REVIEW

The TSP1-CD47-SIRPα interactome: an immune triangle for the checkpoint era

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Abstract

The use of treatments, such as programmed death protein 1 (PD1) or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) antibodies, that loosen the natural checks upon immune cell activity to enhance cancer killing have shifted clinical practice and outcomes for the better. Accordingly, the number of antibodies and engineered proteins that interact with the ligand–receptor components of immune checkpoints continue to increase along with their use. It is tempting to view these molecular pathways simply from an immune inhibitory perspective. But this should be resisted. Checkpoint molecules can have other cardinal functions relevant to the development and use of blocking moieties. Cell receptor CD47 is an example of this. CD47 is found on the surface of all human cells. Within the checkpoint paradigm, non-immune cell CD47 signals through immune cell surface signal regulatory protein alpha (SIRPα) to limit the activity of the latter, the so-called *trans* signal. Even so, CD47 interacts with other cell surface and soluble molecules to regulate biogas and redox signaling, mitochondria and metabolism, self-renewal factors and multipotency, and blood flow. Further, the pedigree of checkpoint CD47 is more intricate than supposed. High-affinity interaction with soluble thrombospondin-1 (TSP1) and low-affinity interaction with same-cell SIRPα, the so-called *cis* signal, and non-SIRPα ectodomains on the cell membrane suggests that multiple immune checkpoints converge at and through CD47. Appreciation of this may provide latitude for pathway-specific targeting and intelligent therapeutic effect.

Introduction

Checkpoint molecules fine-tune immune cells and prevent inappropriate activity [1, 2]. This informed the development of antibodies that interrupt checkpoint pathways to treat cancer 3. In the U.S.A, over 43% of individuals with cancer are eligible to receive checkpoint blocking molecules [4], which will increase as new agents arrive in the clinic. While stimulating attack of cancer cells, checkpoint blocking antibodies were permissive of immune cell injury toward non-cancer cells [5, 6] and associated with adverse events [7] including insulin-dependent diabetes. The cell surface receptor CD47 is a checkpoint molecule, and companies are developing CD47 blocking antibodies with clinical trials proceeding [8, 9]. CD47 has several natural ligands including secreted thrombospondin-1 (TSP1) [10] and cell membrane signal regulatory protein-alpha (SIRPα) [8]. Through interacting with CD47, both ligands restrain immune cells and foster self-tolerance. TSP1 binds with high affinity to CD47 [11] to suppress T [12], natural killer [13], and dendritic cells [14]. SIRPα binds CD47 with less affinity to restrain phagocytosis [15]. Development of CD47 and SIRPα-binding molecules [16] focused on interrupting the binding between macrophage-displayed SIRPα and non-immune cell-displayed CD47. However, the binding interactions of this trio of molecules are only partly characterized. Current CD47 and SIRPα-blocking agents remain untested in relation to the interaction of TSP1 with CD47, the less studied interaction of TSP1 with SIRPα [17] and in regard to

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same-cell *cis* CD47-SIRPα. Of relevance, loss of the SIRPα ectodomain, and thus *cis* signaling, altered inflammation in non-immune human cells [18]. Further, human islet endocrine cells, including beta cells, displayed cell surface CD47 but did not display cell surface SIRPα [19], as had been presumed [20]. Put simply, human islet endocrine cells lack *cis* CD47-SIRPα signaling, a finding with possible implications in view of the increased use of CD47 and SIRPα checkpoint blockers. Adding another layer to this narrative, CD47 was linked to metabolism and glucose homeostasis [21]. These findings occasioned the present appraisal of the TSP1-CD47-SIRPα triad (Fig. 1) to understand the possible impact of intersecting these checkpoints.

