Antibody Therapeutics, 2020, Vol. JNL volume, No. ***1–14***
doi:10.1093/abt/tbaa017
Advance Access Publication on 20 January 2001

Review Article

Preclinical and clinical development of therapeutic antibodies targeting functions of CD47 in the tumor microenvironment

Sukhbir Kaur, Kyle V. Cicalese, Rajdeep Banerjee and David D. Roberts*

Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Received: June 22, 2020; Revised: July 22, 2020; Accepted: August 5, 2020

ABSTRACT

CD47 is a ubiquitously expressed cell surface glycoprotein that functions as a signaling receptor for thrombospondin-1 and as the counter-receptor for signal regulatory protein-α (SIRPα). Engaging SIRPα on macrophages inhibits phagocytosis, and CD47 thereby serves as a physiological marker of self. However, elevated CD47 expression on some cancer cells also protects tumors from innate immune surveillance and limits adaptive antitumor immunity via inhibitory SIRPα signaling in antigen-presenting cells. CD47 also mediates inhibitory thrombospondin-1 signaling in vascular cells, T cells, and NK cells, and blocking inhibitory CD47 signaling on cytotoxic T cells directly increases tumor cell killing. Therefore, CD47 functions as an innate and adaptive immune checkpoint. These findings have led to the development of antibodies and other therapeutic approaches to block CD47 functions in the tumor microenvironment. Preclinical studies in mice demonstrated that blocking CD47 can limit the growth of hematologic malignancies and solid tumors and enhance the efficacy of conventional chemotherapy, radiation therapy, and some targeted cancer therapies. Humanized CD47 antibodies are showing promise in early clinical trials, but side effects related to enhanced phagocytic clearance of circulating blood cells remain a concern. Approaches to circumvent these include antibody preloading strategies and development of antibodies that recognize tumor-specific epitopes of CD47, SIRPα antibodies, and bivalent antibodies that restrict CD47 blockade to specific tumor cells. Preclinical and clinical development of antibodies and related biologics that inhibit CD47/SIRPα signaling are reviewed, including strategies to combine these agents with various conventional and targeted therapeutics to improve patient outcome for various cancers.

Statement of Significance: Preclinical studies defining the function of CD47 in cancer cells and in modulating anti-tumor immunity have led to the development of humanized CD47 antibodies and related biologics. A growing number are entering clinical trials as single agents or used in combination with other therapeutics for treating various cancers.

KEYWORDS: humanized CD47 antibodies; bifunctional antibodies; immune checkpoint; immunotherapy; signal regulatory protein-α

INTRODUCTION

CD47 is a signaling receptor for thrombospondin-1 (TSP1) and the counter-receptor for signal regulatory protein-α (SIRPα) [1–3]. CD47 also associates laterally in the plasma membrane with a subset of integrins and regulates their function [1]. Integrin activation mediates CD47 functions in regulating cell adhesion and migration [1]. TSP1 interaction with CD47 regulates its intrinsic signaling functions in multiple cell types and controls nitric oxide/cGMP

*To whom correspondence should be addressed. David D. Roberts, NIH, Building 10 Room 2S235, 10 Center Drive, Bethesda, MD 20892-1500, USA. Tel: 301-480-4369; Email: droberts@mail.nih.gov

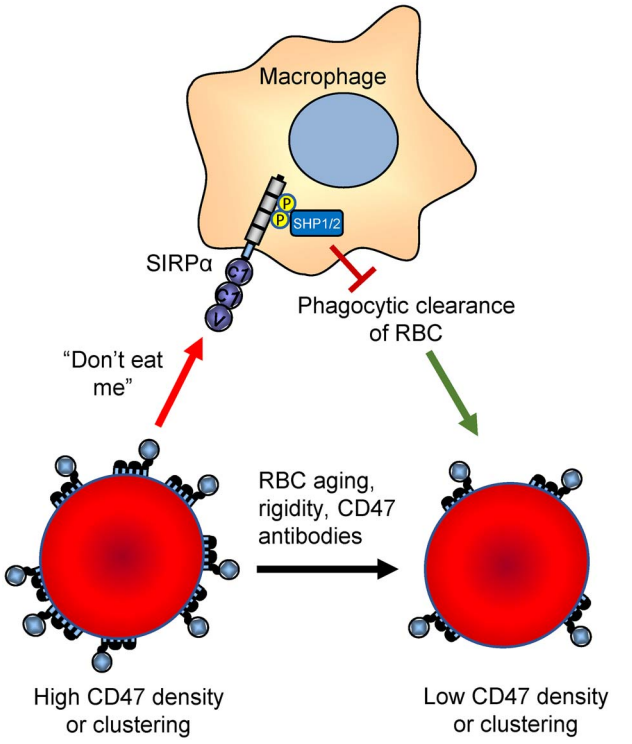

Figure 1. Antiphagocytic function of CD47 on red blood cells (RBCs). Young RBCs express ~25 000 copies of CD47, which inhibits their phagocytic clearance [5]. RBC aging [119], diseases that increase RBC rigidity [120], and exposure to function-blocking CD47 antibodies decrease the SIRPα-mediated "don't eat me" signal and thereby increase erythrophagocytosis.

signaling in vascular cells [1]. The latter has physiological roles in regulating blood pressure, platelet hemostasis, tissue perfusion, and tissue responses to ischemic injuries and genotoxic stress. Conversely, CD47 serves as a ligand to induce inhibitory SIRPα signaling in macrophages [4], which has a physiological role in self-recognition [2]. Loss of inhibitory SIRPα signaling in macrophages results in more rapid turnover of circulating platelets and red blood cells (Fig. 1) [5, 6]. This accounts for the side effects of anemia and thrombocytopenia that are observed in animals and patients treated with CD47-targeted antibodies that block this interaction [7, 8].

The increased CD47 expression on some cancer cells limits their phagocytic clearance by macrophages [2], despite increased expression of pro-phagocytic markers such as calreticulin on cancer cells (Fig. 2A,B) [9, 10] or suppression of the anti-phagocytic markers LILRB1 and CD24/Siglec-10 in cancer cells [11, 12]. Cell-intrinsic inhibitory CD47 signaling in T cells, macrophages, dendritic cells, and NK cells plays additional roles in immune regulation, cell survival, and death signaling (Fig. 2A) [1, 13–15]. Based on the hypothesis that the CD47/SIRPα interaction represents a major innate immune checkpoint in cancer [16], several antibodies and other antagonists of CD47 binding to SIRPα have entered Phase 1 and 2 clinical trials [3] (Table 1). This review focuses on the preclinical development of therapeutic antibodies targeting CD47 and SIRPα, ongoing clinical trials, and future challenges and opportunities for developing effective CD47-targeted cancer therapeutics.

