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Suppressing or Enhancing Macrophage Engulfment through the Use of CD47 and Related Peptides

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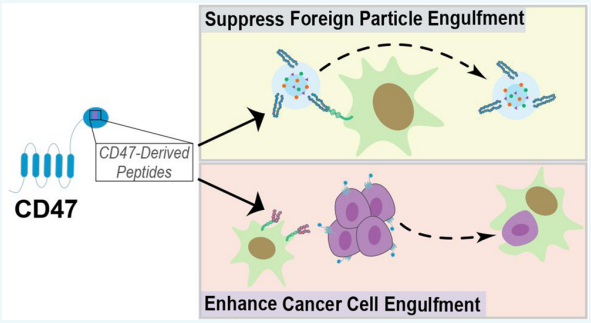
ABSTRACT: Foreign particles and microbes are rapidly cleared by macrophages *in vivo*, although many key aspects of uptake mechanisms remain unclear. "Self" cells express CD47 which functions as an anti-phagocytic ligand for SIRPα on macrophages, particularly when pro-phagocytic ligands such as antibodies are displayed in parallel. Here, we review CD47 and related "Self" peptides as modulators of macrophage uptake. Nanoparticles conjugated with either CD47 or peptides derived from its SIRPα binding site can suppress phagocytic uptake by macrophages *in vitro* and *in vivo*, with similar findings for CD47-displaying viruses. Drugs, dyes, and genes as payloads thus show increased delivery to targeted cells. On the other hand, CD47 expression by cancer cells enables such cells to evade macrophages and immune surveillance. This has motivated development of soluble antagonists to CD47-SIRPα, ranging from blocking antibodies in the clinic to synthetic peptides in preclinical models. CD47 and peptides are thus emerging as dual-use phagocytosis modulators against diseases.



INTRODUCTION

Phagocytosis is an ancient and basic cellular process that refers to the devouring of a target. Bacteria and fungi are targets of phagocytosis by amoebae and require little to no discrimination. However, in animals, phagocytes such as macrophages must identify, attack, and preferentially engulf "foreign" targets while avoiding healthy "Self" cells. These innate immune phagocytes are the host's primary line of defense against various invading microbes, both large and small. Phagocytosis is stimulated by "eat me" signals which initiate actin cytoskeleton remodeling that drives macrophage protrusions to envelop—and subsequently internalize and destroy—a "foreign" target. Relevant factors range from highly specific biomolecular interactors (protein−protein) to less specific surface effectors (charge, adsorbed species, ligand patterns) and physiochemical features (rigidity, shape). Opposing some of these "eat me" pathways are "don't eat me" signaling molecules that can inhibit macrophage uptake. This brief review focuses on some of the latest advances in modulating macrophage elimination of nanoparticles, viruses, and cancer cells through manipulation of the specific "don't eat me" CD47-SIRPα axis.

MACROPHAGE CHECKPOINT, CD47-SIRPα

The "Marker of Self" protein, CD47, is a ubiquitously expressed, integral membrane protein that interacts with the macrophage receptor SIRPα to inhibit phagocytic uptake (Figure 1).[1−3] While an interaction between CD24 and Siglec-10 is potentially another macrophage checkpoint,[4] the CD47-SIRPα interaction is more thoroughly characterized and

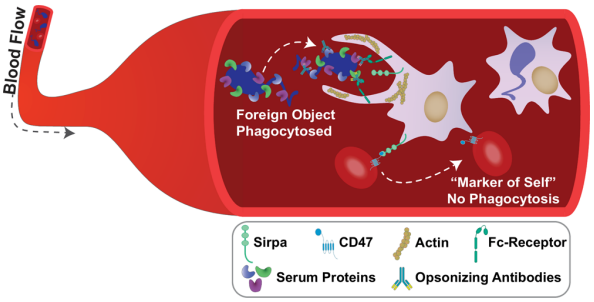


Figure 1. "Marker of Self" CD47 inhibits phagocytosis by macrophages. Serum proteins, such as IgG in blood, adsorb to "foreign" particles or bind specifically, stimulating phagocytosis. Such uptake is inhibited if SIRPα binds its ligand CD47 that is presented on "Self" cells, including red blood cells.

