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REVIEW

CD47 update: a multifaceted actor in the tumour microenvironment of potential therapeutic interest

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CD47 is a ubiquitous 50 kDa five-spanning membrane receptor that belongs to the immunoglobulin superfamily. This receptor, also known as integrin-associated protein, mediates cell-to-cell communication by ligation to transmembrane signal-regulatory proteins SIRPα and SIRPγ and interacts with integrins. CD47 is also implicated in cell-extracellular matrix interactions via ligation with thrombospondins. Furthermore, CD47 is involved in many and diverse cellular processes, including apoptosis, proliferation, adhesion and migration. It also plays a key role in many immune and cardiovascular responses. Thus, this multifaceted receptor might be a central actor in the tumour microenvironment. Solid tumours are composed of not only cancer cells that actively proliferate but also other cell types including immune cells and fibroblasts that make up the tumour microenvironment. Tumour cell proliferation is strongly sustained by continuous sprouting of new vessels, which also represents a gate for metastasis. Moreover, infiltration of inflammatory cells is observed in most neoplasms. Much evidence has accumulated indicating that infiltrating leukocytes promote cancer progression. Given its ubiquitous expression on all the different cell types that compose the tumour microenvironment, targeting CD47 could represent an original therapeutic strategy in the field of oncology. We present a current overview of the biological effects associated with CD47 on cancer cells and stromal cells.

Abbreviations

AML, acute myeloid leukaemia; CBD, cell-binding domain; DC, dendritic cell; Drp-1, dynamin-related protein-1; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HIF, hypoxia-inducible factor; HSC, haematopoietic stem cell; HSPGs, heparan sulfate proteoglycans; IAP, integrin-associated protein; ITIM, immunoreceptor tyrosine-based inhibitory motif; LSC, leukaemia stem cell; mAb, monoclonal antibody; PCD, programmed cell death; PS, phosphatidylserine; RBC, red blood cell; ROS, reactive oxygen species; SCID, severe combined immunodeficiency; SIRP, signal-regulatory protein; Treg, T-regulatory cell; TSP, thrombospondin; VCAM-1, vascular cell adhesion molecule-1

Introduction

It is now widely accepted that solid tumours are not solely composed of proliferating aneuploïd cells particularly resistant to cell death. Thus, solid tumours should be considered as heterogeneous structures containing multiple distinct cell types, including normal cells recruited into tissues surrounding tumours that actively sustain tumourigenesis by

cell-to-cell interactions and secretion of paracrine growth factors. Together with the vascular network provided by angiogenesis that ensures nutrition and oxygenation of tumours, the ensemble of these actors defines the tumour microenvironment. In addition, cancer-associated fibroblasts have been shown to enhance cancer cell proliferation, angiogenesis, invasion and metastasis, especially in the context of chronic inflammation (Tlsty and Coussens, 2006). Finally, many different types of immune cells are also found infiltrated in tumour masses at early stages. Sustained inflammation is one of the hallmarks of tumour promotion and correlates with poor prognosis in many different types of cancer. These infiltrating cells play key roles in both innate and acquired immunity and include dendritic cells (DCs), macrophages, mast cells, neutrophils, and T- and B-cells (Hanahan and Weinberg, 2011).

CD47 is an ubiquitously expressed membrane receptor that has been implicated in many normal and pathophysiological processes including apoptosis, proliferation, cell adhesion, cardiovascular effects, inflammation and immunity. Given this diversity of actions, CD47 appears as a multifaceted receptor that might represent a key target in the tumour microenvironment for development of innovative therapeutic strategies against cancer. In this review, we update the effects associated with activation or inhibition of CD47 in both cancer cells and stromal cells, paying particular attention to vascular cells and immune cells.

CD47 structure and partners

CD47 is an ubiquitous 50 kDa membrane receptor that belongs to the immunoglobulin superfamily (Frazier *et al.*, 2010). This receptor was identified through its association with integrin $\alpha_v\beta_3$ and was therefore initially called integrin-associated protein (IAP; Brown *et al.*, 1990). At that time, an ovarian carcinoma antigen called OA3 was cloned (Campbell *et al.*, 1992) with a proposed structure having five putative transmembrane domains, an extracellular N-terminal IgV domain with five putative N-glycosylation sites and a C-terminal intracellular tail characterized by alternative splicing (Figure 1). A closely similar structure was concomitantly proposed for IAP (Lindberg *et al.*, 1993). The ovarian tumour marker OA3 was subsequently shown to be the same protein as IAP (Mawby *et al.*, 1994). The OA3 marker was also found not to be restricted to ovarian carcinoma, rather exhibiting a larger distribution. CD47 was an orphan receptor until it was reported that this protein was the receptor for the C-terminal cell-binding domain (CBD) of thrombospondins (TSPs) (Gao and Frazier, 1994). Later, structural analysis revealed that a long-range disulfide bond between the extracellular and the fourth membrane-spanning domains was required for CD47 ligand binding, signalling and localization into lipid rafts (Rebres *et al.*, 2001).

Four alternatively spliced forms of CD47 were identified in both murine and human cells, with variants ranging in length from 3 to 36 amino acids in the cytoplasmic tail and

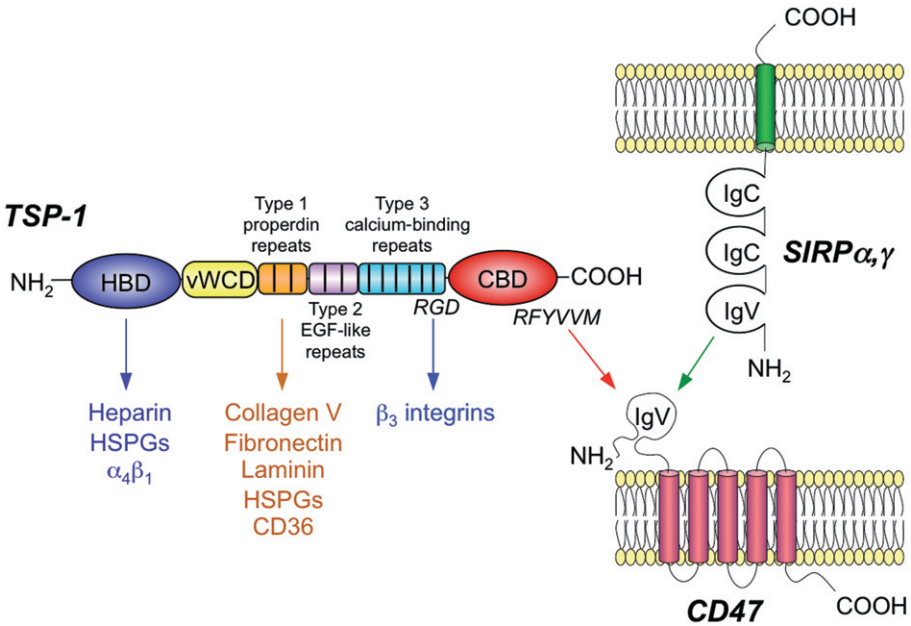

Figure 1

Schematic representation of CD47 and its endogenous ligands TSP-1 and SIRP. CD47 is composed of five transmembrane domains, an extracellular N-terminal IgV domain and an intracytoplasmic C-terminal alternatively spliced domain. TSP-1 comprises several different domains, including the heparin-binding domain (HBD), the von Willebrand C domain (vWCD), three type 1 properdin repeats, three type 2 EGF-like repeats, seven type 3 calcium-binding repeats and the C-terminal CBD. Several domains of TSP-1 interact with different extracellular matrix components or membrane receptors (arrows). Key amino acid sequences responsible for TSP-1 ligation to integrins (RGD) and CD47 (RFYVVMWK) are indicated in italics. SIRPα and γ are single transmembrane spanning proteins with 3 Ig-like motifs, including an extracellular IgV domain that interacts with the IgV domain of CD47.