Thrombospondin-1

TSP1 is the soluble ligand of the interactome and a trimeric ~450 kDa protein secreted by most human cells [22]. It occurs in bodily fluids such as cerebral spinal [23] and plural fluid [24], blood [25], urine [26], and saliva [27] among others. It is also found preformed in platelet alpha granules [28]. Thus, the analysis of soluble TSP1 in body fluids may be complicated by platelet activation. TSP1 modulates cell activity through binding with cell surface receptors including integrins [29], CD36 [30], CD47 [11], and SIRPα [17] and through regulation of growth factors and extracellular matrix [31]. Interestingly, EC retention of TSP1 occurred via the CD47-binding C-terminus of the protein [32], which begs the question if matrix-bound TSP1 can signal through cell surface CD47. Picomolar concentrations of TSP1-activated CD47 [33], suggesting that this interaction dominates under most conditions. In fact, several TSP1-CD36-mediated signals required cross talk with CD47 [34]. The same

may be true for certain integrins [35]. Following secretion, TSP1 is scavenged through cell surface internalization [36] and extracellular protease degradation [37]. Additional paracrine effects might be mediated by exosomes which were found decorated with cell surface TSP1, CD47, and SIRPα [38]. In health, TSP1 is found at low non-signaling concentrations (100 ng/ml and less) but is increased with acute and chronic stress, including aging, where it is largely, although not wholly [39], deleterious [31].

Ligand–receptor interactions

As noted, the secreted protein TSP1 binds with high affinity to CD47 (~K_D of 12 pmol) [11] and to SIRPα [17], although in the latter interaction binding affinities and domain specificity remain to be solved. In fact, exogenous TSP1 blocks CD47 binding to SIRPα. These data were obtained using human protein and cells, an important point as species-specific posttranslational modification of the CD47 ectodomain is essential for TSP1 binding[40]. The human antibody B6H12 blocked TSP1 binding to CD47 and CD47 binding to SIRPα [11]. This is perhaps important, since at least one clinical CD47 checkpoint antibody was inspired by B6H12 [41]. TSP1 is increased by elevated glucose [42], inflammation [43], in experimental [44] and clinical type 1 diabetes [45, 46], and with aging [21]. To wit, aged cells support increased TSP1 binding to CD47 through increased clustering of cell membrane CD47 [47]. This finding speaks to the trimeric structure of TSP1 which theoretically permits a single TSP1 to engage several CD47 simultaneously [48]. Of interest here, fresh human islets secreted substantial amounts of soluble TSP1 [19]. And TSP1 expression was increased by chemotherapy [49] and radiation [50]. Thus,
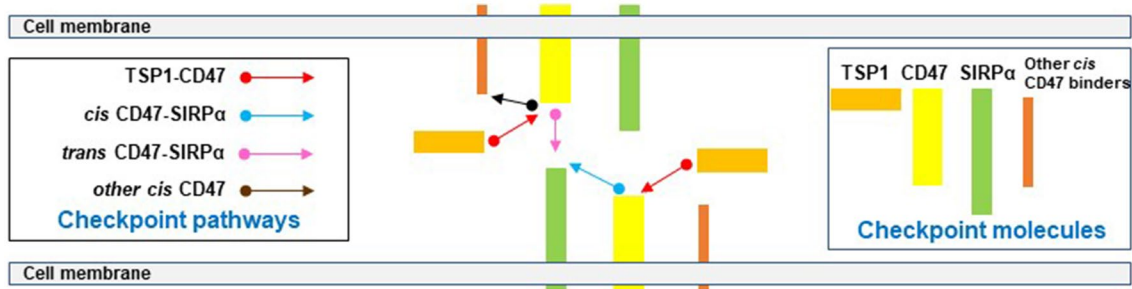

Fig. 1 TSP1-CD47-SIRPα immune checkpoints. CD47 is at the center of multiple immune inhibitory checkpoints: (1) TSP1-CD47 checks T, natural killer and dendritic cells, and perhaps macrophages. (2) *Trans* CD47-SIRPα checks macrophage phagocytosis. (3) *Cis* CD47-SIRPα limits inflammation wherever both occur on the same cell. (4) *Cis* CD47-αMβ2 and *cis* CD47-VEGFR-2, labeled other *cis* CD47, check macrophages and T cells, respectively. Acting through cell surface CD47 or SIRPα, the pictured pathways are inhibitory in immune cells. Not pictured are TSP1-SIRPα and 'reverse'

