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# CD47: A New Target in Cardiovascular Therapy

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## Abstract

CD47, originally named integrin-associated protein, is a receptor for thrombospondin-1. A number of important roles for CD47 have been defined in regulating the migration, proliferation and survival of vascular cells, and in regulation of innate and adaptive immunity. The recent discovery that thrombospondin-1 acts via CD47 to inhibit nitric oxide signaling throughout the vascular system has given new importance and perhaps a unifying mechanism of action to these enigmatic proteins. Here we trace the development of this exciting new paradigm for CD47 function in vascular physiology.

Integrin-associated protein (IAP) was discovered as a contaminant in preparations of αvβ3 integrin from human placenta. Certain monoclonal antibodies raised against these preparations had dramatic effects on integrin-dependent cell behaviors, but bound to a ca. 50 kDa protein rather than α or β integrin subunits [1]. Cloning and sequencing IAP cDNA revealed a new member of the Ig superfamily with a single extracellular IgV domain, a unique 5 membrane-spanning domain and an alternatively spliced cytoplasmic tail [2]Figure 1A. Subsequent experiments found IAP to be identical to CD47, which is widely expressed on tissues, primary cells and nearly all cell lines, with prominent expression on leukocytes, platelets and erythrocytes [3, 4]. At first, CD47 was an orphan receptor apart from its lateral membrane association in *cis* with αvβ3 and αIIbβ3.

## Thrombospondin-1 regulation of integrins requires CD47

Concurrent with the development of the CD47 knockout mouse, thrombospondin-1 (TSP1), a large, secreted glycoprotein, was identified as a potential *trans* ligand of CD47 [5], and signal inhibitory receptor protein α (SIRPα) was recognized as a cell-bound counter-receptor for CD47 [6]. Also called SHPS-1 [7], BIT [8] and p84 [9] by different investigators in different species, SIRPα, is most highly expressed on myeloid lineage cells and functions with CD47 in regulating innate immunity and the transition to the adaptive immune response [10–14].

TSP1 is a major component of platelet α-granules from which it is secreted upon platelet activation [15]. While this localization suggested a role for TSP1 in thrombosis and hemostasis, identification of such a role has been elusive. In addition to blood-borne platelets, TSP1 is expressed at much lower levels in many if not all tissues and is a biosynthetic product of most cultured cells [16]. The TSP family in mammals now has 5 members that consist of group A homotrimers (TSP-1 and -2) and group B homopentamers (TSPs 3–5). TSPs 1 and 2 have broad but distinct tissue expression patterns during development and through adulthood, and TSP1

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is the only TSP found in platelets [17–19]. TSP 1 and 2 subunits have an identical domain organization (Figure 1B), but human TSP1 and 2 share only 54% amino acid sequence identity [20]. The N domains attach TSP1 to the cell surface via several receptors (Figure 1B), while the G domains at the opposite end of each subunit interact functionally with CD47. The binding of TSP1 to so many receptors and matrix ligands serves to concentrate TSP1 at the cell surface and matrix, thereby dramatically enhancing its binding to what otherwise might be sites of rather low affinity. Among this plethora of receptors, none exclusively bind TSP1. This, along with contradictory and confusing reports of cell responses to TSP1, and the subtle phenotype initially described for the TSP1-null mouse[21] have complicated efforts to understand the physiologic functions of TSP1.

While initial efforts to identify TSP1 receptors focused on integrins that might bind to the RGD sequence in the calcium-binding domain of TSP1, we found that cells could attach to a site contained in the C-terminal (now G) domain of TSP1 exclusive of the RGD sequence [22]. Peptides having the cell binding activity were localized within the TSP1 G domain [23, 24], and one of these peptides was used to affinity label a ca. 50 kDa cell membrane receptor candidate [25]. Concurrently, Eric Brown had discovered CD47 [2] and suggested that it might be the TSP1 receptor. We confirmed that the 50 kDa protein was recognized by several CD47 antibodies and that the TSP1 G domain peptides augmented integrin functions such as chemotaxis and cell spreading in a CD47-dependent manner [5, 26]. This activity of CD47 was dependent on functional heterotrimeric Gi [27], suggesting that CD47 might be an unconventional G protein coupled receptor (GPCR) with 5 rather than 7 transmembrane segments. CD47 signaling through Gi can access the “inside-out” pathway used by other GPCRs on platelets to activate αIIbβ3 [28, 29], as well as αvβ3 and some β1 and β2 integrins [30, 31] (and our unpublished data). CD47 can also increase the avidity or clustering of integrins by associating with them in the plane of the membrane, an apparently signaling-independent process [32].