Preclinical studies using CD47 antibodies

Several antibodies have been identified that alter specific functions of CD47 by directly inhibiting its interactions with TSP1, SIRPα, integrins, or growth factor receptors (Table 2). Other CD47 antibodies exhibit agonist or antagonist activities that may result from altering clustering or inducing conformational changes that alter the intrinsic intracellular signaling functions of CD47. B6H12 is a function-blocking antibody that inhibits CD47 interactions with TSP1, SIRPα, some integrins, and EGF receptor [17–20]. B6H12 inhibits TSP1-dependent activation of $\alpha v\beta 3$ integrin while increasing the activation of $\alpha 4\beta 1$ integrin [21, 22]. Relevant to the role of CD47 in tumor immunology, B6H12 directly induces killing of tumor cells by NK cells [23], B cell migration [24], and regulatory T cell differentiation [25]. B6H12 blocks the inhibition of T and NK cell activation by TSP1 [13, 15]. B6H12 inhibits homotypic aggregation of monocytic cells [26], extravasation of neutrophils [27], production of mature IL-1β by macrophages [14], and the maturation and function of dendritic cells [28]. B6H12 also acts on other cell types in the tumor microenvironment, inhibiting nitric oxide signaling and angiogenic responses [29, 30], protecting nonmalignant cells from death and DNA damage caused by ionizing radiation or cytotoxic chemotherapy [31, 32], inhibiting uptake and functional responses in endothelial cells induced by extracellular vesicles released by breast cancer stem cells (CSC) [33], forcing differentiation of CSC [20], and inhibiting CD47-dependent platelet adhesion under flow [34]. Thus, although preclinical cancer studies generally interpret B6H12 inhibition as evidence for SIRPα-mediated functions of CD47 *in vitro* and in xenograft models, such inhibition may result from SIRPα-independent mechanisms including perturbing TSP1- or integrin-mediated activities of CD47.

Several CD47 antibodies directly induce death of cancer cells including 1F7, AD22, MABL, and CC2C6 (Table 2) [35–37]. These antibodies are selective CD47 agonists that in some cases do not induce death of nonmalignant cells and instead exhibit cytoprotective activities in the latter (Fig. 3). CD47 agonist antibodies are also being developed as potential cancer therapeutics [38]. Soluble AD22 and 1F7 antibodies induced apoptosis of Jurkat T lymphoma cells as well as CD3ε-stimulated PBMC [35]. Similar results were reported for CC2C6 [36]. AD22 recognizes the IgV domain of CD47 proximal to epitopes defined by B6H12 and 1F7, whereas 2D3 reacts with a distant epitope. Soluble B6H12 and 2D3 antibodies did not induce cell death, but B6H12 can induce death of some cancer cells when immobilized [35, 39]. 1F7 also induced death of several breast cancer cell lines [40]. 1F7-induced killing of breast cancer cells was not mediated by active caspases, Bcl-2 degradation, or mitochondrial cytochrome *c* release but involved inhibiting protein kinase A via $G_i\alpha$ [40]. Similar induction of apoptosis was observed in B cell chronic lymphocytic leukemia cells exposed to immobilized CD47 antibody BRIC126 but not when the antibody was used in

Table 1. Active and completed clinical trials using CD47 antibodies and related biologics

Antibody	Molecule	Indications	Type of trial	Registry
Hu5F9-G4/Magrolimab (Forty Seven Inc/Gilead)	CD47 mAb	Solid tumors	Single agent	NCT02216409, NCT02678338
Hu5F9-G4/Magrolimab	CD47 mAb	B cell lymphoma	+Rituximab	NCT02953509
Hu5F9-G4/Magrolimab	CD47 mAb	Colorectal cancer	+Cetuximab	NCT02953782
Hu5F9-G4/Magrolimab	CD47 mAb	Hematologic	+Azacitidine	NCT03248479
Hu5F9-G4/Magrolimab	CD47 mAb	Non-Hodgkin's Lymphoma	PRISM Study	NCT03527147
CC-90002 (Celgene)	CD47 mAb	AML, MDS	Single agent	NCT02488811, NCT02641002
CC-90002	CD47 mAb	Solid & hematologic cancers	+Rituximab	NCT02367196
IBI188 (Innovent Biologics)	CD47 mAb	Advanced malignancies	Single agent	NCT03763149
IBI188 (Innovent Biologics)	CD47 mAb	Advanced malignancies	+Rituximab	NCT03717103,
TTI-621 (Trillium Therapeutics)	SIRPα-Ig fusion protein	Solid tumors and mycosis fungoides	Single agent +PD-1/PDL1 Inhibitor +PEG-IFN2a +T-Vec + radiation	NCT02890368
TTI-621	SIRPα-Ig fusion protein	Solid & hematologic cancers	+Rituximab +Nivolumab	NCT02663518
TTI-622 (trillium)	SIRPα-IgG4 fusion protein	Lymphoma or myeloma	Single agent + Rituximab + PD-1 Inhibitor +Proteasome-inhibitor	NCT03530683
SRF231 (Surface Oncology)	CD47 mAb	Solid tumors, hematologic	Single agent	NCT03512340
AO-176 (Arch Oncology)	CD47 mAb	Solid tumors	Single agent	NCT03834948
ALX148 (Alexo Therapeutics)	SIRPα-Fc* fusion protein	Solid tumors and non-Hodgkin's lymphoma	+Pembrolizumab +Trastuzumab +Rituximab +Trastuzumab, Ramucirumab, Paclitaxel	NCT03013218
ALX148	SIRPα-Fc* fusion protein	Myelodysplastic SYNDROME	+ Azacitidine	NCT04417517
CC-95251 (Celgene)	SIRPα mAb	Solid tumors, hematologic cancers	+Rituximab +Cetuximab	NCT03783403
TG-1801 (TG Therapeutics)	CD47/CD19 bsAb	B-cell lymphoma	Single agent	NCT03804996
BI765063/OSE-172 (Boehringer Ingelheim/OSE)	SIRPα mAb	Solid tumors	+ PD-1 mAb	NCT03990233
SGN CD47M (Seattle Genetics)	mAb-drug conjugate	Solid tumors	Single agent	NCT03957096
IBI-322 (Innovent Biologics)	CD47/PD-L1 bsAb	Advanced tumors	Single agent	NCT04338659, NCT04328831
HX-009 (Waterstone Hanxbio)	PD-1/CD47 bsAb	Solid tumors	Single agent	NCT04097769
IMC-002 (ImmuneOncia)	CD47 mAb	Solid tumors and lymphoma	Single agent	NCT04306224

Table 2. Properties of preclinical and clinical CD47 antibodies and related inhibitors of CD47 function