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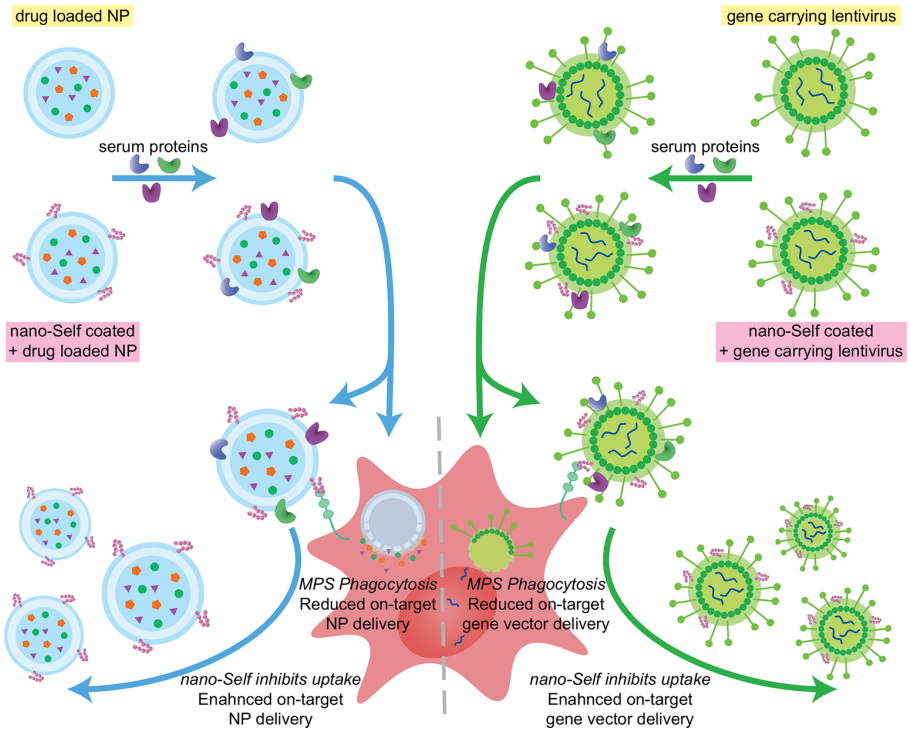

Figure 2. CD47-peptides prolong circulation and increase on-target deliver. Drug-loaded nanoparticles (left) and gene-carrying lentivirus (right) have limited efficacy because of phagocytic uptake. CD47 and related "Self" peptides on nanoparticles and viruses bind SIRPα on macrophages and help recognize the object as "Self", leading to prolonged circulation and enhanced on-target delivery.

conserved across many higher animals. Inhibition of macrophage uptake involves phosphorylation of SIRPα's cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and activating phosphatases SHP-1 and SHP-2.[5−7] The binding interaction between CD47 and SIRPα tends to be species- and even strain-specific,[8,9] with some notable cross-interactions such as human CD47 binding to NOD mouse and pig SIRPα, and pig CD47 binding to human SIRPα.[9−11] Thus, the inhibitory effect of this receptor−ligand interaction is determined by protein sequence and structure.[12]

The high efficiency by which macrophages remove foreign objects circulating in the body often hinders nanoparticle-based drug delivery, with uptake by pervasive macrophages frustrating delivery to intended targets such as cancer cells. This has led to the idea of using CD47 to make solid particles and viruses more tolerable and increase on-target delivery of nanomedicines and genes. Complementary to such efforts has been the goal of antagonizing the CD47-SIRPα axis to enhance phagocytosis, with antibody-based disruption rapidly emerging as a clinically relevant addition to cancer immunotherapy and new possibilities with small, inhibitory peptide designs.

NANOPARTICLES DISPLAYING CD47 AND "SELF" PEPTIDE DELAY CLEARANCE TO ENHANCE DELIVERY

Intravenous administration of nanoparticles has the potential advantage of circulating through every tissue and disease site. Unfortunately, such injected nanoparticles are typically cleared in minutes to hours by the mononuclear phagocyte system (MPS), particularly macrophages in the liver and spleen.[13−15] For comparison, fresh red blood cells (RBCs) can circulate for weeks or longer after infusion but are then also cleared by macrophages, particularly in the spleen.[1] The mechanism(s) by which a macrophage identifies and eliminates a nanoparticle remain unclear. It is known that blood serum proteins physisorb and accumulate on all surfaces to form a "protein corona" that can engage phagocytic receptors—the most notable of which is immunoglobulin-G (IgG) that can bind and activate macrophage Fc-receptors (FcRs).[16−18] The process is often referred to as opsonization and is representative of what has long been described for biomaterials: clean chemistry is invariably "fouled" *in vivo*. PEGylation is a classic approach to prolong nanoparticle circulation and tends to delay protein physisorption to surfaces, but clearance is only delayed.[19−22] Opsonization leads to macrophage interactions, but interactions of cells with materials are further modulated by physical properties, such as rigidity, size, and curvature (shape), which have all proven to be factors that influence nanoparticle clearance by macrophages.[23,24] The limitations of a short circulation half-life of nanoparticles due to macrophage uptake provide an opportunity to modify them in order to make them more like "Self" (Figure 2).