## Biological Roles of CD47

The CD47-null mouse is lethally susceptible to bacterial infections that are of no consequence to wild type mice due to a delay in neutrophil recruitment and a weakened integrin-dependent oxidative burst response [33]. In vitro data also support roles for CD47 in leukocyte adhesion to endothelium [12], leukocyte transmigration [34], and migration of dendritic cells [35]. CD47 is important in the development and function of antigen presenting cells [11, 36], and has a role in immune cell apoptosis [37, 38]. In the context of innate immunity, CD47 functions as a marker of self. When attempts were made to engraft CD47 null marrow cells into WT mice, the CD47 null cells were rapidly engulfed by splenic macrophages and dendritic cells [39]. Experiments by Oldenborg and colleagues revealed that CD47 on circulating cells normally binds SIRPα on phagocytes and delivers a “don’t eat me” signal that prevents their phagocytosis [40, 41]. This signal depends on the docking of SHP-1 phosphatase to phosphorylated ITIMS in the SIRPαcytoplasmic domain leading to SHP-1 activation and presumably dephosphorylation of one or more key components of the phagocytic machinery[42, 43]. A function of this mechanism is suggested by the report that phagocytosis of xenograft cells is augmented by the species incompatibility of donor CD47 with host SIRPα[44].

## TSP1 regulates vascular NO signaling through CD47

The role of CD47 in the regulation of NO signaling was found while focusing on another TSP1 receptor, CD36. A wealth of data both in vitro and in vivo has shown that TSP1, the first identified endogenous angiogenic inhibitor [45], has an important role in opposing tumor-driven angiogenesis and regulating episodes of angiogenesis in the adult [46–49]. Even so, the precise mechanism of this effect has remained elusive. Initial reports indicated that CD36 was the TSP1 receptor that mediates its anti-angiogenic effects [50]. Receptors for several angiogenic

factors such as VEGF can induce NO synthesis, and the NO/cGMP pathway is essential for angiogenesis [51, 52]. Signaling through VEGFR2 activates Akt1 to phosphorylate endothelial nitric oxide synthase (eNOS), which renders it constitutively active, independent of its normal dynamic activation by calcium pulses and shear [53]. This sustained activation of eNOS leads to increased endogenous NO production and a tonic cGMP increase which, through cGMP-dependent protein kinase (cGK) activation, coordinates the signaling pathways needed for endothelial proliferation, migration and survival [54]. In contrast, higher levels of NO, such as that produced by iNOS during an inflammatory response, inhibit these responses or are cytotoxic [55].

Mouse muscle explant cultures provided the first evidence that the anti-angiogenic activity of TSP1 involves regulation of NO signaling [56]. TSP1-null muscle explants produced a more robust vascular outgrowth than WT explants. Addition of NO donors exaggerated this difference, while the NOS inhibitor L-NAME blocked outgrowth. Further, NO-stimulated outgrowth was inhibited by exogenous TSP1. CD36-null muscle explants, though stimulated by exogenous NO, remained sensitive to inhibition by TSP1, demonstrating that CD36 is not necessary for TSP1 inhibition of NO-stimulated vascular cell responses. In contrast, NO-stimulated vascular outgrowth in CD47-null explants was *not blocked by TSP1*[57]. Recombinant TSP1 domains and other specific ligands of CD36 or of CD47 inhibited NO responses in WT vascular cells. However, in cells lacking CD47, ligation of CD36 was unable to block NO signaling. Thus, while CD36 ligation is *sufficient* when CD47 is present, only CD47 ligation is *necessary* for inhibition of NO/cGMP signaling [57]. These results clearly imply coupling of CD36 and CD47 either physically as “co-receptors” in the membrane or via convergent signaling pathways (Figure 2). Further studies in endothelial and vascular smooth muscle cells (VSMC) [57] and in platelets [58], revealed that not only does TSP1 ligation of CD47 block NO stimulation of cGMP production; it also inhibits the direct activation of cGK by cGMP analogs (Figure 2). Angiogenic signaling is only one of several physiologic roles of NO in cardiovascular homeostasis [59]. Thus our data suggested that TSP1 signaling via CD47 might regulate NO responses in a much broader context.