having a very high interspecies conservation with respect to both peptide sequence of the alternatively spliced regions and gene structure (Reinhold *et al.*, 1995). The second-shortest form 2 is the most abundantly expressed isoform while the second most abundant isoform, form 4 (the longest), is predominantly expressed in the brain and in the peripheral nervous system; only keratinocytes expressed significant amounts of form 1 (Reinhold *et al.*, 1995). Little is known about the functional significance of this alternative splicing. Indeed, only a single study has focused on the expression of these alternatively spliced isoforms: during memory consolidation in rats, isoforms 3 and 4 are thought to be closely associated with memory consolidation, while isoform 2 appears to be the principal signal transducer in astrocytes (Lee *et al.*, 2000). Using a yeast model, post-translational modifications such as glycosylation were shown to be necessary for membrane localization of CD47 (Parthasarathy *et al.*, 2006). However, it is still unknown to what extent glycosylation influences CD47 ligand-binding properties and subsequent signalling. Recently, a high molecular weight isoform of CD47 was identified in Jurkat cells (Kaur *et al.*, 2011). In these cells, very little CD47 at its expected molecular weight (55 kDa) was found. Rather, most immunoreactivity in Western blots migrated with an apparent molecular weight >250 kDa, consistent with CD47 also being expressed as a proteoglycan, because this isoform was sensitive to chondroitinase and heparitinase (Kaur *et al.*, 2011). The glycosaminoglycan residues at Ser^{64} are involved in CD47-mediated CD69 induction in T-cells. This high molecular weight isoform is also apparently widely expressed on vascular cells (Kaur *et al.*, 2011).

The first identified endogenous ligand for CD47 was TSP-1 (Gao and Frazier, 1994). Using synthetic overlapping peptides from the C-terminal CBD of TSP-1, a consensus sequence containing the motif VVM was proposed to be essential for binding to CD47. This resulted in the development of several CD47 peptide agonists (Gao *et al.*, 1996a), among which peptides 7N3 (^{1102}FIRVVMYEGKK^{1112}), 4N1 (^{1016}RFYVVMWK^{1024}) and the extended 4N1K (K-^{1016}RFYVVMWK^{1024}-K; i.e. 4N1 flanked by two Lys residues) are still the most widely used experimentally. Nevertheless, it should be noted that, in at least two studies, the CD47-binding peptides 4N1 and 4N1K were reported to induce aggregation of platelets and to enhance Jurkat cell adhesion in CD47-deficient cells (Tulasne *et al.*, 2001; Barazi *et al.*, 2002), raising doubt as to the specificity of these peptides. Thus, results obtained with these peptides alone [i.e. without confirmation using native TSP-1, function-blocking anti-CD47 monoclonal antibodies (mAbs) or CD47-null cells] should be interpreted cautiously because it cannot be ruled out that these peptides might also bind to another yet unidentified receptor. It should also be noted that such controls have in general not been done in published work using 4N1 or 4N1K.

TSP-1 belongs to a family of multidomain calcium-binding glycoproteins that associate as homotrimers (TSP-1 and -2) or homopentamers (TSPs 3–5) in mammals. Most adult tissues co-express one or more TSPs and major alterations occur during pathophysiological conditions. For example, highly expressed levels of TSP-1 and -2 are commonly found in stromal fibroblasts and endothelial cells within tumours (Streit *et al.*, 1999; Hawighorst *et al.*, 2001). In addition to the C-terminal CD47-binding domain, TSP-1 has several other domains that bind to different cell membrane receptors or extracellular matrix, thereby mediating cell-cell and cell-extracellular matrix interactions (Figure 1). The N-terminal domain contains a heparin-binding domain that binds to heparan sulfate proteoglycans (HSPGs) and integrin $\alpha_4\beta_1$. TSP-1 also contains three types of repeats: type 1 properdin repeats that bind to CD36, collagen type V, fibronectin and HSPGs; type 2 EGF-like repeats; and type 3 calcium-binding repeats that contain the arginine-glycine-aspartate (RGD)-sequence involved in ligation of β3 integrin subunits (Figure 1; Adams, 2001). To date, the X-ray structure of the CD47/TSP-1 complex has not been resolved. However, our molecular modelling study has contributed to understanding how the TSP-1 CBD interacts with CD47 (Floquet *et al.*, 2008). Observation of X-ray structure of the CBD of TSP-1 shows that the 4N1 sequence is normally hidden within a hydrophobic pocket, preventing any interaction. By normal mode analysis and energy minimization, it was proposed that the opening of the 4N1 hydrophobic cleft is driven by large amplitude motions when in close proximity to CD47 and the phospholipid bilayer. The main biological effects induced by ligation of TSP-1 CBD to CD47 are summarized in Table 1. Binding of TSP-1 to CD47 influences several fundamental cellular functions including cell migration and adhesion, cell proliferation or apoptosis, and takes part in the regulation of angiogenesis and inflammation.

The second identified endogenous ligand of CD47 was SIRPα (Vernon-Wilson *et al.*, 2000). This protein was alternatively called BIT (Sano *et al.*, 1997), p84 (Jiang *et al.*, 1999), SHPS-1 (Babic *et al.*, 2000) or CD172a. As shown in Figure 1, SIRPα is a transmembrane protein containing three immunoglobulin-like domains in the extracellular N-terminal and four tyrosine phosphorylation sites in the cytoplasmic tail that also bears two immunoreceptor tyrosine-based inhibitory motifs (ITIMs; Brooke *et al.*, 1998). Additionally, two other signal-regulatory protein (SIRP) family members, SIRPβ (also known as CD172b) and SIRPγ (also known as SIRPβ2 and CD172g), have been characterized in man (Kharitonenkov *et al.*, 1997; Ichigotani *et al.*, 2000). The N-terminal IgV domain of SIRPα binds to the IgV domain of CD47 (Vernon-Wilson *et al.*, 2000) and the structure of the CD47/SIRPα complex has been recently resolved by X-ray crystallography (protein database code 2uv3; Hatherley *et al.*, 2008). In spite of the highly conserved structure for SIRP family members, only SIRPα and SIRPγ bind to CD47. Failure of SIRPβ to bind CD47 has been attributed at least in part to the replacement of Val27 by Met in the IgV domain of SIRPα (Hatherley *et al.*, 2008). The structure of their cytoplasmic regions is quite different. SIRPγ has a very short cytoplasmic tail like SIRPβ and lacks a charged amino acid residue in its transmembrane region preventing the association with the adaptor protein DAP12. This molecular adaptor contains an immunoreceptor tyrosine-based activation motif, thought to be responsible for SIRPβ-mediated intracellular signalling. The ligand for SIRPβ is still unknown. CD47 ligation of SIRPα or SIRPγ mediates bidirectional signalling responsible for different cell-to-cell effects including inhibition of phagocytosis, stimulation of cell-cell fusion or T-cell activation (Table 1).