SIRPα-CD47 signaling [110], which have not been assessed within the checkpoint paradigm. Regarding intracellular effects, SIRPα promotes phosphorylation of SHP1 and SHP2 to quell immune cells [111]. However, SHP1 [112] and SHP2 [113] are activated by other than SIRPα, facts not parsed out in relation to the interactome. Cytoplasmic transmission of the TSP1-CD47 signal is via integrins [114], heterotrimeric G proteins [115], and probably other cell surface molecules. The meager cytoplasmic domain of CD47 encourages this [10]

the TSP1-CD47-SIRPα interactome links the endocrine, checkpoint, and cancer worlds.

CD47 is found on all human cells including thymocytes, T and B cells, dendritic cells (DCs), natural killer cells (NKs), monocytes, erythrocytes, and platelets [10]. CD47 is also displayed on cancer cells, and increased expression was associated with worse outcome [51]. Cell membrane SIRPα binds CD47 weakly (K_D of ~ 0.5 to 8.0 uM) [52, 53]. Variation in experimental K_D notwithstanding, there appears to be no latitude in this for optimum effect. Either too much or too little CD47-SIRPα binding increased immune cell activity and allo-rejection [54]. In canonical *trans* signaling, immune cell displayed SIRPα [52], on binding non-immune cell CD47, suppressed macrophage phagocytosis. The separation between the cell-spanning ectodomains is roughly 14 nm, close to the distance of an immune synapse [52]. Crystal structure analysis indicated that the distal IgV portions of the ectodomains effected *trans* binding [55]. Consistent with the specificity of the interaction, known SIRPα polymorphisms are outside of the *trans* binding area [55]. And like TSP1 binding CD47, posttranslation ectodomain modifications impacted SIRPα binding to CD47 [56]. However, some human cell types, such as renal tubular epithelial cells [17] and lung alveolar cells [18], among others, simultaneously display CD47 and SIRPα on their cell membrane permitting lateral *cis* interaction and signaling. The SIRPα ectodomain displays two CD47-binding sites [57], one distal and one lateral, although whether this is material to *cis* or *trans* signaling requires additional study. Immunoprecipitation data found SIRPα dimerization in certain immune cells, but this was not likely part of the *trans* CD47–SIRPα interaction [58] consistent with crystal structure evidence of a 1:1 interaction between the ectodomains [55]. Still, the kinetics of the *cis* CD47–SIRPα interaction has not been fully revealed and could vary secondary to competition between the ectodomains.

Cis CD47 signaling extends beyond its interaction with same-cell SIRPα. CD47 acted in a *cis* fashion with vascular endothelial growth factor receptor two (VEGFR-2) to maximize the pro-angiogenic signal of VEGF [59]. TSP1 and the C-terminus domain interfered with the CD47-VEGFR-2 *cis* interaction. C*is* CD47-αMβ2-integrin regulated macrophage inflammation [60], although whether TSP1 impacts this is unknown. As well, *cis* CD47-α2β1 integrin signaling regulated T-cell adhesion [61]. More to the point, CD47-blocking agents could alter any *cis* CD47 interaction.

As alluded to, a ligand–receptor interaction between TSP1 and SIRPα was revealed [17]. TSP1, but not the C-terminus domain, bound to SIRPα, co-immunoprecipitated with SIRPα, and at low concentrations, activated SIRPα and its downstream Src homology-2 (SH2) domain containing protein phosphatase SHP1, but not SHP2 [17]. Additionally, a SIRPα-specific blocking antibody abolished TSP1-mediated

SIRPα signaling, whereas antisense knockdown or antibody blockade of CD47 did not. However, the implications of this for immune cells await further investigation.