## CD47 inhibits NO action in vivo

NO is a major acute regulator of the cardiovascular system [60, 61]. It plays a role in regulation of blood pressure and vascular tone and also adjusts regional tissue perfusion as the metabolic activity of tissues responds to changing demands. We therefore examined the role of TSP1 and CD47 in the physiological regulation of blood flow. VSMC contraction (and hence blood vessel diameter and flow) is controlled by kinases and phosphatases acting on the regulatory light chain-2 of myosin (MLC-2) [62]. Pulsatile shear and calcium transients activate eNOS, producing NO, which then diffuses across membranes into VSMC, activating soluble guanylate cyclase (sGC). Cyclic GMP in turn, leads to a cGK-dependent activation of myosin light chain phosphatase, a rapid decrease in MLC-2 phosphorylation and VSMC relaxation. NO also activates sGC in the endothelial cell of origin leading to cGK-dependent, inhibitory phosphorylation of eNOS and termination of NO production, thus restoring VSMC contractility [63].

## Blocking TSP1-CD47 alleviates tissue ischemia

TSP1, acting via CD47, inhibits the NO stimulation of sGC in both EC and VSMC [57]. Exogenous NO donors inhibit the contraction of VSMC embedded in a collagen gel induced by serum or sphingosine-1-phosphate [64]. Addition of TSP1 prevents NO-dependent relaxation of VSMC. CD47 null VSMC or WT VSMC in which CD47 expression has been knocked down with an antisense morpholino are insensitive to TSP1 inhibition of NO-stimulated relaxation [65].

These results were found to have implications *in vivo* in several models of tissue injury. Under ischemic challenge, tissue and blood vessels respond by increasing endogenous NO levels, leading to blood vessel dilation and increased tissue perfusion. A well-characterized skin flap model in WT mice routinely results in 40 to 60% necrosis of the ischemic flap within three days [64]. However, skin flaps in TSP1- and CD47-null mice (but not CD36-nulls) experienced markedly enhanced perfusion and survival (Figure 3A) [65]. Full thickness skin grafts (FTSG) are an even more stringent model of ischemia since they initially have no blood supply at all and must initiate neovascularization from the wound bed. Wild type FTSGs failed to survive on WT recipients, but survived and healed nearly completely on TSP1-null recipients (Figure 3B). TSP1-null grafts on WT recipients had intermediate survival[66]. Thus, TSP1/CD47 signaling limits tissue perfusion and survival following partial and complete ischemic challenges.

To explore the therapeutic potential of these discoveries, we targeted the TSP1/CD47 pathway using monoclonal antibodies to both TSP1 and CD47 and knockdown of CD47 expression using an antisense morpholino oligonucleotide. Each of these could be locally applied by injection into the ischemic soft tissues. Importantly, these therapeutics greatly enhanced ischemic tissue survival in WT animals to the level obtained in null animals [65, 66].

