A bispecific antibody targeting CD47 and CD20 selectively binds and eliminates dual antigen expressing lymphoma cells

Emily C Piccione, Silvia Juarez, Jie Liu, Serena Tseng, Christine E Ryan, Cyndhavi Narayanan, Lijuan Wang, Kipp Weiskopf, and Ravindra Majeti*

Department of Medicine; Division of Hematology; Cancer Institute; and Institute for Stem Cell Biology and Regenerative Medicine; Stanford University; Stanford, CA USA

Keywords: bispecific antibody, lymphoma, CD47, phagocytosis, synergy

Abbreviations: SPR, surface plasmon resonance; IgG, immunoglobulin G; VH, immunoglobulin heavy chain variable region; VL, immunoglobulin light chain variable region

Agents that block the anti-phagocytic signal CD47 can synergize with pro-phagocytic anti-tumor antigen antibodies to potently eliminate tumors. While CD47 is overexpressed on cancer cells, its expression in many normal tissues may create an ‘antigen sink’ that could minimize the therapeutic efficacy of CD47 blocking agents. Here, we report development of bispecific antibodies (BsAbs) that co-target CD47 and CD20, a therapeutic target for non-Hodgkin lymphoma (NHL), that have reduced affinity for CD47 relative to the parental antibody, but retain strong binding to CD20. These characteristics facilitate selective binding of BsAbs to tumor cells, leading to phagocytosis. Treatment of human NHL-engrafted mice with BsAbs reduced lymphoma burden and extended survival while recapitulating the synergistic efficacy of anti-CD47 and anti-CD20 combination therapy. These findings serve as proof of principle for BsAb targeting of CD47 with tumor-associated antigens as a viable strategy to induce selective phagocytosis of tumor cells and recapitulate the synergy of combination antibody therapy. This approach may be broadly applied to cancer to add a CD47 blocking component to existing antibody therapies.

Introduction

Monoclonal antibodies hold enormous promise as anti-cancer therapeutics due to their ability to harness the immune system for attack of a highly specific target cell population. Identification of tumor-specific antigens has revolutionized cancer therapy, with ~40 antibodies currently approved and over 300 antibodies undergoing clinical development.1-2 However, while therapeutic antibodies have proved efficacious as molecularly targeted cancer therapies, they are generally administered in combination with chemotherapy due to limited clinical efficacy as monotherapy.3

Immune effector cells are critical to the efficacy of anti-cancer antibodies through a number of mechanisms including antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and priming of antigen-specific T cells through cross-presentation of tumor antigens.1,4 Phagocytosis is partially regulated by SIRPα, a protein expressed on the surface of phagocytic cells, including macrophages and dendritic cells.5 The interaction of SIRPα with its ligand, CD47, a widely expressed transmembrane protein, transmits a “don’t eat me” signal by initiating signaling cascades that ultimately inhibit phagocytosis.6-9 Increased expression of CD47 has been detected on acute myeloid leukemia stem cells (AML LSC), multiple subtypes of B cell non-Hodgkin lymphoma (NHL), and many human solid tumor cells.10-13 CD47 plays an important role in cancer pathogenesis, as increased expression on tumor cells permits evasion of phagocytosis.14 This mechanism can be therapeutically modulated with monoclonal antibodies targeting CD47 or recombinant SIRPα proteins that disrupt the CD47-SIRPα interaction.10,15,16

Therapeutic agents that antagonize the CD47-SIRPα interaction present a unique opportunity to enhance the efficacy of cancer-targeting therapeutic antibodies. Since CD47 blocking antibodies enable phagocytosis by blocking an inhibitory signal, therapeutic synergy can be achieved by combining CD47 blockade with a pro-phagocytic signal elicited by an Fc receptor (FcR)-activating antibody.17 Such synergy was demonstrated through combination of blocking anti-CD47 antibody with rituximab, an approved anti-CD20 antibody known to engage FcRs.11,18 Further evidence of synergy was provided by the demonstration of enhanced trastuzumab-mediated killing of breast cancer cells upon blockade of the CD47-SIRPα interaction with antibodies directed against CD47 or SIRPα.19 Most recently, synergistic induction of
phagocytosis was observed between high affinity SIRPα monomers that antagonize CD47 and tumor-specific monoclonal antibodies including trastuzumab, rituximab, and cetuximab. Collectively, these studies highlight the potential for synergistic elimination of cancer cells by adding a CD47-SIRPα blocking component to existing antibody therapies.

Bispecific antibodies (BsAbs) are an emerging class of antibody therapeutics that exhibit specific binding to 2 different antigens. Many formats of BsAbs have been engineered using recombinant approaches, including IgG-like BsAbs that contain an Fc domain. These BsAbs are desired for many clinical applications because the intact Fc region supports effector functions and confers a long serum half-life. A unique feature of BsAbs is the potential for bispecificity to afford stronger binding to dual antigen-expressing cells relative to single-antigen cells as a result of multivalency leading to higher avidity interactions. This potential has been extensively proposed, but there are few successful examples. Bispecific fragments directed against different epitopes within the same antigen exhibit increased binding, but avidity in these cases is likely dependent upon conformational changes of the antigen. Improved tumor localization was observed with a BsAb targeting ErbB2 and carcinoembryonic antigen, but avid BsAb binding to single antigen-expressing cells indicated a lack of selectivity for dual antigen-expressing cells. Similarly, improved tumor targeting was reported with a BsAb targeting ErbB2 and ErbB3. Notably, both of these BsAbs exhibiting some selectivity for dual antigen-expressing cells were small recombinant BsAb fragments that did not contain an Fc domain. Thus far, IgG-like BsAbs capable of selectively binding dual antigen-expressing cells have not been described.

