CD47-SIRPα Pathway as a Target for Cancer Therapeutics

By: Nicholas Garaffo

Abstract

According to the American Cancer Institute, in 2018, cancer had an estimated 1,735,350 new cases and 609,640 people will die in the United States alone. Like many deadly diseases, cancer has found ways to evade the immune system. Many cancers were recently shown to overexpress CD47, a widely expressed "don't eat me" signal, which interacts with the immune cells' signal receptor protein alpha (SIRPα), to prevent programmed cell removal (PCR) (Oldenborg et al., 2001). Recent advances have been made to target the CD47-SIRPα pathway to prevent the antiphagocytic activity seen in many cancers. The scope of this review is limited to two new methods used to inhibit the CD47-SIRPα pathway: anti-CD47 and SIRPα antibodies, and small peptide inhibitors. The antibodies for CD47 have shown effectiveness in clinical trials. Antibody inhibition for CD47 and SIRPα were compared, and SIRPα produced better cell type specific inhibition, but similar on-target healthy cell phagocytosis caused anemia in both trials. Several factors, including degradation and inability to penetrate dense tumors, hinder antibody treatment in all cancer patients; therefore, small peptide inhibitors offer an alternate route for inhibition to occur.

Introduction

PCR is an efficient and accurate process that clears dead, dying, or infectious cells. Phagocytic macrophages—neutrophils, dendritic cells and monocyte derivatives—perform PCR, and acts independently of apoptosis, thereby, preventing pro-inflammatory molecules from being released (Lagasse and Weissman, 1994; Choa et al. 2011). Cells that are under oxidative stress release chemotactic factors that attract immune cells (Choa et al., 2011). Once the macrophage locates the infected cell, it recognizes the cell through "don't eat me" or, "eat me" ligands to prevent or induce cell engulfment, respectively. The scope of this review is limited to a signal ligand—receptor interaction between cluster of differentiation 47 (CD47)—a widely expressed transmembrane protein (Brown and Frazier, 2001)—and signal receptor protein alpha (SIRPα)—a receptor expressed on phagocytic immune cells. CD47 links to SIRPα and acts as a "don't eat me" signal to prevent cellular phagocytosis.

Macrophages activate specific transcription factors in response to environmental cues. Notch signaling describes the macrophages' internal protein cascade upon receptor-ligand interactions. The macrophage responds by adjusting its polarization into either phagocytic, categorized as the M1 polarization, or non-phagocytic (M2) (Alvey and Discher, 2017). This is important because a macrophages' phenotype is environmentally dependent and plays a critical role in PCR. For example, upon binding CD47, SIRPα initiates an inhibitory signal transduction cascade via src homology-2 domain recruitment that contains the protein tyrosine phosphatases

SHP-1 and SHP-2. Once activated, SHP-1 propagates a downstream antiphagocytic signal (M2) through an unknown mechanism (Barclay and Brown, 2006; Oldenborg et al., 2001; Lin et al., 2018). Naturally, this ensures macrophages do not engulf healthy cells. In fact, a single CD47-SIRPα interaction is capable of preventing phagocytosis (Ho et al., 2015).

One mechanism cancer uses to evade the immune system is through the CD47-SIRPα pathway. For cancer to propagate it must: prevent apoptosis, divide rapidly and evade the immune system (Ottaviano et al., 2019). Many cancers overexpress CD47 and, it is hypothesized that CD47 accumulation acts as a camouflage. Therefore, inhibiting the CD47-SIRPα pathway is a favorable route for therapeutics (Oldenborg et al., 2001). Efforts have been made to target CD47 and SIRPα individually through monoclonal antibodies (mAb) and high-affinity small peptides. These methods, coupled with known cancer therapeutics, have been shown to decrease tumor cell density in vitro, in vivo, and in clinical trials. The main goal here is to assess the potential adverse effects presented in each therapeutic. Major hurdles include the potential for other phagocytic inhibitors, off-target effects, and the lack of long-term effects.

Antibody targeting of CD47 and SIRPα shows inhibition of anti-phagocytic signaling

Antibody targeting of CD47 is an effective therapeutic for specific cancers. Acute myelogenous leukemia (AML) is maintained by self-renewing leukemia stem cells (LSC) which evade phagocytosis through increased CD47 expression (Chao et al., 2011; Oldenborg et al., 2001). By targeting CD47, researchers hope to activate a focused immune response against tumor cells. Both, in vitro and in vivo analysis of an anti-CD47 antibody (B6H12.2) in an AML LSC model reported a 3-5 fold increase in phagocytosis compared to macrophages and tumor cells alone (Majeti et al., 2009). In contrast, an anti-SIRPα antibody reported an increased phagocytosis only when coupled with trastuzumab—a known breast cancer therapeutic (Zhao et al., 2011). This contradiction is important, firstly, because it shows antibodies alone are insufficient to increase phagocytosis. Secondly, it hypothesizes other "don't eat me" signals continue to inhibit phagocytosis after the CD47-SIRPα has been blocked. Lastly, it shows two alternate ways to inhibit the CD47-SIRPα pathway. The anti-SIRPα antibody is argued as a favored cancer therapeutic because CD47 is widely expressed across cell types. Targeting CD47 may cause unwanted on-target CD47 phagocytosis. Despite this possibility, an i*n vivo* analysis of B6H12.2 reported no additional phagocytic activity even with equivalently coated cells (Chao et al., 2011). However, therapeutic exposure only lasted 14 days and animal models were sacrificed afterwards; therefore, long term effects have not been assessed.