To determine if the improved tissue survival in these relatively simple ischemia models would be realized in more complex tissues, we examined a hindlimb ischemia model. Even under the dramatic ischemic challenge of complete femoral artery occlusion, TSP1- and CD47-null mice demonstrated restoration of vascular perfusion of the hindlimb at seven days after surgery to a level much superior to that of ischemic WT hindlimbs [65]. These results further suggest that deleting either TSP1 or CD47 removes a barrier to vascular remodeling of ischemic tissues consistent with the potent inhibitory effects of TSP1 seen in angiogenic explant assays [56]. However, real-time analysis of blood flow by laser Doppler flowmetry and blood oxygen level-dependent (BOLD)-MRI, revealed that increased tissue perfusion in TSP1 and CD47 knockout mice was achieved within minutes following an ischemic insult [65, 67], much too quickly for angiogenesis to occur. Such rapid reperfusion in the face of the permanently ligated femoral artery must require rapid remodeling of existing collateral vessels to bypass the ligation [68]. This startling result indicated that TSP1 and CD47 acutely control blood flow under conditions of ischemic stress. Kopp et al. [69] report that platelet TSP1 is deposited in vessels downstream of femoral artery ligation, suggesting that the TSP1 responsible for the poor perfusion in ischemic WT limbs may be delivered by platelets. However, the extremely low (picomolar) concentrations at which TSP1 can suppress NO signaling in vascular cells in vitro [57, 70] suggests that the low levels of TSP1 present in the vascular wall may also contribute. The fact that both TSP1 and CD47 knockouts show the same rapid improvement in tissue perfusion strongly supports the functional relationship of these two proteins in this regulatory system.

There is a growing literature reporting sudden increases in TSP1 protein and mRNA in ischemic tissues, as much as 20 fold above normal tissue in the case of human legs amputated as a result of chronic ischemia [71] or myocardial infarction in rats [72] with more modest elevations seen in mouse kidney after ischemia/reperfusion [73], or in chronic ischemia due to systemic sclerosis in humans [74]. In a rat middle cerebral artery stroke model, TSP1 increased in a biphasic manner with peaks at 1 and 3 days, while TSP2 appeared much later, peaking 2 weeks after the stroke [75]. The extremely rapid appearance of TSP1 again suggests immediate delivery from platelets or its presence due to infiltrating inflammatory cells such as monocytes and macrophages. However, induction of TSP1 in endothelial, VSMCs, astroglia and kidney tubule cells was also seen. In view of our data above, it would seem that recruitment of TSP1-bearing cells to ischemic tissues or expression of TSP1 in resident cells could only exacerbate a bad situation.

## Long-term effects of TSP1/CD47 signaling

Endothelial dysfunction, secondary to impaired NO bioavailability, is a hallmark of metabolic syndrome and of the aging cardiovascular system [76,61]. We have begun to investigate the long-term consequences of the TSP1/CD47 inhibition of NO signaling. With advanced age (12–16 months) and atherosclerotic vasculopathy ($ApoE^{-/-}$ cross), TSP1- and CD47-null mice continued to demonstrate enhanced tissue perfusion and survival following ischemic challenge comparable that seen in young knockout animals [67]. As expected, aged WT mice suffered much worse ischemia and necrosis and a corresponding drop in tissue cGMP levels compared to young WT animals. In contrast, tissue cGMP levels in aged TSP1- and CD47-null mice remained at levels comparable to those in young animals, suggesting that TSP1/CD47 contribute to the NO-insufficiency characteristic of aging.

Metabolic syndrome is a constellation of cardiovascular risk factors including obesity, hyperlipidemia, hypertension and insulin resistance [77]. Interestingly, C57Bl/6 WT mice spontaneously develop metabolic syndrome [78], and eNOS-null mice develop metabolic syndrome more rapidly than WTs [79]. CD47-null mice are leaner than matched WTs and appear to resist features of metabolic syndrome (our unpublished observations). A primary component of metabolic syndrome is diabetes. Murphy-Ullrich's group reported that an NO donor could block the increase in TSP1 expression caused by high glucose [80], suggesting a mutually antagonistic link between TSP1 and NO signaling that could have important consequences for cardiovascular disease and its treatment.

## TSP1 and the platelet enigma

The above studies indicate a pervasive role for TSP1 and CD47 in regulating NO signaling in blood vessels. Platelet function is also regulated by NO, the overall effect being to oppose activation and aggregation [81]. This is consistent with the global role of NO to maintain a healthy, anti-thrombotic vascular system. However, local differences in the production and availability of NO can alter the fine balance between pro- and anti-thrombotic states.