Antibody Name	Source	Origin	Isotype	Specificity	CD47/SIRPα blockade	Agonist activity
MIAP301	Research	rat	IgG2a	muCD47	Yes	
A4	Research	Alpaca	Nanobody	muCD47	Yes	
A4-Fc	Research	Fusion	IgG2a	nuCD47	Yes	
OX101	Research	Mouse	IgG1	ratCD47	Yes	
B6H12	Research	Mouse	IgG1	huCD47	Yes	Yes/No
2D3	Research	Mouse	IgG1	huCD47	No	
Vx1000R	Arch Oncology	Mouse		huCD47	Yes	
MAB4670	Research	Mouse	IgG1	huCD47	Yes	
BRIC126	Research	Mouse	IgG2b	huCD47	Yes	Yes/No
CC2C6	Research	Mouse	IgG1	huCD47	Yes	Yes
1F7	Research	Mouse	IgG1	huCD47		Yes
Ad22	Research	Mouse	IgG1	huCD47		Yes
mAb400	Research	Mouse	IgG2a	hu/muCD47	Yes	
MIAP410	Research	Mouse	IgG1	hu/muCD47	Yes/No	
MABL sc(Fv)$_2$	Research	Mouse	(scFv)$_2$	huCD47		Yes
MABL scFv	Research	Mouse	scFv	huCD47		No
Hu5F9-G4	47/Gilead	Humanized	IgG4	huCD47	Yes	
AO-176	Arch Oncology	Humanized	IgG2	huCD47	Yes	Yes
AO-104	Arch Oncology	Humanized	IgG4	huCD47	Yes	
SRF231	Surface Oncology	Fully human	IgG4	huCD47	Yes	
SHR-1603	Hengrui	Humanized	IgG4	huCD47	Yes	
CC90002	Celgene	Humanized	IgG4	huCD47	Yes	
HuNb1	Shanghai Novamab	Camel VHH-fusion	IgG4	huCD47	Yes	
C47B	Janssen	Fully human	IgG1	huCD47	Yes	
IBI188	Innovent	Fully human	IgG4	huCD47	Yes	
ZF1	Beijing Inst. of Biotechnology	Fully human	IgG1	huCD47	Yes	
AMMS4-G4	Beijing Inst. of Biotechnology	Fully human	IgG4	huCD47	Yes	
h4C1	CAS Key Laboratory	Humanized		huCD47	Yes	
CD47xPD-L1	CAS Key Laboratory	bsAb from h4C1	IgG4	CD47xPD-L1	Yes	
IBI-322	Innovent	bsAb		CD47xPD-L1	Yes	
HX-009	Waterstone Hanxbio	bsAb		CD47xPD1	Yes	
CD47xMSLN	Novimmune	bsAb	IgG1	CD47xMSLN	Yes	
OV-TL3/CD3	research	bsAb F(ab')2	No Fc	CD47xCD3	?	
CD47xEGFR	Xinxiang Medical University	SIRPαV-bsAb	IgG1	CD47xEGFR	Yes	
HuNb1-rituximab	Shanghai Novamab	Bispecific scFv	No Fc	CD47xCD20	Yes	
RTX-CD47	University of Groningen	Tandem-scFv	No Fc	CD47xCD20	Yes	
TG-1801	TG Therapeutics	Fully human bsAb	IgG1	CD47xCD19	Yes	
CC-95251	Celgene	Humanized		huSIRPα	Yes	
OSE-172	OSE Immunotherapeutics			huSIRPα	Yes	
TTI-621	Trillium	SIRPα fusion	IgG1	huCD47	Yes	
TTI-622	Trillium	SIRPα fusion	IgG4	huCD47	Yes	
ALX148	Alexo	SIRPα-Fc* fusion	Inactive	huCD47	Yes	

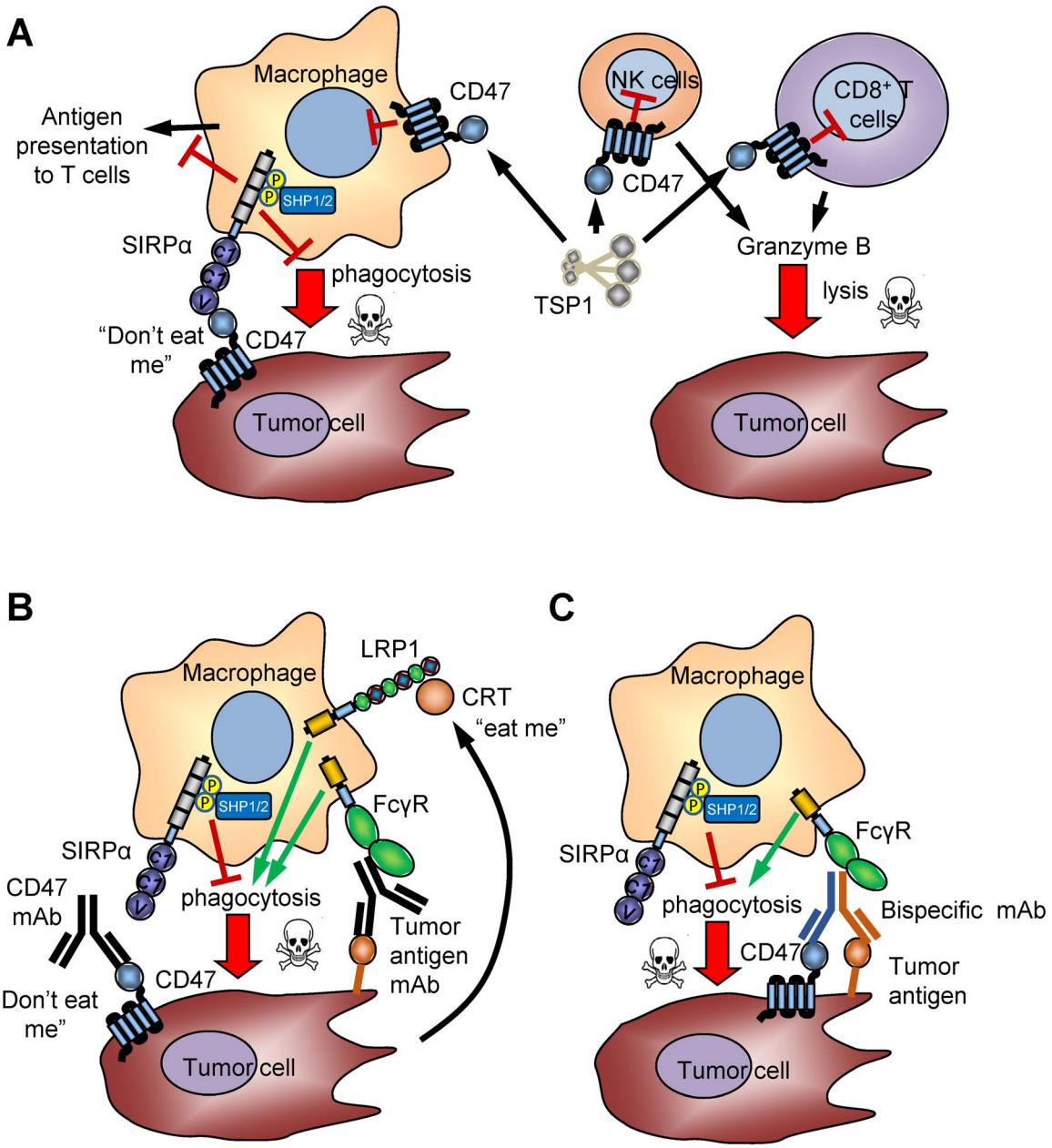

Figure 2. CD47 functions in the tumor microenvironment. **A)** CD47 on tumor cells induces inhibitory SIRPα signaling that prevents macrophage phagocytosis and antigen presentation. Thrombospondin-1 induces CD47 signaling in CD8 T cells and NK cells that inhibits lytic tumor cell killing [15, 113]. **B)** CD47 antibodies that block SIRPα binding relieve the inhibitory signal in macrophages and antigen-presenting cells, enabling pro-phagocytic signals from tumor-secreted calreticulin or tumor-specific antibodies to activate ADCP and ADCC. **C)** Bispecific CD47 antibodies enhance selective blocking of CD47 on tumor cells and induce ADCP and/or ADCC.

solution [39]. This was mediated by changes in the actin cytoskeleton that resulted in type III programmed cell death [41].

