or vaginal administration. Methods of administering therapeutic proteins are known in the art. See, for example, U.S. Patent Nos. 6,174,529, 6,613,332, 8,518,869, 7,402,155, and 6,591,129, and U.S. Patent Application Publication Nos. US20140051634,
25 WO1993000077, and US20110184145, the disclosures of which are incorporated by reference in their entireties. One or more of these methods may be used to administer a pharmaceutical composition of the invention that contains one or more SIRP- α variant constructs of the invention. For injectable formulations, various effective pharmaceutical carriers are known in the art. See, e.g., *Pharmaceutics and*
30 *Pharmacy Practice*, J. B. Lippincott Company, Philadelphia, Pa., Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986).

The dosage of the pharmaceutical compositions of the invention depends on factors including the route of administration, the disease to be treated, and physical

characteristics, e.g., age, weight, general health, of the subject. Typically, the amount of a SIRP- α variant construct of the invention contained within a single dose may be an amount that effectively treats the disease without inducing significant toxicity. A pharmaceutical composition of the invention may include a dosage of a SIRP- α variant construct ranging from 0.001 to 500 mg (e.g., 0.05, 0.01, 0.1, 0.2, 0.3, 0.5, 0.7, 0.8, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 10 mg, 15 mg, 20 mg, 30 mg, 50 mg, 100 mg, 250 mg, or 500 mg) and, in a more specific embodiment, about 0.1 to about 100 mg and, in a more specific embodiment, about 0.2 to about 20 mg. The dosage may be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters of the subject.

A pharmaceutical composition of the invention can be administered in an amount from about 0.001 mg up to about 500 mg/kg/day (e.g., 0.05, 0.01, 0.1, 0.2, 0.3, 0.5, 0.7, 0.8, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 10 mg, 15 mg, 20 mg, 30 mg, 50 mg, 100 mg, 250 mg, or 500 mg/kg/day). Pharmaceutical compositions of the invention that contain a SIRP- α variant construct may be administered to a subject in need thereof, for example, one or more times (e.g., 1-10 times or more) daily, weekly, monthly, biannually, annually, or as medically necessary. Dosages may be provided in either a single or multiple dosage regimens. For example, in some embodiments, the effective amount is a dose that ranges from about 0.1 to about 100 mg/kg/day, from about 0.2 mg to about 20 mg of the SIRP- α variant construct per day, about 1 mg to about 10 mg of the SIRP- α variant construct per day, from about 0.7 mg to about 210 mg of the SIRP- α variant construct per week, 1.4 mg to about 140 mg of the SIRP- α variant construct per week, about 0.3 mg to about 300 mg of the SIRP- α variant construct every three days, about 0.4 mg to about 40 mg of the SIRP- α variant construct every other day, and about 2 mg to about 20 mg of the SIRP- α variant construct every other day. The timing between administrations may decrease as the medical condition improves or increase as the health of the patient declines.

XVII. Methods of treatment

The invention provides pharmaceutical compositions and methods of treatment that may be used to treat patients who are suffering from diseases and disorders associated with SIRP- α and/or CD47 activity, such as cancers and immunological diseases. In some embodiments, the SIRP- α variant constructs

described herein may be administered to a subject in a method of increasing phagocytosis of a target cell (e.g., a cancer cell) in the subject. In some embodiments, the SIRP- α variant constructs may be administered to a subject in a method of eliminating regulatory T-cells in the subject. In some embodiments, the SIRP- α variant constructs may be administered to a subject in a method to kill cancer cells in the subject. In some embodiments, the SIRP- α variant constructs may be administered to a subject in a method of treating a disease associated with SIRP- α and/or CD47 activity in the subject, wherein the SIRP- α variant construct preferentially binds CD47 on diseased cells or at a diseased site over CD47 on non-diseased cells. In some embodiments, the SIRP- α variants may be administered to a subject in a method of increasing hematopoietic stem cell engraftment in the subject, wherein the method includes modulating the interaction between SIRP- α and CD47 in the subject. In some embodiments, the SIRP- α variant constructs may be administered to a subject in a method of altering an immune response (i.e., suppressing the immune response) in the subject.

In some embodiments, before treating a disease (e.g., cancer) in a subject, the amino acid sequence(s) of SIRP- α in the subject are determined, for example, from each of the two alleles encoding the SIRP- α gene. In this method of the invention, the method determines the amino acid sequences of SIRP- α polypeptide in a biological sample from the subject, and then administers to the subject a therapeutically effective amount of a SIRP- α variant construct. In this method, the SIRP- α variant in the SIRP- α variant construct has the same amino acid sequence as that of a SIRP- α polypeptide in the biological sample of the subject, except for the amino acids changes introduced to increase affinity of the SIRP- α variant. The SIRP- α variant construct has minimal immunogenicity in the subject after it is administered.

The SIRP- α variant constructs and pharmaceutical compositions of the invention may be used in various cancer therapies. The cancers amenable to treatment according to the invention include, but are not limited to, solid tumor cancer, hematological cancer, acute myeloid leukemia, chronic lymphocytic leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin lymphoma, Hodgkin lymphoma, multiple myeloma, bladder cancer, pancreatic cancer, cervical cancer, endometrial cancer, lung cancer, bronchus cancer, liver cancer, ovarian cancer, colon and rectal cancer, stomach cancer, gastric cancer,

gallbladder cancer, gastrointestinal stromal tumor cancer, thyroid cancer, head and neck cancer, oropharyngeal cancer, esophageal cancer, melanoma, non-melanoma skin cancer, Merkel cell carcinoma, virally induced cancer, neuroblastoma, breast cancer, prostate cancer, renal cancer, renal cell cancer, renal pelvis cancer, leukemia, lymphoma, sarcoma, glioma, brain tumor, and carcinoma. In some embodiments, cancerous conditions amenable to treatment according to the invention include metastatic cancers. In some embodiments, the cancer amenable to treatment according to the invention is a solid tumor or hematological cancer.

The SIRP- α variant constructs and pharmaceutical compositions of the invention may be used in various therapies to treat immunological diseases. In some embodiments, the immunological disease is an autoimmune disease or an inflammatory disease, such as multiple sclerosis, rheumatoid arthritis, a spondyloarthropathy, systemic lupus erythematosus, an antibody-mediated inflammatory or autoimmune disease, graft versus host disease, sepsis, diabetes, psoriasis, atherosclerosis, Sjogren's syndrome, progressive systemic sclerosis, scleroderma, acute coronary syndrome, ischemic reperfusion, Crohn's Disease, endometriosis, glomerulonephritis, myasthenia gravis, idiopathic pulmonary fibrosis, asthma, acute respiratory distress syndrome (ARDS), vasculitis, or inflammatory autoimmune myositis.

EXAMPLES

Example 1 – Methods

Production of SIRP- α variant constructs

All gene constructs are generated using gene synthesis and codon optimized for expression in mammalian cells (DNA2.0). The genes are cloned into mammalian expression vectors and expressed using CMVa-intron promoter. A leader sequence has been engineered at the N-terminus of the constructs to ensure appropriate signaling and processing for secretion. The expression of SIRP- α fusion proteins is carried out using Expi293F™ cells (Life Technologies). This cell line is adapted to high density, serum-free suspension culture in Expi293F™ Expression Medium and is capable of producing high levels of recombinant proteins. Transfection procedures have been performed according to manufacturer's manual. The supernatant is typically harvested at 5-7 days post transfection. The protein constructs are designed to carry a 6xhistidine affinity tag and this allows purification

by affinity chromatography. The column was first equilibrated with 5 mM imidazole, 20 mM Tris HCl (pH 7.4), 500 mM NaCl. The clarified media expressing the various SIRP- α variant constructs is loaded onto Hi-Trap Ni Sepharose excel affinity resin on Avant 25 (GE Healthcare). Another equilibration step is performed. After that, the column is washed with 40 mM imidazole, 20 mM Tris HCl, 500mM NaCl, and subsequently eluted with 250 mM imidazole, 20 mM Tris HCl, 500 mM NaCl. Eluted fractions containing the SIRP- α variant constructs are pooled and thereafter buffer exchanged into 1X PBS.

10 *In vitro cleavage of SIRP- α proteins*

Recombinant human uPA and matriptase are purchased from R&D systems. 3 μ M of SIRP- α proteins are added to respective amounts of uPA and matriptase (0.1 to 44 ng) in 50 mM Tris HCl (pH 8.5), 0.01% Tween as described. The digestion reactions are typically incubated for 18-24 hours at 37 °C. To stop the reaction, SDS-PAGE loading dye is added to the reaction and heated at 95 °C for 3 minutes. To assess cleavage, the digested samples are separated on a 4-20% Tris-Glycine SDS-PAGE.

20 **Example 2 – Design of SIRP- α variant constructs that will be specifically activated in tumor tissue**

The goal is to design SIRP- α variant constructs that will remain inert until activated locally to bind to CD47 in tumor tissue. This will limit binding of SIRP- α to CD47 on the cell-surface of non-diseased cells and prevent undesirable “on-target” “off tissue” toxicity. To generate such SIRP- α variant constructs, the blocking peptides (e.g., a CD47-based blocking peptide) are genetically fused to the SIRP- α variant by way of a cleavable linker. The blocking peptides explored are based on CD47 interaction sites to SIRP- α and the sequences are described below (sections (a)-(c)). Spacers containing repeated units of GGGGS are designed to flank the cleavable linker, which often encodes a protease recognition site. In some embodiments, the protease cleavage site chosen is LSGRSDNH, but many others are possible. The protease cleavage site LSGRSDNH is selected for its sensitivity to numerous proteases that are up-regulated in a variety of human carcinomas, for example, matriptase (MTSP1), urinary-type plasminogen activator (uPA), legumain, PSA (also called KLK3, kallikrein-related peptidase-3), matrix metalloproteinase-2

(MMP-2), MMP9, human neutrophil elastase (HNE), and proteinase 3 (Pr3) (Ulissee et al., *Curr. Cancer Drug Targets* 9:32-71, 2009; Uhland et al., *Cell. Mol. Life Sci.* 63:2968-2978, 2006; LeBeau et al., *Proc. Natl. Acad. Sci. USA* 110:93-98, 2013; Liu et al., *Cancer Res.* 63:2957-2964, 2003).

5

(a) Blocking SIRP- α by CD47-based blocking peptides

CD47-based blocking peptides are described previously. These peptides bind SIRP- α with different affinities and block its function. The N-terminus of CD47 is important for the interaction with SIRP- α , therefore, structural analysis predicted
10 fusing SIRP- α to the C-terminus of CD47. To better understand the results, both N-terminal and C-terminal fusions are explored with different lengths of spacers. Different CD-47 based blocking peptides (e.g., peptides listed in Table 6) are fused to the N- or C-terminus of a SIRP- α variant with a cleavable linker and one or more spacers. Exemplary sequences of fusion proteins containing a CD47-based blocking
15 peptide fused to a SIRP- α variant by way of a cleavable linker and one or more spacers are shown in sequences of SEQ ID NOs: 48-56, in which single-underlined portion indicates the CD47-based blocking peptide, double-underlined portion indicates the cleavable linker, and bold portion indicates the SIRP- α variant. Sequences of SEQ ID NOs: 48-51 contain CD47-based blocking peptides that
20 include 12 or 21 amino acids and spacers of 2-3 repeats of GGGGS. Sequences of SEQ ID NOs: 52-56 contain CD47-based blocking peptides that include the CD47 IgSF domain (truncated at VVS) having a C15S substitution and spacers of 2-5 repeats of GGGGS or 3-6 repeats of GGS. Additionally, in some embodiments, an HSA may be fused to the C-terminus of any one of the sequences of SEQ ID NOs:
25 48-56. Furthermore, in some embodiments, an Fc domain monomer or an HSA (SEQ ID NO: 68) may be fused to either the N- or C-terminus of any one of the fusion proteins listed in Table 10. Furthermore, in some embodiments, an Fc domain monomer or an HSA may be fused to either the N- or C-terminus of any one of the fusion proteins listed in Table 10.

