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CD47, a multi-facetted target for cancer immunotherapy

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Abstract

CD47 is a ubiquitously expressed immunoregulatory protein best known for its so-called 'don't eat me' function that prevents phagocytic removal of healthy cells by the immune system. Many types of cancer present high levels of this don't eat me signal on their surface, thereby disrupting anti-cancer immune responses. Based on this observation, CD47 has become a prominent target in the field of cancer immunotherapy. Indeed, pre-clinical studies have shown therapeutic benefit of anti-CD47 antibodies in solid cancers and most notably B-cell malignancies. However, CD47 is also involved in various other important cellular processes, such as angiogenesis, cancer cell death and regulation of T-cell immunity, which can be modulated via interactions with thrombospondin-1. The therapeutic outcome of CD47-targeted immunotherapy therefore relies on the combined effects of all these processes. Here we will review the various physiological functions of CD47 and their implications in cancer biology. Further, we will review ongoing efforts and provide perspectives for exploiting CD47 as an immunotherapeutic target in cancer.

Keywords: CD47; Signal regulatory protein α (SIRP α); Thrombospondin-1 (TSP-1); cancer (immune) therapy; phagocytosis; angiogenesis.

Introduction

CD47 is a 50 kDa transmembrane immunoglobulin protein comprising a heavily glycosylated N-terminal IgV domain followed by a pentaspanin transmembrane domain and a short cytoplasmic tail (Lindberg et al., 1993). CD47 is best known for its pivotal role in preventing phagocytic removal of healthy cells by binding to phagocyte-expressed signal regulatory protein alpha (SIRPα). SIRPα is an inhibitory receptor that, once triggered, suppresses phagocytosis. This CD47/SIRPα axis is an important homeostatic mechanism preventing removal of healthy normal cells that express CD47. Reversely, down-regulation of CD47 on damaged, aged and superfluous cells ensures their timely removal. This function of CD47 in cellular turn-over was first established in red blood cells almost 2 decades ago and is now held to be a general homeostatic system (Oldenborg et al., 2000). Both solid and hematologic malignancies overexpress CD47 and, thereby, essentially hijack this homeostatic system to evade phagocytic clearance (Jaiswal et al., 2009; Chao et al., 2011b; Jaiswal et al., 2009; Chao et al., 2010a; Chao et al., 2011a; Zhao et al., 2011; Willingham et al., 2012; Rendtlew Danielsen et al., 2007; Edris et al., 2012). Therapeutic interventions that block CD47-SIRPα interaction have been found to promote phagocytic elimination of such CD47 overexpressing tumor cells and are poised for clinical evaluation (Chao et al., 2010a; Tseng et al., 2013; Theocharides et al., 2012; Majeti et al., 2009;

Chao et al., 2011b; Chao et al., 2011a; Willingham et al., 2012; Edris et al., 2012). CD47 also impacts on various other biological processes via binding to alternate receptors or due to signaling through its intracellular cytoplasmic domain. For instance, interaction of CD47 with thrombospondin-1 (TSP-1) or vascular endothelial growth factor receptor 2 (VEGFR2) inhibits angiogenesis and thereby limits tumor growth. Further, cross-linking of CD47 expressed on tumor cells via e.g. TSP-1 can directly induce cell death in breast cancer cells and leukemia (Mateo et al., 1999; Saumet et al., 2005; Manna and Frazier, 2004). Finally, CD47 expressed on immune cells can both positively and negatively regulates immunity, i.e. innate immune cells such as neutrophils, as well as adaptive immunity, i.e. DC maturation and T-cell activity. Thus, CD47 has a complex and multifactorial role in anti-cancer immunity and cancer biology (figure 1). Correspondingly, the outcome of therapeutic targeting of CD47 will depend on expression, binding affinity and relative levels of its diverse ligands. In this review, we will first review expression patterns of CD47 and its binding partners in normal tissue and cancer. Second, we will discuss the multi-facetted biological effect of CD47 and its ligands in cancer and immunity. Further, we will discuss the current state-of-the-art as well as identify challenges and opportunities CD47 as target in cancer (immuno)therapy.

Tissue distribution and regulation of CD47 and binding partners

Expression of CD47 in normal cells and cancer

CD47 is expressed on almost all normal human cells, as evaluated by mRNA expression and immunohistochemical staining, and occurs in four highly conserved isoforms that differ only in the length of their cytoplasmic domain, ranging from 3 to 36 amino acids (Reinhold et al., 1995). These isoforms arise due to alternative splicing of the CD47 mRNA. Splice form 2 contains the second largest cytoplasmic tail and is most abundantly expressed, whereas neuronal tissue almost exclusively express splice form 4 (Reinhold et al., 1995). Of note, the functional and clinical implications of the respective isoforms in normal biology as well as in cancer remain unclear. CD47 was first identified as an antigen expressed on ovarian carcinoma (Poels et al., 1986), but is overexpressed in many solid tumors and hematologic malignancies (Jaiswal et al., 2009; Chao et al., 2010a; Chao et al., 2011a; Zhao et al., 2011; Willingham et al., 2012; Rendtlew Danielsen et al., 2007; Edris et al., 2012). High levels of CD47 are also found on human bladder tumor-initiating cells and leukemic stem cells (Majeti et al., 2009; Chan et al., 2009). Increased CD47 mRNA levels are an independent negative prognostic factor in multiple types of cancer (Majeti et al., 2009; Chao et al., 2010a; Chao et al., 2010a; Chao et al., 2011a; Willingham et al., 2012). Further, increased protein expression of CD47 associates with poor clinical outcome in among others ovarian cancer and glioblastoma, (Majeti et al., 2009; Chao et al., 2010a; Chao et al., 2011a; Willingham et al., 2012). Moreover, high CD47 levels associate with various adverse characteristics, such as developmental stage of the tumor, adverse molecular subtype, and resistance to therapy (Rendtlew Danielsen et al., 2007; Chao et al., 2010a; Zhao et al., 2011; Edris et al., 2012). Interestingly, high levels of CD47 expression in the bone marrow of breast cancer patients lead to a significant reduction in disease free survival (Nagahara et al., 2010).