Attempts to induce phagocytosis through blockade of CD47-SIRPα signaling with either blocking antibodies or recombinant SIRPα proteins have shown promise, but these strategies fail to address the issue of selective targeting of tumor cells. Expression of CD47 on normal tissues may create an ‘antigen sink’ that prevents anti-CD47 therapeutic antibodies from reaching intended tumor cell targets in vivo. One strategy to circumvent this issue is to employ BsAbs with reduced affinity for CD47 that retain the ability to block the CD47-SIRPα interaction, but require binding to a second tumor antigen for high affinity binding. We hypothesized that such BsAbs targeting CD47 and CD20 would direct CD47-SIRPα blockade specifically to cells that co-express CD20, an established therapeutic target in NHL. Moreover, we hypothesized that such BsAbs would recapitulate the therapeutic synergy observed with anti-CD47 and anti-CD20 combination therapy. Here, we tested these hypotheses by generating BsAbs specific for CD47, with reduced affinity, and CD20, and investigated their binding specificity and functional effects against human NHL.

**Results**

**Bispecific antibodies targeting CD47 and CD20 bind to each antigen alone**

BsAbs were generated using the dual-variable-domain immunoglobulin (DVD-Ig) format that introduces the variable domains from the heavy and light chains of one antibody onto the amino termini of the respective chains of a second antibody through an amino acid linker. Variable domains in the internal position of DVD-Ig molecules often exhibit reduced affinity for their target antigens compared to the parental antibodies. We sought to take advantage of this reduced affinity by engineering the anti-CD47 variable domains into the internal position. Two variants of CD20-CD47 DVD-Igs were generated with either a short (SL) or long (LL) linker sequence between the variable domains. Co-transfection of the heavy and light chains of each DVD-Ig led to the production of a single IgG-like species, with expected molecular weights for each chain (Fig. 1B). The increased size of each chain relative to the parental antibodies is reflective of the additional variable domain on each chain, and is consistent with previously reported molecular weights for DVD-Ig chains. Size exclusion chromatography confirmed that the CD20-CD47 DVD-Ig molecule was produced as a single species with no detectable aggregate formation (Fig. S2).

BsAbs were evaluated for their ability to bind to antigen-expressing cells using a rat cell line engineered to express human CD20 or, separately, human CD47. Binding of each BsAb to CD20 was comparable to the binding of parental anti-CD20 antibody (Fig. 1C) Both BsAbs also bound to CD47, but with compromised affinity relative to the parental anti-CD47 antibody (Fig. 1C). Notably, CD20-CD47 LL exhibited stronger binding to CD47 than CD20-CD47 SL, presumably due to improved accessibility to antigen.

To quantitatively determine the affinity of each antibody for monomeric CD47, binding affinities were determined using surface plasmon resonance (SPR) with Biacore (Fig. 1D; Fig. S3). The affinity of CD20-CD47 LL was reduced ~15-fold, while the affinity of CD20-CD47 SL was reduced 20-fold relative to anti-CD47 (Fig. 1D). The increased affinity observed with the CD20-CD47 LL relative to CD20-CD47 SL is indicative of the greater flexibility and accessibility to antigen allowed by the longer linker, and is consistent with reported data for the DVD-Ig format.