Conjugation of CD47's extracellular domain of ∼100 amino acids (via biotinylation) to avidin-coated, cell-sized, polystyrene beads proved sufficient to inhibit the engulfment of the beads when opsonized with antiavidin IgG.[6] Importantly, CD47 had no effect on beads that lacked IgG. The microbead results encouraged nanobead studies and also motivated synthesis of a related 21-amino-acid "Self" peptide.[25] *In vivo* tests showed CD47 and the "Self" peptide both increased circulation half-life of nanobeads in mice by delaying splenic

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macrophage clearance, greatly enhancing tumor imaging and drug delivery to the tumor.[25] Subsequent studies by a separate lab attached the "Self" peptide to nanosheets of graphene oxide, reporting similar results that concluded that the "Self" peptide is more effective than PEGylation.[26] Other laboratories have also shown that functionalizing nanomaterials with recombinant CD47 or "Self" peptides typically prolonged circulation, suppressed clearance, and improved therapeutic responses.[27−30] One additional application attached the "Self" peptide to nanoliposomes and found that they saturate and passivate liver macrophages, unlike control nanoliposomes, thus increasing the circulation and efficacy of subsequently injected nanoparticles.[31] Whether the deposition of serum-opsonizing IgG on these diverse nanoparticles has a role in the results is generally unclear. Nonetheless, the various studies highlight the utility of conjugating CD47 or shorter "Self" peptides to a diverse range of nanomaterials for many types of applications.

"SELF" ON VIRUSES SUPPRESSES PHAGOCYTOSIS AND ENHANCES GENE DELIVERY

Virus-based gene delivery is in wide clinical use for vaccines (such as Spike protein of SARS-CoV2) and for *ex vivo* engineering of cells (such as CAR T cells). Lentiviral and adeno-associated viral vectors are the most common in efforts to deliver genes to targets intraveneously,[32] but macrophages are again stimulated to eliminate these "natural nanoparticles", possibly resulting in virally induced inflammatory reactions.[33−35] Many groups have tried to suppress MPS-mediated elimination of viruses by conjugating synthetic polymers to minimize opsonization; however, such modifications sterically hinder critical protein interactions for virus binding to the desired target.[36,37]

Lentivirus is generally harvested after exocytosis from a cell line, and so overexpression of the membrane protein CD47 by a suitably engineered cell line can in principle generate CD47 displaying Lentivirus. Two separate studies have indeed generated CD47-Lenti and shown reduced macrophage interactions and improved delivery of genes. The first study delivered red fluorescent protein (RFP) with control or CD47-Lenti to differentiated human macrophage cultures and showed the following: (1) transduction by CD47-Lenti was ∼3-fold lower than the control, and (2) SIRPα-expressing A549 lung adenocarcinoma cells were preferentially transduced by CD47-Lenti.[38] The latter suggests that SIRPα serves as a docking receptor for CD47-mediated attachment and infection. Similar results were observed *in vivo*: transgene expression was higher in A549 tumors with CD47-Lenti, while liver and spleen macrophages showed significantly decreased expression relative to controls. Additional experiments, such as antibody-based inhibition of SIRPα interactions, were conducted to validate specificity. A second study used human-CD47 to increase the efficiency of the liver gene transfer by lentivirus.[39] After determining that liver macrophages clear intravenously administered lentivirus, CD47-Lenti increased gene transfer to hepatocytes and decreased transfer to macrophages. The assays were done in both NOD mice, which express SIRPα that binds to human-CD47, and C57BL/6 mice with weaker affinity. Clearance of CD47-Lenti proved greater in the C57BL/6 mice. Safety and efficacy were further demonstrated in nonhuman primates that have higher sequence homology of CD47 and SIRPα to humans. These studies show that displaying CD47 protects a membrane-enveloped virus such as lentivirus from macrophage elimination, thereby enhancing the efficacy of gene transfer therapies.