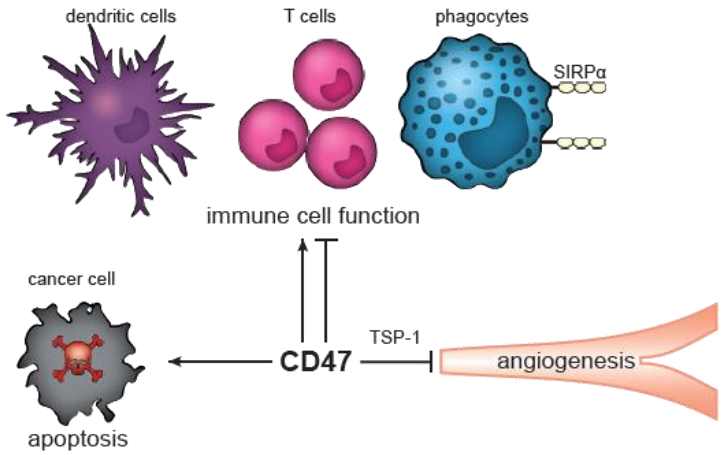

Figure 1: CD47 has a complex and multifactorial role in anti-cancer immunity and cancer biology. CD47 is involved in regulation of the activity of different immune cell types, and can induce direct cancer cell death when it is crosslinked. In addition, CD47 is involved in angiogenesis. All these aspects are discussed in this review.

Expression of CD47 binding partners SIRPα and TSP-1 in normal cells and cancer

The best characterized binding partner of CD47 is SIRPα (also termed CD172a or SHPS-1). SIRPα is a transmembrane protein consisting of three extracellular Ig-like domains, a transmembrane domain and an intracellular tail containing four immunoreceptor tyrosinebased inhibitory motifs (ITIMs) (Barclay and Van den Berg, 2014). SIRPα belongs to the signal regulatory protein receptor family, which is subdivided in a SIRPα and SIRPβ subgroup. Expression of SIRPα is restricted to phagocytes (macrophages, granulocytes and DCs) and neuronal cells (Fujioka et al., 1996). In addition to SIRPα, CD47 can bind to SIRPβ2 (also termed SIRPγ) albeit with a lower affinity than SIRPα (Piccio et al., 2005). SIRPβ2 is among others expressed on CD3-positive T-cells (Seiffert et al., 2001). SIRPα expression in cancer has not been extensively evaluated, but in primary brain tumor biopsies and astrocytoma cell lines SIRPα expression was detected. Of note, this tumor expressed SIRPα was underglycosylated compared to SIRPα expressed on Chinese hamster ovary (CHO) cells (Chen et al., 2004), suggestive of a higher affinity for CD47 (see also section of posttranslational glycosylation below). In contrast to solid tumors, SIRPα is notably down-regulated in primary hematopoietic cells and myeloid blasts from AML patients (Seiffert et al., 1999). This down-regulation may be related to the reported induction of apoptosis and growth inhibition by SIRPα in AML cells (Irandoust et al., 2013). Another important binding partner of CD47 is TSP-1, the first endogenous ligand identified for CD47. TSP-1 is a large matricellular and homotrimeric glycoprotein (430kDa) comprising at least six different structural domains of which the C-terminal domain binds to CD47 (Roberts, 2005). TSP-1 itself is a pleiotropic protein important for platelet aggregation, cell-cell and cell-matrix interactions, and negative regulation of (neo)vascularization. TSP-1 is secreted by various normal cell types, i.e. endothelial cells, smooth muscle cells and monocytes/macrophages. Further, TSP-1 is a major constituent of the extracellular matrix in normal tissues and cancer. Preclinical evidence suggests that there is an inverse correlation with a decrease in TSP-1 and an increase in CD47 mRNA levels in a prostate cancer model (Vallbo and Damber, 2005). Similarly, TSP-1 expression is inversely correlated with malignant progression in preclinical models as well as in patients with various types of cancer, including melanoma, breast, lung and bladder cancer (Papadaki et al., 2009; Ioachim et al., 2012; Zabrenetzky et al., 1994; Grossfeld et al., 1997). In line with this, TSP-1 over-expression reduced the tumorigenic potential of human cutaneous squamous cell carcinoma (Streit et al., 1999) and breast carcinoma (Weinstat-Saslow et al., 1994). Of note, oncogenic transformation of the well-established tumor suppressor p53 negatively affects TSP-1 expression, with reduced TSP-1 expression levels in ovarian carcinoma being associated with overexpression of p53 (Alvarez et al., 2001). Similarly, p53 mutation correlated with low TSP-1 levels in bladder cancer (Grossfeld et al., 1997). Further, survival of p53-null/TSP-1-null mice was significantly reduced survival compared to TSP-1-expressing p53-null mice due to naturally arising tumors (Lawler et al., 2001). Thus, TSP-1 has tumor suppressor activity in certain cancers and its expression may be deregulated by oncogenic p53 mutation.

Regulation of CD47 expression and activity

Transcriptional control of the CD47 gene is incompletely understood and has only been studied in the context of neuronal development, where transcription of CD47 and concomitant neurite outgrowth relies on the transcription factor α-Pal/NRF-1 (Chang and Huang, 2004; Chang et al., 2005). Similarly, the mechanism underlying constitutive upregulation of CD47 during transformation and progression is as yet unclear, although CD47 can be transiently upregulated by mobilizing cytokines in hematopoietic stem cells (Jaiswal et al., 2009). The latter has been speculated to be a physiological response mechanism exploited by hematologic malignancies. CD47 expression is also subject to post-transcriptional regulation by micro RNAs (miRNAs). Aberrant overexpression of CD47 correlates with downregulation of miRNA-133a in esophageal squamous cell carcinoma and colorectal cancer (Suzuki et al., 2012; Dong et al., 2013). Reporter construct studies validated the ability of miR-133a to directly inhibit CD47 transcription in vitro. Several other regulatory miRNAs were identified, i.e. miR-155 in multiple sclerosis (MS) and miR-141 in Hirschsprung's disease (Junker et al., 2009; Tang et al., 2013). Both miRs were found to target the 3'UTR of the CD47 mRNA. In MS lesions, upregulation of microRNAs was proposed to reduce CD47 thereby releasing macrophages from inhibitory control and promoting phagocytosis of myelin (Junker et al., 2009). Further, hypermethylation of a CpG Island in the promoter region of miR-141 has been linked to increased expression of CD47 (Tang et al., 2013). CD47 is also subject to post-translational modifications, most notably glycosylation. CD47 has a number of N-terminal glycosylation sites that directly affect cell surface display and regulate interaction with extracellular ligands. For instance, deglycosylated CD47 has a higher avidity for SIRPα than glycosylated CD47 and, vice versa, deglycosylated SIRPα has a higher avidity for CD47 (Subramanian et al., 2007; Subramanian et al., 2006). Reversely, hyperglycosylated SIRPα can disrupt CD47/SIRPα interactions (Ogura et al., 2004). Of note, site-