The epitope recognized by the 2B8 anti-CD20 clone is dependent upon the conformation of a loop structure that is created between transmembrane domains, and is therefore not possible to mimic with a recombinant fusion protein for use in SPR studies. To assess the strength of BsAb binding to the CD20 antigen on the cell surface, rat cells engineered to express human CD20 were stained with test antibodies prior to staining with a fluorescently conjugated anti-CD20 antibody generated from the same 2B8 clone (Fig. 1E). Both BsAbs blocked the fluorescent anti-CD20 signal with an identical pattern as observed with the anti-CD20 parental antibody, indicating a comparable affinity. The reciprocal experiment was conducted using cells engineered to express human CD47 and fluorescently conjugated anti-CD47 antibody (Fig. 1F), and only the parental anti-CD47 blocked binding of labeled anti-CD47 antibody. Given the ability of the BsAbs to bind these cells in the absence of competition with anti-CD47 antibody (Fig. 1C), the lack of blocking with the BsAbs is indicative of a weaker interaction with CD47 that is outcompeted by labeled anti-CD47.
**Figure 1.** Bispecific antibodies targeting CD47 and CD20 bind to each antigen alone: (A) Schematic of a CD20-CD47 DVD-Ig molecule with each variable domain of anti-CD20 (red) connected to each variable domain of anti-CD47 (blue) through a short or long amino acid linker. Constant regions are human Constant Light (kappa) and C\(_{H1}, C_{H2}, C_{H3}\) from human IgG1. (B) SDS-PAGE analysis of purified antibodies under non-reducing (left) and reducing (right) conditions. Sizes of heavy chain (HC) and light chain (LC) for each antibody type are indicated. (C) Antibodies were used to stain rat YB2/0 cells engineered to express human CD20, but not human CD47 (left) or YB2/0 cells engineered to express human CD47, but not human CD20 (right) prior to detection with PE-conjugated anti-human secondary antibody by flow cytometry. The experiment was performed 3 times with similar results. (D) Kinetic association and dissociation parameters, along with calculated affinity (K\(_D\)) were measured by surface plasmon resonance using Biacore for anti-CD47 (B6H12-hIgG4), CD20-CD47 SL, or CD20-CD47 LL for human CD47. (E) CD20-CD47 YB2/0 cells were incubated with the indicated antibodies over a range of concentrations prior to staining with 10 \(\mu\)g/ml DyLight 488. Mean fluorescence intensity (MFI) of the DL488 signal was measured by flow cytometry. Data are representative of 3 independent experiments performed in triplicate. (F) CD20-CD47 YB2/0 cells were incubated with the indicated antibodies over a range of concentrations prior to staining with 10 \(\mu\)g/ml Alexa Fluor 647. Mean fluorescence intensity (MFI) of the AF647 signal was measured by flow cytometry. Data are representative of 3 independent experiments performed in triplicate.
Simultaneous binding to CD47 and CD20 contributes to BsAb binding to cells

We next wanted to address whether a single BsAb molecule could simultaneously bind to both antigens. Cells expressing CD20 but not CD47 were coincubated with test antibodies and complexes containing the recombinant biotinylated CD47 antigen bound to neutravidin-fluorescent conjugates. Antibody binding to cells was then detected by flow cytometry with a secondary antibody, and double positive live cell events indicated simultaneous binding to CD20 and CD47 (Fig. 2A). CD20-CD47 SL and CD20-CD47 LL were capable of simultaneous binding to both antigens, with CD20-CD47 LL demonstrating more double-positive events, consistent with its greater affinity for CD47 (Fig. 2B, Fig. S4). Having demonstrated that both BsAbs are capable of binding...
simultaneously to each antigen in trans, we next sought to determine if simultaneous binding occurred in the context of antigens expressed on the cell surface. This is particularly important as the BsAbs retain a high affinity to CD20 (Fig. 1E) that could potentially drive BsAb binding to dual-antigen cells independent of CD47 binding. Raji cells, a human lymphoma cell line that expresses both CD20 and CD47, were used to stain cells in this assay because high expression of CD47 has been observed in this tumor type (cells in this assay because high expression of CD47 has been observed in chronic lymphocytic leukemia (B-CLL) were used as dual antigen-expressing cells). They are abundant, accessible to antibody in the bloodstream, and highly express CD47, which is involved in RBC clearance.

To determine whether BsAbs selectively bind dual-antigen-expressing cells in the presence of an ‘antigen sink’

A major reason for generating BsAbs targeting CD20 and CD47 is to achieve the therapeutic benefit of blocking the CD47-SIRPα interaction on tumor cells while avoiding a potential ‘antigen sink’ created by normal cells expressing CD47. Red blood cells (RBCs) are a likely candidate for an antigen sink as they are abundant, accessible to antibody in the bloodstream, and highly express CD47, which is involved in RBC clearance. To determine whether BsAbs selectively bind dual-antigen-expressing cells in the presence of excess CD47-only expressing cells, CD20+CD47+ tumor cells were labeled with CFSE and mixed with a 20-fold excess of unlabeled RBCs prior to incubation with antibody (Fig. 3A). Cells from 2 patients with B cell chronic lymphocytic leukemia (B-CLL) were used as dual antigen cells in this assay because high expression of CD47 has been observed in this tumor type (Fig. 3B). To determine the relative strength with which each antibody bound to each cell type, events were gated by cell type and antibody binding was assessed. BsAbs, as well as the control anti-CD47 and anti-CD20, bound to tumor cells, whereas only anti-CD47 and CD20-CD47 LL bound appreciably to RBCs (Fig. 3C). Importantly, CD20-CD47 SL did not bind strongly to RBCs, indicating that reduced affinity for CD47 successfully reduced binding to RBCs. To determine whether each antibody preferentially bound to tumor cells or RBCs, cells within the PE-positive gate were discriminated as either CLL (CFSE+) or RBC (CFSE−). The majority of cells bound by anti-CD47 and CD20-CD47 LL were RBC (Fig. 3D). In contrast, anti-CD20 and CD20-CD47 SL bound preferentially to tumor cells (Fig. 3D). To confirm that BsAbs were engaging CD47 on tumor cells, the ability of the antibody to block subsequent binding of labeled anti-CD47 was determined as in Figure 2C (Fig. 3A). Both BsAbs blocked staining with this antibody, indicating that the BsAbs were binding to the CD47 antigen on tumor cells (Fig. 3E). Similar findings were observed with Raji cells (Fig. S5). Notably, at a lower concentration, both BsAbs blocked the labeled anti-CD47 more completely than anti-CD47 (Fig. S5), suggesting that antibodies capable of dual binding to both antigens exhibit higher avidity binding to Raji cells than antibody that bound to CD47 alone.