Anti-CD47 antibody development towards human variant

A limitation to antibody therapeutics is inter-species variation. B6H12.2s' affinity decreased from mice to humans due to CD47 variation. Therefore, a human anti-CD47 antibody (5F9) was produced and grafted to immunoglobulin G4 scaffold (IgG4) (Liu et al., 2015). The resulting antibody (Hu5F9-G4) was tested in vitro for its affinity towards human CD47 and

revealed strong attraction (K_D= $1x10^{-12}$). Hu5F9-G4 was further tested in cynomolgus monkeys to assess potential toxicity in a human-like model. No serious adverse events were characterized except dose dependent anemia which, was expected and reverted naturally after antibody treatment (Oldenborg et al., 2001). However, using healthy monkeys was a limitation to this study; tumor cell phagocytosis was not assessed in vivo. Furthermore, the toxic effects were only tested in a three week period and no long-term effects were characterized.

Clinical trials for the human CD47 antibody variant

Clinical trials of Hu5F9-G4 antibody coupled with rituximab are currently being conducted. Toxicity and effectiveness were assessed in 22 patients with aggressive and indolent lymphoma (Advani et al., 2018). From this sample, 50% had an objective response and 36% had a complete response. Furthermore, by day 28, white and red blood cells had, an approximate, 100% of their CD47 receptors occupied. This is important because blocking all CD47-SIRPα interactions is needed for effective results (Ho et al., 2015) and, since all cells are not degraded, other signals must be preventing phagocytosis on healthy cells. As seen in other animal models, dose-dependent anemia was the most common side-effect but, it reverted to baseline at lower dosages or after the treatment period (Oldenborg et al., 2001). This coupled treatment showed promising results for patients with aggressive and indolent lymphoma.

High-affinity small peptides as an alternate CD47-SIRPα inhibitor

Another issue with antibody therapeutics is their poor permeability into dense tumors (Chames et al., 2009). Given this hurdle, an alternate route is small peptide inhibitors against the CD47-SIRPα pathway. By antagonizing CD47 or SIRPα, the small peptides should block any anti-phagocytic signaling and allow PCR to occur. Small peptides are highly specific antagonists modeled after invariable regions of their target. By analyzing the human SIRPαs' binding domain, a competitive antagonist for human CD47 was produced (Weiskopf et al., 2013). The high-affinity SIRPα monomer (CV1) was tested in vitro to assess its affinity towards human and mouse CD47. CV1 presented the same inhibition between human and mouse CD47 variants (50,000-fold affinity increase and K_D=34.0 pm). Since small peptides are modeled after invariable regions, their affinities are similar between species. This is important because affinity testing for humans can now be estimated through animal models; thereby, eliminating toxic and costly human trials. Furthermore, ex vivo co-treatment of CV1 with anti-Her2/neu—a well studied breast cancer antibody—increased phagocytosis of human breast cancer cells compared to anti-Her2/neu alone. This coupled treatment was tested in vivo and revealed increased anti-tumor responses in a mouse breast cancer model. Co-treatment illustrates the possibility for more "don't-eat-me" signals present on cancer cells. Despite CV1s' efficacy, its high affinity caused on-target CD47 binding across all cell types. Although this high-affinity is wanted in therapeutics, unwanted red blood cell phagocytosis occurred and resulted in anemia. This

side-effect, however, is common between all CD47 inhibitors and reverted after treatment (Willingham et al., 2012; Weiskopf et al., 2013).

A solution to CD47 on-target side-effects is antagonizing SIRPα instead. CD47 is expressed widely across cell lines, while SIRPα is present on a subset of macrophages; therefore, SIRPα is arguable the favored target for cancer therapeutics (Zhao et al., 2011). One potential SIRPα antagonist, which showed similar potency as CV1, is Velcro-CD47- a high-affinity CD47 variant synthesized through a novel protein "velcro" technique (Ho et al 2015). Through in vitro analysis, Velcro-CD47 enhanced mAb-mediated phagocytosis by inhibiting anti-phagocytic signals. It is important to note that the small peptide inhibitors do not, by themselves, promote phagocytosis. While antibodies illicit a targeted immune response, small peptides rely on the immune systems' natural clearance or other cancer therapeutics to clear cancer cells.