directed mutagenesis of N-linked glycosylation sites inhibited cell surface localization of CD47 in yeast models (Parthasarathy et al., 2006), although similar mutagenesis did not affect membrane localization of human CD47 in CHO cells (Subramanian et al., 2006). Aberrant glycosylation of either CD47 or SIRPα can also alter downstream responses, with differentially glycosylated SIRPα rendering B16 melanoma cells resistant to CD47-induced inhibition of motility (Ogura et al., 2004). In addition, a heavily glycosylated (>250 kD) form of CD47 has been detected in primary and transformed T-cells, endothelial cells and vascular smooth muscle cells (Kaur et al., 2011). This modification was located distally from the SIRPα binding site, but was required for TSP-1 mediated inhibitory signaling in T-cells. Although not evaluated in the context of cancer as of yet, deregulation of these mechanisms may play a role in cancer pathogenesis.

The diverse immunoregulatory effects of CD47 in cancer

Controlling phagocytic activity through CD47/SIRPα interaction

SIRPα is an important negative regulator of phagocyte activity that, upon binding by CD47 to its N-terminal IgV domain, is phosphorylated on ITIM motifs leading to concomitant activation of SHP-1 and SHP-2 phosphatases (Hatherley et al., 2008; Kharitonenkov et al., 1997; Okazawa et al., 2005). Downstream events include inhibition of myosin IIA accumulation at the phagocytic synapse (Tsai and Discher, 2008) and suppression of respiratory burst in phagocytes (van Beek et al., 2012). In line with the hypothesis that CD47 overexpression suppresses phagocytosis, ectopic overexpression of CD47 in $CD47^{lo}$ MOLM-13 myeloid leukemia cells inhibited in vitro and in vivo phagocytosis and increased tumor outgrowth (Jaiswal et al., 2009). Reversely, dissemination of Raji NHL cells was strongly reduced after shRNA knockdown of CD47 (Chao et al., 2011b). In addition, disruption of CD47-SIRPα signaling by either mutagenesis of macrophage-expressed SIRPα or by treatment with recombinant SIRPα-Fc eliminated AML xenografts (Theocharides et al., 2012). Thus, cancer cells escape from phagocytic removal by upregulation of CD47 expression, which inhibits myeloid cell activity by binding to SIRPα (figure 2A).

Controlling T-cell differentiation through "reverse" CD47 signaling

Whereas regulatory effects by CD47 on phagocytes is due to SIRPα signaling, regulatory effects on T-cells mainly stem from signaling through T-cell expressed CD47. Specifically, CD47 binding by TSP-1 (or SIRPα) can also trigger CD47 intracellular signaling in immune cells and thereby affect the immunological outcome in cancer. Treatment of naïve T-cells ($CD4^+CD25^-$) with TSP-1 or an anti-CD47 mAb upregulated expression of transcription factor FoxP3 and promoted the formation of regulatory T-cells (Tregs) (Grimbert et al., 2006; Baumgartner et al., 2008). Correspondingly, elevated serum TSP-1 levels positively correlated with the percentage of Tregs in peripheral blood of advanced melanoma patients (Baumgartner et al., 2008).

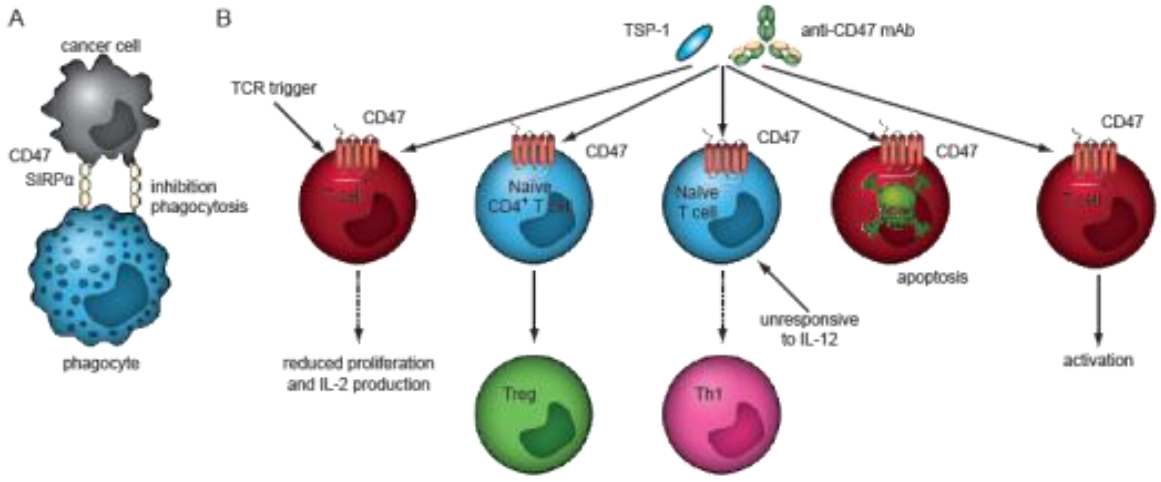

Figure 2: The diverse immunoregulatory effects of CD47. A. The interaction of CD47 (over) expressed on cancer cells with signal regulatory protein α (SIRPα) on phagocytes results in inhibition of phagocytosis. **B.** CD47 is also expressed on T-cells where it regulates diverse processes upon ligation by TSP-1 or anti-CD47 antibodies. Most of these are anti-inflammatory, as the differentiation of naïve T-cells into Th1 is inhibited, whereas Treg differentiation is induced. Further, binding of CD47 results in reduced proliferation or even T-cell death. However, depending on the context, CD47 ligation can also induce T-cell proliferation and activation.