One of the puzzling properties of the TSP1-null mouse was its apparent lack of a platelet phenotype even thought its platelets are completely devoid of TSP1 (or TSP2) [21]. Earlier studies using TSP1 antibodies [82, 83] and peptides [29] suggested that TSP1 could facilitate platelet activation or aggregation in vitro, but this remained controversial [84, 85]. In light of the newly discovered role for TSP1 as a regulator of NO signaling, we re-examined the function of TSP1 in platelets [58]. The aggregation of human platelets activated with thrombin is delayed by fast acting NO donors, but this delay, and the NO-stimulated increase in platelet cGMP are abolished by adding exogenous TSP1. Freshly isolated TSP1-null mouse platelets have higher resting levels of cGMP, and addition of NO donors or supplementation of the traditional Tyrode's buffer with L-arginine induces greater cGMP synthesis in TSP1 null platelets compared to WT. When small amounts of thrombin are added sequentially to stirred platelet suspensions, TSP1 null platelets require 2 to 3 times more thrombin for activation than WT platelets and are much more sensitive to inhibition by NO donors and cGMP analogs [58]. A primary effect of NO in platelets is to prevent GTP loading of the small G protein Rap1b, which, upon binding GTP, activates αIIbβ3 [81]. TSP1 prevents the inhibition of Rap1b GTP loading by NO, thus facilitating αIIbβ3 activation, binding of fibrinogen, and aggregation. As in other vascular cell types, TSP1 binding to CD36 or CD47, both of which are highly expressed on platelets, is sufficient to inhibit NO signaling and thereby promote platelet activation and aggregation [58]. Traditional methods for assessing platelet aggregation deplete platelets of both NO and the NOS substrate L-arginine, leading to progressive loss of endogenous NO and cGMP thereby lowering the barrier to activation. It is important to emphasize that NO does not prevent platelet activation, but only increases the level of agonist required to initiate the

process. In a similar vein, TSP1 is not required for platelet activation, it simply lowers the threshold for platelet agonists. The massive amount of TSP1 discharged from activating platelets and the large number of CD47 receptors on the platelet surface combine to effectively abolish NO inhibition.

## Why did “we” need TSP1 and CD47?

If NO is important in promoting cardiovascular health, why was the TSP1/CD47 inhibitory system selected during evolution and allowed to persist? What we see as maladaptive in the context of current longevity and lifestyle must have once provided an important selective advantage for our early vertebrate ancestors. We believe that the essential selective advantage of TSP1/CD47 antagonism of NO signaling was its ability to limit life-threatening hemorrhage. NO is an ancient signaling molecule, but the unique NO-responsive soluble GC of vertebrates may have evolved more recently[86], perhaps coincident with the divergence of TSP1 from the ancestral TSP gene in lower animals [87] and the appearance of CD47 [13]. Closed, pressurized circulatory systems require finely-regulated hemostasis to control hemorrhage yet maintain flow. Packaging TSP1 in platelet α-granules allows a bandaid to be applied locally at a site of wounding, while permitting the rest of the circulatory system to continue functioning. Platelets secrete several compounds that further stimulate platelet activation, thus reinforcing the initial platelet layers at the blood-wound interface and recruiting more platelets from flowing blood. TSP1, unlike the other prothrombotic agents released from platelets, binds firmly to the platelet surface via a number of receptors (Fig. 1). TSP1 also binds to components of the clot matrix such as fibrinogen, fibronectin, and vWF. Thus, TSP1 is a *tethered* prothrombotic agent that is long-lived relative to compounds such as prostacyclins and ADP, which are rapidly degraded and/or diluted by blood flow. It could also be significant that TSP1 is a trimer with long and flexible subunits. In addition to acting on platelets, the released TSP1 can suppress NO signaling in cells exposed in the wounded vessel wall, promoting local vasoconstriction to further decrease bleeding. Local TSP1 binding to leukocyte CD47 will also attract and promote transmigration and oxidative bursting of neutophils [13], the first responders to infection.