30

Table 10

SEQ ID NO	Fusion protein of a CD47-based blocking peptide and a SIRP-α variant
48	<u>SEVTELTREGETGGGSGGGGSLSGRSDNHGGGSGGGGSEELQIIQPDKSVLVAA</u> <u>GETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRVTTVSDTTKRNNMDF</u> <u>SIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPS</u>
49	<u>SEVTELTREGETGGGSGGGGSGGGGSLSGRSDNHGGGSGGGGSEELQIIQPDKS</u> <u>VLVAAGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRVTTVSDTTKR</u> <u>NNMDFSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPS</u>
50	<u>GQYTSEVTELTREGETIIELKGGGSGGGGSLSGRSDNHGGGSGGGGSEELQIIQ</u> <u>PDKSVLVAAGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRVTTVSD</u> <u>TTKRNNMDFSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPS</u>
51	<u>GQYTSEVTELTREGETIIELKGGGSGGGGSGGGGSLSGRSDNHGGGSGGGGSEEE</u> <u>LQIIQPDKSVLVAAGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRV</u> <u>TTVSDTTKRNNMDFSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAK</u> <u>PS</u>
52	<u>QLLENKTKSVEFTFSNDTVVIPCFVTNMEAQNTTEVYVKWKFKGRDIYTFDGALNKS</u> <u>TVPTDFSSAKIEVSQLLKGDAKMDKSDAVSHTGNYTCEVTELTREGETIIELKYR</u> <u>VVSGGGSGGGSGGGGSLSGRSDNHGGGSGGGGSEELQIIQPDKSVLVAAGETA</u> <u>TLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRVTTVSDTTKRNNMDFSIRI</u> <u>GNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPS</u>
53	<u>QLLENKTKSVEFTFSNDTVVIPCFVTNMEAQNTTEVYVKWKFKGRDIYTFDGALNKS</u> <u>TVPTDFSSAKIEVSQLLKGDAKMDKSDAVSHTGNYTCEVTELTREGETIIELKYR</u> <u>VVSGGGSGGGSGGGGSLSGRSDNHGGGSGGGGSEELQIIQPDKSVLVA</u> <u>AGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRVTTVSDTTKRNNMD</u> <u>FSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPS</u>
54	<u>QLLENKTKSVEFTFSNDTVVIPCFVTNMEAQNTTEVYVKWKFKGRDIYTFDGALNKS</u> <u>TVPTDFSSAKIEVSQLLKGDAKMDKSDAVSHTGNYTCEVTELTREGETIIELKYR</u> <u>VVSGGGSGGGSGGGGSLSGRSDNHGGGSGGGGSEELQIIQPDK</u> <u>SVLVAAGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRVTTVSDTTK</u> <u>RNNMDFSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSVRAKPS</u>
55	<u>EEELQIIQPDKSVLVAAGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGP</u> <u>PRVTTVSDTTKRNNMDFSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSV</u> <u>RAKPSGGSGGSLSGRSDNHGGSGGSGGSGGSLLENKTKSVEFTFSNDTVVIP</u> <u>FVTNMEAQNTTEVYVKWKFKGRDIYTFDGALNKSTVPTDFSSAKIEVSQLLKGDAK</u> <u>KMDKSDAVSHTGNYTCEVTELTREGETIIELKYRVVS</u>
56	<u>EEELQIIQPDKSVLVAAGETATLRCTITISLFPVGPIQWFRGAGPGRVLIYNQRQGP</u> <u>PRVTTVSDTTKRNNMDFSIRIGNITPADAGTYCYIKFRKGSPPDVEFKSGAGTELSV</u> <u>RAKPSGGSGGSLSGRSDNHGGSGGSGGSGGSLLENKTKSVEFTF</u> <u>SNDTVVIPCFVTNMEAQNTTEVYVKWKFKGRDIYTFDGALNKSTVPTDFSSAKIEVS</u> <u>QLLKGDAKMDKSDAVSHTGNYTCEVTELTREGETIIELKYRVVS</u>

(b) Blocking SIRP-α variant by a low affinity CD47 mutant having an extended N-terminus

Due to the high affinity of the SIRP- α variant for CD47, there is a chance that after cleaving the linker, the fusion protein may not dissociate and the SIRP- α variant may remain blocked. To solve this, we studied the structure of the SIRP- α -CD47 complex and designed CD47 mutants with reduced binding affinity for the SIRP- α variant relative to the binding affinity for a wild-type SIRP- α . Thus, after protease cleavage of the linker, the CD47 mutant will dissociate away from the SIRP- α variant. The designed low affinity CD47 mutants having an extended N-terminus are described below. Initial experiments will be performed fusing the SIRP- α variant to the wild-type CD47. These SIRP- α variant constructs including a SIRP- α variant and a wild-type CD47 or CD47 mutant will be cleaved *in vitro*, analyzed by SDS-page to ensure cleavage, and measured by biacore to see their capacity of binding to CD47 (i.e., the binding of the SIRP- α variant to wild-type CD47 after the cleaved CD47 is dissociated from the SIRP- α variant). If the initial SIRP- α variant constructs containing a SIRP- α variant fused to the wild-type CD47 are expressed, able to block CD47 binding before protease cleavage, and able to bind CD47 after protease cleavage, CD47 mutants may not be needed. If these initial SIRP- α variant constructs are inactive (i.e., can be cleaved but do not bind CD47 after protease cleavage due to the lack of dissociation), then other fusion proteins containing a SIRP- α variant fused to the low affinity CD47 mutants will be tested.

In the co-crystalized structure of CD47:SIRP- α (PDB: 4KJY, 4CMM), the N-terminus of CD47 exists as a pyro-glutamate and makes hydrogen bonding interactions with Thr66 of a SIRP- α variant and Leu66 of a wild-type SIRP- α (FIG. 1). It is hypothesized that extending the N-terminus of CD47 by adding an amino acid, e.g., a glycine, will prevent cyclization of glutamine to pyroglutamate and therefore will likely disrupt the hydrogen bonding interactions with Thr66 or Leu66, and perturb binding of CD47 to SIRP- α . Sequences of the fusion proteins containing a low affinity CD47 IgSF domain mutant and a SIRP- α variant are shown in SEQ ID NOs: 57-59 in Table 11, in which single-underlined portion indicates the low affinity CD47 IgSF domain mutant containing amino acids 1-118 and C15S, relative to SEQ ID NO: 46 in Table 6, double-underlined portion indicates the cleavable linker, and bold portion indicates the SIRP- α variant. SEQ ID NOs: x10-x12 also include spacers of 3-5 repeats of GGGGS. Sequences similar to SEQ ID NOs: x10-x12 may be designed and expressed in which a low affinity CD47 IgSF domain mutant is fused to the C-terminus of a SIRP- α variant by way of a cleavable linker and one or more

spacers. Furthermore, in some embodiments, an Fc domain monomer or an HSA may be fused to either the N- or C-terminus of any one of the fusion proteins listed in Table 11.

Table 11

SEQ ID NO	Fusion protein of a low affinity CD47 IgSF domain mutant having an extended N-terminal glycine and a SIRP-α variant
57	<u>GQLLFNKT</u> KSVEFTFSNDTVVIPCFTVNMEAQNTTEVYVKWKFKGRDIYTFDGAL NKSTVPTDFSSAKIEVSQLLKGDASLKMDKSDAVSHTGNYTCEVTELTREGETII ELKYRVVSGGGGSGGGGSGGGGSLSGRSDNHGGGGSGGGGSEEEELQIIQPDKSVL VAAGETATLRCTITSLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRTTVSDTTKR NNMDFSIRIGNITPADAGTYCYCIKFRKGSPPDVEFKSGAGTELSVRAKPS
58	<u>GQLLFNKT</u> KSVEFTFSNDTVVIPCFTVNMEAQNTTEVYVKWKFKGRDIYTFDGAL NKSTVPTDFSSAKIEVSQLLKGDASLKMDKSDAVSHTGNYTCEVTELTREGETII ELKYRVVSGGGGSGGGGSGGGGSLSGRSDNHGGGGSGGGGSEEEELQIIQP DKSVLVAAGETATLRCTITSLFPVGPIQWFRGAGPGRVLIYNQRQGPFPRTTVS DTTKRNNMDFSIRIGNITPADAGTYCYCIKFRKGSPPDVEFKSGAGTELSVRAKPS
59	<u>GQLLFNKT</u> KSVEFTFSNDTVVIPCFTVNMEAQNTTEVYVKWKFKGRDIYTFDGAL NKSTVPTDFSSAKIEVSQLLKGDASLKMDKSDAVSHTGNYTCEVTELTREGETII ELKYRVVSGGGGSGGGGSGGGGSLSGRSDNHGGGGSGGGGSEEEEL QIIQPDKSVLVAAGETATLRCTITSLFPVGPIQWFRGAGPGRVLIYNQRQGPFP RTTVSDTTKRNNMDFSIRIGNITPADAGTYCYCIKFRKGSPPDVEFKSGAGTELSV RAKPS

(c) Blocking SIRP-α by a low-affinity CD47 IgSF domain mutant having amino acid substitutions

CD47 binds to a deep pocket to SIRP-α (PDB code: 4KJY and 4CMM). Computational modeling has been performed to identify amino acid residues in the pocket region of CD47, which, when mutated would reduce the binding affinity of CD47 to SIRP-α variant, but maintain the binding affinity of CD47 to a wild-type SIRP-α. The CD47 residues identified are L101Q, L101H, L101Y, T102Q, and T102H. It is hypothesized that a low-affinity CD47 IgSF domain mutant containing one of these substitutions will be able to block the SIRP-α variant efficiently in the tethered mode. However, upon reaching the tumor site and cleavage by proteases at the linker locally, the low-affinity CD47 IgSF domain mutant will dissociate from the SIRP-α variant to bind to a wild-type SIRP-α, leaving the SIRP-α variant free to bind CD47 on the cell-surface of tumor cells. The dissociated, low-affinity CD47 IgSF domain mutant can now block activity of wild-type SIRP-α. This will potentially result in enhanced double-blocking activity from the released low-affinity CD47 IgSF domain mutant and the SIRP-α variant. To illustrate how the amino acid residues

are selected and the principle of how this may result in differential blocking of wild-type SIRP- α and SIRP- α variant, an example is shown using Ala27 of a wild-type SIRP- α (FIG. 2). For instance, Ala27 of the wild-type SIRP- α is a smaller residue than Ile27 in the SIRP- α variant. Therefore, by mutating Thr102 in the wild-type CD47 to a larger amino acid such as Gln102, Gln102 in the low-affinity CD47 IgSF domain mutant may result in a steric clash with Ile27 in the SIRP- α variant at the corresponding interaction site. However, the interaction between the CD47 mutant having Thr102Gln substitution and the wild-type SIRP- α having Ala27 would be preserved. Accordingly, the CD47 mutant would have a low binding affinity to the SIRP- α variant and a relatively higher binding affinity to the wild-type SIRP- α . Sequences of some exemplary low-affinity CD47 IgSF domain mutants are shown in SEQ ID NOs: 41-45 in Table 6. Sequences of the SIRP- α variant constructs containing a low affinity CD47 IgSF domain mutant having amino acid substitutions and a SIRP- α variant are shown in SEQ ID NOs: 60-63 in Table 12, in which single-underlined portion indicates the low affinity CD47 IgSF domain mutant containing amino acid substitution L101Q, L101Y, T102Q, and T102H, respectively, double-underlined portion indicates the cleavable linker, and bold portion indicates the SIRP- α variant. SEQ ID NOs: 60-63 also include spacers of 3-5 repeats of GGGGS. Sequences similar to SEQ ID NOs: 60-63 may be designed and expressed in which a low affinity CD47 IgSF domain mutant is fused to the C-terminus of a SIRP- α variant by way of a cleavable linker and one or more spacers.