CD47 activation on naïve T-cells also inhibited the differentiation of these cells into T helper 1 (Th1) effector cells (Avice et al., 2000). Specifically, incubation of umbicilical cord blood mononuclear

cells with a CD47 antibody in the presence of Th1-differentiating conditions (IL-12+anti-IL4 mAb) reduced both IFN-γ and IL-2 production. This inhibition was also obtained when using $F(ab')_2$ fragments of the CD47 mAb or an TSP-1 derived CD47-binding peptide. Mechanistically it was uncovered that the reduced Th1 differentiation upon CD47 ligation was caused by T-cell unresponsiveness toward IL-12 (Avice et al., 2000; Latour et al., 2001). In line with this, murine $CD47^{-/-}$ T-cells had elevated levels of Th1-lineage transcription factor Tbet, leading to higher levels of IFNγ production and Th1 differentiation than CD47 expressing cells, both in vitro and in vivo (Bouguermouh et al., 2008). Thus, CD47-signaling on T-cells has a two-fold effect, namely the enhanced differentiation of naïve T-cells into Tregs and reduced differentiation into Th1-cells (figure 2B). Of note, Th1 cells are the most effective helper T-cells during anti-tumor immune responses that control development and persistence of cytotoxic tumor-specific T-cells (Knutson and Disis, 2005). Therefore, the anti-cancer effect of CD47-targeted agents may be partly attributable to CD47-mediated reduction in Treg formation and an enhanced induction of Th1 cells via T-cell expressed CD47.

Controlling T-cell activity through "reverse" CD47 signaling

CD47-signaling can have a diverse and paradoxical outcome ranging from induction of T-cell death to activation of T-cells. For instance, CD47 ligation e.g. through soluble or immobilized TSP-1 induced a state of T-cell anergy characterized by unresponsiveness to T-cell receptor (TCR) stimulation and a lack of proliferation and IL-2 production (Avice et al., 2001; Li et al., 2001). In addition, CD47 ligation on T-cells can directly induce cell death, although contrasting data has been reported. Specifically, in one study anti-CD47 antibodies did not affect resting T-cells, but induced cell death in anti-CD3 stimulated T-cells (Pettersen et al., 1999). Reversely, in a second study, resting T-cells were sensitive toward CD47 crosslinking, whereas anti-CD3 T-activated T-cells proved resistant (Mateo et al., 2002). The reason for this discrepancy is not known, but might relate to different isolation methods and/or use of different soluble or cross-linked anti-CD3 mAbs. These studies indicate that CD47 ligation mainly serves to shut-down T-cell immune responses (figure 2B). However, CD47 can also act as T-cell co-stimulator leading to enhanced proliferation upon cross-linking of anti-CD47 and anti-CD3 antibodies (Reinhold et al., 1997; Waclavicek et al., 1997). Notably, the anti-CD47 and anti-CD3 antibodies needed to be on the same surface, with a total lack of T-cell co-stimulation when one or both antibodies were provided in solution. Hence, it was hypothesized that anti-CD47 antibodies mimick a co-stimulatory signal provided by antigen presenting cells like DCs. In line with this, a soluble anti-CD47 antibody inhibited T-cell proliferation in co-cultures of T-cells and monocyte-derived DCs (Waclavicek et al., 1997). Further, in Jurkat leukemic T-cells interaction of CD47 with TSP-1 or SIRPα on inflammatory vascular endothelium induced recruitment of lymphocytes into inflammatory tissues (Ticchioni et al., 2001). Of note, CD47 was also recently reported to directly associate with vascular endothelial growth factor (VEGF) receptor-2 (VEGFR-2) expressed on T-cells (Kaur et al., 2014). In T-cells, VEGF induced VEGFR phosphorylation inhibits T-cells proliferation and TCR signaling and thus acts as an inhibitory pathway. As also reported in endothelial cells (see anti-angiogenic effects via CD47), VEGFR-2/CD47 interaction was disrupted by TSP-1 or CD47 binding peptide. TSP-1 or the CD47 binding peptide blocked VEGFR phosphorylation in wildtype, but not $CD47^{-/-}$ T-cells. Of note, CD47 also regulated expression of both VEGF and VEGFR, with $CD47^{-/-}$ cells having significantly higher levels of both proteins. Thus, CD47 appears to control an autocrine feedback loop in T-cells involving VEGF and VEGFR.

Controlling DC and neutrophil activity through "reverse" CD47 signaling

Like most cells, DCs are also characterized by surface expression of CD47. DC-expressed CD47 was found to be required for DC entry into lymphatic vessels and for DC migration under inflammatory conditions in mice (Van et al., 2006). Since pre-treatment with CD47-Fc did not further inhibit DC migration in CD47-deficient mice, these effects were due to DC-expressed CD47 and through negative signaling via e.g. SIRPα ligation. In addition, entry of $CD47^{-/-}$ DCs into the marginal zone of the spleen was impaired, with a reduced number of DCs in the splenic marginal zone in CD47-deficient mice (Hagnerud et al., 2006). Of note, the injection of $CD47^{+/+}$ DCs but not $CD47^{-/-}$ DCs triggered efficient T-cell priming in $CD47^{-/-}$ mice, demonstrating that DC-expressed CD47 is crucial (Van et al., 2006). Thus, signaling through the intracellular CD47 domain on DCs is needed for efficient migration and entry of DCs to lymphoid organs. On neutrophils CD47 appears to be similarly important for migration, with blocking anti-CD47 mAbs delaying neutrophil transmigration and the rate of migration correlating with neutrophil surface-expressed CD47 (Parkos et al., 1996; Liu et al., 2001). Thus, CD47 expressed on DCs and neutrophils is required to efficiently induce immune responses. The role of CD47 signaling on DCs and neutrophils in anti-cancer immune responses has not been evaluated yet, but will need to be taken into account when therapeutic targeting of CD47 is to be considered.