## A new target for cardiovascular therapeutics?

Hemostasis may have been the critical, evolutionarily selected function of TSP1 and/or CD47 in our ancestors. However, our present-day well-fed, sedentary, but nonetheless, long-lived contemporaries may have become unwitting victims of the TSP1/CD47 antagonism of NO signaling. The global role of NO regulation in the cardiovascular system is now well documented, but the beneficial effects of NO signaling are relatively easily overwhelmed by stressors such as hyperlipidemia, diabetes, smoking and others that disrupt this balance [61, 76, 88]. Overcoming the beneficial effects of NO is made easier because TSP1 and CD47 continuously oppose NO signaling in circulating cells and blood vessels. The genetic knockout of TSP1 or CD47 relieves this antagonism, but has few discernable effects when mice are kept in a safe, pathogen-free environment. However, when stressors such as vessel injury, ischemia, hyperlipidemia and even old age are applied, we see that the knockouts fare substantially better than WT mice. Furthermore, therapeutic interventions that interrupt TSP1/CD47 signaling provide dramatic improvement in the WT response to ischemic stress. The previously unsuspected role for CD47 in limiting NO signaling provides a novel and accessible target to augment the beneficial effects of NO for the treatment of cardiovascular disease.

The data obtained so far present us with a new way of looking at the function and mechanism of TSP1 and CD47. However, many questions remain to be answered: What functions of TSP1 and CD47 are shared, and which are independent, i.e. due to TSP1’s binding to other receptors? What is the mechanism by which CD47 blocks NO signaling? Can CD47 regulate targets of NO that are independent of sGC and cGK? Answers to these questions will help to guide and

define the role of CD47-targeted therapies, placing them in context with existing therapeutic approaches to improve the treatment of cardiovascular disease.

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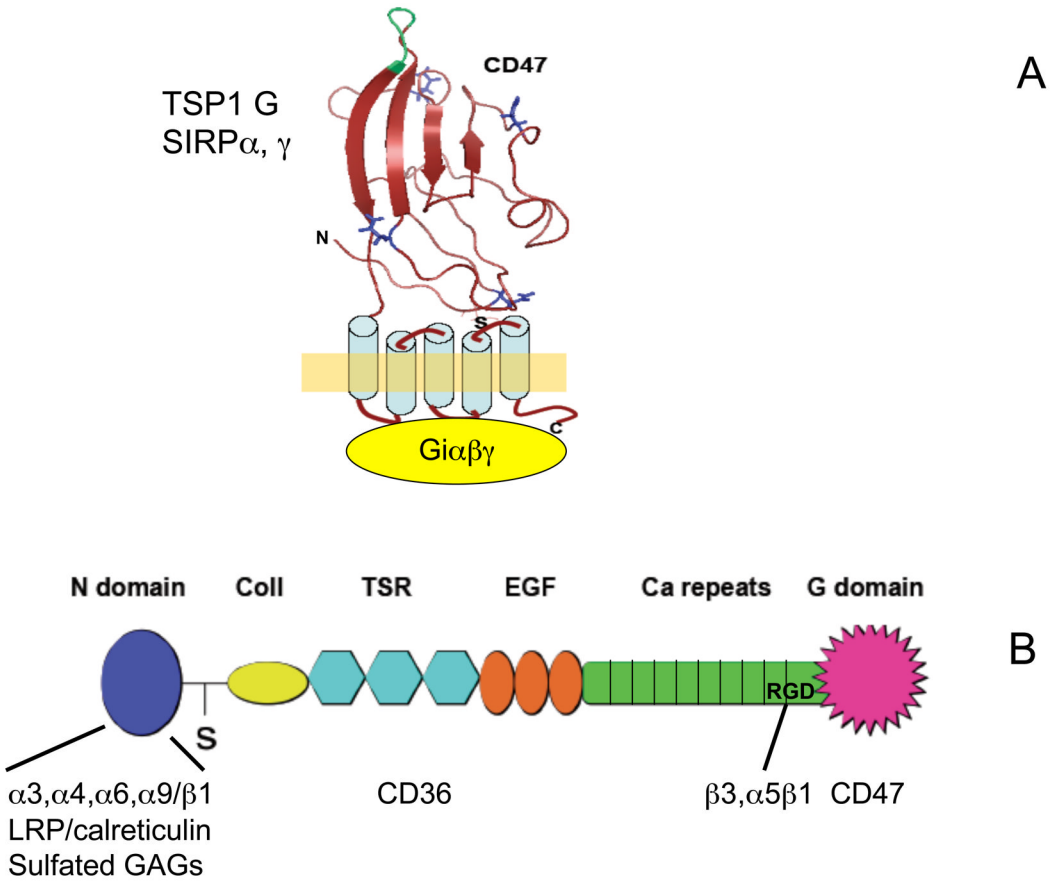