Table 12

SEQ ID NO	Fusion protein of a low affinity CD47 IgSF domain mutant having amino acid substitutions and a SIRP- α variant
60	<u>QLLFNKT</u> <u>KSVEFTFS</u> <u>NDTVV</u> <u>IPCFVTN</u> <u>MEAQNTTE</u> <u>VYVKW</u> <u>KFKGRDI</u> <u>YTFD</u> <u>GALNKS</u> <u>TVPTDF</u> <u>SSAKIEVS</u> <u>QLLKG</u> <u>DASLKMDK</u> <u>SDAVS</u> <u>HTGNYT</u> <u>CEVTEQ</u> <u>TREGETI</u> <u>IELKYR</u> <u>VVSGGGG</u> <u>SGGGG</u> <u>SGGGG</u> <u>SGGGG</u> <u>SLSGR</u> <u>SDNH</u> <u>GGGGG</u> <u>SGGGG</u> <u>SEELQ</u> <u>IIQPD</u> <u>KSVLVA</u> <u>AGETAT</u> <u>LRCTIT</u> <u>SLFPV</u> <u>GPIQW</u> <u>FRGAG</u> <u>PGRVLI</u> <u>YNQRQ</u> <u>GPFPR</u> <u>VTTVSD</u> <u>TTKRNNMD</u> <u>FSIRIGNIT</u> <u>PADAGT</u> <u>YYCIK</u> <u>FRKGS</u> <u>PDDVE</u> <u>FKSGAG</u> <u>TELSV</u> <u>RAKPS</u>
61	<u>QLLFNKT</u> <u>KSVEFTFS</u> <u>NDTVV</u> <u>IPCFVTN</u> <u>MEAQNTTE</u> <u>VYVKW</u> <u>KFKGRDI</u> <u>YTFD</u> <u>GALNKS</u> <u>TVPTDF</u> <u>SSAKIEVS</u> <u>QLLKG</u> <u>DASLKMDK</u> <u>SDAVS</u> <u>HTGNYT</u> <u>CEVTEY</u> <u>TREGETI</u> <u>IELKYR</u> <u>VVSGGGG</u> <u>SGGGG</u> <u>SGGGG</u> <u>SGGGG</u> <u>SLSGR</u> <u>SDNH</u> <u>GGGGG</u> <u>SGGGG</u> <u>SEELQ</u> <u>IIQPD</u> <u>KSVLVA</u> <u>AGETAT</u> <u>LRCTIT</u> <u>SLFPV</u> <u>GPIQW</u> <u>FRGAG</u> <u>PGRVLI</u> <u>YNQRQ</u> <u>GPFPR</u> <u>VTTVSD</u> <u>TTKRNNMD</u> <u>FSIRIGNIT</u> <u>PADAGT</u> <u>YYCIK</u> <u>FRKGS</u> <u>PDDVE</u> <u>FKSGAG</u> <u>TELSV</u> <u>RAKPS</u>

62	<u>QLLFNKT</u> <u>KSVEFTFS</u> <u>NDTVVI</u> <u>PCFVTN</u> <u>MEAQNT</u> <u>TEVYVK</u> <u>WKFKGR</u> <u>DIYTFD</u> <u>GALNKS</u> <u>TVPTDF</u> <u>SSAKIEVS</u> <u>QLLKGD</u> <u>ASLKMD</u> <u>KSDAVS</u> <u>HTGNYT</u> <u>CEVTEL</u> <u>QREGET</u> <u>IIELKYR</u> <u>VVSGGG</u> <u>GSGGGG</u> <u>GSGGGG</u> <u>GSGGGG</u> <u>SLSGR</u> <u>SDNH</u> <u>GGGGG</u> <u>GSGGGG</u> <u>SEELQI</u> <u>IQPD</u> <u>KSVLVA</u> <u>AGETAT</u> <u>LRCTIT</u> <u>SLFPV</u> <u>GPIQW</u> <u>FRGAG</u> <u>PGRVLI</u> <u>YNQR</u> <u>QGPF</u> <u>PRVTT</u> <u>VSDDT</u> <u>TKRNNMD</u> <u>FSIRIG</u> <u>NITPAD</u> <u>AGTYYC</u> <u>IKFRKG</u> <u>SPDDV</u> <u>EFGSG</u> <u>AGTEL</u> <u>SVRAKPS</u>
63	<u>QLLFNKT</u> <u>KSVEFTFS</u> <u>NDTVVI</u> <u>PCFVTN</u> <u>MEAQNT</u> <u>TEVYVK</u> <u>WKFKGR</u> <u>DIYTFD</u> <u>GALNKS</u> <u>TVPTDF</u> <u>SSAKIEVS</u> <u>QLLKGD</u> <u>ASLKMD</u> <u>KSDAVS</u> <u>HTGNYT</u> <u>CEVTEL</u> <u>HREGET</u> <u>IIELKYR</u> <u>VVSGGG</u> <u>GSGGGG</u> <u>GSGGGG</u> <u>GSGGGG</u> <u>SLSGR</u> <u>SDNH</u> <u>GGGGG</u> <u>GSGGGG</u> <u>SEELQI</u> <u>IQPD</u> <u>KSVLVA</u> <u>AGETAT</u> <u>LRCTIT</u> <u>SLFPV</u> <u>GPIQW</u> <u>FRGAG</u> <u>PGRVLI</u> <u>YNQR</u> <u>QGPF</u> <u>PRVTT</u> <u>VSDDT</u> <u>TKRNNMD</u> <u>FSIRIG</u> <u>NITPAD</u> <u>AGTYYC</u> <u>IKFRKG</u> <u>SPDDV</u> <u>EFGSG</u> <u>AGTEL</u> <u>SVRAKPS</u>

Example 3 – Expression and production of SIRP-α variant constructs for *in vitro* studies

Various SIRP-α variant constructs (SEQ ID NOs: 48-56) including a SIRP-α variant and a CD47-based blocking peptide were expressed in Expi293-F mammalian cells. All the constructs were designed with a leader sequence that enabled their expression as secreted proteins into the media. As an example to demonstrate the protein profile of isolated SIRP-α variant constructs, SDS-PAGE analyses of SIRP-α variant constructs of SEQ ID NOs: 48-56 are shown in FIG. 3. For instance, FIG. 3A shows a reduced, SDS-PAGE gel of SIRP-α variant constructs of SEQ ID NOs: 48-56 and FIG. 3B shows a non-reduced, SDS-PAGE gel of the SIRP-α variant constructs. Size exclusion data indicated that the SIRP-α variant constructs are not aggregated (data not shown).

Example 4 – *In vitro* cleavage of SIRP-α and CD47 fusion proteins

To determine whether the SIRP-α variant constructs (e.g., SEQ ID NOs: 48-63) would be cleaved specifically *in vivo* at tumor tissues, *in vitro* experiments were performed using proteases uPA and matriptase, which are commonly known to be up-regulated in cancers, to cleave the SIRP-α variant constructs. Initial experiments were performed using SIRP-α variant construct (SEQ ID NO: 54) to determine protease cleavability and optimize cleavage conditions. FIG. 4A shows the results of testing cleavability by uPA and matriptase. 3 μM SIRP-α variant construct (SEQ ID NO: 54) was incubated for 18 hrs at 37 °C using excess uPA or matriptase. Lane 1 of FIG. 4A shows the control experiment with no addition of protease and lanes 2 and 3 show incubation of the SIRP-α variant construct (SEQ ID NO: 54) with uPA and matriptase, respectively. Data obtained as shown in FIG. 4A clearly demonstrate that the SIRP-α variant construct (SEQ ID No: 54) can be cleaved and released *in*

vitro by digesting the SIRP- α variant construct with excess uPA and matriptase for 18 hrs at 37 °C. The cleaved SIRP- α variant migrates as a ~17 KDa molecular weight band. Cleaved CD47 migrates with smeary banding, most likely due to glycosylation, around 36-40 kDa. By comparing the amount of uncut SIRP- α variant construct in lanes 2 and 3 of FIG. 4A, it appears that a more complete cleavage was achieved using matriptase compared to using uPA.

Therefore further optimization of cleavage conditions was performed only using matriptase and the results are shown in FIG. 4B. Different amounts of matriptase were tested and the cleavage was performed for 18 hrs at 37 °C. Lane 1 of FIG. 4B shows the control experiment with no addition of matriptase and lanes 2-4 each shows cleavage performed with 44 ng, 0.44 ng, and 0.167 ng of matriptase, respectively. The data obtained indicate that 0.44 ng enzyme is sufficient for complete cleavage under current conditions. Next, we tested cleavage of remaining SIRP- α variant constructs using the optimized cleavage conditions. As an example and shown in FIG. 4C, respective SIRP- α variant constructs (SEQ ID NOs: 57-63) were cleaved successfully *in vitro* by matriptase using the optimized cleavage conditions. Lanes 1-7 of FIG. 4C correspond to the uncleaved fusion proteins of SEQ ID NOs: 57-63, respectively. Lanes 8-14 of FIG. 4C correspond to the fusion proteins of SEQ ID NOs: 57-63, respective, cleaved with matriptase.

Example 5 – Binding affinities of SIRP- α variant constructs

Binding of human CD47-hFc (R & D Systems, catalog number 4670-CD) to SIRP- α variant constructs was analyzed on a Biacore T100 instrument (GE Healthcare) using phosphate buffered saline (pH 7.4) supplemented with 0.01% Tween-20 as running buffer.

370 Resonance Unit (RU) of CD47-hFc were immobilized on flow cell 2 of a CM4 sensor chip (GE Healthcare) by standard amine coupling. Flow cell 1 was activated with EDC/NHS and blocked (with ethanolamine) to serve as a reference. All SIRP- α variant constructs were injected at 50 nM or 100 nM for two minutes at a flow rate of 30 μ L/min and followed by ten minutes of dissociation time. After each injection, the surface was regenerated using a 2:1 mixture of Pierce IgG elution buffer (Life Technologies, catalog number 21004) and 4 M NaCl. Complete regeneration of the surface was confirmed by injecting the SIRP- α variants at the

beginning and end of the experiment. All sensorgrams were double-referenced using flow cell 1 and a buffer injection.