Therapeutic targeting of CD47 activity in cancer immunity

The therapeutic potential of targeting the immunoregulatory role of CD47 has been mainly investigated in the context of its anti-phagocyte activity (figure 3A) with a series of studies using monoclonal antibodies that disrupt CD47/SIRPα interaction. Anti-CD47 monoclonal antibody (mAb) B6H12 inhibited in vivo outgrowth and dissemination of xenotransplanted solid tumors and metastatic leiosarcoma as well as primary human NHL (Chao et al., 2011b; Willingham et al., 2012; Edris et al., 2012) (figure 3B). Further, in vivo outgrowth of human leukemia cells was inhibited by either CD47 or SIRPα blocking antibodies (Jaiswal et al., 2009; Majeti et al., 2009; Chao et al., 2011a). This therapeutic effect required macrophage effector cells since clonodrate depletion of macrophages abrogated any response (Jaiswal et al., 2009; Majeti et al., 2009; Chao et al., 2011a). Nevertheless, the mechanism of CD47 antibody-mediated in vivo tumor depletion remains debated. Specifically, the dominant therapeutic mode-of-action of intact CD47 antibodies may be antibody dependent cellular cytotoxicity (ADCC) and FcR-dependent phagocytosis instead of disruption of CD47/SIRPα interaction (figure 3B). Indeed, injection of murine anti-CD47 mAb clone MIAP410 that does not affect CD47/SIRPα interaction (Han et al., 2000) also significantly inhibits tumor growth in immune competent mice (Willingham et al., 2012). Nevertheless, inhibition of CD47/SIRPα interaction alone can potentiate phagocytosis since CD47 targeted F(ab')$_2$ fragments did induce phagocytosis of NHL cells by mouse macrophages in vitro (Chao et al., 2010a), whereas rituximab derived F(ab')$_2$ fragments did not induce phagocytosis (figure 3C). Further, treatment of transgenic mice lacking SIRPα inhibitory signaling (by deletion of its cytoplasmic domain) with suboptimal concentrations of an anti-melanoma therapeutic antibody (mAb TA99) yielded effective anticancer activity, indicating that SIRPα-derived negative signaling limits antibody-mediated phagocytic elimination of target cells in vivo (figure 3D) (Zhao et al., 2011). Thus, the activity of anti-CD47 blocking antibodies may partly be attributed to blocking of the CD47 don't eat me signal but perhaps for a large part also due to typical antibody effector functions upon binding to tumor-overexpressed CD47. Interestingly, in the above-described SIRPα-signaling deficient mouse model, tumor outgrowth was not affected in the absence of therapeutic antibody, indicating that relieving CD47/SIRPα signaling is not sufficient for elimination of cancerous cells (Zhao et al., 2011; Zhao et al., 2012; Soto-Pantoja et al., 2012) (figure 3D). Based on these data, the blocking of the CD47 anti-phagocytic signal may only effectively elicit phagocytosis of target cells when combined with a prominent pro-phagocytic (therapeutic antibody) signal (Chao et al., 2010b). Preclinical data support this premise, with for instance anti-CD47 mAb blocking or CD47 knockdown in SKBR3 breast cancer cells potentiating the cytotoxicity of anti-Her-2 antibody trastuzumab (Zhao et al., 2011).

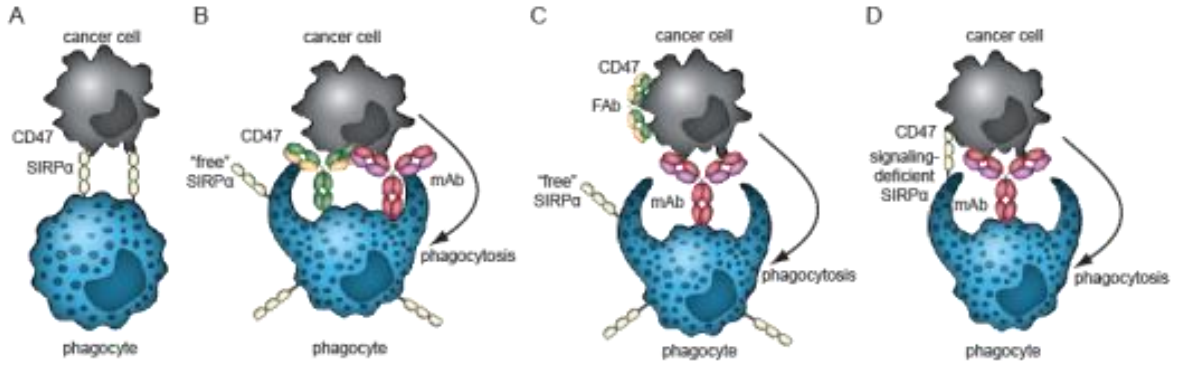

Figure 3: Therapeutic targeting of CD47 activity in cancer immunity. A. The interaction of CD47 (over) expressed on cancer cells with signal regulatory protein α (SIRPα) on phagocytes results in inhibition of phagocytosis. **B.** The use of full anti-CD47 antibodies (containing an Fc-domain) prevents the interaction of CD47 with SIRPα, whereby phagocytosis is restored. This is also partly mediated by induction of ADCC via the Fcdomain of the antibody. The addition of a therapeutic antibody enhances the pro-phagocytic effect of anti-CD47 blockage. **C.** The use of F(ab')2 fragments of the anti-CD47 antibody (lacking the Fc-domain) showed efficacy in some studies, whereas others showed the requisite for the presence of a complete functional antibody. The addition of a therapeutic antibody enhanced the therapeutic effect of F(ab')2 fragment-mediated CD47-blockage. **D.** Disrupting the CD47/SIRPα signaling pathway by expressing a signaling deficient form of SIRPα (by deletion of its cytoplasmic domain), was not sufficient to eliminate cancer cells in mice. However, when these SIRPα-signaling deficient mice were treated with an therapeutic antibody, this yielded effective anticancer activity.

Moreover, combination treatment of NHL cells with rituximab and anti-CD47 potentiated their in vivo phagocytosis and elimination compared to rituximab alone (figure 3B) (Chao et al., 2011a). This synergistic enhancement also took place when an anti-CD47 F(ab')$_2$ was co-administered with rituximab (Chao et al., 2011a) (figure 3C). Thus, probably the most effective use of releasing the

brake on the immune response by blocking CD47 is in the context of combinatorial treatment with a therapeutic anti-cancer antibody.

Challenges to CD47-targeting in cancer immune evasion

From the above it appears straightforward that the CD47/SIRPα interaction and reverse CD47 signaling in immune cells are prominent target for antibody-based approaches. However, there are a few open questions that remain to be addressed. First, several reports suggest CD47 can also function as an "eat me" signal in certain circumstances. For instance, a subset of old erythrocytes present in whole blood was shown to bind and to be phagocytosed via CD47-SIRPα interactions (Burger et al., 2012). Moreover, CD47/SIRPα interaction was shown to promote engulfment of apoptotic splenocytes by BAM3 macrophages (Tada et al., 2003). Transformation with CD47 also augmented phagocytosis of a CD47 negative lymphoma cell line after the induction of apoptosis. Similarly, trans-interaction of CD47 and SIRPα resulted in endocytosis of ligand-receptor complex by SIRPα-expressing cells (Kusakari et al., 2008). Second and as also discussed earlier, CD47 can bind to SIRPβ2 (Piccio et al., 2005). The SIRPβ subfamily comprises SIRPβ1 and SIRPβ2 and has a short intracellular domain of only a few amino acids (e.g. 4 for SIRPβ1). Despite this short domain, SIRPβ family members can transmit signals, with e.g. a positively charged lysine in the transmembrane domain of SIRPβ1 mediating interactions with an immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor protein. Of note, whereas SIRPβ1 does not bind to CD47 (Seiffert et al., 2001), interaction of SIRPβ2 on CD3-positive T-cells with endothelial CD47 is required for human T-cell trans-endothelial migration (Stefanidakis et al., 2008). Further, SIRPβ2 ligation by CD47 expressed on antigen-presenting cells induces T-cell proliferation (Piccio et al., 2005). Therefore, the use of CD47 blocking antibodies may also affect T-cell responses, e.g. by negatively regulating tumor-infiltration of T-cells. Finally, it will be important to assess whether such CD47 antibodies have any effect on DC or T-cell activity through activation of reverse CD47 signaling. Of note, on DCs this CD47 signaling is required for migration and should thus be activated. In contrast, on T-cells the major effect of CD47 signaling appears to be inhibitory with induction of T-cell arrest and promotion of regulatory T-cell differentiation and should thus be inhibited. If and how to reconcile these various requisites is an outstanding question.