CD47 was initially characterized as associated with $\alpha_v\beta_3$ (Brown *et al.*, 1990) integrins. Since then, numerous

Table 1

Partners of CD47 and their associated actions

CD47 partners		Main biological and cellular events	References
Extracellular partners			
Thrombospondin-1 (C-terminal domain)		Apoptosis, cell proliferation, cell survival, cell adhesion, inhibition of angiogenesis, pro- and anti-inflammatory effects, platelet activation and aggregation	Dorahy *et al.* (1997); Manna and Frazier (2004); Rath *et al.* (2006a); Lamy *et al.* (2007); Isenberg *et al.* (2009); Xing *et al.* (2009); Sick *et al.* (2011)
Serpin A1 (C-terminal domain)		Cell proliferation	Congote and Temmel (2004)
SIRPα1		Inhibition of phagocytosis, stimulation of cell-cell fusion, T-cell activation, neutrophil transepithelial migration	Han *et al.* (2000); Oldenborg *et al.* (2000); Seiffert *et al.* (2001); Liu *et al.* (2002)
SIRPγ		Leukocyte transendothelial migration, T-cell proliferation	Piccio *et al.* (2005); Stefanidakis *et al.* (2008)
Membrane partners			
*Direct partners (*demonstrated experimentally by immunoprecipitation or FRET in the case of VEGFR-2)*			
Integrins	α_2*	COX-2 expression and intestinal epithelial cell migration	Broom *et al.* (2009)
	$\alpha_2\beta_1$*	Migration and proliferation of smooth muscle cells	Chung *et al.* (1997); Wang and Frazier (1998)
	$\alpha_{IIb}\beta_3$*	Platelet activation	Chung *et al.* (1997)
	$\alpha_4\beta_1$*	Adhesion of sickle reticulocytes, B-cell migration	Yoshida *et al.* (2000); Brittain *et al.* (2004)
	α_5	Glycosaminoglycan synthesis by chondrocytes	Holledge *et al.* (2008)
	$\alpha_6\beta_1$	Fibrillar beta amyloid-mediated microglia activation and phagocytosis	Bamberger *et al.* (2003); Koenigsknecht and Landreth (2004)
	$\alpha_M\beta_2$	Neutrophil transepithelial migration	Hofman *et al.* (2000)
	$\alpha_v\beta_3$*	C32 human melanoma cells spreading, pro-inflammatory cytokine synthesis in human monocytes, *Coxiella burnetii* phagocytosis, promyelocytic leukaemia cell death	Gao *et al.* (1996a); Capo *et al.* (1999); Hermann *et al.* (1999); Saumet *et al.* (2005)
VEGFR-2*		Inhibition of VEGFR-2 downstream signalling	Kaur *et al.* (2010)
Indirect partners			
CD36		Amyloid β-induced inhibition of NO signalling, inhibition of NO-stimulated vascular cell responses and cGMP signalling	Isenberg *et al.* (2006); Miller *et al.* (2010a)
Fas/CD95		Stimulation of Fas-mediated apoptosis	Manna *et al.* (2005)
Intracellular partners			
Direct partners			
Gi proteins*	Syk	Platelet activation (Lyn and FAK phosphorylation)	Chung *et al.* (1997)
	AC/PKA	Platelet aggregation (decrease in cAMP), smooth muscle cell migration, T-cell death	Frazier *et al.* (1999); Wang *et al.* (1999); Manna and Frazier (2003)
	ERK	Jurkat T-lymphoma cell adhesion (phosphorylation); inhibition of smooth muscle cell migration	Wang *et al.* (1999); Wilson *et al.* (1999)
	PI3K	C32 melanoma cell spreading, T-cell migration, astrocytoma cell proliferation	Gao *et al.* (1996b); Li *et al.* (2005); Sick *et al.* (2011)
PLIC-1*		Jurkat T-cells spreading, regulation of heterotrimeric G-protein function	Wu *et al.* (1999); N'Diaye and Brown (2003)
BNIP3*		T-cell apoptosis	Lamy *et al.* (2003)
Indirect partners			
Rac		Neurite and filopodium formation, neuronal development	Miyashita *et al.* (2004); Murata *et al.* (2006)
Cdc42		B-cell migration, neurite and filopodium formation, neuronal development	Miyashita *et al.* (2004); Yoshida *et al.* (2000); Murata *et al.* (2006
Src and MEK kinases		Epithelial cell spreading and migration	Shinohara *et al.* (2006)
Drp1		Caspase-independent cell death of normal and leukaemic cells	Bras *et al.* (2007)
Protein 4.2		Rh complex integrity on red blood cells	Bruce *et al.* (2002)
GC		Inhibition of NO signalling (decrease in cGMP)	Isenberg *et al.* (2006)

interactions between CD47 and many different integrin subunits have been reported (Table 1). For example, in association with $\alpha_2\beta_1$ integrins, CD47 mediates migration and proliferation of smooth muscle cells (Wang and Frazier, 1998). In cooperation with $\alpha_v\beta_3$, CD47 mediates diverse functions such as cell spreading, cytokine synthesis, phagocytosis and cell death (Gao *et al.*, 1996b; Capo *et al.*, 1999; Hermann *et al.*, 1999; Saumet *et al.*, 2005). Interaction between $\alpha_v\beta_3$ integrin and subsequent intracellular signalling was dependent on the IgV domain of CD47 but did not require the transmembrane domain (Lindberg *et al.*, 1996). However, CD47-mediated cellular effects may occur independently of integrins, especially in red blood cells (RBCs). This is why the designation CD47 is now considered more appropriate than the initial name IAP. CD47 has also been shown to cooperate with the Fas pathway in mediating apoptosis (Manna *et al.*, 2005). Moreover, recent advances describing the role of CD47 in the field of cardiovascular diseases (Table 1) have underlined strong cooperation of CD47 with CD36 (Isenberg *et al.*, 2006) and VEGFR-2 (Kaur *et al.*, 2010).

Many of the cellular effects of CD47 are mediated by Gi proteins (Table 1). For example, apoptosis induced by the peptide agonist 4N1K involves inhibition of AC and a subsequent decrease in intracellular cAMP levels (Manna and Frazier, 2004). CD47-associated platelet activation requires Gi proteins with activation of Syk kinases that induce phosphorylation of Lyn and focal adhesion kinase (FAK) (Chung *et al.*, 1997). Other kinases identified in the signalling pathway downstream of Gi proteins include PI3K and MAPK. Taking into account such G-protein-dependent signalling associated with CD47 activation, Brown and Frazier (2001) proposed that CD47 with five transmembrane domains associated to an integrin dimer might mimic classical heptahelical GPCRs. In agreement, CD47-dependent signalling involves not only Gi proteins, but also direct activation of small G-proteins like Rac and Cdc42, especially in neurones where CD47 plays an important role in neurite formation and more generally in neuronal development (Table 1). Furthermore, CD47 also associates with protein linking integrin-associated protein to cytoskeleton-1 protein (PLIC-1) known to regulate G-protein signalling. Several studies have also identified cytoplasmic Ca^{2+} as an important mediator of CD47 signalling. TSP-1 was reported to induce an increase in intracellular Ca^{2+} in fibroblasts via its RGD and C-terminal domain (Tsao and Mousa, 1995). CD47 has been shown to mediate fibronectin-induced intracellular Ca^{2+} rises in HUVECs (Schwartz *et al.*, 1993). CD47 cross-linking by mAb 1/1A4 resulted in a strong increase in intracellular Ca^{2+} in Jurkat cells (Waclavicek *et al.*, 1997). Peptide 4N1 also induced an increase in intracellular Ca^{2+} responsible for mast cell exocytosis (Sick *et al.*, 2009). In Jurkat T-cells, a recombinant TSP-1 C-terminal fragment (E3CaG1) inhibited sGC via increased intracellular Ca^{2+} (Ramanathan *et al.*, 2011). In Jurkat cells, increases in Ca^{2+} were CD47 dependent but did not involve Gi protein activation, contrary to what we found in mast cells (Sick *et al.*, 2009). In contrast, in HUVECs, TSP-1 via CD47 is thought to inhibit acetylcholine-induced increases in intracellular Ca^{2+} (Bauer *et al.*, 2010). Because decreased Ca^{2+} concentrations inhibit endothelial nitric oxide synthase (eNOS) Ca^{2+}-dependent activation, TSP-1 contributes to regulate blood pressure by limiting eNOS activation and endothelial-dependent vasorelaxation (Bauer *et al.*, 2010). Several previous studies have also confirmed that CD47 acts as a key regulator of the NO/cGMP pathway in vascular smooth muscle cells, endothelial cells and platelets (Isenberg *et al.*, 2006; 2008a).