Oxidative stress is a feature of cancer and a consequence of chemotherapy and radiation [62]. CD47 is sensitive to oxidation [63] which may alter *trans* and *cis* ligand–receptor binding. For example, high glucose-mediated oxidative stress enforced CD47-SIRPα binding [64]. It is fair to speculate that diabetes-associated oxidative stress will impact the binding and activity of CD47 and SIRPα checkpoint blockers.

Immune and inflammatory implications

The TSP1-CD47 checkpoint suppresses most immune cells including T cells [65], NKs [13], DCs [14], and macrophages (Fig. 2). The CD47 binding domain of TSP1, but not the SIRPα ectodomain, stimulated human T-cell apoptosis [66]. An oligonucleotide CD47 translation blocker partially decreased total CD47 protein and increased CD8-positive T-cell killing in radiated tumors [67]. Among T cells, the suppressive effect of TSP1 was partly secondary to the inhibition of IL-2 mRNA expression and biogas hydrogen sulfide [68]. This expands upon the known inhibitory effect that TSP1-CD47 has on vascular biogas nitric oxide [33, 69]. And in CD47-null mice, CD4- and CD8-positive T cells were increased [70]. Interestingly, TSP1-treated human T cells showed increased expression of programmed death-ligand 1 (PD-L1) [71] suggesting that TSP1 may co-opt other checkpoints to limit immune activity further.

In human NKs, TSP1 limited transforming growth factor beta (TGF-β)-driven proliferation [72], a process likely involving CD47 [73]. NKs express CD47-SIRPα, which limits cell killing [74]. In line with this, SIRPα-null NKs showed increased cell killing[74]. Interruption of NK cell TSP1-CD47 signaling improved cell homing and increased granzyme B and interferon-γ levels [13]. Although CD47-null NKs had under some circumstances less capacity, consistent with the role CD47 has in metabolic regulation.

TSP1 inhibited human DC activation and cytokine production which was restored by a CD47-blocking antibody [14]. Exhibiting the nuanced effects of TSP1, the CD47-binding C-terminus forced DC tolerance induction, whereas the heparin-binding N-terminus promoted phagocytosis [75]. Here too, a CD47-blocking antibody undid tolerance. TSP1-CD47 signaling decreased human DC differentiation while circulating levels of TSP1 correlated with decreased immune cell response [76]. In this situation, inhibitory SHP1 was activated, suggesting an overlap between TSP1-CD47 and CD47-SIRPα.

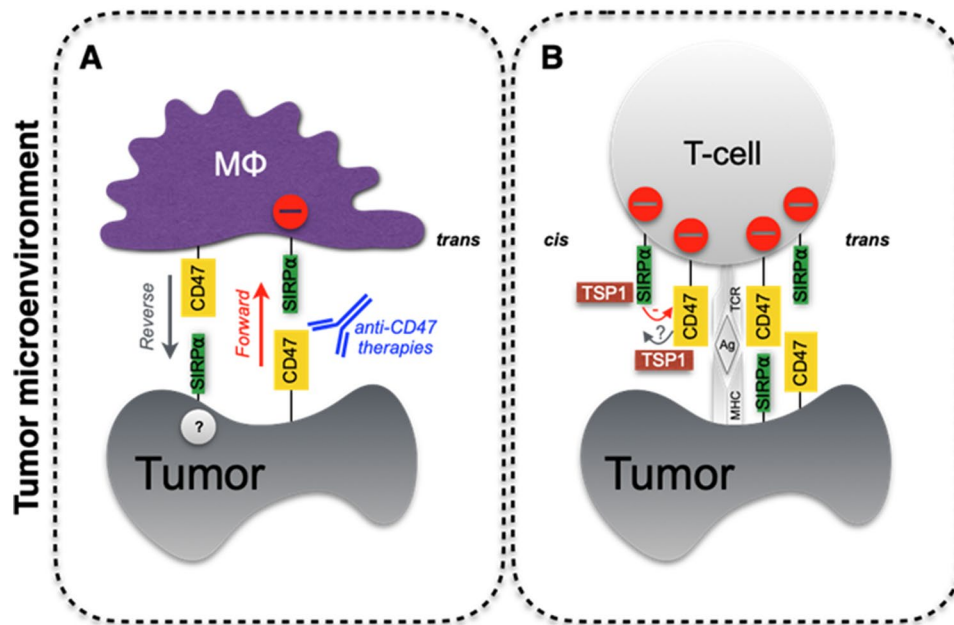Monocytes produced more anti-inflammatory factors in the presence of TSP1, but this involved CD36, an alternative