Anti-angiogenic and direct anti-cancer effects mediated by CD47

CD47 does not only affect the (cancer) immune response at various levels, but can also directly affect cancer cell biology and (neo)vascularization, i.e. tumor growth. Many of these effects can be attributed to interaction of tumor cell or endothelial cell-expressed CD47 with TSP-1. Thus, the CD47/TSP-1 axis represents an important tumor-suppressor pathway in cancer and is a prominent target for therapeutic intervention.

CD47/TSP-1 mediated anti-angiogenic activity

The inhibition of angiogenesis is one of the best studied effects of CD47/TSP-1 and is due to TSP-1 mediated modulation of endothelial cell adhesion, migration and proliferation (Lawler and Lawler, 2012). Indeed, vascular outgrowth of explants from melanoma cells grown in TSP-1 knock-out mice in type I collagen matrices was better compared to explants derived from wildtype animals (Isenberg et al., 2008). Exogenous addition of TSP-1 to TSP-1$^{-/-}$ melanoma explants reduced vascular outgrowth to levels comparable to wildtype explants. Reversely, cutaneous squamous cell carcinoma cells that overexpressed TSP-1 had a decreased tumor vessel number and size in mice (Streit et al., 1999). Similarly, overexpression of TSP-1 reduced vascularization in spontaneous mammary tumors, whereas vascularization was significantly increased in TSP-1 deficient mice (Rodriguez-Manzaneque et al., 2001). The anti-angiogenic effects of TSP-1 were initially attributed to its binding to endothelial-expressed CD36 (Dawson et al., 1997). Specifically, native TSP-1 bound to surface immobilized CD36, an interaction that was blocked by anti-angiogenic TSP-1 peptides (Dawson et al., 1997). However, in three-dimensional collagen cultures, the anti-angiogenic effect of exogenous TSP-1 was abrogated in CD47$^{-/-}$ cells, but retained in CD36$^{-/-}$ cells (Isenberg et al., 2006), with ligation of CD36 also failing to inhibit NO-stimulated proliferation in CD47$^{-/-}$ cells. Furthermore, anti-angiogenic effects of TSP-1 were inhibited by a TSP-1 peptide that recognizes CD47, leading to inhibition of vascular outgrowth in TSP-1 wildtype muscle explants. Similarly, TSP-1 peptide-mediated ligation of CD47 was sufficient to inhibit NO-stimulated vascular cell responses. In line with this, overexpression of TSP-1 by tumor cells decreases tumor blood flow in response to NO in vivo, which was abrogated in mice expressing a truncated TSP-1 lacking affinity for CD47 (Isenberg et al., 2008). Importantly, inhibitory signaling of TSP-1 via CD47 takes place at relevant physiological levels of TSP-1 (picomolar range), whereas TSP-1 mediated inhibitory signaling via CD36 requires higher concentrations of TSP-1 (nanomolar range) (Isenberg et al., 2006). Thus, TSP-1 mediated inhibition of angiogenesis is regulated via interactions with both CD36 and CD47, whereby CD47 likely acts downstream of CD36 in endothelial signaling. Therefore, CD47 is the dominant anti-angiogenic receptor for TSP-1 mediated inhibition of angiogenesis (figure 4A).

CD47 mediated regulation of angiogenesis via VEGFR-2

In addition to the above-described TSP-1/CD47-mediated inhibition of blood vessel formation, CD47 directly interacts with VEGFR-2 on endothelial cells (Kaur et al., 2010). This direct interaction between CD47 and VEGFR-2 was demonstrated using immunofluorescent co-localization analysis and co-immunoprecipitation. The association of CD47 with VEGFR was abrogated by TSP-1 or TSP-1 derived peptides that bind to CD47, but not by CD36 binding peptides. Furthermore, TSP-1 binding to CD47 prevented phosphorylation of VEGFR-2 and its downstream target Akt (Kaur et al., 2010). Thus by binding to CD47, TSP-1 prevents CD47/VEGFR interaction and subsequent downstream signaling. In addition, TSP-1 itself also directly interacts with VEGF, thereby preventing angiogenic VEGF/VEGFR interaction (Gupta et al., 1999). In line with this, knockdown of TSP-1 increased the association of VEGF with VEGFR in spontaneous murine mammary tumors (Rodriguez-Manzaneque et al., 2001). Thus, the anti-angiogenic effect of TSP-1 is also partly due to disturbing VEGF/VEGFR interaction either directly by binding to VEGF or by inhibition of CD47/ VEGFR interaction, an interaction crucial for downstream signaling (figure 4A).