For all samples, the binding signal at 100 nM after 50 seconds of association was determined, normalized by the molecular weight of the SIRP- α variant construct, and expressed as percent of maximal binding response. The binding of the SIRP- α variant (SEQ ID NO: 31) at 100 nM normalized by its MW was used as maximal binding response. Results in FIG. 5A show that SIRP- α variant constructs (SEQ ID NOs: 48-51) do not block SIRP- α variants from binding to CD47 on the chip. After cleavage of the linker, the binding activity modestly increases. SIRP- α variant constructs (SEQ ID NOs: 52-54) efficiently blocked binding of the SIRP- α variant to CD47 on the chip. However, after cleavage of the linker, the binding activity of the SIRP- α variant to CD47 on the chip only modestly increased, suggesting that the high affinity of interaction between the SIRP- α variant and the IgSF domain of CD47 keeps the complex together and therefore the IgSF domain of CD47 continues block the SIRP- α variant even after the linker is cleaved. Surprisingly, when the IgSF domain of CD47 is fused to the C-terminus of SIRP- α (SEQ ID NO: 55), the intact SIRP- α variant construct is efficiently blocked from binding to CD47 on the chip (same as fusion proteins of SEQ ID NOs: 52-54), but cleavage of the linker restores 100% of the binding of the SIRP- α variant to CD47 on the chip, suggesting that the IgSF domain of CD47 dissociated from the SIRP- α variant after linker cleavage, thus, the SIRP- α variant is free to bind to CD47 on the chip. Another construct with CD47 fused to the C-terminus of a SIRP- α variant was tested later (see FIG. 5B). This construct (SEQ ID NO: 56), which contains a longer spacer, also recovered activity after cleaving, confirming the general approach of linking the N-terminus of a CD47-based blocking peptide to the C-terminus of a SIRP- α variant obtain SIRP- α constructs in which the CD47-based blocking peptide efficiently blocks the SIRP- α variant and dissociates after cleaving of the cleavable linker.

To further examine the binding affinity of SIRP- α variant constructs, SIRP- α variant constructs of SEQ ID NOs: 52-63 were analyzed on the Biacore instrument following the same protocol described previously. SIRP- α variant constructs of SEQ ID NOs: 52-54 contain the CD47 IgSF domain having amino acids 1-117 and C15S, relative to wild-type CD47 (SEQ ID NO: 35) fused to the N-terminus of the SIRP- α variant (SEQ ID NO: 31) through the cleavable linker LSGRSDNH and multiple spacers of different lengths. SIRP- α variant constructs of SEQ ID NOs: 55 and 56

contain the CD47 IgSF domain having amino acids 1-117 and C15S, relative to wild-type CD47 (SEQ ID NO: 35) fused to the C-terminus of the SIRP- α variant (SEQ ID NO: 31) through the cleavable linker LSGRSDNH and multiple spacers of different lengths. SIRP- α variant constructs of SEQ ID NOs: 57-59 contain the CD47 IgSF domain having amino acids 1-118 and C15S, relative to SEQ ID NO: 46 in Table 6 fused to the N-terminus of the SIRP- α variant (SEQ ID NO: 31) through the cleavable linker LSGRSDNH and multiple spacers of different lengths. SIRP- α variant constructs of SEQ ID NOs: 60-63 contain the CD47 IgSF domain having amino acids 1-117 of SEQ ID NOs: 41, 42, 44, and 45 in Table 6, respectively, fused to the N-terminus of the SIRP- α variant (SEQ ID NO: 31) through the cleavable linker LSGRSDNH and multiple spacers of different lengths.

FIG. 5B shows that SIRP- α variant constructs of SEQ ID NOs: 55 and 56 were efficiently blocked from binding to CD47 on the chip before linker cleavage, but cleavage of the linker restores 100% of the binding activity. Similar results were observed for SIRP- α variant constructs of SEQ ID NO: 57-63. We observed that by extending the N-terminus of the CD47-based blocking peptide by one glycine residue generated a SIRP- α variant construct that was efficiently blocked before the linker was cleaved and subsequently recovered close to 100% of the CD47 binding activity after protease treatment (SEQ ID NOs: 57-59), demonstrating dissociation of the CD47-based blocking peptide from the SIRP- α variant after linker cleavage.

This result suggests SIRP- α variant fused to the N-terminus of CD47-based blocking peptide through a cleavable linker and spacers works well. The cleavable linker stabilizes the fusion complex and once cleaved, the extended N-terminus of the CD47-based blocking peptide, which includes a fragment of the cleavable linker attached to the N-terminus of the CD47-based blocking peptide, prevents binding of the CD47-based blocking peptide to the SIRP- α variant. The same effect is obtained by fusing a CD47-based blocking peptide having one or more amino acid additions, e.g., one glycine addition (e.g., sequences of SEQ ID NO: 46 in Table 6), at the N-terminus of the CD47-blocking peptide to the C-terminus of a SIRP- α variant by way of a cleavable linker and one or more spacers. This same effect is also observed by fusing a CD47-based blocking peptide having one or more amino acid substitutions, e.g., L101Q, L101Y, L101H, T102Q, or T102H (e.g., sequences of SEQ ID NOs: 41-45 in Table 6), to the C-terminus of a SIRP- α variant by way of a cleavable linker and one or more spacers. We have demonstrated that CD47-based blocking peptides

can be fused to the C-terminus of a SIRP- α variant and can block SIRP- α variant binding to CD47 before linker cleavage and release SIRP- α variant after linker cleavage (see, e.g., SEQ ID NO: 55 in FIG. 5A, and SEQ ID NOs: 55 and 56 in FIG. 5B).

5 Based on this information, we can create fusion proteins of CD47-blocked SIRP- α variants, i.e., fusing an Fc domain monomer or HSA to a SIRP- α variant, by choosing the orientation (e.g., N- or C-terminal fusion) that gives better results in pharmacokinetics, efficacy, safety, production, and stability of the product.

10 **Example 6 - Specific targeting of SIRP- α variants through antibody-binding peptide**

First, we used Cetuximab (Absolute Antibody, Ab00279-10.0), which is known to contain a binding site for the DLP having the sequence of SEQ ID NO: 64 and 65, to check if the SIRP- α variant construct including a SIRP- α variant and the DLP is
15 able to concentrate on bound antibody. We immobilized Cetuximab using EDC/NHS chemistry on a CM4 biacore chip (2000RU) and flowed the SIRP- α variant construct (SEQ ID NO: 66) at 100 nM and 50 nM using PBS 0.01% P20 as running and sample buffer at 30 μ L/min onto the chip (biacore T100). This SIRP- α variant construct (SEQ ID NO: 66) is designed to have two DLP sequences linked at N- and
20 C-terminal ends. FIG: 6 shows the binding of the SIRP- α variant construct, but not the SIRP- α variant alone, onto the chip. We then injected CD47-ECD and saw binding of CD47 in the case where the SIRP- α variant construct was used, demonstrating that the SIRP- α variant construct can bind EGFR and CD47 simultaneously (FIG. 6). Therefore, the SIRP- α variant construct including a SIRP- α
25 variant and a DLP injected to a cancer patient would concentrate at the site where the therapeutic antibody (e.g., Cetuximab) accumulates, increasing efficacy and reducing toxicity.

Secondly, we demonstrated that the SIRP- α variant construct including a
30 SIRP- α variant and a DLP can first bind Cetuximab that is bound to EGFR, and then bind CD47. A scheme of the binding complex is shown in FIG. 7A. We immobilized 3000 RUs of hrEGFR-Fc (R&D Systems) to a CM4 chip using EDC/NHS chemistry. Using PBS 0.01% P20 as sample and running buffer at 30 μ L/min (biacore T100), we injected different concentrations (4, 20, and 100 nM) of Cetuximab. Binding of

Cetuximab to the immobilized hrEGFR-Fc was observed. We then injected the SIRP- α variant construct (SEQ ID NO: 66) at 100 nM and observed binding. Binding was not observed when the SIRP- α variant was injected alone. We then injected CD47-ECD at 100 nM and observed binding. The data is shown in FIGs. 7B and 7C.

5 Therefore, we demonstrated that the formation of the quaternary complex EGFR-Cetuximab-SIRP- α variant construct of SEQ ID NO: 66-CD47 is possible. The SIRP- α variant construct including a SIRP- α variant and a DLP is able to bind and inhibit CD47 when the construct pre-concentrates at the diseased site by binding specifically to a tumor-specific antibody (e.g., Cetuximab). Furthermore, following
10 the same concept, a SIRP- α variant construct including a SIRP- α variant, a DLP, and a CD47-based blocking peptide is also able to bind and inhibit CD47 when the construct pre-concentrates at the diseased site by binding specifically to a tumor-specific antibody (e.g., Cetuximab).

15 **Example 7 – Phagocytosis assay**

SIRP- α variant construct (SEQ ID NO: 66), which includes a SIRP- α variant attached to DLPs through spacers, and a SIRP- α variant (SEQ ID NO: 31) were tested in a phagocytosis assay on DLD1 cells (FIG. 8). Phagocytosis assay was performed using methods modified from that described in Weiskopf et al, *Science*
20 341:88-91, 2013.

Buffy coats were obtained from the Stanford Blood Center from anonymous donors, and peripheral blood mononuclear cells were enriched by density gradient centrifugation over Ficoll-Paque Premium (GE Healthcare). Monocytes were purified using Macs Miltenyi Biotec Monocyte Isolation Kit II according to the manufacturer's
25 instructions. This is an indirect magnetic labeling system for the isolation of monocytes from human PBMCs. The isolated monocytes are differentiated into macrophages by culturing in RPMI 1640 media supplemented with 10% heat-inactivated human AB serum and 1% GlutaMax and 1% penicillin and streptomycin (GIBCO Life Technologies) for 6-10 days. For phagocytosis assay, 100,000 GFP+
30 DLD-1 cells are plated onto wells of Ultra low attachment U bottom 96 well plate (Corning 7007). 50 μ L/well of either 4 μ g/ml IgG1k isotype control or 4 μ g/ml Cetuximab (Absolute Antibody, Ab00279-10.0) are added to DLD-1 tumor cells and pre-incubated for 30 minutes at room temp. After that, 50 μ L/well SIRP-a variants are added and 50 μ L/well macrophages (1×10^6 /ml) (50,000 macrophages) are also

added to each well. Final dilution of antibodies and SIRP- α construct samples is 1:4. Cetuximab final concentration is 1 μ g/ml. The co-culturing of macrophages, tumor cells, antibodies and SIRP- α variant constructs are carried out for 2 hours at 37 °C. For analysis, cell samples were fixed, stained and analyzed by BD FACS Canto.

5 Primary human macrophages were identified by flow cytometry using anti-CD14, anti-CD45, or anti-CD206 antibodies (BioLegend). Dead cells were excluded from the analysis by staining with DAPI (Sigma). Phagocytosis was evaluated as the percentage of GFP+ macrophages and normalized to the maximal response by each independent donor against each cell line.