BsAbs induce phagocytosis of target cells in an FcR-dependent manner and recapitulate the synergy of antibody combinations

We hypothesized that CD20-CD47 BsAbs would enable phagocytosis of tumor cells as a result of blocking the CD47-SIRPα interaction and engaging FcRs on macrophages. To test this, CD20+CD47+ CFSE-labeled cell lines were co-incubated with unlabeled human macrophages in the presence of different antibodies, and phagocytosis of target cells was assessed by flow cytometry. Antibodies directed against CD47 or CD20 induced significant phagocytosis relative to the baseline level observed with isotype control antibody (Fig. 4A). Both BsAbs recapitulated the synergy of anti-CD47 and rituximab combination treatment and increased phagocytosis relative to treatment with anti-CD47 alone in most cell lines tested.

To explore whether FcR interactions contributed to the pro-phagocytic effect of the BsAbs, phagocytosis was measured in the presence of inhibitors of FcR binding. All antibody-mediated phagocytosis was potently inhibited by pre-incubation with a cocktail of FcR-blocking anti-CD16 and anti-CD32 antibodies, indicating a requirement of FcR interaction for induction of phagocytosis by the BsAbs (Fig. 4B).

CD20-CD47 SL reduces lymphoma burden and extends survival in vivo

We sought to pursue CD20-CD47 SL as our lead candidate for in vivo studies because its reduced affinity for CD47 successfully reduces binding to the CD47-expressing antigen sink (Fig. 3). To test the in vivo therapeutic potential of CD20-CD47 SL, we utilized localized and disseminated human NHL models that we previously described. To model localized disease, NSG mice, which lack T, B, and NK cells but retain phagocytic cells, were transplanted subcutaneously with luciferase-expressing Raji cells. Upon establishment of engraftment, mice were administered daily antibody treatments. Anti-CD47 antibody and rituximab alone each decreased lymphoma burden (Fig. 5A–B) and marginally extended survival relative to control mice (Fig. 5C). As we previously reported, the combination of anti-CD47 and rituximab eliminated detectable lymphoma and led to long-term disease-free survival (Fig. 5A–C).

Strikingly, CD20-CD47 SL recapitulated the effect of combination therapy and significantly prolonged survival relative to mice treated with either anti-CD47 or rituximab alone (Fig. 5A–C). Similar findings were observed in a disseminated lymphoma model, in which 3 different doses were tested in order to identify conditions with DVD-Ig serum levels comparable to rituximab (Fig. S6). This was best achieved with 1 mg of DVD-Ig and 200 micrograms of rituximab (Fig. S6). Notably, the improved outcome with CD20-CD47 SL relative to anti-CD47 or rituximab in both models occurs despite lower serum levels of CD20-CD47 SL (Fig. 5D–E).
Discussion

We report here the generation of BsAbs that target CD47 along with CD20 and recapitulate the benefits of combination antibody treatment within a single molecule. Specifically, we established a BsAb format that exhibited reduced affinity for CD47 relative to the parental anti-CD47 antibody, permitting selective binding to dual antigen-expressing cells in the presence of an excess of CD47-only expressing cells.

**Figure 3.** CD20-CD47 SL selectively binds to dual antigen-expressing cells in the presence of an antigen sink: (A) Schematic of the experimental design to assay for selectivity in binding to dual antigen-expressing cells in the presence of an excess of CD47-only expressing cells. CLL cells were labeled with CFSE and mixed with a 20-fold excess of CD20-CD47+ human red blood cells (RBCs). Cell mixtures were incubated with primary antibody prior to staining with PE anti-human Fc secondary and AF647 anti-CD47, and analysis by flow cytometry. (B) Two primary CLL samples were stained for CD47 and CD20 expression to identify the percentage of dual antigen-expressing cells by flow cytometry. (C) CLL cells were distinguished from RBCs on the basis of CFSE uptake. Primary antibody was used at 10 μg/ml and binding to cells was detected with PE secondary antibody staining. (D) Percentages of CLL cells (CFSE+) and RBC (CFSE−) within the antibody bound (PE+) population. A single replicate from each donor was averaged together (n = 2). (E) Binding of AF647 anti-CD47 to CLL cells from panel C is reported as MFI normalized to isotype control. A single replicate from each donor was averaged together (n = 2).
of a large antigen sink. Moreover, our CD20-CD47 BsAb was able to simultaneously bind to both antigens and retain the therapeutic benefit of tumor cell phagocytosis. Importantly, the CD20-CD47 SL BsAb was able to recapitulate the in vivo therapeutic synergy obtained through combination of anti-CD47 and rituximab. Together, these results establish BsAb targeting of CD47 along with a tumor antigen as a viable strategy for directing the synergistic benefits of combination therapy specifically toward tumor cells.