Direct anti-cancer effects via cross linking of tumor expressed CD47

The engagement of cancer cell-expressed CD47 by soluble TSP-1 strongly inhibited in vitro growth of a panel of breast cancer cell lines or pro-myelocytic leukemia cells through induction of caspase-independent cell death (Saumet et al., 2005; Manna and Frazier, 2004). In breast cancer cell lines, cell death induction by CD47 requires Gi-mediated inhibition of protein kinase A (Manna and Frazier, 2004). Similarly, B-cell chronic lymphocytic leukemia cells undergo caspase-independent cell death dependent on cytoskeletal reorganization upon treatment with soluble TSP-1 or anti-CD47 antibody (Mateo et al., 1999; Mateo et al., 2002) (figure 4B). Of note, TSP-1 or a CD47-blocking peptide reduced cell viability and in vivo growth of cells expressing oncogenic RAS, but did not affect immortalized nontumorigenic parental cells (Kalas et al., 2013). This tumoricidal activity of TSP-1 was dependent on CD47 cross-linking and activation of cytotoxic autophagy. These experimental results are in line with the near complete loss of TSP-1 expression in RAS-transformed cells. TSP-1/CD47 interaction can also differentially affect cancer cell sensitivity toward chemotherapeutics. For instance, TSP-1 sensitized taxol-resistant prostate cancer cells to taxol treatment (Lih et al., 2006), whereas a blocking anti-CD47 antibody decreases cytotoxicity of taxol treatment. Of note, taxol resistance is partly regulated by the txr1 gene, a gene known to down-regulate TSP-1 expression. Correspondingly, treatment with a TSP-1 mimetic peptide sensitized cells to taxol by activating CD47 signaling (Lih et al., 2006). In contrast, treatment of thyroid carcinoma cells with a CD47-binding peptide reduced doxorubicin and camtothecin cytotoxicity (Rath et al., 2006).

In line with this, pro-apoptotic activity of camtothecin and doxorubicin relied on down-regulation of TSP-1 expression in thyroid carcinoma (Rath et al., 2006). Therefore, the effect of TSP-1/CD47-signaling on apoptosis is likely dependent on cancer type as well as type of therapy. In this respect, blocking of either CD47 or TSP-1 increased radiosensitivity of tumors, whereas it induced radioprotection in normal endothelial cells (Maxhimer et al., 2009).

Although the mechanism of this differential response between normal and transformed cells is unknown, the in vitro suppression of CD47 did not increase the sensitivity of melanoma cells to radiation. Therefore, it was hypothesized that the observed enhanced anti-tumor effects were due to induction of tumor-specific immune responses. Thus, although cancer cells often up-regulate CD47 expression to escape from the immune system presumably via SIRPα-mediated inhibitory signaling, cancer cells may also benefit from loss of expression of CD47. In this respect, loss of CD47 expression was also found to up-regulate c-Myc, induce cell proliferation and upregulate the self-renewal potential of endothelial cells (Kaur et al., 2013).

Correspondingly, treatment with TSP-1 or the TSP-1 derived peptide 7N3, inhibited c-Myc expression in Jurkat cells, but did not effect c-Myc expression in $CD47^{-/-}$ cells (Kaur et al., 2013). In contrast, the use of the TSP-1 derived peptide enhanced proliferation in human astrocytoma cell lines via an Akt-dependent pathway (Sick et al., 2011).

Taken together, CD47-signaling via binding to TSP-1 is involved in both tumorigenic as tumoricidal processes, although the latter seem to be the most prevalent outcome.

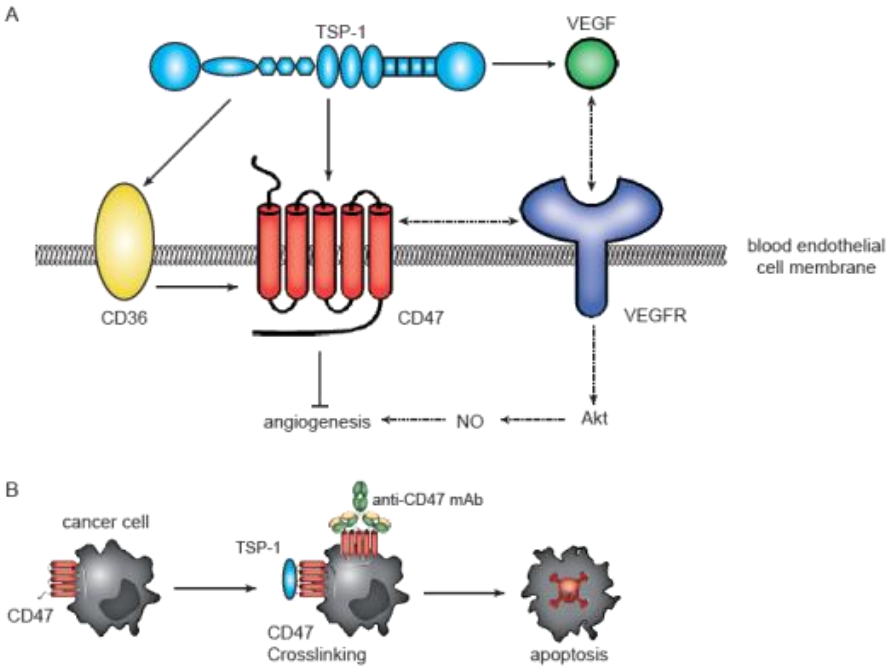

Figure 4: Anti-angiogenic and direct anti-cancer effects mediated by CD47. A. TSP-1 inhibits angiogenesis via binding to CD36 and CD47. However, TSP-1 mediated inhibition of angiogenesis by binding to CD36 is also regulated via CD47. In addition, CD47 directly interacts with vascular endothelial growth factor receptor-2 (VEGFR-2) on endothelial cells. By binding to CD47, this interaction is abrogated by TSP-1, whereby angiogenesis is inhibited. Further, TSP-1 can directly bind to VEGF, thereby preventing its interaction with VEGFR-2. **B.** Crosslinking of CD47 by antibodies or TSP-1 can lead to caspase-independent cancer cell death.

Therapeutic exploitation of CD47-mediated anti-angiogenic and direct anti-cancer effects

Based on the above, the therapeutic exploitation of CD47/TSP-1 interaction might prevent or reduce angiogenesis and tumor progression through both anti-angiogenic and possibly direct anti-cancer effects. Proof of concept for the former mechanism has been generated using a synthetic peptide, designated ABT-510, that mimics the anti-angiogenic activity of TSP-1. ABT-510 treatment significantly increased the number of patients with stable disease in patients with advanced solid malignancy (12 different types of advanced cancer, including colorectal cancer, non-small-cell-lung cancer, renal cell cancer and sarcoma) (Hoekstra et al., 2005). Further, ABT-510 was well tolerated without significant toxicity in phase I trials upon subcutaneous application in patients with advanced stages of solid cancer (Gordon et al., 2008). However, minimal antitumor activity was detected in various phase II clinical trials including in advanced renal cell carcinoma (Ebbinghaus et al., 2007), metastatic melanoma (Markovic et al., 2007) and advanced soft tissue sarcoma (Baker et al., 2008). Optimization of peptide design may be used to further increase efficacy, as evidenced by a comparative study in dogs where better responses were detected with the second-generation TSP-1 peptide ABT-898 (Sahora et al., 2012). However, most anti-angiogenic agents are best suited in combinatorial strategies with e.g. chemotherapeutics (Ma and Waxman, 2008). In line with this, ABT-510 increased the uptake and effectiveness of cisplatin and paclitaxel in a mouse model of epithelial ovarian cancer (Campbell et al., 2010). Further, combination treatment with ABT-510 and bevacizumab prolonged the duration of stable disease in patients with advanced solid tumors (Uronis et al., 2013). Thus, blocking of TSP-1/CD47 interaction e.g. using anti-CD47 antibodies, in further combination with anti-angiogenic and chemotherapeutic regimens may well have clinical potential. However, clinical application of this strategy will need to take into account that tumors can upon prolonged exposure eventually by-pass anti-angiogenic effects of TSP-1 (Filleur et al., 2001). In this respect, an optimal CD47-targeted strategy would provide a dual hit approach that would trigger not only anti-angiogenic activity but also direct CD47-mediated anti-cancer signaling that would sensitive cells for combinatorial strategies such as standard-of-care chemo/radiotherapy.