Fig. 2 Re-thinking CD47 targeting checkpoint inhibition for cancer immunotherapy. **A** The conventional 'don't eat me' signal mediated by the 'forward' negative effect in *trans* of macrophage-displayed CD47. This signal is postulated to be interfered with by clinical blocking anti-CD47 antibodies. The potential 'reverse' effect of macrophage CD47 on tumor-expressing SIRPα is unknown. The TSP1-CD47-SIRPα interactome effect on adaptive immunity is ill-defined. **B** Current data support a predominantly negative effect on T cells in *cis*, which may represent a natural mechanism of homeostasis to maintain self-tolerance. It could be reinforced by acting in *trans*, resulting in an additional tumor escape mechanism. And data suggest that TSP1 interferes with CD47 binding to SIRPα presumably in *trans* and *cis*. MΦ, macrophage; TCR, T cell receptor; MHC, major histocompatibility complex; Ag, antigen; mAb, monoclonal antibody

TSP1 receptor [77]. However, the possibility for CD36-CD47 cross talk [78] was not tested. Inflammatory macrophage adhesion was decreased by antibody blockade of TSP1-CD47 [79]. Consistent with these findings, TSP1-null macrophages showed better phagocytosis of sheep red blood cells [80]. Further, *trans* CD47-SIRPα decreased human macrophage phagocytosis [81]. Independent of CD47, the N-terminal domain of TSP1 increased macrophage activation and superoxide production [82]. Whether N- and C-terminus TSP1 signaling simultaneously act upon cells is unknown.

Upending this, tissues [83] and vital organs [84] lacking CD47 fared better than CD47-expressing tissues and organs when transplanted into SIRPα-replete locations. These findings question the primacy of CD47-SIRPα as a checkpoint and delineator of self.

The implications of *cis* CD47-SIRPα signaling for immune cells, especially in correspondence with *trans* signaling, is daunting to sort out (Fig. 2). This is not surprising since there are practical barriers to testing *cis* signaling when the same molecules also interact in a *trans* manner. Macrophage *cis* CD47-SIRPα is a low-affinity interaction ($\sim K_D$ 1.6 to 2 μM) and acts, separate from *trans* signaling, as a suppressant of phagocytosis [85]. Loss of *cis* signaling by elimination of macrophage cell surface CD47 increased

phagocytosis and correlated with less SIRPα phosphorylation. The effect was enhanced when CD47 was blocked on target non-immune cells [85]. Acute CD47 suppression did not alter same-cell SIRPα levels. But this should be determined in somatic human CD47-null T cells, which are employed in the study of these interactions, as compensatory changes might be found. *Cis* CD47-SIRPα signaling was also demonstrated in human epithelial cells [18], albeit from the direction of SIRPα. Loss of functional SIRPα ectodomain in CD47 expressing human lung alveolar epithelial cells increased inflammatory JAK/STAT activity suggesting *cis* CD47-SIRPα limits inflammation in non-immune cells. This was found true for THP-1 human monocytes as well [18]. Thus, loss of same-cell CD47 or SIRPα increases inflammation. SIRPα dimerization was shown in neutrophils [58], but whether this alters *trans* or *cis* interactions with CD47 is unknown. It was proposed that *cis* SIRPα might sequester CD47, rendering non-immune cells 'CD47 low' as seen by SIRPα-expressing immune cells [85]. Extending this, *cis* CD47 on immune cells might sequester SIRPα away from the *trans* interaction. One might wonder if *cis*-driven lowering of available CD47 on non-immune cells or available SIRPα on immune cells lowers the overall *trans* signal.