CD47 antibodies that induce apoptosis only when immobilized may act by inducing clustering of CD47 in the membrane. This is consistent with the ability of dimeric bivalent CD47 scFv derived from MABL but not a MABL scFv monomer to induce apoptosis of leukemic cells *in vitro* and inhibit multiple myeloma (MM) growth in a mouse xenograft model [37, 42].

Another issue for evaluating CD47 antibodies is that binding may depend on or be limited by specific post-translational modifications of CD47. For example, the antibody CC2C6 binds selectively to CD47 with an N-terminal pyroglutamyl residue, which is formed by QPTCL-mediated enzymatic cyclization of the N-terminal Gln and is required for SIRPα binding [43]. CC2C6 resembles B6H12 in blocking binding to SIRPα [18], but CC2C6 differs from B6H12 in that only soluble CC2C6 antibody induces caspase-independent programmed cell death in

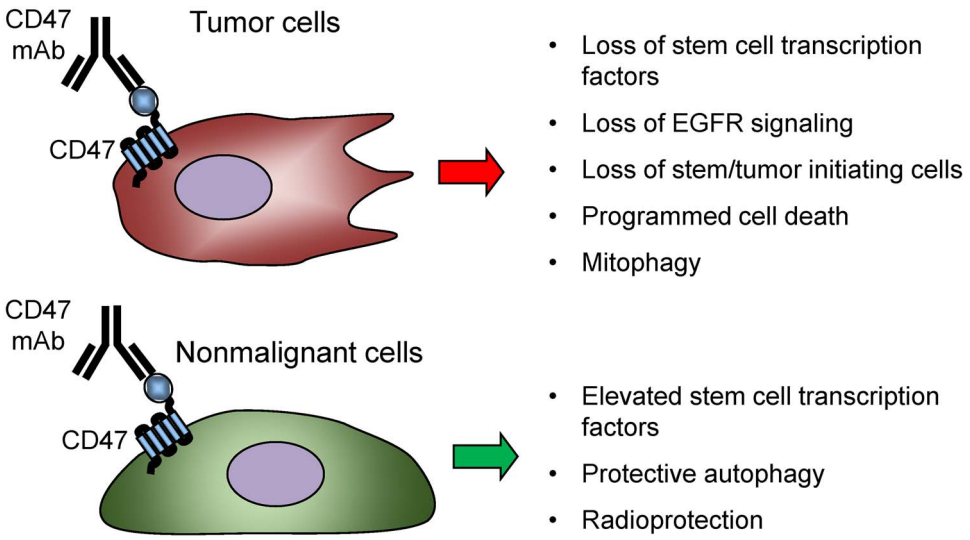

Figure 3. Direct effects of CD47 antibodies on tumor cells. Several CD47 antibodies induce signaling that causes cancer cell death (1F7, AD22, CC2C6, and AO-176) or suppress cancer stem cells (B6H12). In contrast, blocking CD47 in nonmalignant cells preserves stem cells and can be cytoprotective [31, 121].

acute lymphoblastic leukemia (ALL) cells [36]. CC2C6 also synergized with low dose cytotoxic chemotherapeutic agents to enhance apoptosis.

Preclinical CD47 antibodies that induce tumor cell phagocytosis by blocking SIRPα binding

B6H12. B6H12 was one for the first CD47 antibodies shown to inhibit its binding to SIRPα and thereby enhance macrophage phagocytosis [18]. This was extended to cancer cells with the observation that B6H12 enhanced antibody-dependent cellular phagocytosis (ADCP) of human acute myeloid leukemia (AML) cells *in vitro* by human and mouse macrophages [44]. This prophagocytic activity of B6H12 extends to many cancer cell types [2]. In mice, tolerance to human cell xenografts is conferred by the *NOD* mutation of murine SIRPα, which enables human CD47 to inhibit phagocytosis by engaging murine SIRPα with high affinity [45, 46]. Thus, B6H12 inhibited AML tumor growth in NOD/SCID/IL2Rγ^- (NSG) mice by blocking this "don't eat me" signal [44]. The antitumor activity of B6H12 in NSG mice extends to a number of other hematologic malignancy and solid tumor xenografts [2, 44, 47–59]. Conjugating B6H12 with a near-infrared photosensitizer enhanced its pro-phagocytic activity *in vitro* and efficacy in a bladder carcinoma xenograft photoimmunotherapy model [60]. B6H12 was also effective when combined with the small molecule TLR4 agonist pyrimido [5,4-b] indole [61]. Combination therapy resulted in enhanced macrophage-mediated ADCP of Daudi cells relative to pyrimido [5,4-b] indole or B6H12 monotherapy.

B6H12 was used in several preclinical studies to explore combining CD47 blockade with therapeutic antibodies that target specific cancers. B6H12 synergized with the CD20 antibody rituximab to promote phagocytosis *in vitro* and eradicated non-Hodgkin lymphoma in NSG mice [62]. The proposed mode of action of B6H12 (IgG1) as monotherapy and in this combination (Fig. 2A) involves increased ADCP but was independent of antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or apoptosis induced by B6H12. However, SIRPα signaling enhances ADCC of both malignant and nonmalignant cells [63–65]. F(ab′)$_2$ fragments of B6H12 potently and synergistically enhanced trastuzumab-mediated ADCC of breast cancer cells by neutrophils [65]. Combining B6H12 and the CD19/CD3-bispecific T cell engager antibody blinatumomab to treat Raji cell non-Hodgkin's lymphoma (NHL) xenografts in NSG mice almost completely suppressed tumor growth if administered with human PBMCs [66]. Mono- and dual-therapies were significantly less effective in inhibiting tumor growth in the absence of PBMC injection or removal of macrophages via clodronate liposomes. These findings demonstrate roles for adaptive (CD8 T cell cytotoxicity) and innate (macrophage phagocytosis) immune responses in the anticancer activity of B6H12. Similar enhancement of NK cell-mediated ADCC by B6H12 was reported for ALL cells [67]. Treatment of CD16$^+$ NK cells derived from cord blood with B6H12 increased their interferon-γ production. B6H12 similarly increased CD16$^+$ NK cell-mediated ADCC of ALL cells.

Several studies reported beneficial direct effects of B6H12 to limit the maintenance of CSC. B6H12 inhibited CSC maintenance in aggressive breast cancer cell lines [20]. B6H12 treatment decreased mRNA expression of the EGF receptor and acutely inhibited EGF-induced phosphorylation of the receptor. B6H12 inhibited breast CSC proliferation, asymmetric division, and expression of the stem cell transcription factor KLF4. Changes in KLF4 and EGFR mRNA expression induced by B6H12 were mediated in part by induction of miR7. Similar suppression of CSC and tumor growth was reported when human hepatocellular carcinoma (HCC) cells were treated with B6H12 or CD47 expression was

suppressed using a shRNA [68, 69]. B6H12 suppressed the tumorigenicity, self-renewal, and chemoresistance of HCC cells, mediated by suppression of cathepsin S. B6H12 also enhanced macrophage-independent chemosensitivity of HCC cells to doxorubicin and cisplatin *in vitro* [69]. Combining B6H12 and doxorubicin suppressed patient-derived HCC xenografts in a mouse model. Treating pancreatic carcinomas in mice with B6H12 also suppressed CSC abundance, but in this case, *in vitro* studies indicated that the antibody reduced viability of the CSC by inducing apoptosis [70].