Similar to lentivirus, display of the "Self" peptide on the clinically relevant adeno-associated virus vector (AAV) resulted in reduced phagocytic susceptibility of the AAV *in vitro*.[40] Because AAVs do not have a membrane envelope, the "Self" peptide was directly introduced into an AAV2 capsid protein and flanked by glycine-serine linkers to ensure capsid stability and minimize viral titer loss. Such insertion had little to no impact on transduction efficiency but reduced virus uptake up to 10-fold in human macrophages when compared to control AAV2. This difference was again lost upon blocking with anti-SIRPα antibody. AAV is only 20 nm in size, whereas lentivirus is ∼100 nm, and since CD47-SIRPα is a specific inhibitor of phagocytic uptake and not endocytosis, the results to date with CD47-conjugated viruses underscore the high efficiency of macrophage phagocytosis of nanoparticles.

Phagocytosis is often cited in the cell biology literature as relevant only for larger entities (particles, apoptotic cells, or microbes), but early experiments with particles of widely different sizes did not adequately consider particle buoyancy differences and other size effects (per discussion elsewhere[25]). If few small particles settle, then few small particles will be taken up. However, buoyancy is unimportant *in vivo*. Macrophages in the liver and spleen are prominent in the above studies with nanoparticles and viruses because macrophages line the blood vessels in these tissues, facilitating direct and immediate access to intravenously injected particles. Macrophages are nonetheless resident in all tissues and are commonly a major cell presence at disease sites such as tumors or at sites of puncture or injury.[41] Uptake pathways are also important to end points: for example, phagosomes are more oxidative and destructive to cargoes than endosomes. All of these factors have implications for the billions of viral doses being injected as vaccines, such as the DNA delivered by adenovirus in the SARS-CoV2 vaccines from Johnson & Johnson or Oxford AstraZeneca.

SOLUBLE ANTAGONISTS OF CD47-SIRPα ENHANCE PHAGOCYTOSIS

CD47 is ubiquitously expressed, but not only overexpression of CD47 on ovarian cancer was documented many decades ago, but antibody targeting for tumor imaging before CD47 was also sequenced and eventually shown to inhibit phagocytosis.[42] Antibody targeting of CD47 on other cancers subsequently followed with evidence of a therapeutic window for human tumor xenografts, although it was initially unclear whether the IgG inhibited phagocytosis and/or activated FcR-driven phagocytosis.[43−45] Moreover, the single study[45] of syngeneic mouse tumors in mice treated with anti-mouse CD47 was the subject of a focused reproducibility study that showed no hint of any antitumor effect with anti-CD47 but did show anemia.[46] This latter, negative result for *monotherapy* has largely translated to anticancer efforts in the clinic and seems consistent with the fact that CD47-knockout mice are nearly normal, with minimal defects, and no measurable anemia.[1] This latter observation by an immunology lab raised considerable doubt among hematologists about CD47's claimed "Marker of Self" function.

Antagonizing the CD47-SIRPα macrophage checkpoint in combination with an "eat me" signal is promising, in contrast to monotherapy, and has prompted an explosion of work on

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soluble antagonists. These range from various IgG designs and recombinant proteins in the clinic to small peptides, all of which serve as possible drugs with varying efficacies against numerous liquid and solid malignancies (Figure 3).[12,47−49]