Together these data suggest co-stimulatory roles for TSP1, CD47, and SIRPα. We conceptualize co-stimulatory to

include enhancement or suppression of inflammation. Other ligand–receptor pathways such as CD6 showed co-stimulatory effects in T cells based on the binding of ligands CD166 [86] and CD318 [87]. Nonetheless, CD6 was successfully targeted with the immunomodulatory antibody itolizumab [88]. This encourages efforts to optimize therapeutic agents against TSP1, CD47, and SIRPα.

Interactome pathway disruption

Antibodies to checkpoint CD47 are being tested in numerous trials [89]. The most clinically advanced of these is magrolimab [90]. The CD47 antibody B6H12 was produced by immunizing mice with RGD-binding human placental protein [91] and was a muse for the development of magrolimab [41]. B6H12 blocked RGD binding and immune cell activation [91]. This is important, as TSP1 contains an RGD sequence in its CD47-binding C-terminus [92]. Consequently, magrolimab may block TSP1 binding to CD47. One might surmise that magrolimab also interferes with *cis* CD47-SIRPα (Fig. 2).

Magrolimab binding was tested in rat lymphoblast YB2/0 cells transfected with human CD47. However, rodent cells do not carry out human-specific posttranslational protein modifications that are important for ligand binding [40]. The binding affinity of magrolimab to monomeric human CD47 was estimated to be a K_D 8 nM [41], much less than that of TSP1. Magrolimab binding to SIRPα was tested with ELISA and plate-bound protein [41]. Details on several clinical CD47 and SIRPα-blocking molecules that are in or completed trial or that are in development are found in Table 1. Overall, binding information is incomplete. It is fair to say that the therapeutic interruption of checkpoint CD47 remains focused upon *trans* SIRPα. Analysis of *cis* SIRPα, soluble TSP1, and other *cis* CD47-interacting ectodomains is warranted. Similar recommendations apply to SIRPα-targeting molecules.

The CD47 ectodomain is also a therapeutic target for peptides [93] and small molecules [94]. Even these agents may overlap *trans* and *cis* CD47-SIRPα, TSP1, and other same-cell CD47 interactions. Screening assays [95] and protocols that encompass the multiple interactions would be useful in the development of CD47- and SIRPα-binding agents. Acute knockdown techniques and existent somatic mutant CD47-null cells [96] may help discriminate between *trans* and *cis* CD47-SIRPα [85].

Perspectives and interesting questions

Clinical results suggest that this is reasonable to target CD47. But this will likely come with side effects. CD47 on non-immune cells such as red blood cells and platelets soak up

CD47 blockers leading to anemia and thrombocytopenia [97]. Because of this, CD47 blockers must be given in large amounts. And CD47 blockers might interfere with blood banking techniques used in cross-matching [98]. Complications from other therapeutic checkpoint molecules are mostly secondary to inflammation and immune injury [99]. Such occurrences may eventually be seen in individuals administered CD47- and SIRPα-targeting agents, especially if used in combination with other checkpoint blockers. Pertinent to this, humanized diabetic mice administered magrolimab lost CD47-overexpressing islet-like grafts and metabolic control [100]. Human islet endocrine cells were found not to have *SIRPA* mRNA or to display cell surface SIRPα protein, even after exposure to diabetes-associated cytokines [19]. It is unknown if the lack of SIRPα is a feature of human endocrine cell types in general and if this deficit sensitizes or protects endocrine cells from CD47 or SIRPα checkpoint blockers. Hypothetically, the lack of SIRPα on islet endocrine cells could increase available CD47 to increase the *trans* CD47-SIRPα signal as further protection from autoimmune injury.