Taken together, these data indicate that CD47 signalling may differ according to cell type. Given the ubiquitous expression of CD47, its intracellular and membrane-associated partners are likely to be crucial in defining a given specific cellular response both in physiological and also pathophysiological conditions.

CD47 and tumour cells

Tumour cell apoptosis

Tumour growth is considered, at least in part, to result from dysfunctions in the balance between apoptosis and cell proliferation. CD47 actively takes part in apoptosis and therefore plays a key role in maintaining tissue homeostasis. Exposure to soluble anti-CD47 mAbs resulted in rapid (within 3 h) apoptosis of Jurkat E6 cells and activated T-cells (Pettersen *et al.*, 1999). However, this first report underlines the complexity and ambiguity concerning the functional effects of anti-CD47 mAbs. Indeed, soluble anti-CD47 mAbs Ad22 and 1F7 induced Jurkat cell apoptosis whereas mAbs B6H12 and 2D3 were without effect. Ad22 was reported to react with the IgV domain of CD47, in close proximity to epitopes defined by B6H12 and 1F7, whereas 2D3 reacts with a distant region. Thus, CD47-induced apoptosis was thought to require ligation of distinct epitopes on the IgV domain. Interestingly, immobilized Ad22 and 2D3 had no influence on Jurkat cell proliferation, suggesting that the mode of presentation of anti-CD47 mAbs is critical with respect to functional responses. Furthermore, the effects of anti-CD47 mAbs also depend on the cell line tested and on the activation state of the cell. For example, Ad22 induced cell death of Jurkat cells and CD3-activated T-cells but not resting T-cells. In these cells, CD47-induced apoptosis was thought to be independent of Fas and the tumour necrosis factor receptor-dependent pathway (Pettersen *et al.*, 1999). Subsequently, CD47-mediated apoptosis induced by TSP-1 or immobilized (B6H12 and BRIC126) but not soluble anti-CD47 mAbs was reported in many leukaemic cells, including B-chronic lymphocytic leukaemia (B-CLL) cells (Mateo *et al.*, 1999), promyelocytic leukaemia NB4 cells (Saumet *et al.*, 2005) and Jurkat T-cells (Roué *et al.*, 2003). Activation of CD47 apparently leads to a particular type of cell death called type-III programmed cell death (PCD) in normal as well as leukaemia cell lines. Type-III PCD is caspase independent and is characterized by cytoplasmic effects such as cell shrinkage, phosphatidylserine (PS) exposure and mitochondrial matrix swelling, which result in disruption of mitochondrial transmembrane potential ($\Delta\psi m$) and subsequent reactive oxygen species (ROS) production, as described for B-cells cultured on immobilized B6H12 mAbs (Mateo *et al.*, 2002). Neither cytochrome *c* and apoptosis-inducing factor nor release of other mitochondrial intermembrane proteins were required for CD47-dependent PCD (Roué *et al.*, 2003; Saumet *et al.*, 2005). Mitochondrial transmembrane potential loss and PS externalization were prevented by cytochalasin D, suggesting that actin cytoskeleton

rearrangement was necessary in CD47-dependent B-CLL cell death (Mateo *et al.*, 2002; Barbier *et al.*, 2009). Additionally, immobilized anti-CD47 mAbs induced morphological and functional changes in B-cell leukaemia cell lines that involve Rac1 and Cdc42 GTPases (Yoshida *et al.*, 2000). Furthermore, CD47 ligation failed to induce characteristic type-III PCD cytoplasmic events in patients who do not express Wiskott–Aldrich syndrome protein (WASP), indicating that both Cdc42 and WASP are key components of the signalling pathway responsible for actin polymerization in CD47-induced caspase-independent apoptosis (Mateo *et al.*, 2002).

Nevertheless, the molecular basis of CD47-induced type-III PCD is still incompletely understood. Recently, dynamin-related protein-1 (Drp-1) was identified as a key molecular relay in activating PCD (Bras *et al.*, 2007). Chymotrypsin-like protease activation after CD47 ligation by immobilized B6H12 mAbs or immobilized TSP-1 was shown to induce Drp-1 translocation from cytosol to mitochondria, thus leading to ROS production and ATP loss independently of its GTPase activity in primary B-cells (Barbier *et al.*, 2009). Of note, in addition to induction of type-III PCD, immobilized anti-CD47 1F7 mAbs have been reported to promote death of Jurkat T-cells and normal murine T-cells by classical caspase-dependent apoptosis. Indeed, the extracellular IgV domain of CD47 might interact with Fas receptors to induce apoptosis without mobilization of CD47-dependent signalling or localization of CD47 in rafts (Manna *et al.*, 2005).

Considering the key role of TSP-1/CD47 interactions in leukaemia, the putative therapeutic potential of anti-CD47 mAbs was investigated. A bivalent single-chain Ab fragment (scFv) of a murine mAb was first shown to promote apoptosis of hCD47-transfected L1210 murine leukaemic cells without causing haemagglutination of RBCs (Kikuchi *et al.*, 2004). Bivalent single-chain Ab fragments were also effective against the human myeloma cell line KPMM2 implanted in severe combined immunodeficiency (SCID) mice (Kikuchi *et al.*, 2005). Antitumour activity of CD47 mAbs was reported in xenograft models of acute lymphoblastic leukaemia and B-cell chronic lymphocytic leukaemia. Interestingly, no apoptotic side effects on CD34+ haematopoietic progenitor/stem or human endothelial cells were observed in these models (Uno *et al.*, 2007). Recently, a disulfide-linked dimer of a single-chain Ab fragment against CD47 induced prolonged survival of SCID mice engrafted with JOK-1 leukaemic cells (Sagawa *et al.*, 2011). *In vitro* results indicate that this effect involved the hypoxia-inducible factor (HIF)-1α-induced genes *BNIP3* and *RTP801*.

The role of CD47 in cancer development is not restricted to only leukaemia, but has also been shown for vascularized solid tumours. Triggering of CD47 by 4N1K induces caspase-independent apoptosis in several human breast cancer cell lines. Indeed, CD47 activation stimulates cell death of both non-invasive (MCF-7 and AU-565) as well as invasive (MDA-MB-231) cell lines through Gi signalling, leading to subsequent decrease in cAMP levels and inhibition of PKA (Manna and Frazier, 2004).

Interestingly, CD47 has been reported to be involved in resistance of cancer cells to taxol (Lih *et al.*, 2006). The resistance of human prostate cancer cells to taxanes was correlated with overexpression of a recently identified protein Txr1, reported to dramatically down-regulate TSP-1 expression. Stimulation of CD47 with either TSP-1 or 4N1K in resistant cells increased taxol-induced apoptosis (Lih *et al.*, 2006). Whether this mechanism is directly or indirectly mediated by CD47 is unknown. In addition, primary vascular cells cultured from TSP-1-null mice and CD47-null mice were shown to be resistant to high-dose radiation injury (Isenberg *et al.*, 2008b). CD47 suppression in mice resulted in increased radiation sensitivity of implanted melanoma and squamous cell lung tumours, while conferring radioresistance in normal soft tissues, bone marrow and tumour-associated leukocytes (Maxhimer *et al.*, 2009a). Radiation-induced cell death was similar in HUVECs when treated either by mAbs directed against TSP-1 (clone A6.1) or CD47 (clone B6H12) or by antisense CD47 morpholinos, consistent with blockade of the TSP-1/CD47 interaction being responsible for the decrease in radiation sensitivity. Radioprotection in normal tissues may be attributable at least in part to restoration of NO-mediated cytoprotective activity (Isenberg *et al.*, 2008b; Maxhimer *et al.*, 2009a).