Other small peptide therapeutics for CD47-SIRPα inhibition include 4N1K and its derivative PKHB1. There has been substantial evidence that 4N1K increases PCR *in vivo (*Martinez-Torres et al., 2015; Soto-Pantoja et al., 2013; Kanda et al., 1999; Kalas eat al., 2013). Several papers highlight a difference between CD47 +/+ and CD47 -/- tumor cells removal upon 4N1K treatment (Fujimoto et al., 2003). Unlike B6H12/Hu5F9, 4N1K is able to potentiate PCR of chronic lymphocytic leukemia (CLL) in soluble conditions; however, in human serum, 4N1K is degraded by proteases faster than antibodies-more than 90% was degraded in an 1-hour incubation (Martinez-Torres et al., 2015). This therapy, therefore, requires more injections for an accurate response. Furthermore, 4N1K has conflicting evidence for its CD47 specificity, and may cause off-target effects (Jeanne et al., 2015). In order to combat these issues, two terminal residues were replaced on 4N1K with their D analogues. This new therapeutic, PKHB1, lasted longer in human serum, maintained its solubility, and continued to bind CD47. PKHB1 was then tested *in vivo* and showed higher rates of CLL PCR (Martinez-Torres et al., 2015). PKHB1 is currently in pre-clinical trials for CLL treatment.

Conclusion

Cancer therapeutics continue to progress towards more accurate and less toxic forms. In turn, this eliminates the need for deleterious options like chemotherapy. CD47-SIRPα presents a target for future immunological therapeutics. Although anemia and off-target effects must be further assessed, CD47-SIRPα inhibitors present a feasible and effective option.

CD47-SIRPα pathway is an inhibitor signal pathway that acts as a "don't-eat-me" signal for healthy cells. Upon binding to CD47, SIRPα causes a signal cascade within the immune cell, and shifts carbon metabolism resulting in a M1 phenotype (Alvey and Discher, 2017). Cancer cells were shown to over express CD47 and it is believed that this alone can prevent PCR and allow tumor progression (Oldenborg et al., 2001; Ho et al., 2015) . However, inhibiting the CD47-SIRPα pathway alone did not cause an increase in tumor cell phagocytosis forcing co-treatments, and providing evidence for other inhibitors signals (Zhao et al., 2011; Chao et al., 2011).

Anti-CD47 antibodies increase tumor cell phagocytosis in a coupled therapy with Rituximab and show accurate responses in Phase I clinical trials (Advani et al., 2018). However, CD47 is widely expressed across cell lines, and its therapeutics cause on-target healthy cell phagocytosis. In response to this concern, anti-SIRPα antibodies have been developed which illustrated similar phagocytic responses *in vivo* with higher cell type specificity (Zhao et al., 2011). Regardless, blocking the CD47-SIRPα pathways still causes anemia in patients. This is an expected and treatable side-effect that naturally reverts after a short-term treatment. No long-term effects of antibody treatments have been assessed and remains a limitation to these studies.

To combat the limitations seen in antibody treatments, small peptide inhibitors are being developed for the CD47-SIRPα pathway. While this division of therapeutics do not, by themselves, cause phagocytosis, they enhance treatment of known cancer drugs. Velcro-CD47 presented a novel protein manufacturing technique and provided a high-affinity peptide to prevent inhibitor signals (Weiskopf et al., 2013). 4N1K has been shown to increase tumor cell phagocytosis between CD47 +/+ and CD47-/- but remains controversial for off-target effects (Fujimoto et al., 2003; Jeanne et al., 2015). Despite the progress, small peptide inhibitors are hindered by their short half-life in blood serum due to protease activity. Further development of 4N1K produced PKHB 1, which lasted longer in human serum, maintained its solubility, and has entered pre-clinical trials (Martinez-Torres et al., 2015).

By blocking the CD47-SIRPα pathway and other inhibitor signals, researchers can trigger a natural immune clearance of cancer cells. Although differences between CD47 and SIRPα therapeutics, long-term effects, and 4N1K off-target effects must be further assessed, preliminary research indicates this pathway as a potential target for future therapeutics.

References:

Advani R et al. “CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin’s Lymphoma.” *N Engl J Med*. 2018 Nov 1;378(18):1711-21.

Alvey C, and Discher DE. “Engineering macrophages to eat cancer: from “marker of self” CD47 and phagocytosis to differentiation.” *J Leukoc Biol*. 2017;102: 31–40.

Barclay AN, and Brown MH. “The SIRP family of receptors and immune regulation.” *Nat Rev Immunol.* 2006 Jun;6(6): 457-64

Beatty, Gregory L et al. “CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans.” *Science.* 2011.