10 The results for the phagocytosis experiments are shown in Fig. 8. As shown, the SIRP- α variant construct (SEQ ID NO: 66) in combination with Cetuximab showed higher potency in inducing phagocytosis in DLD-1 cells than the SIRP- α variant alone (SEQ ID NO: 47) in combination with Cetuximab. This presumably works via the mechanism as we have conceptualized and it is probably due to higher
15 accumulation of SIRP- α variant construct (SEQ ID NO: 66) and Cetuximab on disease cells.

Example 8 – Modeling pH dependent binding of SIRP- α variants to CD47

To engineer pH-dependent binding of a SIRP- α variant of the invention,
20 histidine mutagenesis may be performed on the SIRP- α , especially on the region of SIRP- α that interacts with CD47. Crystal structures of a SIRP- α and CD47 complex (see, e.g., PDB ID No. 2JJS) and computer modeling may be used to visualize the three-dimensional binding site of SIRP- α and CD47. Computational design and modeling methods useful in designing a protein with pH-sensitive binding properties
25 are known in the literature and described in, e.g., Strauch et al., *Proc Natl Acad Sci* 111:675-80, 2014, which is incorporated by reference herein in its entirety. In some embodiments, computer modeling may be used to identify key contact residues at the interface of SIRP- α and CD47. Identified key contact residues may be substituted with histidine residues using available protein design software (e.g.,
30 RosettaDesign), which can generate various protein designs that can be optimized, filtered, and ranked based on computed binding energy and shape complementarity. Therefore, energetically favorable histidine substitutions at certain amino acid positions may be identified using computational design methods. Computer modeling may be also be used to predict the change in the three-dimensional

structure of SIRP- α . Histidine substitutions that generate a significant change in the three-dimensional structure of SIRP- α may be avoided.

Once energetically and structurally optimal amino acid substitutions are identified, the amino acids may be systematically substituted with histidine residues.

5 In some embodiments, one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum of 20) amino acids of SIRP- α may be substituted with histidine residues. In particular, amino acids located at the interface of SIRP- α and CD47, preferably, amino acids directly involved in the binding of SIRP- α to CD47, may be substituted with histidine residues. The SIRP- α variants of
10 the invention may include one or more (e.g., one, two, three, four, five, six, seven, eight, nine, ten, etc, with a maximum of 20) histidine residue substitutions. In other embodiments, naturally occurring histidine residues of SIRP- α may be substituted with other amino acid residues. In yet other embodiments, one or more amino acids of SIRP- α may be substituted with non-histidine residues in order to affect the
15 binding of naturally occurring or substituted histidine residues with CD47. For example, substituting amino acids surrounding a naturally occurring histidine residue with other amino acids may “bury” the naturally occurring histidine residue. In some embodiments, amino acids not directly involved in binding with CD47, i.e., internal amino acids (e.g., amino acids located at the core of SIRP- α) may also be
20 substituted with histidine residues. Table 4 lists specific SIRP- α amino acids that may be substituted with histidine residues. Contact residues are the amino acids located at the interface of SIRP- α and CD47. Core residues are the internal amino acids not directly involved in the binding between SIRP- α and CD47. The SIRP- α variants of the invention may include one or more (e.g., one, two, three, four, five,
25 six, seven, eight, nine, ten, etc, or all) of the substitutions listed in Table 4.

Example 9 – Generating and screening SIRP- α variants with pH-dependent binding to CD47

The SIRP- α variants containing one or more (e.g., one, two, three, four, five,
30 six, seven, eight, nine, ten, etc, with a maximum of 20) substitutions of amino acids with histidine residues may be generated using conventional molecular cloning and protein expression techniques. A nucleic acid molecule encoding a SIRP- α variant of the invention may be cloned into a vector optimized for expression in bacteria using well known molecular biology techniques. The vector can then be transformed

into bacteria cells (e.g., *E. coli* cells), which may be grown to optimal density prior to protein expression induction. After protein expression induction (i.e., using IPTG), bacterial cells may be allowed to grow for an additional 24 hours. Cells can be collected and the expressed SIRP- α variant protein may be purified from the cell culture supernatant using, e.g., affinity column chromatography. Purified SIRP- α variant may be analyzed by SDS-PAGE followed by Coomassie Blue staining to confirm the presence of protein bands of expected size.

Purified SIRP- α variants may be screened for pH-dependent binding to CD47 using available techniques in the art, such as phage display, yeast display, surface plasmon resonance, scintillation proximity assays, ELISA, ORIGEN immunoassay (IGEN), fluorescence quenching, and/or fluorescence transfer. Binding may also be screened using a suitable bioassay. The desired SIRP- α variant binds with higher affinity to CD47 under acidic pH (e.g., less than pH 7 (e.g., pH 6)) than under neutral pH (e.g., pH 7.4). The K_D of a SIRP- α /CD47 complex at pH 6 would be lower than K_D of a SIRP- α /CD47 complex at pH 7.4.

Example 10 – Testing SIRP- α variants with pH-dependent binding to CD47 in mice

Genetically engineered mouse models of various cancers, e.g., solid tumor and hematological cancer, may be used to test the pH-dependent binding of SIRP- α variants of the invention to CD47 at a diseased site in a mouse model. A SIRP- α variant may be injected directly or indirectly to the diseased site in a mouse, which may be dissected at the later time to detect the presence of the complex of SIRP- α variant and CD47 at the diseased site. Antibodies specific to SIRP- α variant or CD47 may be used in the detection.

Other Embodiments

All publications, patents, and patent applications mentioned in the above specification are hereby incorporated by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the

described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention. Other embodiments are within the following claims.

What is claimed is:

5

CLAIMS

What is claimed is:

1. A construct comprising a SIRP- α polypeptide or a fragment thereof, wherein said SIRP- α polypeptide or said fragment thereof preferentially binds CD47 on diseased cells or at a diseased site as compared to CD47 from non-diseased cells or at a non-diseased site.
2. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof binds to CD47 on diseased cells or at a diseased site with a higher affinity than it binds CD47 on non-diseased cells.
3. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof is attached to a blocking peptide.
4. The construct of claim 3, wherein said blocking peptide binds with higher affinity to a wild-type SIRP- α than to said SIRP- α polypeptide or a fragment thereof.
5. The construct of claim 3, wherein said SIRP- α polypeptide or a fragment thereof binds with higher affinity to a wild-type CD47 than to said blocking peptide.
6. The construct of claim 3, wherein said blocking peptide is a CD47-based blocking peptide.
7. The construct of claim 6, wherein said CD47-based blocking peptide has at least 80% amino acid sequence identity to the sequence of the wild-type, IgSF domain of CD47 (SEQ ID NO: 35), or a fragment thereof.
8. The construct of claim 7, wherein said CD47-based blocking peptide has the sequence of SEQ ID NO: 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, or 46.
9. The construct of claim 3, wherein said SIRP- α polypeptide or a fragment thereof is attached to said blocking peptide by a cleavable linker and optionally one or more spacers.
10. The construct of claim 9, wherein said cleavable linker is cleaved under acidic pH and/or hypoxic conditions.
11. The construct of claim 9, wherein said cleavable linker is cleaved by a tumor-associated enzyme.
12. The construct of claim 11, wherein said tumor-associated enzyme is a protease.
13. The construct of claim 12, wherein said protease is selected from the group consisting of matriptase (MTSP1), urinary-type plasminogen activator (uPA), legumain, PSA (also called KLK3, kallikrein-related

peptidase-3), matrix metalloproteinase-2 (*MMP-2*), MMP-9, human neutrophil elastase (HNE), and proteinase 3 (Pr3).

14. The construct of claim 9, wherein said cleavable linker has the sequence of L/S/G/R/S/D/N/H (SEQ ID NO: 47); /K/R/KQ/gAS/RK/A (SEQ ID NO: 76); ---/-/-/N/-/-/- (SEQ ID NO: 78); SI/SQ/-/YQR/S/S/-/- (SEQ ID NO: 81); S/S/K/L/Q (SEQ ID NO: 82); -/P/-/-/LI/-/-/- (SEQ ID NO: 83); G/P/L/G//A/G/Q (SEQ ID NO: 85); P/V/G/L//G (SEQ ID NO: 86); H/P/V/G/L/L/A/R (SEQ ID NO: 87); -/-/-/MIAT/-/-/-/- (SEQ ID NO: 88); -/Y/Y/NTA/-/-/-/- (SEQ ID NO: 89); PRFKIIGG (SEQ ID NO: 90); PRFRIIGG (SEQ ID NO: 91); SSRHRRALD (SEQ ID NO: 92); RKSSIIIRMRDVVL (SEQ ID NO: 93); SSSFDKGKYKKGDDA (SEQ ID NO: 94); SSSFDKGKYKRGDDA (SEQ ID NO: 95); IEGR (SEQ ID NO: 95A); IDGR (SEQ ID NO: 96); GGSIDGR (SEQ ID NO: 97); PLGLWA (SEQ ID NO: 98); or DVAQFVLT (SEQ ID NO: 99).

15. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof is attached to an antibody-binding peptide.

16. The construct of claim 15, wherein said antibody-binding peptide binds to a constant region of an antibody reversibly or irreversibly or to a fragment antigen-binding (Fab) region of an antibody reversibly or irreversibly or to a variable region of an antibody reversibly or irreversibly.

17. The construct of claim 15, wherein said antibody-binding peptide has at least 75% amino acid sequence identity to the sequence of a disease localization peptide (DLP) (SEQ ID NO: 64, 65 or 66).

18. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof is attached to an Fc domain monomer, an Fc domain, a human serum albumin (HSA), an albumin binding peptide or a polymer, wherein said polymer comprises a polyethylene glycol (PEG) chain or a polysialic acid chain.

19. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof is attached to an antibody or a fragment thereof.

20. The construct of claim 19, wherein said antibody binds to one or more of the following: 4-1BB, 5T4, ALK1, ANG-2, B7-H3, B7-H4, c-Met, CA6, CCR4, CD123, CD19, CD20, CD22, CD27, EpCAM, CD30, CD32b, CD33, CD37, CD38, CD40, CD52, CD70, CD74, CD79b, CD98, CEA, CEACAM5, CLDN18.2, CLDN6, CS1, CTLA-4, CXCR4, DLL-4, EGFR, EGP-1, ENPP3, EphA3, ETBR, FGFR2, fibronectin, FR-alpha, frizzled receptor, GCC, GD2, glypican-3, GPNMB, HER2, HER3, HLA-DR, ICAM-1, IGF-1R, IL-3R, LIV-1, mesothelin, MUC16, MUC1, NaPi2b, Nectin-4, Notch 2, Notch 1, OX-40, PD-1, PD-L1, PD-L2, PDGFR- α , PS, PSMA, SLTRK6, STEAP1, TEM1, VEGFR, CD25, DKK-1, and/or CSF-1R.

21. The construct of claim 19, wherein said antibody is cetuximab, necitumumab, pembrolizumab, nivolumab, pidilizumab, MEDI0680, atezolizumab, avelumab, durvalumab, MEDI6383, MEDI6469, RG7888, ipilimumab, tremelimumab, urelumab, PF-05082566, enoblituzumab, vantictumab, varlilumab,

mogamulizumab, SAR650984, daratumumab, trastuzumab, trastuzumab emtansine, pertuzumab, elotuzumab, rituximab, ofatumumab, obinutuzumab, RG7155, FPA008, anti-HER2 antibody, anti-CD20 antibody, anti-CD19 antibody, anti-CS1 antibody, anti-CD38 antibody, panitumumab, or brentuximab vedotin.

22. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof has at least 80% sequence identity to any of SEQ ID NOs: 3-12 and 24-34.

23. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof is one of or a fragment of SEQ ID NOs: 13-23.

24. The construct of claim 1, wherein said SIRP- α polypeptide or a fragment thereof comprises at least one amino acid substitution with a histidine residue.

25. The construct of claim 24, wherein said at least one amino acid substitution occurs at one or more of the following amino acid positions: 29, 30, 31, 32, 33, 34, 35, 52, 53, 54, 66, 67, 68, 69, 74, 93, 96, 97, 98, 100, 4, 6, 27, 36, 39, 47, 48, 49, 50, 57, 60, 72, 74, 76, 92, 94, 103, relative to a sequence of any one of SEQ ID NOs: 3-12.

26. The construct of claim 2, wherein said affinity is at least two, at least four, or at least six-fold higher.

27. The construct of claim 2, wherein said SIRP- α polypeptide or a fragment thereof binds with at least two, at least four, or at least six-fold higher affinity to CD47 under hypoxic condition than under physiological condition.

28. The construct of claim 2, wherein said SIRP- α polypeptide or a fragment thereof binds with at least two, at least four, or at least six-fold higher affinity to CD47 under acidic pH than under neutral pH.

29. A pharmaceutical composition comprising a therapeutically effective amount of the construct of claim 1.

30. A method of increasing phagocytosis of a target cell in a subject, comprising administering to said subject a construct according to claim 1 or a pharmaceutical composition according to claim 29.

SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF

ABSTRACT

The invention relates to compositions and methods of constructs comprising a SIRP- α polypeptide, including SIRP- α variants. The constructs may be engineered in a variety of ways to respond to environmental factors, such as pH, hypoxia, and/or the presence of tumor-associated enzymes or tumor-associated antigens. The constructs of the invention may be used to treat various diseases, such as cancer, preferably solid tumor or hematological cancer.

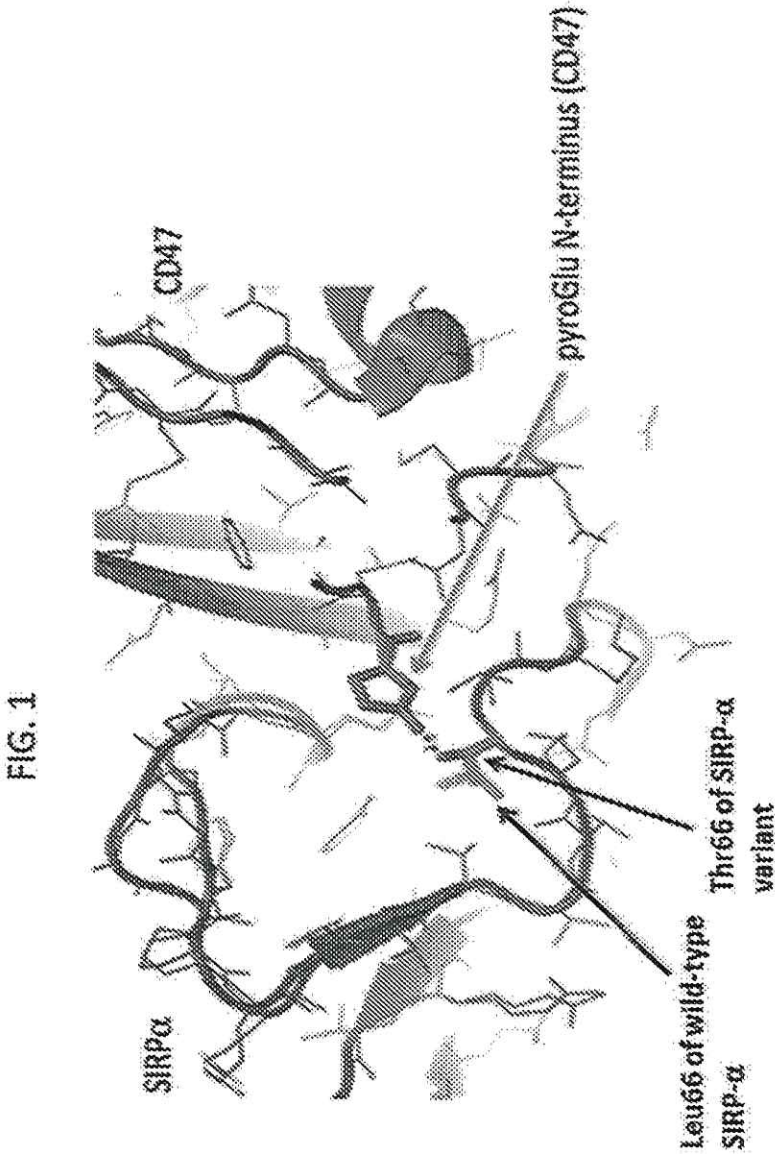
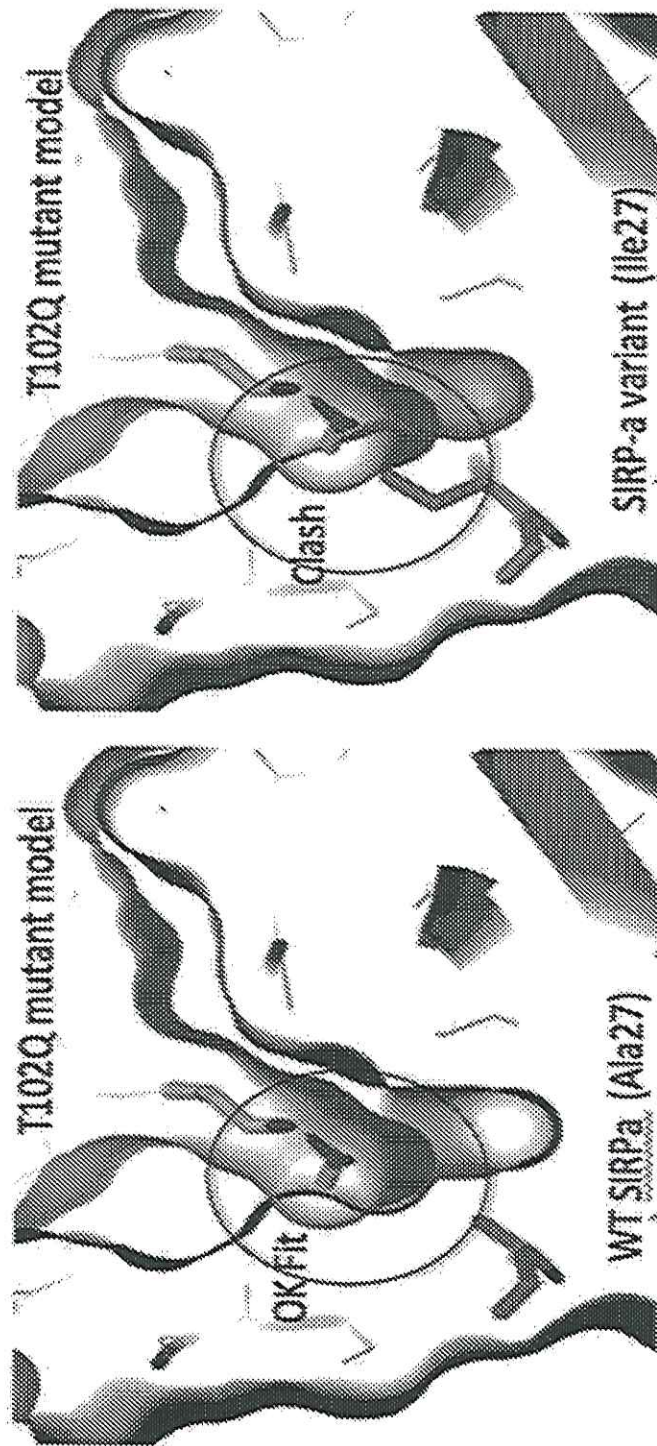


FIG. 2



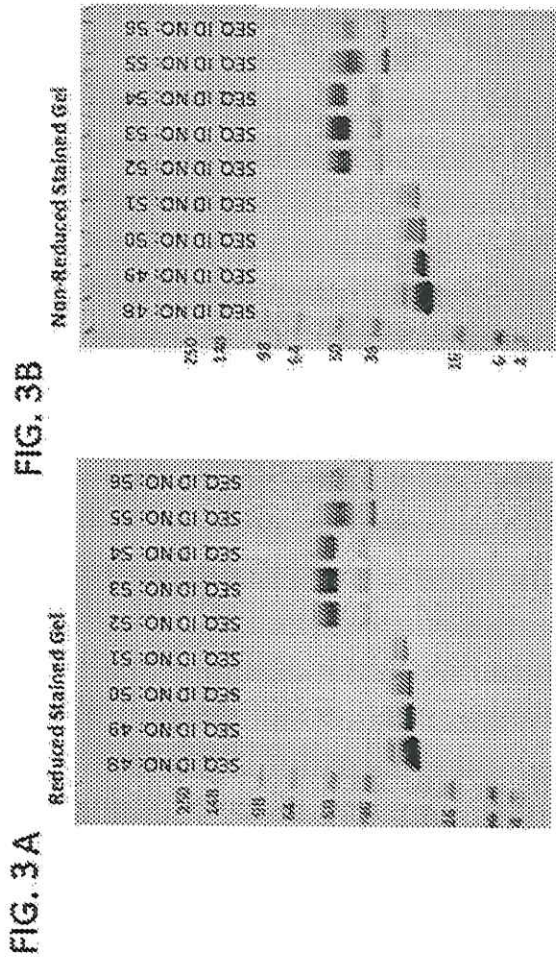


FIG. 4A

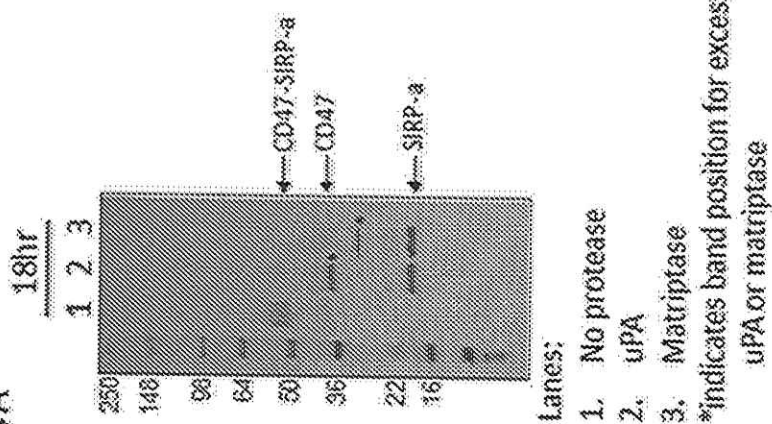
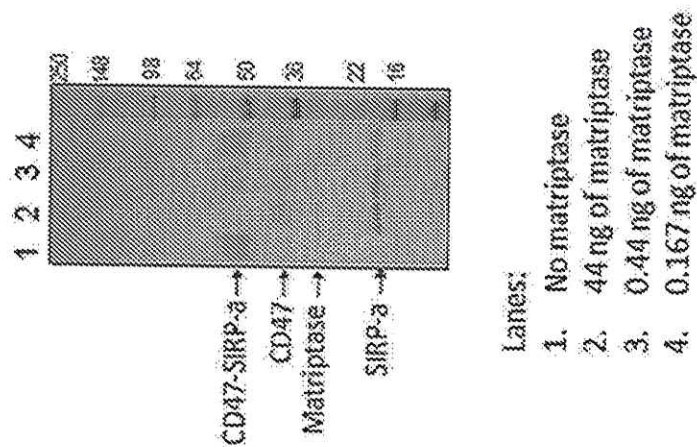