Tumour cell survival and proliferation

Besides its well-described role in inducing apoptosis, CD47 has been paradoxically shown to modulate cell survival and proliferation. Indeed, we demonstrated that activation of CD47 by 4N1 results in protection of thyroid cells from apoptosis induced by C_2-ceramides by stimulating the cAMP/PKA pathway (Rath *et al.*, 2006a). Moreover, 4N1-mediated activation of CD47 inhibited camptothecin- and doxorubicin-induced apoptosis of human follicular thyroid carcinoma FTC-133 cells (Rath *et al.*, 2006b).

Furthermore, we showed that CD47 stimulated by the agonist 4N1 induces the proliferation of astrocytoma cells but not normal astrocytes, with downstream signalling subsequent to CD47 activation involving Gβγ dimer-dependent activation of the PI3K/Akt pathway in astrocytoma cells (Sick *et al.*, 2011). As well, a peptidic fragment resulting from serpin A1 proteolytic cleavage exhibits CD47-dependent mitogenic activity in both Hep G2 liver cancer cells and MCF-7 breast cancer cells (Congote and Temmel, 2004).

Migration

The implication of CD47 in cell migration was first demonstrated for neutrophils, with blocking Abs against CD47 inhibiting transmigration of neutrophils and monocytes through endothelium (Cooper *et al.*, 1995; de Vries *et al.*, 2002). Increased levels of CD47 expression were correlated to improved rates of neutrophil migration during inflammatory responses (Liu *et al.*, 2001). It was also found that CD47 plays a major role in smooth muscle cell proliferation and chemotaxis towards soluble collagen, in part via association with $\alpha_2\beta_1$ integrins, subsequent Gi-mediated decrease in cAMP levels and inhibition of ERK activity (Wang and Frazier, 1998; Wang *et al.*, 1999). Recent data suggest that CD47 stimulates intestinal epithelial cell migration on collagen-I through COX-2 expression (Broom *et al.*, 2009). Moreover, increased expression of CD47 in fibroblasts promotes intercellular adhesion and decreases the spontaneous migration of Jurkat lymphocytes through a fibroblast monolayer. This effect was dependent on Rho family GTPases and Gi proteins (Rebres *et al.*, 2005). These data suggest that CD47 may restrict the

infiltration of cancer cells in surrounding tissues and development of metastasis. Paradoxically, CD47 was reported to induce epithelial cell spreading and migration. Indeed, CD47 co-localizes with E-cadherin at cell-cell adhesion sites and forced expression of CD47 induces epithelial cell spreading and leads to a partial disruption of cell-cell adhesion (Shinohara *et al.*, 2006).

Surprisingly, very few data are available regarding the role of CD47 in the regulation of cancer cell migration. CD47 was shown to stimulate migration of several lymphoma cell lines, but not myeloma cell lines, involving the Cdc42 GTPase and a tight molecular cooperation with $\alpha_4\beta_1$ integrins (Yoshida *et al.*, 2000). In addition, CD47 was demonstrated to decrease Melan-a cell migration but not that of B16F10 melanoma cells. Aberrant N-glycosylation of SIRPα was observed in B16F10 melanoma cells and results in impaired binding to CD47. Binding of CD47 to SIRPα in Melan-a cells is supposed to down-regulate SIRPα expression levels on adjacent cells by stimulation of proteosomal degradation. Increased proteosomal degradation of SIRPα resulted in inactivation of SHP-2 phosphatase, inhibition of the dephosphorylation of FAK, subsequent disassembly of focal adhesions and thereby to inhibition of Melan-a cell motility (Ogura *et al.*, 2004). Resistance to this molecular mechanism in B16 melanoma is thought to contribute, at least in part, to the more aggressive behaviour of these cells (Ogura *et al.*, 2004). Blockade of CD47 by neutralizing Abs reduced migration and chemotaxis in response to collagen IV of melanoma, prostate cancer and ovarian cancer-derived cells. These effects were shown to be dependent on $\alpha_v\beta_3$ integrins and intracellular calcium (Shahan *et al.*, 2000). Finally, CD47 was reported to stimulate osteoclastogenesis and its disruption may protect against metastatic tumours in bone (Uluçkan *et al.*, 2009). Taken together, these data are consistent with activation of CD47 contributing to cell migration, and it therefore may be that CD47 antagonism represents a promising therapeutic strategy to limit infiltration at tumour sites and to decrease cancer cell dissemination and formation of metastases.

Cell adhesion and spreading

CD47 is required for post-adhesive events after neutrophil transmigration across intestinal epithelial cells (Parkos *et al.*, 1996). C32 melanoma cell spreading onto vitronectin was stimulated by CD47 through cooperation with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and involved activation of Gi proteins, FAK and PI3K (Gao *et al.*, 1996b). This effect apparently depends on cholesterol, which was thought to maintain the structural and functional integrity of the CD47/$\alpha_v\beta_3$/G-protein complex in lipid rafts (Green *et al.*, 1999). However, adhesion may also be stimulated by CD47 independently of cholesterol content and G-protein signalling. Indeed, CD47 improves $\alpha_v\beta_3$ avidity in human ovarian carcinoma cells after cholesterol depletion in the cell membrane and similarly, $\alpha_4\beta_1$ avidity for vascular cell adhesion molecule-1 (VCAM-1) and TSP-1 in sickle reticulocytes (Brittain *et al.*, 2004; McDonald *et al.*, 2004). Thus, CD47 can also transmit inside-out signals contributing to the regulation of integrin activation and clustering. Different pools of integrins appear to be differentially regulated by CD47 and this could be related to the localization of CD47 in specific microdomains at the plasma membrane. CD47 can also control cell-to-cell adhesion by interaction with SIRPγ, for example, in T-cell adhesion to antigen-presenting cells (Piccio *et al.*, 2005). Moreover, in CD47-deficient fibroblasts, CD47 expression induces intercellular adhesion, resulting in cell aggregation even in the absence of active integrins, SIRPα1 or TSP-1 binding (Rebres *et al.*, 2005). This effect is proposed to involve CD47 homophilic interaction, but unlike other homophilic interactions (e.g. involving platelet/endothelial cell adhesion molecule-1 or cadherins), expression of CD47 is not sufficient to induce aggregation and may require activation by serum (concentration above 4%) or by 4N1K. It is also interesting to note that anti-CD47 2D3 mAbs, which inhibit CD47-dependent cell aggregation, enhanced SIRPα1 binding. This effect was also mediated by Fab fragments, and suggests that even monovalent binding causes CD47 to undergo conformational or clustering changes that are relevant for SIRPα binding (Rebres *et al.*, 2005). Even if the homotypic interaction of CD47 with itself was not further explored, this study underlines two important features that may explain some of the reported paradoxical results, namely the influence of serum concentration on the aggregation state of cells and differential SIRP binding in the presence of CD47 mAbs. CD47 also influences cell adhesion indirectly. Indeed, TSP-1 induced the up-regulation of the adhesion molecules intercellular adhesion molecule-1 and VCAM-1 in human brain microvascular endothelial cells (Xing *et al.*, 2009). In view of these results, CD47 modulation by blocking strategies may have detrimental effects resulting in increased cell migration depending on the cell type considered. Such effects have to be taken into account in developing anti-metastatic strategies where CD47 is the target.

CD47 and stromal cells

Angiogenesis

Angiogenesis represents a key component of solid tumour development, allowing both nutrition and oxygenation of tumour cells, thus subsequently contributing to dissemination and metastasis. Strategies targeting angiogenesis have emerged over the past decade based on a better understanding of molecular mechanisms underlying angiogenesis. This has led to the development of anti-angiogenic approaches for the treatment of cancer, with notably several vascular endothelial growth factor (VEGF)-neutralizing mAbs being approved for clinical use in cancer (for review, see Chames *et al.*, 2009). However, even if effective in certain cancers, significant problems have appeared. For example, many patients with metastatic diseases are refractory or acquire resistance towards VEGF inhibitors (Bergers and Hanahan, 2008). Tumour vessel abnormalities resulting in heterogeneous and tortuous vessel networks are also believed to decrease the efficiency of anti-VEGF strategies (Carmeliet and Jain, 2011). To date, even if current anti-VEGF agents have proved to be less than ideally efficacious, targeting angiogenesis still represents a most promising therapeutic strategy.