Conclusions and perspectives

CD47 is a prominent target for cancer therapy and has three main effects that should be considered in the design of CD47-based cancer therapy. The first and perhaps most studied effect is the inhibitory effect on anti-cancer immunity, which occurs through overexpression of CD47 on tumor cells and

inhibition of phagocytes through SIRPα binding (figure 5A).
The second effect of CD47 is its reverse signaling activity through neutrophil, DC, or T-cell expressed CD47 that can both inhibit and activate immune responses. Finally, CD47 has direct anti-cancer and anti-angiogenic activity through its interaction with TSP-1 (figure 5B).
All of these different aspects have to be carefully characterized for each malignancy and possibly in each patient using appropriately identified predictive biomarkers for CD47-based immunotherapy.
Therapeutic anti-CD47 antibodies have shown promising pro-phagocytic activity in preclinical models. Further integration of CD47-targeting into bi-functional immunotherapeutics that combine CD47 blockade with alternate effector moieties may help to expand on this therapeutic effect. In this respect, we recently described an anti-CD47:TRAIL fusion protein that both induced phagocytosis via CD47 inhibition as well as induced CD47-restricted cell death in malignant B-cells (Wiersma et al., 2014) (figure 5C). In an analogous fashion, it would be interesting to evaluate whether a bispecific antibody comprising a CD47 blocking antibody fragment and antibody fragment targeting a tumor overexpressed antigen could trigger tumor-localized accretion and inhibition of negative immunoregulatory signaling by CD47. Further, an approach combining both the anti-angiogenic effects of e.g. TSP-1 peptides and the pro-phagocytic activity of anti-CD47 mAbs may yield synergistic direct and immunostimulatory effects.
Finally, CD47 overexpression in cancer can also be targeted with siRNA or miRNAs that down-regulate CD47.
The potential of such an approach is highlighted by the delivery of liposome encapsulated CD47 siRNA, which effectively inhibited melanoma outgrowth and metastasis (Wang et al., 2013), whereas CD47-targeted siRNA or shRNA treatment reduced migration of colon cancer cells (Broom et al., 2009; Zhang et al., 2013) and prevented in vivo dissemination of Non-Hodgkin lymphoma cells (Chao et al., 2011b).
Finally, the transfection or injection of miR-133a, known to regulate CD47 expression, into mouse tumor xenografts significantly inhibited tumor outgrowth (Suzuki et al., 2012; Dong et al., 2013). Thus, siRNA or miRNA-mediated down-regulation of CD47 is a potentially interesting approach, which will however need to be performed using tumor-selective delivery systems in order to prevent systemic side-effects. In conclusion, many different therapeutic strategies that target CD47 have already proven effective in preclinical models.
The next few years are likely to witness the translation of CD47-targeting approaches into the clinic.
This may hinge on development of strategies that have increased the tumor-selectivity of CD47 blocking as well as identification of optimal combinatorial strategies with e.g. standard chemo/radiotherapy or combined triggering of anti-angiogenic TSP-1 signaling.

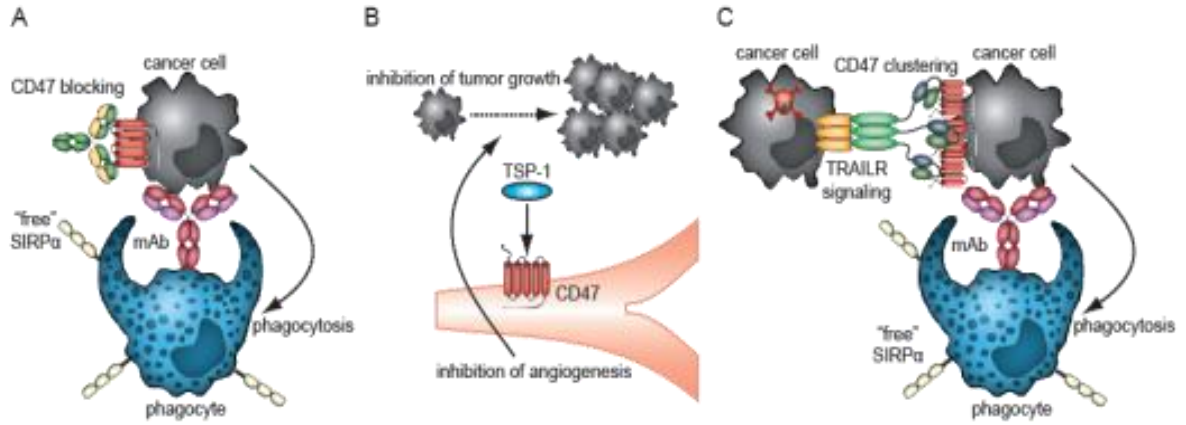

Figure 5: Perspectives in CD47-targeted cancer therapy. A. Blocking CD47/SIRPα signaling by use of anti-CD47 antibodies is effective, especially when combined with other therapeutic antibodies. **B.** Inhibition of angiogenesis via TSP-1/CD47 mediated signaling has potent anti-cancer effects. Combining the enhancement of phagocytosis and inhibition of angiogenesis would be of interest. **C.** Combining CD47 blockade with alternate effector moieties may help to expand on the therapeutic effect of this approach. In this respect, the anti-CD47:TRAIL fusion protein induced phagocytosis via CD47 inhibition, especially when combined with therapeutic antibodies, as well as induced CD47-restricted cell death in malignant B-cells.

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