Behrens EM et al. “Repeated TLR9 stimulation results in macrophage activation syndrome-like disease in mice.” *J Clin Invest*. 2011 Jun;121(6):2264-77.

Brown EJ, and Frazier WA. “Integrin-associated protein (CD47) and its ligands.” *Trends Cell Biol*. 2001 Mar;11(3): 130-5.

Chames P, Van Regenmortel M, Weiss E, Baty D. “Therapeutic antibodies: successes, limitations and hopes for the future.” *Br J Pharmacol*. 2009 May;157(2): 220-33.

Chao MP, Alizadeh AA, Tang C, Jan M, Weissman- Tsukamoto R, Zhao F, Park CY, Weissman IL, Majeti R. “Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia.” *Cancer Res*. 2011 Feb 15; 71(4): 1374-84.

Fujimoto T.-T., Katsutani S., Shimomura T., Fujimura K. “Thrombospondin-bound integrin-associated protein (CD47) physically and functionally modifies integrin alphaIIbbeta3 by its extracellular domain.” *J Biol Chem*. 2013;278 26655–26665

Ho CC, Guo N, Sockolosky JT, Ring AM, Weiskopf K, Ozkan E, Mori Y, Weissman IL, Garcia KC. “‘Velcro’ engineering of high affinity CD47 ectodomain as signal regulatory protein α (SIRPα) antagonists that enhance antibody-dependent cellular phagocytosis.” *J Biol Chem*. 2015 May 15;290(20): 12650-63.

Jeanne A, Schneider C, Martiny L, Dedieu S. “Original insights on thrombospondin-1-related antireceptor strategies in cancer.” *Front Pharmacol*. 2015;6: 252.

Kalas W et al. “Thrombospondin-1 receptor mediates autophagy of RAS-expressing cancer cells and triggers tumour growth inhibition.” *Anticancer Res*. 2013;33(4):1429-38.

Kanda S, Shono T, Tomasini-Johansson B, Klint P, Saito Y. “Role of Thrombospondin-1-Derived Peptide, 4N1K, in FGF-2-Induced Angiogenesis.” *Exp Cell Res*. 1999;252, 262–272.

Lagasse E, and Weissman IL. “bcl-2 inhibits apoptosis of neutrophils but not their engulfment by macrophages.” *J Exp Med*. 1994 Mar 1;179(3):1047-52.

Lin Y, Zhao JL, Zheng QJ, Jiang X, Tian J, Liang SQ, Guo HW, Qin HY, Liang YM, Han H. “Notch Signaling Modulates Macrophage Polarization and Phagocytosis Through Direct Suppression of Signal Regulatory Protein α Expression.” *Front Immunol.* 2018 July 30.

Liu J et al. “Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential.” *PLoS ONE*. 2015.

Liu M et al. “Metabolic rewiring of macrophages by CpG potentiates clearance of cancer cells and overcomes tumor-expressed CD47-mediated ‘don’t-eat-me’ signal.” *Nat Immun.* 2019 Jan 21;265-275.

Majeti R, Chao MP, Alizadeh AA, Pang WW, Siddhartha J, Gibbs Jr. KD, Rooijen N, and Weissman IL. “CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells.” *Cell.* 2009 July 13;138(2): 286-299.

Martinez-Torres AC, Quiney C, Attout T, et al. “CD47 agonist peptides induce programmed cell death in refractory chronic lymphocytic leukemia B cells via PLCγ1 activation: evidence from mice and humans.” *PLoS Med*. 2015;12(3): e1001796.

Oldenborg PA, Zheleznyak A, Fang Y, Lagenaur CF, Gresham HD, Lindberg FP. “Role of CD47 as a Marker of Self on Red Blood Cells.” *Science*. 2001;288: 2051- 54

Ottaviano M, De Placido S, Ascierto PA. “Recent success and limitations of immune checkpoint inhibitors for cancer: a lesson from melanoma.” *Virchows Arch.* 2019 Feb 12.

Soto-Pantoja DR, et al. “Therapeutic opportunities for targeting the ubiquitous cell surface receptor CD47.” *Expert Opin Ther Targets*. 2013 Jan;17(1):89-103.

Weiskopf K et al. “Engineered SIRPα variants as immunotherapeutic adjuvants to anti-cancer antibodies.” *Science*. 2013 July 5;341(6141).

Willingham SB, et al. “The CD47-signal regulatory protein alpha (SIRPα) interaction is a therapeutic target for human solid tumors.” *Proc Natl Acad Sci*. 2012;109:6662.

Zhao XW et al. “CD47-signal regulatory protein-α (SIRPα) interactions form a barrier for antibody-mediated tumor cell destruction.” *Proc Natl Acad Sci USA*. 2011 Nov 8;108(45): 18342-7.