The first evidence for CD47 involvement in the vascular system was described in platelets where 4N1 was shown to stimulate platelet activation and aggregation (Dorahy *et al.*, 1997). The signalling pathway responsible for platelet activation involves a Gi protein-mediated decrease in cAMP

production (Frazier *et al.*, 1999). More recently, several groups have focused on the putative role of CD47 in angiogenesis. *In vitro*, TSP-1 and 4N1K were reported to induce cytotoxicity of brain endothelial cells and inhibit endothelial cell migration and tube formation (Xing *et al.*, 2009). These effects were confirmed *in vivo*. Using recombinant fragments, the anti-angiogenic properties of TSP-1 were demonstrated to be mediated by the interaction between its C-terminal domain and CD47 (Isenberg *et al.*, 2006), with this interaction being responsible for the inhibition of NO-induced GC activation. Therefore, CD47 can modulate blood flow and tissue perfusion by interfering with the NO-dependent balance of the relative state of constriction and relaxation of arteries.

Although CD36 ligation by TSP-1 inhibits NO-stimulated vascular cell responses and cGMP signalling, the anti-angiogenic effects of TSP-1 at picomolar physiological concentrations are driven solely by CD47 ligation. Moreover, angiogenic responses following CD36 ligation are also dependent on CD47. The NO-driven delay in thrombin-induced platelet activation is blocked by TSP-1 ligation to CD47 and/or CD36 as shown using, respectively, TSP-1 C-terminal domain and type 1 repeat recombinant fragments (Isenberg *et al.*, 2008a).

Restoration of NO signalling consecutive to CD47 antagonism that results in a rapid restoration of NO-driven blood flow would be essential for graft survival. Consistently, in a murine skin graft model, graft survival was dramatically increased by both morpholino suppression of CD47 expression and CD47 blockade by miap 301 mAbs (Isenberg *et al.*, 2008c). In a comparable murine ischaemic model of full thickness flaps, perfusion, survival and angiogenesis were optimized using combined treatment with nitrite and TSP-1/CD47 blockade via anti-TSP-1 A6.1 mAbs or gene silencing (Isenberg *et al.*, 2009). In this study, a survival rate of almost 100% was attained, indicating that optimal results were obtained by combined treatment. The efficiency of such an approach is especially promising for the treatment of burns and diverse pathologies requiring skin grafts. In support, an increase in tissue survival following myocutaneous flap surgery was observed when using CD47-blocking mAbs (clone miap 301), antisense morpholino or CD47 silencing in mice (Isenberg *et al.*, 2007). Moreover, increased survival consecutive to CD47-blocking mAb treatment was described in CD36-null mice, suggesting that this response is CD36 independent. Flap survival was also increased by CD47-blocking OX101 mAbs in a rat model (Maxhimer *et al.*, 2009b). In addition to flap reperfusion enhancement, CD47 blockade contributed to a decrease in the inflammatory response in flap tissues after ischaemia-reperfusion injury. Random cutaneous flap survival, blood vessel permeability and blood flow were also drastically increased by antisense CD47 morpholino and anti-TSP mAbs (A6.1) in a porcine model (Isenberg *et al.*, 2008d). Considering the high level of homology between porcine and human tissues, the potential therapeutic targeting of CD47 is supported by these results. Finally, TSP-1 and CD47 suppression or pretreatment with a CD47-antagonist mAb miap 301 was shown to enhance tissue survival and perfusion after ischaemia-reperfusion injury in a murine liver model, suggesting the inhibition of CD47 could not only enhance skin graft but also organ survival after transplantation surgery (Isenberg *et al.*, 2008e).

CD47 ligation by TSP-1 was reported to inhibit the NO-dependent and NO-independent activation of sGC in platelets as well as in smooth muscle cells (Miller *et al.*, 2010b). Furthermore, CD47 activation by TSP-1 controls intracellular cAMP levels in smooth muscle, directly via Gi-dependent inhibition of adenylate cyclase and indirectly through PDE3 inhibition in a cGMP-dependent manner (Yao *et al.*, 2011). In addition to the modulation of cyclic nucleotide levels, CD47 ligation by native TSP-1 or CBD recombinant domain disrupted the constitutive association of VEGFR-2 and CD47 in endothelial cells. This in turn inhibits VEGFR-2 phosphorylation and its downstream signalling, without affecting VEGF binding (Kaur *et al.*, 2010). Finally, CD47 silencing was recently described to result in enhancement of the pro-angiogenic properties of endothelial colony forming cells by interfering with the SDF-1 chemokine pathway (Smadja *et al.*, 2011).

The role of NO is quite ambiguous in tumours. Indeed, NO is reported to increase tumour perfusion via induction of local vasodilatation. On the contrary, NO is also thought to decrease tumour perfusion by preferential relaxation of the normal vascular network outside the tumour, resulting in blood redistribution away from the tumour (Isenberg *et al.*, 2008f). Even if the capacity of tumour vasculature to respond to vasoactive agents is quite limited, recovery of basal perfusion after a vasoactive treatment (vasoconstrictor or vasodilatator) is modulated by TSP-1 through CD47 interaction. Indeed, overexpression of truncated TSP-1 lacking parts of its CBD domain failed to reproduce this activity (Isenberg *et al.*, 2008f). These data indicate that TSP-1/CD47 interaction may regulate long-term vascular responses in the tumour context.

Inflammation/immune responses

The concept that inflammation sustains cancer development is not new. Indeed, in 1863, Rudolf Virchow first hypothesized that the origin of cancer was at sites of chronic inflammation (Balkwill and Mantovani, 2001). After a long eclipse, this hypothesis has been more recently supported by clinical and experimental studies demonstrating that infiltration of leukocytes into the tumour microenvironment is a key event in promoting cancer growth (for review, see Tlsty and Coussens, 2006). In this context, cytokines and chemokines as well as activation of transcription factors such as NF-κB, Stat3 or HIF play major roles in tumour progression. Moreover, tumour cells are thought to have altered self-antigens that impair recognition by the immune system, leading to tumour escape from immune surveillance (for review, see Dunn *et al.*, 2004). Accordingly, inflammation and escape of tumoural cells from surveillance by the immune system are fundamental steps in tumour promotion and, therefore, targeting immune cells may represent a promising therapeutic strategy against cancer.

Among tumour infiltrating leukocytes, macrophages represent the main actors of tumour promotion (for review, see Mantovani *et al.*, 2008). Several studies have underlined a role for CD47 as a marker of 'self', initially in RBCs. Thus, RBCs from $CD47^{-/-}$ mice were rapidly cleared by macrophages when transfused into wild-type mice, whereas they circulated normally in $CD47^{-/-}$ mice (Oldenborg *et al.*, 2000). This occurred independently of complement and antibodies and was mediated by the interaction between SIRPα and CD47.