Vx1000R (Vasculox Inc., currently Arch Oncology)

The mouse anti-human CD47 antibody Vx1000R induced phagocytosis and killing of human MM cells *in vitro* but did not affect survival of MM cells in the absence of macrophages [71]. Vx1000R antibody showed similar activity to suppress MM growth in xenograft models to previously reported CD47 antibodies [37, 56].

BRIC126

BRIC126 inhibits CD47 binding to SIRPα [18]. BRIC126 enhanced phagocytosis of ALL cells by human macrophages *in vitro* [72]. However, only modest activity was reported in a laryngeal squamous cell carcinoma model in mice, and B6H12 had significantly greater tumor inhibitory activity relative to BRIC126 [73].

mAb400

CD47 mAb400 recognizes both human and murine CD47 and was compared to B6H12 in a HCC xenograft model in NSG mice [74]. B6H12 and mAb400 significantly increased macrophage-mediated ADCP of human HCCs *in vitro* but not in a cytotoxic manner. Both antibodies significantly inhibited tumor growth in heterotopic and orthotopic HepG2 xenograft models and increased macrophage recruitment into the tumors. However, mAb400 was significantly more potent relative to B6H12 in the heterotopic HepG2 model [74].

MIAP301

MIAP301 is specific for murine CD47 and blocks its binding to murine SIRPα [63]. This antibody has been useful to examine the efficacy of CD47 blockade in immunocompetent syngeneic mouse tumor models. Consistent with its documented blocking of SIRPα binding [75], MIAP301 enhanced phagocytosis of GL261 glioma cells and CD133$^+$ glioma stem cells by mouse macrophages [76]. MIAP301 inhibited tumor growth and increased survival in an orthotopic glioma/glioma stem cell mouse model by upregulating both innate and adaptive immune responses. However, treating mice bearing MT1A2 mouse breast carcinoma tumors with MIAP301 yielded no significant inhibition of tumor growth relative to an IgG control [47]. This lack of activity is consistent with the observation that genetic deletion of SIRPα signaling by truncation of its cytoplasmic domain did not impair growth of a syngeneic melanoma [65]. Therefore, antibody blocking or genetic ablation of CD47/SIRPα signaling is not sufficient to enhance phagocytic clearance of all tumors in the absence of additional pro-phagocytic signals [75]. Such additional signals can include expression of cell damage markers, calreticulin, or engagement of activating Fc receptors (FcR). In contrast to MIAP301, MIAP410 (IgG1), an anti-mouse CD47 antibody previously reported to not inhibit SIRPα binding [75], inhibited growth of MT1A2 tumors [47], but a replication study failed to reproduce this result [77]. In another study, combination therapy using MIAP301 F(ab')$_2$ and anti-CD19 (ID3-IgG2a) increased mouse macrophage-mediated ADCP of B cells *in vitro*, but the combination did not differ from ID3-IgG2a monotherapy *in vivo* [78]. These reports suggest that CD47 neutralizing antibodies can sensitize tumors in an immune competent host by mechanisms other than SIRPα-mediated activation of ADCP (Fig. 2A). One critical difference between these syngeneic tumor models and the widely used NSG xenograft models is the absence of xenoantigens that can activate phagocytosis of human tumor cells by mouse macrophages.

As noted above for B6H12, MIAP301 interacts with multiple immune effector cells (Fig 2A). MIAP301 treatment of mice bearing B16 melanomas inhibited tumor growth, accompanied by increased tumor infiltration of NK cells that were positive for interferon-γ and granzyme B [15]. Treatment of isolated NK cells with MIAP301 *in vitro* produced a similar enhancement of interferon-γ expression. MIAP301 also alters CD47 functions by SIRPα-independent mechanisms including elevating blood pressure when administered intravenously in mice [79] and maintaining tissue perfusion and enhancing protective autophagy in ischemic tissues [80, 81]. Therefore, antitumor activities of MIAP301 may not be exclusively mediated by the SIRPα pathway.

MAB4670

MAB4670 (IgG1) blocks human CD47 binding to SIRPα. The antibody was used in combination with the CD38 antibody daratumumab to treat CD14$^+$ bone marrow mononuclear cells and MM cells (CD138$^+$) obtained from 29 patients with MM [82]. CD47 was significantly upregulated on CD138$^+$ MM cells relative to CD138$^-$ MM cells. Treatment with daratumumab upregulated autologous *ex-vivo* killing of CD138$^+$ MM cells by CD14$^+$ and CD14$^+$/CD16$^+$ monocytes. Monocyte-mediated killing of MM cells was further upregulated by combination therapy with MAB4670.

A4 (CD47 nanobody)

CD47 nanobodies with picomolar affinity were developed as an approach to evade the CD47 antigen sink on RBC [83, 84]. The alpaca anti-mouse CD47 nanobody A4 inhibited SIRPα interaction but lacked effector function due to the absence of an antibody Fc-domain. A4 synergized with anti-PD-L1, but not anti-CTLA4, therapy in the syngeneic B16F10 melanoma model. However, A4 synergized with

an anti-TRIP-1 (TA99, IgG2a) to promote ADCP of B16-F10 cells *in vitro* but not with either anti-CD200 (OX-90) or anti-EGFR (cetuximab) to promote ADCP of Tubo-EGFR cells. A4 synergized with anti–PD-L1 to increase ADCP of B16F10 cells treated with IFN-γ to induce PD-L1 expression. A4-secreting A12 CAR-T cells combined with melanoma-specific TA99 significantly decreased tumor growth rate and increased survival of immunocompetent hosts bearing syngeneic B16F10 tumors. This combination treatment did not affect the persistence of the CAR T cells. An IgG2A/A4 fusion protein (A4Fc) was constructed to enhance phagocytosis but induced severe anemia in mice that limited its utility [84]. A4Fc A12 CAR-T cells did not have cytotoxic effects, but decreased tumor volume and increased M1-macrophage infiltration in the mice bearing MC38 tumors [85].

CD47 antibody conjugates and nanoparticles

Azide-modified exosomes derived from M1 macrophages were conjugated with dibenzocyclooctyne-modified CD47 and SIRPα antibodies through pH-sensitive linkers. The modified exosomes enhanced phagocytosis *in vitro* and increased the survival of BALB/c mice bearing 4 T1 breast carcinoma tumors relative to animals treated with exosomes or CD47 antibody alone [86]. A CD47 antibody drug conjugate, SGN CD47M, has been developed by Seattle Genetics and is enrolling cancer patients for a phase 1 trial (NCT03957096), but no preclinical data have been published.