CD47-mediated activation of SIRPα signaling results in inhibition of phagocytosis, and reagents that antagonize this interaction are potential therapeutics that act by enabling phagocytosis. Importantly, CD47-targeting agents have the potential to augment existing antibody therapies by adding the benefit of blocking the CD47-mediated inhibitory signal to the established therapeutic effect of pro-phagocytic anti-cancer antibodies. We originally tested this hypothesis using rituximab, an FcR-activating pro-phagocytic antibody, in combination with a blocking anti-CD47 antibody, and observed synergistic phagocytosis and elimination of human NHL in vivo. This synergy mechanism was further demonstrated with several approaches to disrupt CD47-SIRPα interactions resulting in increased antibody-mediated elimination of melanoma cells in vivo and enhanced trastuzumab-mediated ADCC in vitro. Notably, interference of the CD47-SIRPα interaction with F(ab’)2 fragments of anti-CD47 synergized with trastuzumab, but had no effect alone, suggesting a requirement in this setting for an Fc domain in addition to CD47-SIRPα blockade for cancer cell elimination. In agreement with these findings, a third study showed that high affinity SIRPα variants increased phagocytosis when presented as Fc fusion proteins, but failed to effectively induce phagocytosis in monomeric form or when expressed as dimers lacking Fc domains. Interestingly, this property allowed SIRPα monomers to function as adjuvants to increase the efficacy of the tumor-specific monoclonal antibodies trastuzumab, rituximab, and cetuximab.

While our in vivo experiments demonstrate potent anti-tumor efficacy with CD20-CD47 SL that is comparable to combination therapy, they do not address the issue of selectivity in the presence of an antigen sink as CD20-CD47 SL does not cross-react with mouse CD47. One approach to test this hypothesis would be to create transgenic mice that express human CD47 in all cells, but such a strain is currently available. Alternatively, immunocompromised mice could be engrafted with human hematopoietic cells prior to engraftment with tumor cells and antibody treatment. However, this approach does not lead to engraftment with RBCs that are the primary physiological antigen sink, and mice do not maintain human RBCs after transfusion (data not shown). In light of these limitations, the most feasible approach to test this hypothesis would be to create a CD20-CD47 SL DVD-Ig using VH and VL from an anti-CD47 antibody that cross-reacts with mouse CD47. While this reagent may provide insight in proof-of-concept studies, prior observations with DVD-Ig molecules using different antibodies targeting the same antigen demonstrate that binding properties are empiric to each DVD-Ig. Thus, findings from such a molecule would not necessarily extrapolate to the CD20-
Figure 5. CD20-CD47 SL reduces lymphoma burden and extends survival in vivo: (A) NSG mice transplanted subcutaneously with Raji-luciferase cells were treated with daily injections of 200 µg mouse IgG control, anti-CD47 antibody, rituximab, or 200 µg anti-CD47 antibody + 200 µg rituximab. CD20-CD47 SL was administered at 1 mg to account for the increased molecular weight (n = 10 per treatment group). Luciferase imaging of representative mice from pre-treatment and after 14 doses are shown. (B) Bioluminescence values were normalized to pre-treatment values for each mouse. p values were derived by one-way ANOVA with Holm-Sidak’s correction. *p < 0.05, **p < 0.01, ****p < 0.0001, line denotes mean. (C) Survival of engrafted mice. Arrows indicate the start (day 7) and stop (day 21) of treatment. Statistical analysis was performed by Mantel-Cox test. (D) Serum concentration pharmacokinetic profiles of each antibody throughout the course of treatment. Each line represents an individual mouse. Arrows indicate start (day 7) and stop (day 21) of treatment. Immobilized antibody directed against the human or mouse Fc region was used to capture the treatment antibodies from mouse serum prior to detection with HRP-conjugated antibody against the human or mouse kappa light chain of the treatment antibodies. Standard curves using known concentrations of antibodies were used to derive the serum levels. Antibody concentration is represented as µM of anti-CD20 F(ab’2) (left) or as µM of anti-CD47 F(ab’2) (right).
CD47 SL that we have developed. Given these limitations, the in vitro systems that we have used represent the best available strategy to test the hypothesis that CD20-CD47 SL avoids an antigen sink presented by human blood cells that would be prohibitive to combination therapy. Importantly, these experiments demonstrate that CD20-CD47 SL, by nature of its reduced affinity for CD47, binds poorly to RBC (Fig. 3) and this property suggests a potential advantage over combination therapy.

The CD20-CD47 SL BsAb developed here is a potential therapeutic for NHL, where targeting of CD20 via rituximab is an established therapy and co-expression of CD20 and CD47 has been reported.11 Our demonstration of the use of a BsAb in the DVD-Ig format to achieve highly selective tumor targeting while retaining CD47 binding is proof of principle for future CD47 targeted therapies that incorporate other tumor antigens. For example, we have demonstrated that BsAbs with similar reduced affinity for CD47 relative to parental antibody can be generated in the DVD-Ig format targeting CD47 and CD33, an antigen expressed on AML cells, thus illustrating the adaptability of this approach to other antigen pairs. (Fig. S1) In addition to its high expression on NHL and AML, CD47 expression has been observed on most human solid tumor types evaluated, including ovarian, bladder, colon, brain, breast, and hepatocellular carcinoma.10,11 There are clinically approved therapeutic antibodies for many of these cancer types, in addition to many other antibodies in clinical development that could be used to construct DVD-Igs.40 We feel it is likely that the approach described here can be used to enhance the specificity and efficacy of most anti-tumor antibodies that engage Fc receptors including trastuzumab, cetuximab, and others. Ultimately, we anticipate that BsAb targeting of CD47 along with tumor-associated antigens will be a viable therapeutic strategy for preferentially inducing the phagocytosis of tumor cells that can be broadly applied to cancer.