Such a molecular interaction is also responsible for maintaining platelet homeostasis (Olsson *et al.*, 2005). The ITIM domains of SIRPα become phosphorylated upon ligation with CD47 and recruit SHP phosphatases, resulting in deactivation of myosin-II without affecting F-actin (Tsai and Discher, 2008). The absence of self-recognition at the cell surface thus allows contractile engulfment. Several paired receptors engaged at the immunological synapse trigger phagocytosis but CD47/SIRPα is the only identified negative regulator. Interestingly, the expression level of CD47 is likely to vary under pathophysiological situations. Indeed, CD47 expression is up-regulated on normal haematopoietic stem cells (HSCs) when they are recruited to the periphery upon exposure to pro-inflammatory stimuli like granulocyte colony stimulating factor or LPS (Jaiswal *et al.*, 2009). Increased CD47 at the cell surface is therefore thought to protect haematopoietic cells from phagocytosis during their trafficking from bone marrow to the periphery. On the contrary, decreased expression of CD47 was observed in apoptotic neutrophils and fibroblasts and correlated with the degree of apoptosis. Apoptotic cells lose their ability to activate SIRPα and are engulfed, unlike viable cells expressing normal amounts of CD47. Moreover, on apoptotic cells, CD47 is redistributed on the plasma membrane to form patches. This is thought to decrease the ability of CD47 to interact with SIRPα. Additionally, CD47 becomes segregated away from calreticulin and PSs, which are 'eat me' stimuli triggering low-density lipoprotein receptor-related protein 1-dependent efferocytosis (Gardai *et al.*, 2005). Interestingly, CD47 expression levels were shown to increase in several leukaemic cell lines (Jaiswal *et al.*, 2009), as well as in bladder tumour-initiating cells (Chan *et al.*, 2009), non-Hodgkin lymphoma (Chao *et al.*, 2010a) and acute lymphoblastic leukaemia (Chao *et al.*, 2011). CD47 is thus considered as an adverse prognostic factor. Minimally expressed on most normal cells, calreticulin levels are increased at the cell surface of several cancer cells and are commonly correlated with enhanced CD47 expression. This is thought to protect tumour cells from calreticulin-mediated phagocytosis (Chao *et al.*, 2010a,b). Increased levels of CD47 expression observed in leukaemic mice were confirmed for dysplastic human haematopoietic cells as compared with normal umbilical cord blood and peripheral blood stem cells. Indeed, granulocyte-macrophage progenitor populations were expanded in myeloproliferative disorders such as atypical chronic myeloid leukaemia, proliferative phase chronic myelomonocytic leukaemia and acute myeloid leukaemia (AML) (Jaiswal *et al.*, 2009). MOLM-13 cells having low CD47 levels derived from a patient with AML failed to engraft when implanted in immunocompromised mice. In contrast, implanted MOLM-13 cells overexpressing CD47 lead to development of tumours, consistent with CD47 overexpression enabling these cells to escape from phagocytosis by macrophages. Moreover, macrophages were shown to exert a selective pressure for high-expressing CD47 clones *in vivo*. Thus, leukaemic progenitors are thought to hijack the protective effect of overexpressing CD47 used by normal HSCs to escape from macrophage-dependent killing observed during inflammatory states. Consistently, a blocking antibody directed against CD47 was reported to trigger phagocytosis of human bladder cancer cells *in vitro* (Chan *et al.*, 2009). Finally, anti-CD47-blocking mAbs (clones BRIC126 and B6H12) were shown to promote macrophage-mediated phagocytosis of acute lymphoblastic leukaemia cells and leukaemia stem cells (LSCs) and to inhibit tumour engraftment *in vivo* (Majeti *et al.*, 2009; Chao *et al.*, 2011).

The interaction of CD47 with SIRPα contributes to fusion of rat alveolar macrophages and the generation of multinucleated cells (Han *et al.*, 2000) that can differentiate into osteoclasts in bone. Formation of such giant multinucleated cells was strongly inhibited both *in vivo* and *in vitro* in the absence of SIRPα/CD47 interaction (Lundberg *et al.*, 2007). The distance between two cells interacting via CD47 and SIRPα is supposed to be around 14 nm, a distance similar to that observed during the establishment of the immunological synapse (Hatherley *et al.*, 2009). However, this distance may be reduced to 5–10 nm in macrophages by interaction of CD47 with a short form of SIRPα that lacks the C1 domain, thereby leading to fusion (Han *et al.*, 2000). To date, even if the mechanism is poorly documented, several receptors including CD44 (Sterling *et al.*, 1998), DC-STAMP (Yagi *et al.*, 2006), RANK/RANKL and tetraspanin CD9 (Ishii *et al.*, 2006) have been reported to take part in the macrophage fusion process leading to formation of osteoclasts or giant cells. The fusion of cancer cell with macrophages is believed to represent a putative mechanism for dissemination and development of metastasis, as described for melanoma cells fused with macrophages (Rachkovsky *et al.*, 1998; Chakraborty *et al.*, 2000). Nevertheless, to date, this proposed mechanism remains controversial because of the absence of proof of concept *in vivo*. However, fusion between intestinal epithelial cells and macrophages was very recently shown to occur *in vivo* in a cancer context and was related to nuclear reprogramming resulting in a transcriptomic identity similar to the parental cells but also with a unique subset of transcripts (Powell *et al.*, 2011). Thus, cancer cell fusion with circulating bone marrow-derived cells contributes to masking the cancer phenotype, thereby permitting cancer cells to escape from immune surveillance and to infiltrate in a distant niche. In this context, even if poorly characterized at present, therapeutic inhibition of CD47/SIRPα interactions might provide not only enhanced clearance and degradation of cancer cells but also limited metastatic potential.

DCs represent another key class of cells in the tumour microenvironment, being the most potent antigen-presenting cells of the immune system and thus playing a critical role in establishing innate and adaptive immune responses. Tumour-bearing mice as well as patients with breast cancer, prostate cancer and glioma present a decreased number of DCs in lymph nodes spleen and skin (Rabinovich *et al.*, 2007). Indeed, cancer patients have significantly decreased numbers of circulating and peripheral DCs whereas immature DCs accumulate. Immature DCs have a reduced capacity for capturing antigens and a reduced capacity to stimulate naïve T-cells (Petersen *et al.*, 2010). Interestingly, stimulation of CD47 by 4N1K inhibits secretion of pro-inflammatory cytokines like IL-12, TNFα, IL-6 and GM-CSF by maturing DCs (Demeure *et al.*, 2000). Furthermore, CD47 activation inhibits maturation (i.e. up-regulation of major histocompatibility complex class II antigens, co-stimulatory molecules, acquisition of a potent T-cell stimulatory activity) of immature DCs. CD47 stimulation by 4N1K also directly

affects DC populations by inducing apoptosis-like cell death (Johansson and Londei, 2004). Thus, CD47 may participate in reducing the efficiency of immune responses against tumour by inducing T-cell tolerance towards tumours.

Various and sometimes contradictory functional effects have been reported when CD47 is activated on T-cells. It was reported that CD47-deficient animals exhibit sustained inflammation related to decreased local apoptosis of T-cells (Lamy *et al.*, 2007). SIRPγ expressed on T-cells which interacts with CD47 on endothelial cells was critical for T-cell transendothelial migration and in inducing apoptosis of Jurkat cells, similar to what has been described for SIRPα ligation (Brooke *et al.*, 2004; Stefanidakis *et al.*, 2008). Furthermore, TSP-1 may be a potent inhibitor of T-cell receptor (TCR)-mediated T-cell activation. Indeed, TSP-1 signalling through CD47 and HSPGs inhibits TCR signalling including suppression of the autocrine growth factor IL-2 and early activation markers such as CD69, Egr-1 and PAC-1 (Li *et al.*, 2001). T-regulatory cells (Tregs) are critical in the limitation of damage induced by exacerbated responses to 'self' or 'foreign' antigens, but in cancer, they have been shown to proliferate. Because tumour antigens are derived from the host, tumours are more likely regarded as self by Tregs that actively promote their tolerance. Increased infiltration of Tregs in tumours is thus correlated with poor prognosis in several types of cancers (Elkord *et al.*, 2010). CD47 stimulation by TSP-1 or 4N1K was demonstrated to trigger the conversion of naïve or memory CD4+ CD25- T-cells into Tregs (Grimbert *et al.*, 2006). Furthermore, CD47 cross-linking was shown to induce apoptosis of normal T- and B-cells when cultured on immobilized B6H12 mAbs (Mateo *et al.*, 2002) or in the presence of soluble anti-CD47 Ad22 mAbs (Lamy *et al.*, 2003). Interestingly, haematopoietic progenitors CD34+ and immature DCs were described to be resistant to CD47-induced apoptosis (Mateo *et al.*, 2002). CD47 was also reported to induce the development of hyporesponsive or anergic T-cells (Avice *et al.*, 2001).