Chitosan/hyaluronic acid polyelectrolyte complex nanoparticles were loaded with CD47 antibody and administered to atherosclerotic $ApoE^{-/-}$ mice fed a high fat diet [87]. The particles significantly inhibited atherosclerotic plaque development. The particles were also used to coat foam cells before administration, which increased targeted inhibition of atherosclerotic plaques. Potential applications for cancer remain to be examined.

HUMANIZED THERAPEUTIC CD47 ANTIBODIES

Hu5F9/Magrolimab (Forty Seven, Inc./Gilead)

Enhanced macrophage phagocytosis and the antitumor efficacy of humanized Hu5F9/magrolimab (IgG4) were demonstrated using various tumor cell types in NSG xenograft models [88, 89]. Intravenous administration of magrolimab to cynomolgus monkeys resulted in dose-dependent anemia, but a protocol utilizing a 1–3 mg/kg priming dose, to occupy the RBC sink and induce compensatory erythropoiesis, followed by 30 mg/kg maintenance dosing achieved a sustained effective circulating concentration [88]. The same protocol was effective to decrease anemia in a Phase 1 human trial (Table 1), and receptor occupancy in a patient tumor was documented with a 30 mg/kg dose following priming [8]. A Phase 1b clinical trial combining magrolimab and rituximab enrolled 22 patients: 15 with diffuse large B cell lymphoma (DLBCL) and 7 with follicular lymphoma [7]. Patients had received a median of four prior therapies, and 95% had disease that was resistant to rituximab. Eleven patients responded to the drug, with eight complete responses, five in DLBCL and three in follicular lymphoma. Additionally, at a median follow-up of 6.2 months in the DLBCL group and 8.1 months in follicular lymphoma group, 91% of patients continued to respond. The most common side effects (chills, headache, and anemia) were observed in 41% of patients; one patient had grade 4 neutropenia and one grade 4 thrombocytopenia. Magrolimab combined with azacytidine increased tumor cell calreticulin expression and phagocytosis by macrophages *in vitro* and yielded increased survival in an AML xenograft model in NSG mice [89]. Recently presented Phase 1b results demonstrate tolerability of this combination and suggest efficacy [90]. Additional combination trials are in progress (Table 1).

SHR-1603

SHR-1603 is a humanized CD47 IgG4 antibody that includes active Fc domains to mediate CDC/ADCC functions [91]. This antibody blocked the interaction of CD47 with SIRPα and enhanced phagocytosis. SHR-1603 is being tested in a Phase 1 trial (Table 1).

CC-90002 (Celgene)

CC-90002 is an IgG4 high-affinity CD47 antibody that blocks its binding to SIRPα and enhanced macrophage-dependent killing of tumor cells in preclinical studies [92]. A Phase I Study of CC-90002 was completed in patients with relapsed and/or refractory AML and high-risk myelodysplastic syndromes (MDS). A Phase 1 trial using CC-90002 in combination with rituximab is ongoing for patients with $CD20^{+}$ NHL (Table 1). CC-90002 antibody did not induce hemagglutination of red blood cells or hemolysis in preclinical studies. CC-90002 treatment combined with rituximab demonstrated tolerability and modest clinical activity in this early-phase study of heavily pretreated refractory/recurrent NHL subjects, with adverse events predominantly grade 1/2 cytopenias and dose-limiting thrombocytopenia [92].

AMMS4-G4

The fully human IgG4 CD47 antibody ZF1 blocked interaction between CD47 and human or mouse SIRPα slightly weaker than B6H12 but induced *in vitro* macrophage-mediated phagocytosis as robust as B6H12 [93]. ZF1 induced phagocytic killing of ALL or CML leukemic cells in mouse xenografts and increased survival comparable to B6H12 and significantly greater than control IgG. However, neither CD47 antibody completely eradicated the tumors. A fully human anti-CD47 antibody, AMMS4-G4, was derived from ZF1 using affinity maturation [94]. AMMS4-G4 had higher affinity for human CD47, blocked CD47/SIRPα interaction, and significantly increased macrophage recruitment, tumor phagocytosis, and survival in mice engrafted with human hematologic cancers. AMMS4-G4 inhibited tumor growth through increased macrophage recruitment in mice engrafted with SKOV3 ovarian cancer solid tumors. Combination therapy with

the VEGF antagonist bevacizumab modestly enhanced this anti-tumor activity. Combination therapy with the EGFR antibody AC21 (IgG1) significantly inhibited tumor growth in mice bearing LOVO colon cancer xenografts. As observed for other CD47 antibodies, AMMS4-G4 infusion resulted in reversible anemia in cynomolgus monkeys, but no hemagglutination was observed.

HuNb1

HuNb1 is a high affinity nanobody that is specific for human CD47 but exhibits low binding to human RBC CD47. HuNb1 was identified by phage display screening [95]. It exhibited safety in cynomolgus monkeys. A recombinant HuNb1-IgG4 induced phagocytosis *in vitro* and showed anti-tumor activity in a human ovarian carcinoma xenograft model.

SRF231

SRF231 is a fully human IgG4 CD47 antibody that inhibits binding to SIRPα but does not agglutinate RBC or induce their clearance by macrophages [96]. In addition to enhancing macrophage phagocytosis of tumor cells by blocking SIRPα, SRF231 activated macrophages by its binding to the activating FcγR CD32a. Compared to the antibody CC2C6, which directly increased tumor cell apoptosis when used in solution, SRF231 induced less direct cytotoxicity. However, SRF231 exhibited stronger proapoptotic activity than CC2C6 when immobilized on a scaffold, and this activity was inhibited by a pan-caspase inhibitor. Therefore, SRF231 has a context-dependent ability to directly induce tumor cell death. SRF231 treatment reduced tumor growth in multiple mouse xenograft models. Treatment was associated with increased tumor macrophage infiltration and induction of the macrophage cytokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α. SRF231 is currently in a Phase 1 clinical trial (Table 1).

Therapeutic SIRPα antibodies and decoys

Monoclonal antibodies specific for SIRPα that block its interaction with CD47 represent another strategy to enhance tumor ADCP while avoiding the RBC sink, thus permitting use of lower dosages than CD47 antibodies. Prophagocytic and antitumor efficacy has been demonstrated in several preclinical models [65, 97, 98]. Two such antibodies have entered phase 1 trials (Table 1), but preclinical data for these antibodies have not been published.

A decoy strategy has also been advanced using the CD47-binding domains of SIRPα fused to an immunoglobulin Fc region [99, 100]. These offer a potential advantage over the first generation of humanized CD47 antibodies in that they exhibit minimal binding to RBC. TTI-621 is a bivalent human SIRPα construct with a IgG1 Fc that enhances macrophage-mediated phagocytosis *in vitro* and growth of several xenograft tumor models *in vivo* [99, 101]. TTI-621 and TTI-622, with a IgG4 Fc, are currently in Phase 1 trials (Table 1). ALX148 contains the D1 domain of SIRPαf used to an inactive human IgG1 Fc [100]. ALX148 enhances phagocytosis of tumor cells in vitro, is active in several tumor models, and enhances the activities of other immunotherapy antibodies. It is currently in phase 1 clinical testing (Table 1).