Other means are available to turn down CD47-SIRPα signaling. For example, a translation blocking oligonucleotide to *CD47* mRNA partially lowered total protein, and in combination with radiation, increased T-cell-mediated killing of cancer [67, 101]. While assumed at the time, it is unclear if such an approach decreased cell surface CD47 expression. Alternatively, CRISPR/Cas9 lowered cell surface CD47 and increased phagocytosis [85]. Be that as it may, use of any method targeting TSP1, CD47, and SIRPα should be complemented by the characterization of cell surface molecule copy number (B_{max}). And if it is the case that certain knockdown approaches do not substantially alter cell surface CD47 expression, this opens the door for other possible mechanisms of action.

Taking a reverse position, overexpression of cell membrane CD47 [102] was employed to provide a defense against immune cells. Decorating non-animate surfaces with CD47 was also tried [103]. In view of the other homeostatic mechanisms that CD47 impinges upon [10], this strategy may not be benign. For instance, TSP1-CD47 signaling promotes aging in human cells and tissues [104] and animals [31, 105], and limits the Yamanaka self-renewal transcription factors in human cells [106]. In fact, CD47-null cells grown in serum-free medium de-differentiation [107]. Thus, forced CD47 expression may prematurely drive stem cells out of the cell cycle and into senescence [108].

Conclusion

The TSP1-CD47-SIRPα interactome is a multi-tier check on immune cells. Agents that attempt to intervene on one of these will probably alter or undo other interactome

Table 1 CD47- and SIRPα-targeting molecules

Name	Source	Origin	Isotype	Blocks	Kinetics	cis/trans
Magrolimab[1]	Gilead	Humanized	IgG4	CD47-SIRP	$4.4E^{-11}$ M	? /+
AO-176[2]	Arch Oncology	Humanized	IgG2	CD47-SIRP	?	? /+
AO-104	Arch Oncology	Humanized	IgG4	CD47-SIRP	?	? /+
CC90002[3]	Celgene	Humanized	IgG4	CD47-SIRP	?	? /+
SRF231[4]	Surface Oncology	Fully human	IgG4	CD47-SIRP	?	? / ?
IBI188[5]	Innovent	Fully human	IgG4	CD47-SIRP	?	? /+
IBI-322	Innovent	Bi-specific	?	CD47 x PD-L1	?	? /+
TG-1801	TG Therapeutics	Bi-specific	IgG1	CD47 x CD19	?	?
SGN CD47M	Seattle Genetics	Conjugate	?	?	?	?
HX-009	Waterstone Hanxbio	Bi-specific	?	CD47 x PD-1	?	?
IMC-002	Immune-Onco Therapeutics	?	?	?	?	?
AK117[6]	Akeso Biopharma	?	?	CD47-SIRP	$1.5E^{-10}$ M	? /+
STI-6643[7]	Sorrento Therapeutics	Fully human	IgG4	CD47-SIRP	$7.6 E^{-10}$ M	? /+
TKKTLRT-SIRPαFc[8]	Immune-Onco Therapeutics	Collagen-SIRPαFc	?	CD47	$1.4E^{-9}$ M	? / ?

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signals. This suggests opportunity to refine the approach to targeting these molecules. Further, checkpoint blocker-sensitive islet endocrine cells are devoid of SIRPα. This occasions the question if this is a protective adaptation. Like beta cells, thyroid cells are derived from endoderm109. A closer look at SIRPα in other endocrine organs should prove rewarding.

Declarations

Competing interests J.S.I. is a consultant to San Rocco Therapeutics. E.M. declares no conflicts of interest regarding the work.

Conflict of interest J.S.I. is a consultant to San Rocco Therapeutics, Tampa, FL. E.M. declares no conflicts of interest regarding the work.

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