Finally, levels of CD47 expression in head-and-neck squamous cell carcinoma apparently influence the cytotoxicity of NK cells, with increased expression of CD47 being related to a decrease in NK cytotoxicity (Kim *et al.*, 2008). The mechanism remains unknown but may represent a possible supplementary pathway for immune escape in cancer.

Taken together, these data indicate that blockade of CD47 receptors apparently triggers immune system responses against tumours at many different levels. This may thus represent a promising therapeutic strategy resulting in increased tumour cell phagocytosis, enhanced DC maturation and subsequently augmented T-cell-mediated cytotoxicity.

Conclusion

This overview of CD47 indicates that CD47 very likely plays a central role in the tumour microenvironment. Considering its ubiquitous expression, its ability to modulate tumour cell growth and migration and to limit angiogenesis, and the development of immune responses against tumours, CD47 therefore appears to represent an exciting target candidate for new therapeutic approaches against cancer (Figure 2).

A number of recent studies using function-blocking anti-CD47 mAbs are consistent with an eventual therapeutic application in the field of leukaemia. For example, single-chain Ab fragments against CD47 were successfully used to induce apoptosis of malignant lymphoid cells isolated from patient with B-CLL and to significantly increase survival rate of mice implanted with human lymphocytic leukaemia cell lines (Sagawa *et al.*, 2011). In addition to directly inducing apoptosis, other mechanisms of action for anti-CD47 mAbs include the stimulation of complement or antibody-dependent cytotoxicity and the induction of leukaemic stem cell phagocytosis by disruption of CD47/SIRPα interaction. A demonstration of this latter effect was the phagocytosis of human AML LSCs treated with B6H12 mAbs by mouse macrophages *in vivo* (Majeti *et al.*, 2009). Of note, the mouse anti-CD47-blocking mAb miap 301 promoted phagocytosis of mouse AML while not depleting normal HSCs *in vivo* (Majeti *et al.*, 2009), consistent with the feasibility of therapeutic targeting of CD47. A synergic effect of anti-CD47 mAbs in combination with rituximab, an anti-CD20 mAb, in promoting phagocytosis was described for the eradication of non-Hodgkin lymphoma (Chao *et al.*, 2010a). To date, most of the studies reporting successful eradication of leukaemia cells by anti-CD47 mAbs were done using human tumour xenograft models in immunodeficient mice. Because only human leukaemic cells were targeted by the human anti-CD47 mAbs used in such experimental models, interpretation of the observed depletion of leukaemic cells is somewhat hampered. Notably, characterization of possible side effects that could occur in immunocompetent mice or in humans is not accessible with these models.

To our knowledge, very few studies have addressed the role of CD47 in solid tumours, which are highly vascularized, and for which little is known about pathological cell-to-cell interactions in tumour stroma. An accurate description of CD47 function during vascularization at the site of tumours is still lacking and available data sometimes appear contradictory or paradoxical. TSP-1, a well-known inhibitor of angiogenesis, acts physiologically via CD47 through inhibition of NO signalling. However, little is known about TSP-1 responsiveness in tumour vasculature where NO could act as a friend or a foe, either by dilatation of blood vessels in the tumour or dilatation of peripheral blood vessels resulting in redistribution of blood away from the tumour. Thus, in this context, it is currently very difficult to predict outcomes if systemic blocking strategies against CD47 were to be tested.

Furthermore, as described beforehand, depending on the cell type considered, CD47 activation is reported either to induce apoptosis or in contrast to enhance proliferation and survival. On the other hand, cell migration appears to be quite universally stimulated by CD47 ligation and activation (Figure 2). Thus, antagonism of CD47 should reduce cell migration, thereby representing a plausible strategy to fight against cancer cell dissemination. Blockade of SIRPα/CD47 interaction that subsequently promotes immune system responses against tumours (Figure 2), especially in cases where CD47 is highly overexpressed, likewise appears to be a promising possibility.

Given the ubiquitous expression of CD47, specific targeting will be a critical issue to resolve. This might be addressed using mAbs directed against both CD47 and a specific tumour cell marker. For example, because high levels of CD47 are

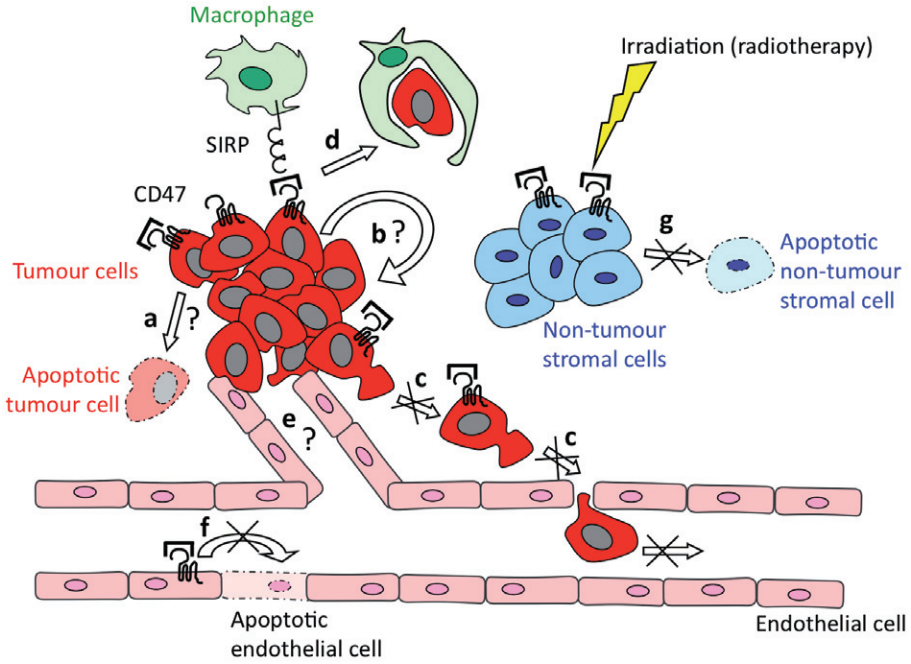

Figure 2

Potential consequences of therapeutic blockade of CD47 in the tumour microenvironment. (a) Modulation of apoptosis of tumour cells. (b) Modulation of tumour cell proliferation or survival. (c) Inhibition of tumour cell migration and transmigration across the endothelium. (d) Stimulation of tumour cell phagocytosis by disruption of CD47/SIRPα interaction. (e) Modulation of angiogenesis. (f) Inhibition of endothelial cell apoptosis. (g) Prevention of apoptosis of normal cells after irradiation. The question marks indicate effects that might also be influenced by the cellular environment.

found in prostate cancer (Vallbo and Damber, 2005), development of blocking CD47 mAbs that also target the prostate-specific antigen could be of particular therapeutic interest. It should nevertheless be pointed out that strategies using anti-CD47 mAbs may also mask binding sites for different endogenous ligands or partners, which might prevent some of their beneficial effects against tumours. Therefore, the development of alternative strategies using synthetic competitive antagonists of CD47 is likely required. In this context, detailed X-ray analysis and molecular modelling studies of the TSP-1/CD47 complex would be helpful. Indeed, this might lead to the characterization of non-evident binding sites or other sites of interaction, thereby perhaps contributing to the development of new highly specific and efficacious small molecule antagonists of CD47.

In conclusion, it thus may be that CD47 represents an interesting therapeutic target for the treatment of cancer. However, evaluation is clearly hampered, given the current lack of candidate molecules and accordingly, the absence of clinical trials.

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Conflict of interest

None.

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