**FIGURE 1. Models of CD47 and TSP1**

**A:** CD47 (integrin-associated protein) is an atypical member of the Ig superfamily with a single IgV type domain connected to a multiple membrane spanning segment rather than a single transmembrane segment. It is also an atypical G protein-coupled receptor (GPCR) having only 5 transmembrane segments instead of seven. The C-terminal cytoplasmic tail of CD47 is alternatively spliced, giving rise to 4 isoforms that are expressed differentially in various tissues [13]. In addition to its role in TSP1 signaling, CD47 binds the N-terminal or membrane distal, IgV domain of SIRPαand SIRPγ. In some systems, it has been shown that CD47 couples to and signals via heterotrimeric Gi [27].

**B:** A domain model of TSP1 (after 89). The N-terminal (N) domain binds sulfated glycosaminoglycans and glycolipids and contains binding sites for four β1 integrins, calreticulin and LRP1 (LDL receptor-related protein1) [90, 91, 89]. The N-domain is followed by a segment containing the trimer-forming cysteines and heptad repeats, a von Willebrand C domain, 3 TSRs (TSP type 1 repeat domains or properdin-like repeats) containing CD36 [50] and additional β1 integrin [92] binding sites, 3 EGF-like domains and a highly repetitive and extensible calcium-binding domain [93]. At the C terminus is the β-sheet rich G domain [94, 95]. The RGD site in the last of the calcium-binding repeats is cryptic in the fully calcium loaded protein, but may become available when TSP1 is bound to other cellular or matrix receptors. The C-terminal G domain contains the CD47 agonist peptide 4N1, which is routinely used as 4N1K (kRFYVVMWKk, lowercase k = non-native lysine).