Humanized CD47 antibodies with cell-autonomous anticancer activities

AO-176. AO-176 is a humanized mouse anti-human CD47 IgG2 antibody with κ-light chain that inhibits SIRPα binding [38]. AO-176 exhibited negligible interaction with RBCs, platelets, and endothelial cells and did not induce RBC agglutination. AO-176 also bound well to cynomolgus monkey and murine CD47. Intravenous administration of AO-176 in cynomolgus monkeys was well tolerated without any hematologic effects, with stable RBC counts and hemoglobin values even after repeated dosing. This contrasts with other CD47 blocking antibodies including the control humanized CD47 antibody AO-104, which exhibited high binding to RBCs and induced the typical transient anemia in monkeys. Compared to AO-104, AO-176 also demonstrated significantly lower binding affinity to naïve and activated T-cells and other non-malignant cells. In addition to preferential binding to cancer cells, AO-176 selectively induced death in Jurkat T-ALL and OV90 ovarian cancer cells involving late and early apoptosis. AO-176 promoted human macrophage phagocytosis of hematologic (Jurkat T-ALL, Raji B-cell lymphoma) and solid tumor cells (OV90, Detroit 562, and FaDu). AO-176 inhibited growth in Raji lymphoma xenografts. A 5 mg/kg dose of AO-176 remained detectable in circulation after 18 days, while AO-104 did not.

The mechanism by which AO-176 selectively binds CD47 on to tumor cells remains unclear but may involve cell-specific posttranslational modifications or masking of the AO-176 epitope by CD47 binding partners (eg. components of the Rh complex in the erythrocyte membrane) [1]. Conversely, AO-176 binding may depend on a specific lateral CD47-interacting protein in cancer cells, differences in the surface mobility of CD47 [102], differential densities, or differential partitioning of CD47 into lipid rafts between normal and tumor cells [103].

CD47 bispecific antibodies (bsAb). Based on the homeostatic function of CD47 on RBC to limit clearance of aging RBCs and the ability of some CD47 antibodies to induce hemagglutination, anemia is a common reported side effect for humanized CD47 antibodies including Hu5F9 [8]. One approach to minimize these adverse effects is to construct bsAb wherein the second epitope is a tumor-specific surface antigen that directs the antibody to bind preferentially to tumor cells and in some cases activate ADCC or ADCP mediated via its Fc region, while simultaneously blocking the do not eat me signal (Fig. 2C). Such CD47 bsAb generally exhibit decreased anemia [104, 105].

CD3xCD47 (OV-TL3/CD3). The first CD47 bsAb was reported in 1994 and contained variable regions recognizing CD47 and CD3 (Fig. 4A) [106]. The antibody

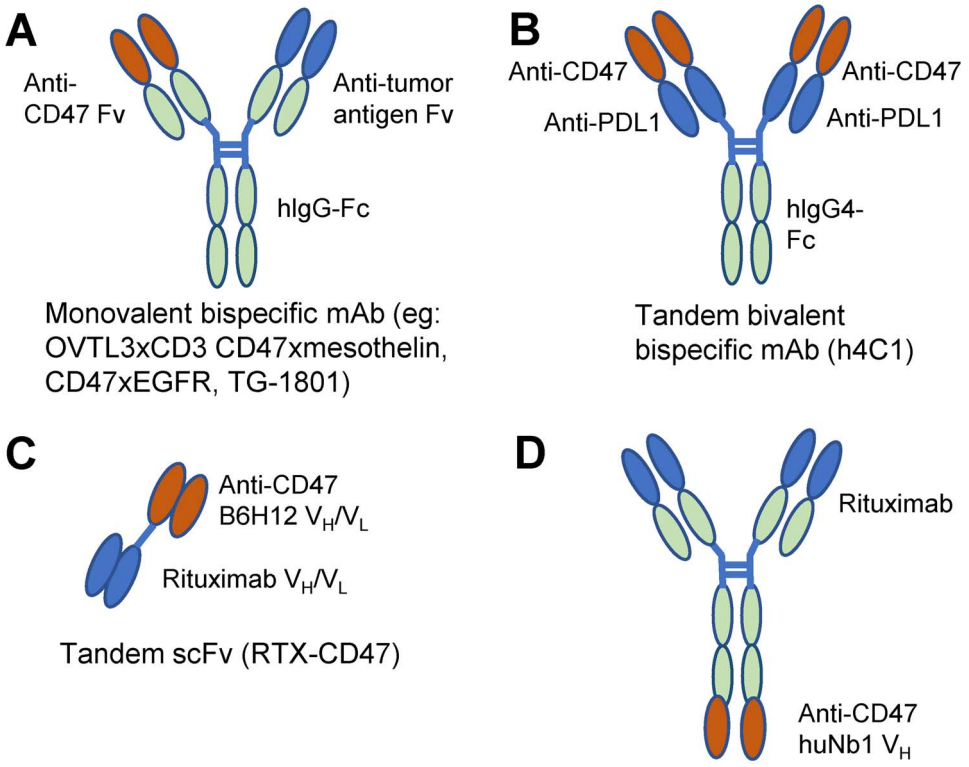

Figure 4. Structures of therapeutic bispecific CD47 antibodies. **A)** Conventional bispecific antibodies combine Fv regions from two antibodies and are monovalent for CD47 and a tumor-specific or immune cell antigen. **B)** A tandem bispecific that is bivalent for CD47 and PD-L1 [109]. **C)** A bispecific antibody containing scFv domains recognizing CD47 and CD20 [108]. **D)** A bispecific combining rituximab with the CD47 nanobody huNb1 [95].

OV-TL3/CD3 enhanced killing of human ovarian cancer cells by peripheral blood leukocytes (PBL) *in vitro*. Treatment of nude mice bearing ovarian carcinoma ascites tumors with bivalent OV-TL3/CD3 F $(ab')_2$ combined with PBL and IL-2 increased survival relative to antibody or PBL + IL-2 alone. This $F(ab')_2$ resembles the current bispecific T cell enhancers [107].

CD20xCD47 (RTX-CD47, HuNb1-rituximab). A bispecific tandem scFv antibody lacking an Fc region (RTX-CD47) was constructed from B6H12 and rituximab Fv regions (Fig. 4C) [108]. RTX-CD47 had enhanced avidity for $CD20^+/CD47^+$ tumor cells, but CD47/SIRPα blockade depended on CD20-restricted cooperativity. RTX-CD47 enhanced ADCP of primary malignant B cell lines by both macrophages and granulocytes in a CD20-restricted cooperative manner. Combination therapy with RTX further enhanced phagocytosis of some cell lines. Combining the CD20 antibody obinutuzumab with RTX-CD47 further improved ADCP without competing for the CD20 epitope for the RTX portion of RTX-CD47. The absence of a Fc-domain allowed this bispecific scFv to avoid triggering of FcR-mediated ADCC, ADCP, or CDC.

Another CD47xCD20 bsAb was constructed using the CD47 nanobody HuNb1 (IgG4) and the variable domain from rituximab (Fig. 4D), which exhibited increased efficacy in mouse xenograft B cell lymphoma models relative to parental HuNb1 [95].