Materials and Methods

Bispecific antibody construction and production

The VH and VL of 2B8, huM195, or B6H12.2 were synthesized using custom gene synthesis (MCLAB).41-43 Each VH and VL was subcloned into the pCEP4 mammalian expression vector (Invitrogen) containing the human CH1 or CK genes, respectively, to create the heavy and light chain for expression of B6H12-hIgG1 (anti-CD47), 2B8-hIgG1 (anti-CD20), or huM195-hlgG1 (anti-CD33). To create BsAbs in the DVD-Ig format, the amino terminus of each B6H12 variable domain was fused to the carboxyl terminus of each 2B8 (or huM195) variable domain by overlapping PCR. A linker separating each variable domain was introduced into the PCR primers in this process. The short linker (SL) and long linker (LL) on the light chain are TVAAP and TVAAPSVFIFPP, respectively. On the heavy chain, the SL was ASTKG and the LL was ASTKGPSVFPLAP. These linkers are derived from the amino termini of human CH1 and human CK chains. Plasmids containing each chain were co-transfected into Freestyle 293 cells using 293 Fectin (Invitrogen). Antibody was purified from expression media on Protein A Sepharose (GE Healthcare Lifesciences), dialyzed against PBS, and analyzed by 10% SDS-PAGE (Invitrogen) under reducing and non-reducing conditions followed by Coomassie Brilliant Blue staining. Purified antibody was quantified by A280.

Therapeutic antibodies

Rituximab (anti-CD20, human IgG1) was obtained from the Stanford University Medical Center, mouse IgG was purchased from Innovate Research, and B6H12.2 (anti-CD47, mouse IgG1) was described previously.10

Cell lines

The YB2/0, Raji, Daudi, Ramos, ST486, and J774 cell lines were obtained from American Type Culture Collection. Raji cells expressing modified firefly luciferase and eGFP were described previously.11 YB2/0 cells were engineered to stably express human CD20 cDNA (Genecopoeia) using an engineered transposable element.44 YB2/0 cells were engineered to stably express human CD47 by lentiviral transduction with virus generated from human CD47 cDNA (Open Biosystems) in the pCDH backbone (System Biosciences).

Biacore

SPR-based measurements were performed by Biosensor Tools, LLC with a Biacore 2000 instrument. A CM5 sensor chip was coated with an anti-human capturing agent prior to capture of anti-CD47, CD20-CD47 SL, or CD20-CD47 LL. The CD47 antigen was a His-tagged monomer containing the epitope for B6H12 to allow for affinity measurements in the absence of avidity contributions. The antigen was injected over reaction matrices in a 3-fold dilution series. Each sample was injected across the antibody surface for 460 seconds. The association and dissociation rate constant, ka (M⁻¹s⁻¹) and kd (s⁻¹), respectively, were monitored and a KD value was determined.

Xenograft models

Mice were maintained in a barrier facility under the care of the Stanford Veterinary Services Center and handled according to protocols approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC protocol number 22264). 1 × 10⁶ luciferase-labeled Raji cells were injected intravenously into the tail vein or subcutaneously into the hind flank of 6 to 10 week old NOD.Cg-PrkdcsidIl2tg1WjI/J/Sj (NSG) mice. Mice were monitored for engraftment via bioluminescent imaging and daily intraperitoneal injections of 200 µg mouse IgG control, anti-CD47, rituximab, or anti-CD47 antibody + rituximab (200 µg each) were administered for 3 weeks (intravenous model) or 2 weeks (subcutaneous model). Mice treated with CD20-CD47 SL were administered 300, 500, or 1000 µg antibody to account for the increased molecular weight and reduced serum levels of the DVD-Ig molecule relative to conventional antibodies. Mice were monitored with weekly
bioluminescent imaging analysis and were followed for overall survival. Luciferase imaging analysis was performed as described previously.\textsuperscript{11}

**Human samples**

Normal human peripheral blood and salvage red blood cell product were obtained from the Stanford Blood Center. Mononuclear cells were prepared from leukocyte reduction system (LRS) chambers using Ficoll-Paque Plus (GE Healthcare). Human CLL samples (Fig. 3B) were obtained from patients at the Stanford Medical Center with informed consent, under Stanford IRB # 6453.

**Disclosure of Potential Conflicts of Interest**

RM is a co-inventor of U.S. Patent No. 8,562,997 entitled “Methods of Treating Acute Myeloid Leukemia by Blocking CD47” and U.S. Patent No. 8,758,750 entitled “Synergistic Anti-CD47 Therapy for Hematologic Cancers.” RM and JL have filed U.S. Patent Application Serial No. 13/675,274 entitled “Humanized and Chimeric Monoclonal Antibodies to CD47.”