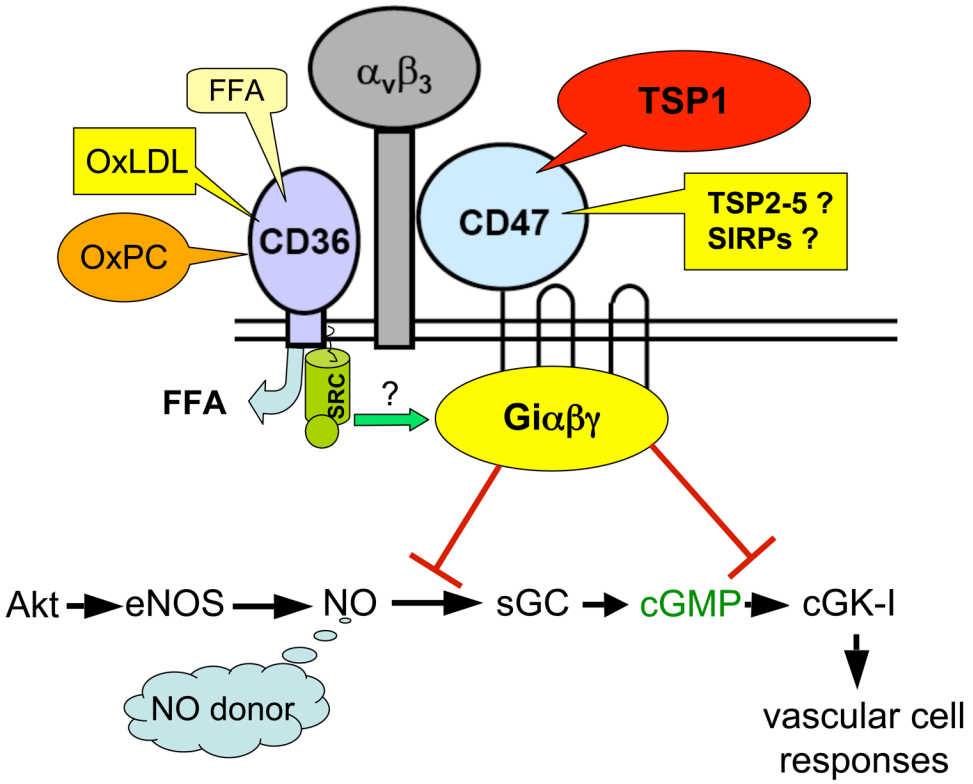


**Figure 2. Model of CD47-dependent regulation of NO signaling**

CD47 can associate with integrins (here αvβ3) and CD36 in the plasma membrane and is coupled to heterotrimeric Gi through which at least some effects on NO signaling appear to be mediated. Known and potential ligands of CD36 and CD47 are indicated. It is not yet known if TSPs 2–5 or any SIRPs can impact NO signaling. CD36 associates with Src kinases, activation of which may impact CD47 signaling. CD36 also takes up free fatty acids (FFAs), among them myristate, which via N-myristoylation of src kinases, can promote eNOS activation. Binding of TSP1 to CD36 blocks FFA uptake thus inhibiting eNOS and subsequent NO-dependent responses [96]. CD47 can inhibit the effect of NO, generated by NOS or supplied via a chemical donor, on sGC (soluble guanylyl cyclase) and the effect of 8Br-cGMP on cGK-I (cyclic GMP kinase I); these effects may be mediated by activation of heterotrimeric Gi.

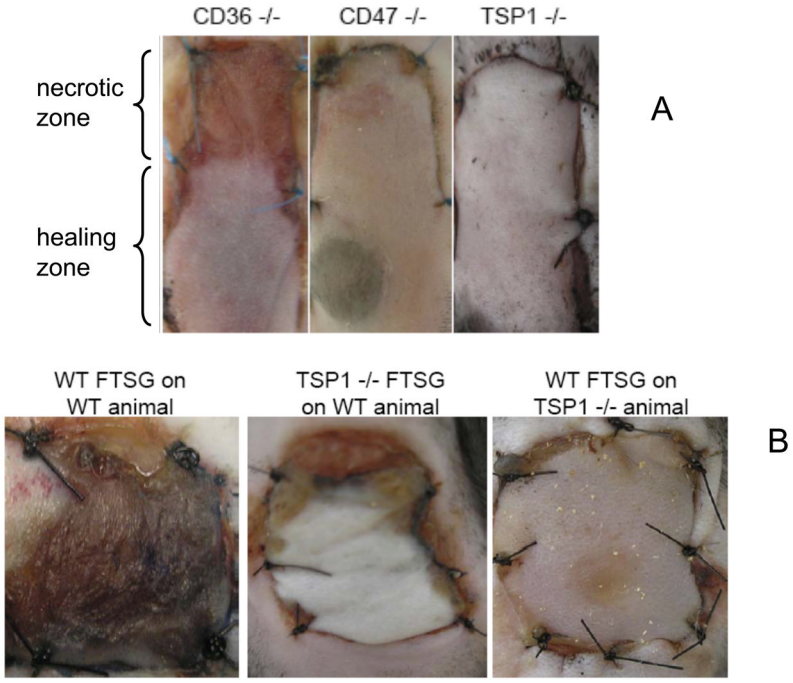


**FIGURE 3. Improved healing of skin flaps and grafts in TSP1- and CD47-null mice**

**A:** Appearance of random cutaneous McFarlane flaps created on the dorsum of CD36, CD47 and TSP1 null mice after 3 days of healing. The hinge of skin left intact is at the bottom of each panel shown. CD36 null flaps, like WT flaps (not shown), routinely undergo necrosis in the distal portion (ca 40 to 50%) of flap, while CD47 and TSP1 null flaps heal nearly completely (90 to 100%). (from 65).

**B:** Appearance of full thickness skin grafts 3 days after surgery. The host was a WT mouse (1st and 2nd panels) or a TSP1-null mouse (3rd panel) and the graft was from a WT donor (1st and 3rd panels) or a TSP1-null donor (middle panel). While a TSP1-null graft does much better than a WT graft on WT hosts, a WT graft completely heals on a TSP1-null host. (from 66).