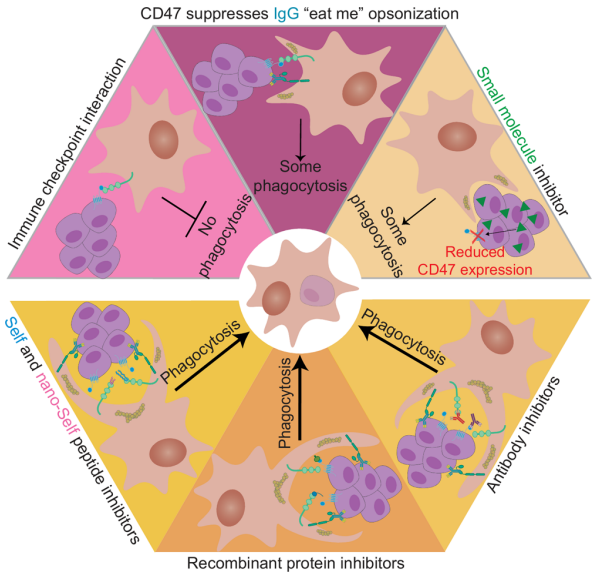

Figure 3. Soluble antagonists of SIRPα-CD47 for immunotherapy. Tumor cells express macrophage checkpoint CD47, which inhibits phagocytosis. IgG opsonization alone is insufficient to prompt efficient phagocytosis due to the CD47 "don't eat" signal, but various strategies can antagonize this inhibition. At least three immunotherapeutic strategies are currently pursued in preclinical and clinical studies: antibodies against CD47 or SIRPα, soluble versions of these proteins as inhibitors, and related "Self" peptide antagonists. Small molecules (green triangles) might eventually be developed to suppress transcription of CD47 but would still require an "eat me" signal.

The most advanced anti-CD47 treatment is a humanized IgG4 monoclonal antibody named magrolimab (or Hu5f9-G4) that binds CD47 and inhibits its binding to SIRPα without soliciting macrophage activation due to weak IgG4 affinity for macrophage FcRs.[50−52] Nonetheless, expression of CD47 on virtually every cell in the body constitutes an "antigen sink" with indiscriminate binding of magrolimab and other CD47-targeting inhibitors resulting in unavoidable on-target binding toxicities, such as anemia and thrombocytopenia.[53,54] Ongoing efforts to address this safety concern include development of nanobodies with strong CD47 binding and antitumor activity but low affinity for human RBCs.[55]

Targeting the SIRPα receptor might prove safer, as its expression is more restricted, although SIRPα expression extends beyond myeloid lineages to cells such as epithelial cells.[56,57] Some studies have indeed suggested that the anti-SIRPα blockade is as effective as anti-CD47 but maintains safe hematological profiles.[58,59] An engineered macrophage approach further demonstrated that a blockade of SIRPα coupled with priming of FcRs with tumor targeting IgG's is efficacious in shrinking established tumors.[41]

Multivalent 8-amino-acid "nano-Self" antagonists have recently been made based on CD47's β-hairpin loop that binds SIRPα. Variants of the peptides potently blocked CD47-SIRPα interactions and increased internalization of antibody-opsonized human erythroleukemia cells by human macrophages at concentrations as low as 5 nM.[60] Additional observations in the same study included further evidence for CD47 on the macrophage interacting in *cis* with SIRPα on the same cell, conveying an autoinhibition signal in agreement with earlier observations.[61] However, not all studies with soluble CD47 polypeptides added to cultured macrophages have shown increased phagocytosis. Curiously, an early investigation with bacterially expressed human-CD47 protein reported that *soluble* CD47 reduced *in vitro* phagocytosis by mouse macrophages of colloidal emulsions (for 2 h).[62] Subsequent studies by other groups have shown that the CD47 interaction improves with a post-translational N-terminal modification that is lacking in bacteria,[47] that the particular human−mouse CD47-SIRPα interactions are especially weak, and that IgG opsonization of the target is likely needed to reveal the effect of CD47-SIRPα blockade. All of these remain important considerations for the field going forward.

CONCLUSIONS

The SIRPα-CD47 axis is an increasingly appealing candidate for a diverse range of delivery and therapy applications. Nanoparticles and viruses that display CD47 or related peptides are recognized as "Self" by macrophages, delaying phagocytosis of these particles, prolonging circulation, and increasing on-target delivery of dyes, drugs, and genes. Further studies are needed to understand pro-phagocytic signals (i.e., opsonization and protein corona formation) on these nanoparticles and viruses. Soluble antagonists of this axis continue to be developed and explored to enhance phagocytosis, particularly of cancer cells, and illustrate the dual uses of developments in this area of research. Challenges remain in limiting off-target effects after systemic injection of antagonists such as anti-CD47 IgG. At least one recent and interesting effort used nanoparticles to both block CD47 and opsonize cancer cells,[63] but of course this requires nanoparticle avoidance of macrophages as well as access to tumor cells. Small size helps with permeation into solid tumors, and a recently synthesized and compact, cyclic version of "nano-Self" was shown to enhance engulfment *in vitro* of mAb-targeted melanoma by primary macrophages, setting the stage for efficacy tests *in vivo*.[64]

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Notes

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