**Acknowledgments**

The authors acknowledge Adriel Cha, Stephen Willingham, Aaron Ring, and Ryan Corces-Zimmerman for reagents, Feifei Zhao and Melissa Stafford for lab management, and Irv Weissman for discussion.

**References**

1. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol 2010; 10:317-27; PMID:20414205; http://dx.doi.org/10.1038/nri2744
2. Reichert JM. Which are the antibodies to watch in 2013? MAbs 2013; 5:1-4; PMID:23254906; http://dx.doi.org/10.4161/mbx.22976
3. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. Nat Biotechnol 2005; 23:1147-57; PMID:16151408; http://dx.doi.org/10.1038/nbt1137
4. Cheson BD, Leonard JP. Monoclonal antibody therapy for B-cell non-Hodgkin’s lymphoma. N Engl J Med 2008; 359:613-26; PMID:18687642; http://dx.doi.org/10.1056/NEJMra0708875
5. Brown EJ, Frazier WA. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol 2001; 11:130-5; PMID:11396274; http://dx.doi.org/10.1016/S0962-8924(00)01906-1
6. Jiang P, Lagenauf CF, Narayanan V. Integrin-associated protein is a ligand for the P84 neural adhesion molecule. J Biol Chem 1999; 274:559-62; PMID:9872987; http://dx.doi.org/10.1074/jbc.274.2.559
7. Seifert M, Bossart P, Canz C, Cellia M, Colonna M, Brugger W, Kanz U, Ulrich A, Buhling HJ. Signal-regulatory protein alpha (SIRPalpha) but not SIRPbeta is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34\(^+\) (CD47) and its ligands. Trends Cell Biol 2001; 11:130-5; PMID:11396274; http://dx.doi.org/10.1016/S0962-8924(00)01906-1
8. Barclay AN, Brown MH. The SIRP family of receptors and immune regulation. Nat Rev Immunol 2006; 6:457-64; PMID:16991243; http://dx.doi.org/10.1038/nri1859
9. Tsai RK, Discher DE. Inhibition of “self” engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. J Cell Biol 2008; 180:989-1003; PMID:18332220; http://dx.doi.org/10.1083/jcb.200708043
10. Majeti R, Chao MP, Alizadeh AA, Pang WW, Jaiwal S, Gibbs KD, Jr., van Rooijen N, Weissman IL. CD47 is an alternate prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell 2009; 138:286-99; PMID:19632179; http://dx.doi.org/10.1016/j.cell.2009.05.045
11. Chao MP, Alizadeh AA, Tang C, Myklebust JS, Zhao F, Park CY, Weissman IL, Majeti R. The CD47-SIRPalpha signaling in macrophages eliminates human acute myeloid leukemia stem cells and leukemia cells to avoid phagocytosis. Cancer Res 2011; 71:1374-84; PMID:21177380; http://dx.doi.org/10.1159/000101046
12. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, Wang J, Gomez-Torrijil H, Martin R, Cohen JD, et al. The CD47-signal regulatory protein alpha (SIRPalpha) interaction is a therapeutic target for human solid tumors. Proc Natl Acad Sci U S A 2012; 109:6662-7; PMID:22451913; http://dx.doi.org/10.1073/pnas.1106550108
13. Chao MP, Alizadeh AA, Tang C, Jan M, Weissman IL, Tsukamoto R, Zhao F, Park CY, Weissman IL, Majeti R. Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. Cancer Res 2011; 71:1374-84; PMID:21177380; http://dx.doi.org/10.1159/000105808-5472.CAN-10-2238
14. Jaiswal S, Jamieson CH, Pang WW, Park CY, Chao MP, Majeti R, Traver D, van Rooijen N, Weissman IL. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. Cell 2009; 138:271-85; PMID:19632178; http://dx.doi.org/10.1016/j.cell.2009.05.046
15. Weiskopf K, Ring AM, Ho CC, Volkmer JP, Levin AM, Volkmer AK, Ozkan E, Fernhoff NB, van der Mijn M, Weissman IL, et al. Engineered SIRPalpha Variants as Immunotherapeutic Adjuvants to Anticancer Antibodies. Science 2013. 341:88-91; PMID:23722425; http://dx.doi.org/10.1126/science.1238856
16. Techoharies AP, Jin L, Cheng PY, Prasolova TK, Malko AV, Ho JM, Poeppl AG, van Rooijen N, Minden MD, Danska JS, et al. Disruption of SIRPalpha signaling in macrophages eliminates human acute myeloid leukemia stem cells in xenografts. J Exp Med 2012; 209:1883-99; PMID:22945919; http://dx.doi.org/10.1083/jem.20120902
17. Chao MP, Weissman IL, Majeti R. The CD47-SIRPalpha pathway in cancer immune evasion and potential therapeutic implications. Curr Opin Immunol 2012; 24:225-32; PMID:22310103; http://dx.doi.org/10.1016/j.coi.2012.01.010
18. Winiarska M, Gliedkowska-Mrowka E, Bil J, Golab J. Molecular mechanisms of the antitumor effects of anti-CD20 antibodies. Front Biosci 2011; 16:277-306; PMID:21190171; http://dx.doi.org/10.2741/3688