CD47xPD-L1

The humanized IgG4 SIRPα-blocking CD47 mAb h4C1 increased phagocytosis of Raji cells by bone marrow-derived macrophages and improved survival and enhanced suppression of Raji cell xenografts in mice relative to a Hu5F9 positive control and IgG negative control. h4C1 binds to the FG loop of CD47, which interacts with SIRPα and several other known CD47/SIRPα-blocking mAb. Binding of h4C1 to CD47 was not affected by glycosylation modifications of CD47. The variable-domain from the PD-L1-blocking mAb #18 (CN Patent: 201810952740.3) was introduced in tandem onto h4C1 to generate a dual variable domain bsAb that binds bivalently to both CD47 and PD-L1 (Fig. 4B). CD47 binding affinity was reduced compared to h4C1, but PD-L1 binding remained comparable to monovalent PD-L1 mAb [109]. The construct retained blocking activity for both interactions but lacked the hemagglutinating activity of the parent h4C1.

IBI-322 is another bsAb that recognizes PD-L1 and CD47 and is in phase 1 trials for treating advanced tumors (Table 1). IBI-322 was also tested as a positron emission tomography imaging agent after conjugation with p-SCN-deferoxamine and loading with ^{89}Zr [110].

CD47xEGFR

A CD47xEGFR bsAb was engineered from the EGFR antibody Pan and a SIRPα variant-Fc with a human IgG1 Fc [105]. The bsAb enhanced macrophage phagocytosis

of human A431 epidermoid carcinoma cells *in vitro* and inhibited A431 xenograft tumor growth in mice. RBC counts assessed 20 days following injection in mice were higher than in mice injected with bivalent SIRPα variant-Fc, indicating reduced toxicity, although acute anemia at earlier times were not examined. Phagocytic activity and tumor inhibition were comparable to SIRPαV-Fc and Pan monotherapies, respectively.

CD47xMesothelin (Novimmune)

A bsAb recognizing CD47 and mesothelin increased ADCC and ADCP by promoting FcγR-IIIA signaling in NK cells and macrophages, respectively, relative to IgG1 control or either monospecific antibody [111]. Targeting a membrane-proximal epitope on mesothelin alone led to increased ADCC but not ADCP activity. However, the corresponding bsAb significantly increased ADCC and ADCP in an FcγR-IIIA-dependent manner and completely suppressed mesothelin-expressing HepG2 xenografts in mice.

CD47xCD19 (TG-1801/NI-1701, TG Therapeutics/Novimmune)

The CD47xCD19 bsAb TG-1801/NI-1701 induced potent ADCP against B cell malignancies *in vitro* and *in vivo* [104]. In contrast to either monospecific antibody, TG-1801/NI-1701 inhibited B cell receptor (BCR)-induced B cell proliferation in a non-cell death-dependent manner and inhibited BCR signaling, internalization, and cytokine production [112]. Simultaneously engaging CD47 was proposed to restrict mobility of CD19 to cluster and associate with the BCR. Combination therapy with the anti-CD20 rituximab further enhanced tumor growth inhibition in Raji cell xenograft mouse models. *In vitro* binding assays in whole blood as well as *in vivo* measurements in cynomolgus monkeys showed minimal-to-no binding of NI-1701 to RBCs, T cells, and platelets. Furthermore, no hematological or other adverse side effects were seen in cynomolgus monkeys at doses reaching 100 mg/kg. The high affinity of the anti-CD19 binding arm may specifically restrict CD47 blockade by NI-1701 to B-cells, and its IgG1 Fc may contribute to its ADCP potency. A clinical trial using TG-1801 for B cell lymphoma is in progress (Table 1).

CONCLUSIONS

The ubiquitous expression of CD47 on normal as well as tumor cells creates challenges for developing CD47 antibodies for cancer therapy. CD47 expressed on circulating blood cells and vascular endothelium serves as a sink for intravenously administered therapeutic CD47 antibodies and leads to the commonly observed side effects of transient anemia, hyperbilirubinemia, thrombocytopenia, and lymphopenia. Several strategies are emerging to circumvent these issues by achieving selective binding to CD47 on tumor cells, including identifying tumor-specific CD47 epitopes and developing bispecific antibody designs. A better understanding of the variations in posttranslational modifications of CD47 will facilitate this effort and may identify additional molecular targets that could selectively impair the function of CD47 on tumor cells. The finding that the glutaminyl cyclase QPCTL mediates a posttranslational modification of CD47 that is required for SIRPα binding suggests QPCTL is a relevant molecular target for future therapeutics [43].

Although antibodies targeting the CD47/SIRPα interaction may have significant efficacy as single agents for treating some cancers, data from immune-competent mouse and xenograft models indicate that efficacy for most cancers will require combination therapies. Such synergies have proven effective in preclinical models wherein CD47 therapeutics are combined with ionizing radiation or chemotherapy [31, 113–115] and immune checkpoint inhibitors targeting the PD-1/PD-L1 and CTLA4 pathways [116, 117]. Consequently, several new clinical trials are examining such therapeutic combinations (Table 1). In addition to minimizing binding to circulating blood cells, the emerging bispecific antibodies may provide specific targeting of CD47 blockade to tumor cells and effector functions to stimulate antitumor immunity.

The potential for CD47 therapeutics to achieve cancer-autonomous suppression of CSC or selectively induce programmed death of malignant cells is another promising path forward. Further investigation of how CD47 signaling controls the balance between protective autophagy and programmed cell death pathways in malignant versus non-malignant cells is needed to realize this potential.

To date, CD47 therapeutics that have entered clinical testing are intended to block its engagement of SIRPα [2, 3]. However, preclinical studies have demonstrated that blocking TSP1 interactions also provides therapeutic benefits, especially when combined with radiation, chemotherapy, or adoptive immunotherapy [31, 32, 113, 114]. Because CD47 serves as an inhibitory signaling receptor on dendritic cells, T cells, and NK cells [15, 28, 113], therapeutics targeting the CD47/TSP1 axis could have broad immune-activating activities. This is supported by tumor models in immune competent mice that established the necessary role for CD8 T cell immunity and parallel increases in activation of adaptive immunity in *Thbs1* and *Cd47*-null mice [83, 113, 118]. Based on the evidence that B6H12 inhibits both SIRPα and TSP1 binding, steric blocking of TSP1 binding by some antibodies intended to block SIRPα binding may be expected and merits further study for the antibodies currently in clinical trials. Further development of nanobody technologies may also enable more selective targeting of each CD47 function.

ABBREVIATIONS

ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; bsAb, bispecific antibody; CDC, complement-dependent cytotoxicity; CML, chronic myeloid leukemia; CSC, cancer stem cells; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DLBCL, diffuse large B cell lymphoma; EGFR, epidermal growth factor receptor; HCC,

hepatocellular carcinoma; MM, multiple myeloma; NK, natural killer; NSG, nonobese diabetic/severe combined immunodeficient/IL2Rγ^{-}; PD-L1, programmed death ligand 1; QPTCL, glutaminyl-peptide cyclotransferase-like protein; RBC, red blood cells; scFv, single-chain variable fragment; SIRPα, signal regulator protein-α (CD172a); TSP1, thrombospondin-1.

Conflict of interest statement. None declared.

FUNDING

This work was supported by the Intramural Research Program of the NIH/NCI (ZIA SC009172).

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