FIG. 4B



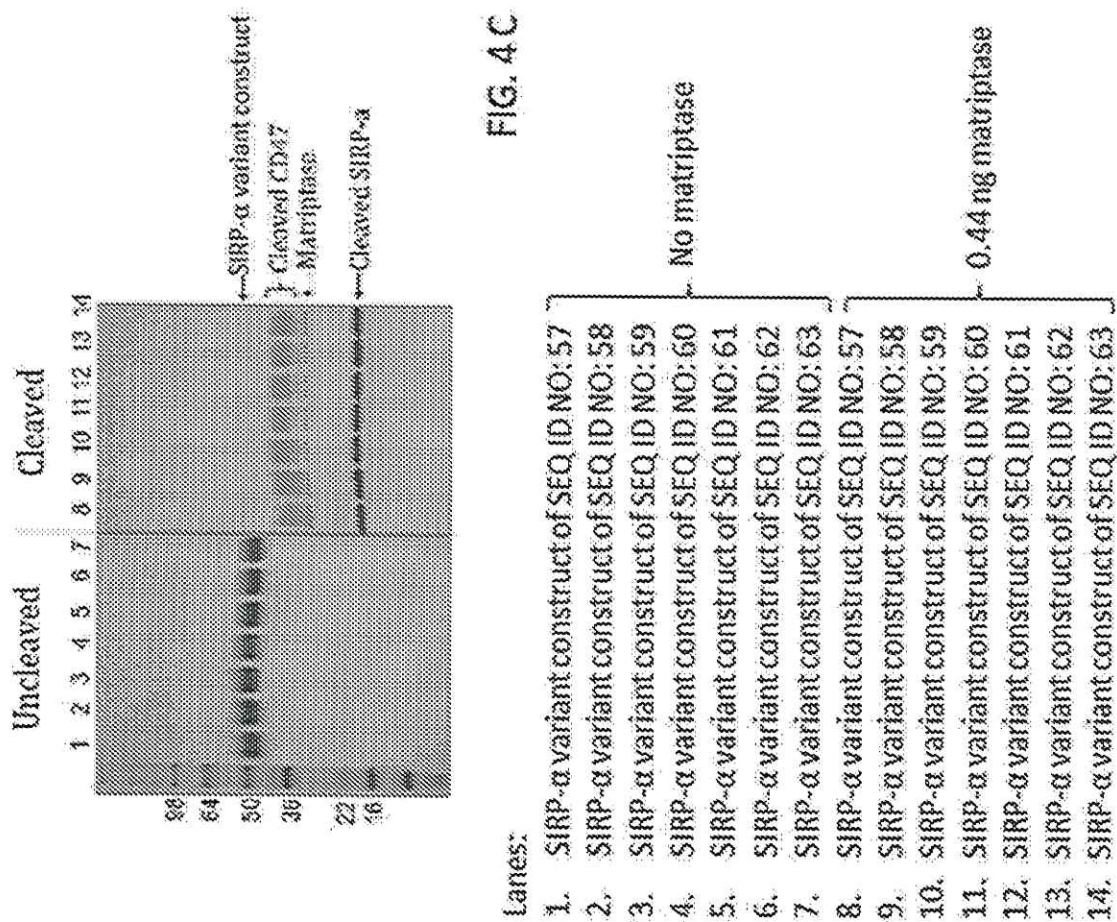


FIG. 4 C

FIG. 5A

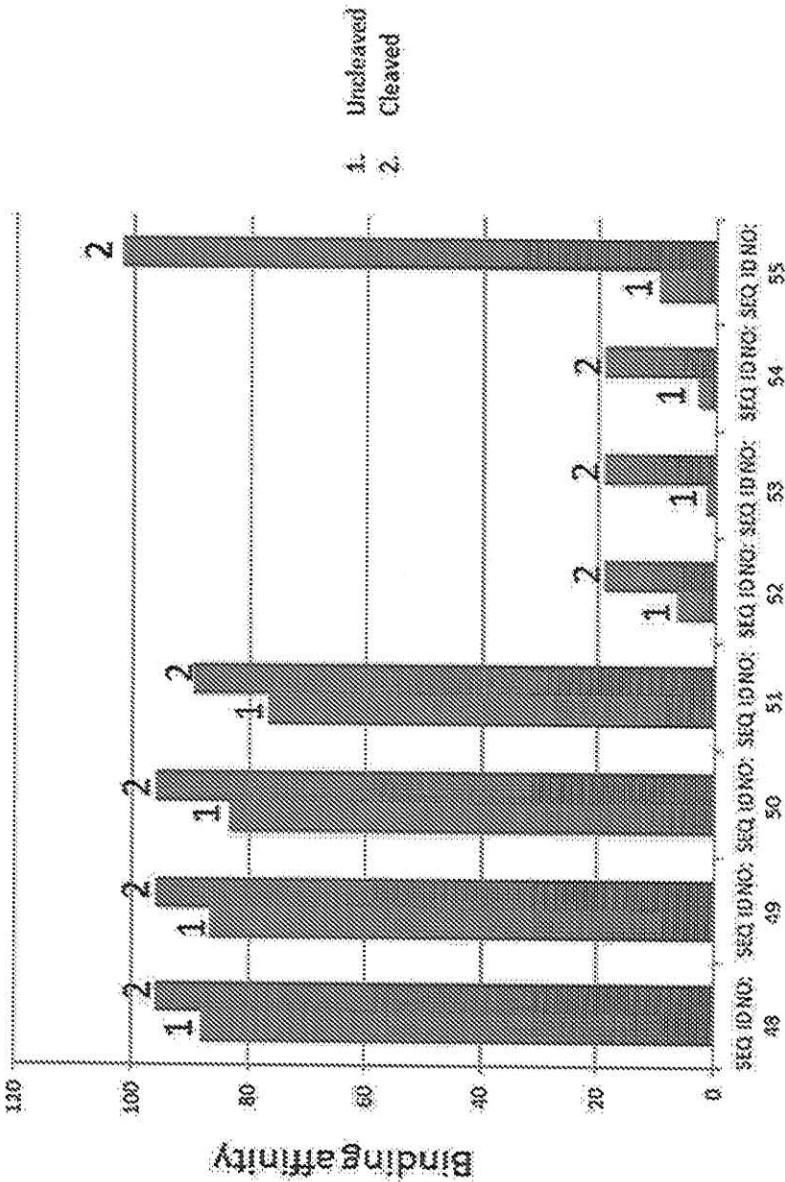


FIG. 5B

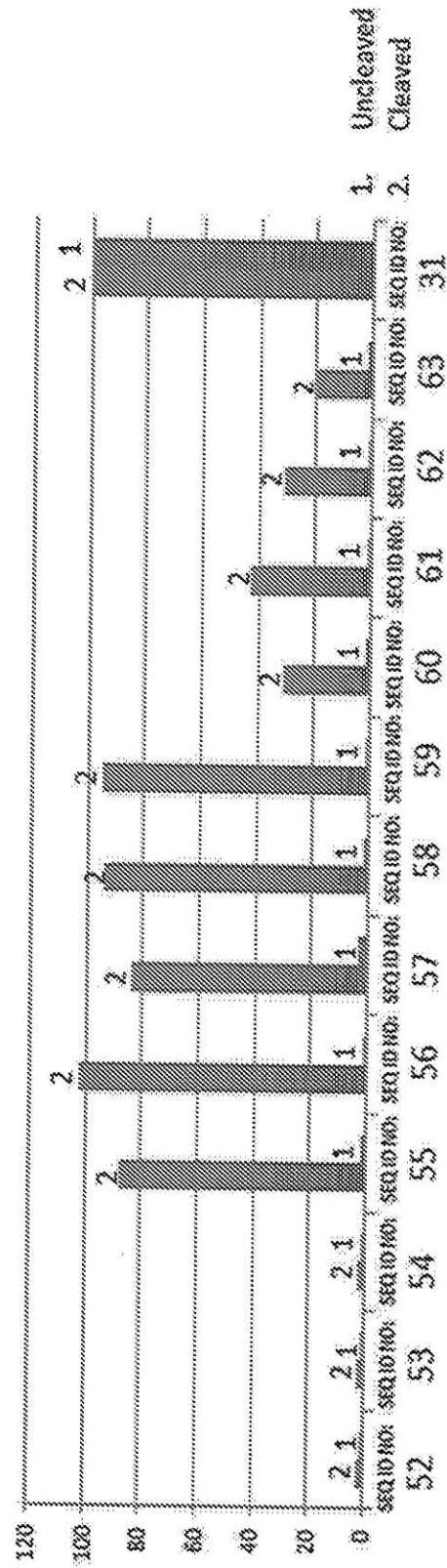


FIG. 6

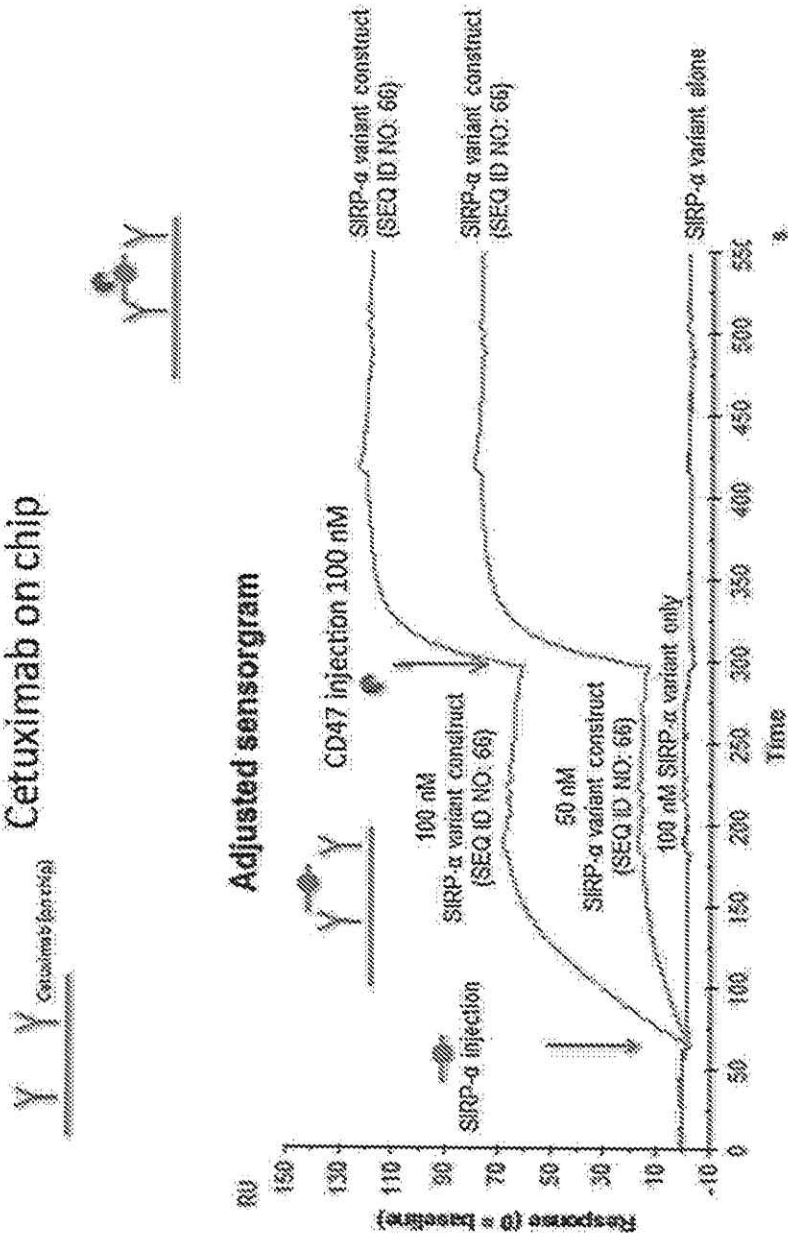


FIG. 7A



FIG. 7B

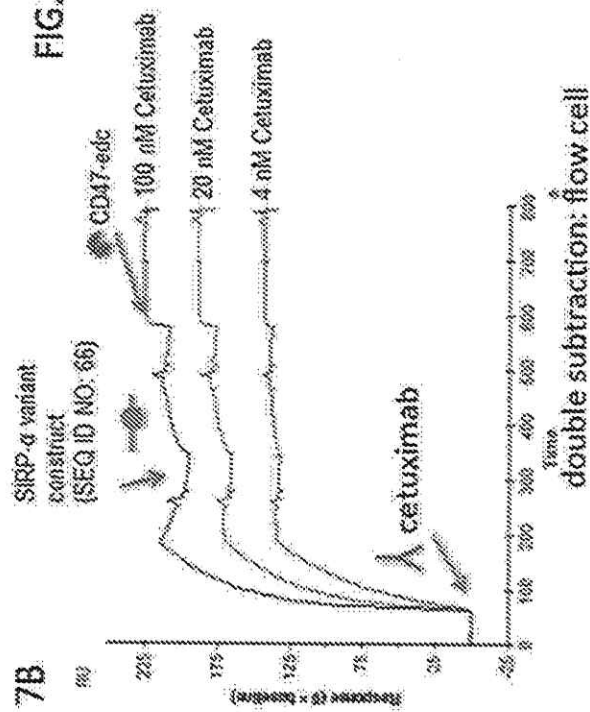


FIG. 7 C

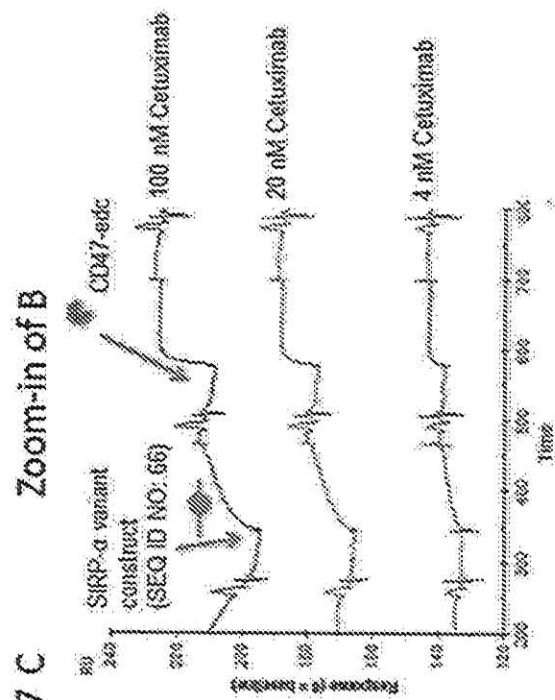
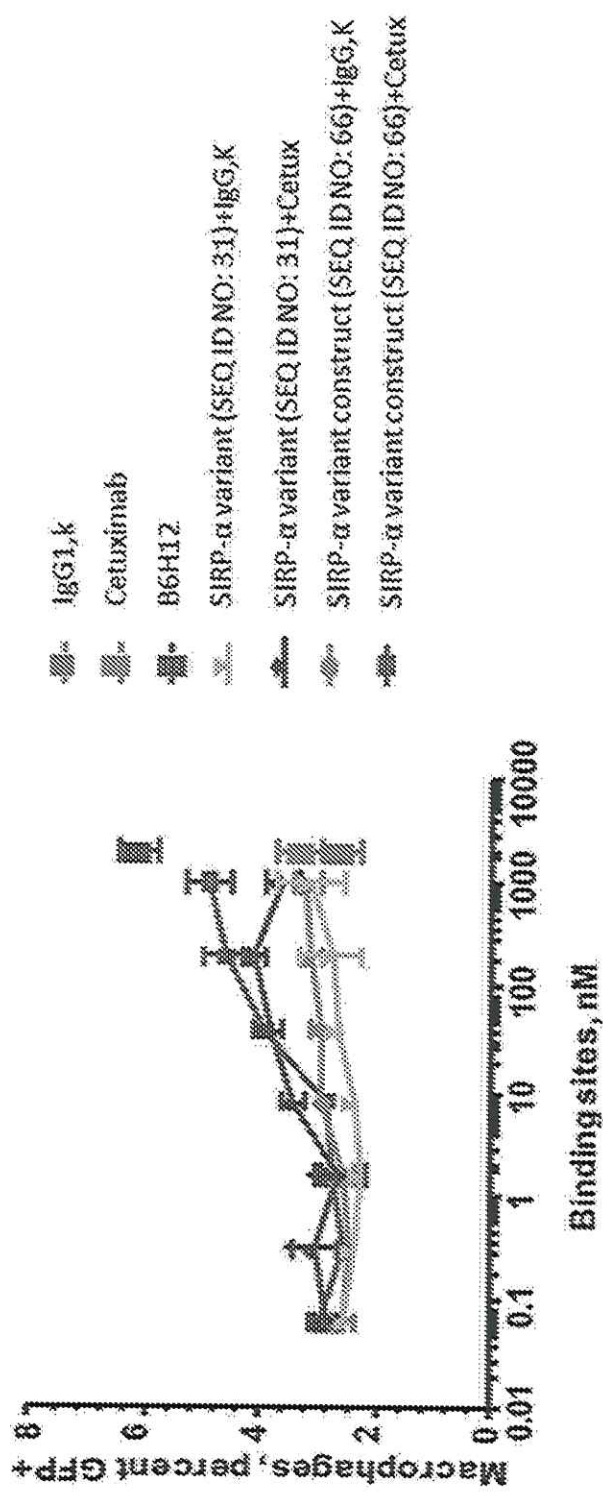


FIG. 8



Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		
The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.			

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Laura		Deming		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Palo Alto	State/Province	CA	Country of Residence	US
Mailing Address of Inventor:					
Address 1		555 Bryant Street, #517			
Address 2					
City	Palo Alto	State/Province	CA		
Postal Code	94301	Country	US		
Inventor 2					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Corey		Goodman		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Marshall	State/Province	CA	Country of Residence	US
Mailing Address of Inventor:					
Address 1		PO Box 803			
Address 2					
City	Marshall	State/Province	CA		
Postal Code	94940	Country	US		
Inventor 3					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Jaume		Pons		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		47819-702.301	
		Application Number			
Title of Invention		SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF			
City	San Francisco	State/Province	CA	Country of Residence	US
Mailing Address of Inventor:					
Address 1		4598 17th Street			
Address 2					
City	San Francisco	State/Province	CA		
Postal Code	94114	Country i	US		
Inventor 4					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Bang	Janet	Sim		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	Brisbane	State/Province	CA	Country of Residence	US
Mailing Address of Inventor:					
Address 1		58 Sierra Point Road			
Address 2					
City	Brisbane	State/Province	CA		
Postal Code	94005	Country i	US		
Inventor 5					Remove
Legal Name					
Prefix	Given Name	Middle Name	Family Name	Suffix	
	Marija		Vrljic		
Residence Information (Select One) ☒ US Residency ☐ Non US Residency ☐ Active US Military Service					
City	San Mateo	State/Province	CA	Country of Residence	US
Mailing Address of Inventor:					
Address 1		130 Barneson Avenue, Apt. 4			
Address 2					
City	San Mateo	State/Province	CA		
Postal Code	94402	Country i	US		
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.					
Add					

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).	
☐ An Address is being provided for the correspondence Information of this application.	

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		
Customer Number	21971		
Email Address	patentdocket@wsgr.com	Add Email	Remove Email

Application Information:

Title of the Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		
Attorney Docket Number	47819-702.301	Small Entity Status Claimed ☐	