19. Zhao XW, van Bekkum EM, Schonekens K, Van der Maar H, Van Houdt M, Otten MA, Finetti P, Van Egmond M, Matzokzi T, Kraal G, et al. CD47-signal regulatory protein-alpha (SIRPalpha) interactions form a barrier for antibody-mediated tumor cell destruction. Proc Natl Acad Sci U S A 2011; 108:18342-7; PMID:22048261; http://dx.doi.org/10.1073/pnas.1106550108
20. Muller D, Kontermann RE. Bispecific antibodies for cancer immunotherapy: Current perspectives. BioDrugs 2010; 24:89-98; PMID:20139912; http://dx.doi.org/10.1007/s40252-014-0000-0
21. May C, Supra P, Gerber HP. Advances in bispecific biotherapeutics for the treatment of cancer. Biochim Pharmacol 2012; 84:1105-12; PMID:22858161; http://dx.doi.org/10.1016/j.bcp.2012.07.011
22. Fischer N, Leger O. Bispecific antibodies: molecules that enable novel therapeutic strategies. Pathobiology 2007; 74:3-14; PMID:17496428; http://dx.doi.org/10.1159/000101046
23. Marvin JS, Zhu Z. Recombiant approaches to IgG-like bispecific antibodies. Acta Pharmacol Sin 2005; 26:649-58; PMID:15916729; http://dx.doi.org/10.1111/j.1745-727X.2005.00119.x
24. Holguer P, Prospero T, Winter G. Diabodies: small bivalent and bispecific antibody fragments. Proc Natl Acad Sci U S A 1993; 90:6444-8; PMID:8341653; http://dx.doi.org/10.1073/pnas.90.14.6444
25. Deyov SM, Lobsendko-EN. Multivalence: the hallmark of antibodies used for optimization of tumor targeting by
32. Wu C, Ying H, Bose S, Miller R, Medina L, Santora L, Ghayur T. Molecular construction and optimization of anti-human IL-1α/β dual variable domain immunoglobulin (DVD-Ig) molecules. MAbs 2009; 1:339-47; PMID:20068402; http://dx.doi.org/10.4161/mabs.1.4.8755
33. Ernst JA, Li H, Kim HS, Nakamura GR, Yansura DG, Vandlen RL. Isolation and characterization of the B-cell marker CD20. Biochemistry 2005; 44:15150-8; PMID:16285718; http://dx.doi.org/10.1021/bi0511078
34. Khandelwal S, van Rooijen N, Saxena RK. Reduced expression of CD47 during murine red blood cell (RBC) senescence and its role in RBC clearance from the circulation. Transfusion 2007; 47:1725-32; PMID:17725740; http://dx.doi.org/10.1111/j.1537-2995.2007.01348.x
35. Shultz LD, Lyons BL, Burzynski LM, Gert B, Chen X, Challeff S, Korb M, Gillies SD, King M, Mangada J, et al. Human lymphoid and myeloid cell development in NOD/SCID congenic IL2Rγc gamma null mice engrafted with mobilized human hematopoietic stem cells. J Immunol 2005; 174:6477-89; PMID:15879151; http://dx.doi.org/10.4049/jimmunol.174.10.6477
36. Michaelis K, Chao MP, Majeti R, Weissman IL. Macrophages as mediators of tumor immunosurveillance. Trends Immunol 2010; 31:212-9; PMID:20452821; http://dx.doi.org/10.1016/j.it.2010.04.001
37. Duslova S, Norta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. Cell Stem Cell 2012; 10:120-36; PMID:22305562; http://dx.doi.org/10.1016/j.stem.2012.01.006
38. Jaiswal S, Chao MP, Majeti R, Weissman IL. Macrophages as mediators of tumor immunosurveillance. Trends Immunol 2010; 31:212-9; PMID:20452821; http://dx.doi.org/10.1016/j.it.2010.04.001
39. Duslova S, Norta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. Cell Stem Cell 2012; 10:120-36; PMID:22305562; http://dx.doi.org/10.1016/j.stem.2012.01.006
40. Reichert JM. Antibodies to watch in 2013: Mid-year update. MAbs 2013; 5:513-7; PMID:23727858; http://dx.doi.org/10.4161/mabs.23990
41. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman BA, Hanna N, Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 1994; 83:435-45; PMID:7506851
42. Gresham HD, Goodwin JL, Allen PM, Anderson DC, Brown EJ. A novel member of the integrin receptor family mediates Arg-Gly-Asp-stimulated neutrophil phagocytosis. J Cell Biol 1989; 108:1935-43; PMID:2785522; http://dx.doi.org/10.1083/jcb.108.5.1935
43. Caron PC, Co MS, Bull MK, Avaldovic NM, Queen C, Scheinberg DA. Biological and immunological features of humanized M195 (anti-CD33) monoclonal antibodies. Cancer Res 1992; 52:667-67; PMID:1458463
44. Huang X, Wilber AC, Bao L, Tuong D, Tolar J, Orchard PJ, Levine BL, June CH, McVor RS, Blazar BR, et al. Stable gene transfer and expression in human primary T cells by the Sleeping Beauty transposon system. Blood. 2006; 107:483-91; PMID:16189271; http://dx.doi.org/10.1182/blood-2005-05-2133