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	10	Suggested Figure for Publication (if any)	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
☐ Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.			
Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	21971		

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending	Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS1544528	Claims benefit of provisional	62138257	2015-03-25
Prior Application Status	Pending	Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS1544528	Claims benefit of provisional	62138306	2015-03-25
Prior Application Status	Pending	Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS1544528	Claims benefit of provisional	62138282	2015-03-25
Prior Application Status	Expired	Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS1544528	Claims benefit of provisional	62035057	2014-08-08
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.			

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Remove
PCT/US15/44528	US	2015-08-10	Access Code ^j (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

☒ Assignee

☐ Legal Representative under 35 U.S.C. 117

☐ Joint Inventor

☐ Person to whom the inventor is obligated to assign.

☐ Person who shows sufficient proprietary interest

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name

Alexo Therapeutics International

Mailing Address Information For Applicant:

Address 1

c/o Alexo Therapeutics Inc.

Address 2

951 Gateway Blvd, Suite 201

City

South San Francisco

State/Province

CA

Country

US

Postal Code

94080

Phone Number

Fax Number

Email Address

Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	47819-702.301
	Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF	

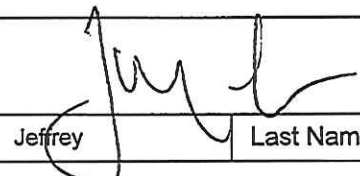
Assignee 1				
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.				
If the Assignee or Non-Applicant Assignee is an Organization check here. ☐				
Prefix	Given Name	Middle Name	Family Name	Suffix
Mailing Address Information For Assignee including Non-Applicant Assignee:				
Address 1				
Address 2				
City		State/Province		
Country i		Postal Code		
Phone Number		Fax Number		
Email Address				
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.				

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).

This Application Data Sheet must be signed by a patent practitioner if one or more of the applicants is a juristic entity (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, all joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of all joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature				Date (YYYY-MM-DD)	2015-12-16
First Name	Jeffrey	Last Name	Morton	Registration Number	L1070
Additional Signature may be generated within this form by selecting the Add button.					

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47819-702.301
		Application Number	
Title of Invention	SIRP-ALPHA VARIANT CONSTRUCTS AND USES THEREOF		

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**