Minimal "Self" Peptides That Inhibit Phagocytic Clearance and Enhance Delivery of Nanoparticles

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Foreign particles and cells are cleared from the body by phagocytes that must also recognize and avoid clearance of "self" cells. The membrane protein CD47 is reportedly a "marker of self" in mice that impedes phagocytosis of self by signaling through the phagocyte receptor CD172a. Minimal "Self" peptides were computationally designed from human CD47 and then synthesized and attached to virus-size particles for intravenous injection into mice that express a CD172a variant compatible with hCD47. Self peptides delay macrophage-mediated clearance of nanoparticles, which promotes persistent circulation that enhances dye and drug delivery to tumors. Self-peptide affinity for CD172a is near the optimum measured for human CD172a variants, and Self peptide also potently inhibits nanoparticle uptake mediated by the contractile cytoskeleton. The reductionist approach reveals the importance of human Self peptides and their utility in enhancing drug delivery and imaging.

Macrophages evolved to engulf and clear invading microbes and dying cells, but they respond similarly to injected particles, viruses, and implants, which hampers delivery of therapeutics and imaging agents. Coating of nanoparticles or liposomes with polyethylene glycol (PEG) creates "stealth" brushes that mimic a cell’s glycolalx and that delay immune clearance of foreign particles (1–3), but brushes can also hinder uptake by diseased cells (4). Neither a polymer brush nor a glycolalx stops adsorption of abundant serum proteins, such as immunoglobulin G (IgG) (table S1), which promote clearance (e.g., (1–3, 5)), and any foreign polymer can also be immunogenic (6). Targeting diseased cells with ligand-modified particle constructs might make brushes unnecessary, but some ligands also promote rapid clearance by phagocytes (2, 7). In the reductionist approach here, we make a synthetic, human-based "Self" peptide that specifically binds and signals to phagocytes to inhibit clearance of particles as small as viruses.

CD47 glycoprotein is a putative "marker of self" in mice (8) and is expressed on all cell membranes in humans, mice, and other mammals (9). It associates in cis with integrins (10) and other species-specific, immunogenic complexes on cells (11–13). Mouse knockouts of CD47 (mCD47) are viable, but when red blood cells (mRBCs) from these mice are injected into the circulation of control mice, the deficient cells are cleared within hours by splenic macrophages, whereas normal mRBCs circulate for weeks (8). CD47's extracellular domain interacts with CD172a (also known as signal regulatory protein-α, SIRPα) on phagocytes (10). Although binding is typically species-restricted (13), SIRPα is highly polymorphic, even within a species (14). Macrophages in nonobese diabetic/severe combined immunodeficient (NOD/SCID) strains of mice express a SIRPα variant that cross-reacts with human CD47 (hCD47), which explains why human hematopoietic cells engraft and circulate in NOD/SCID better than any other mice (14, 15). In vitro, the CD47-SIRPα interaction inhibits mouse macrophage uptake of antibody-coated mRBCs (8), as well as human macrophage uptake of both human RBCs (hRBCs) and hCD47-coated microparticles (16). This is not surprising as SIRPα signaling inhibits contractility-driven uptake of micron-size cells and particles (16). However, contractile forces are widely considered unimportant to internalization of nanoparticles and viruses, and so it is unclear whether this inhibitory interaction could be exploited in nanoparticle-based therapeutics.

We addressed whether hCD47 and a synthetic Self peptide can minimize phagocytic uptake of nanoparticles and thereby enhance delivery in NOD/SCID mice with X-linked severe combined immunodeficiency (il2rg−/−) mice (NSG). We first showed that blocking mCD47 accelerates clearance of mRBCs in NSG mice. Cells (or nanobeads) were split into two samples, with one sample labeled by red fluorophore and the other sample labeled by far-red fluorophore plus antibody against mCD47. The samples were mixed 1:1 for injection into the same mouse, and blood samples at subsequent time points were analyzed by flow cytometry for both colors, which produced a ratio (fig. S1A) that minimizes mouse-to-mouse variations. IgG and other serum proteins physiobor in vivo to RBCs (17), viruses (18), and PEGylated nanoparticles (19) (table S1), but in NSG mice, IgG is very low or not detectable (versus ~100 μM in normal animals). Controlled opsonization with IgG was therefore used in most of our studies to better mimic immune-competent animals. With RBCs, mRBC-specific antibody was added to promote clearance via phagocytosis (20). Consistent with a marker-of-self function of mCD47, the persistence ratio for the mixed sample [mRBCs/mRBCs with blocked mCD47] increased exponentially with a doubling time (T) of 33 min (Fig. 1B); single-color analyses also give T = 30 min (Fig. S1B).

RBC membranes have hundreds of different interacting proteins, and many involved in clearance are different for mouse and humans (12, 21). To give a better-defined surface for reductionist studies in vivo and also to begin testing the marker-of-Self concept on foreign particles of potential use for imaging and therapy, the extracellular immunoglobulin-like domain of hCD47, which binds SIRPα, was recombinantly expressed; site-specifically biotinylated; and then bound to streptavidin-coated, 160-nm polystyrene nanobeads. Beads were also labeled with red or near-infrared dyes (or left unlabeled) and controllably opsonized with either antibody against streptavidin (fig. S1C) or a biotinylated antibody for targeting (fig. S1D). After injection into an NSG mouse, blood analysis by flow cytometry clearly identified nanobeads on the basis of both distinctive scatter and fluorescence detection of the opsonizing antibody + hCD47 (Fig. 1A and fig. S1, A and C). A persistence ratio for [(nanobead + hCD47)/nanobead] was well-controlled at every time point and again increased exponentially with a doubling time (T) of 33 ± 8 min (Fig. 1C). Mice injected with a single color of nanobead gave similar results (T = 31 min) (fig. S1E). PEG-biotin nanobeads that were also preopsonized showed a flat persistence curve (T > 200 min), consistent with the fact that PEG brushes alone do not directly inhibit clearance by macrophages (fig. S2, A to C). In the absence of preopsonization, PEG-nanobeads circulated for hours as expected, but hCD47, once again, enhanced circulation (fig. S2D). hCD47 on virus-size particles is thus an inhibitor of in vivo clearance and thereby prolongs circulation.

Minimization of the 117–amino acid immunoglobulin-like domain of hCD47 to a small, binding-site SeSelf peptide could provide key evidence that signaling to mouse SIRPα (mSIRPα) is part of the molecular mechanism for inhibiting clearance in vivo. A crystal structure of hCD47–mSIRPα suggests three distinct binding sites (22), but the highest density of interactions are in one loop in hCD47 between canonical β strands F and G, where a nine–amino acid sequence constitutes 40% of hCD47’s contacting residues (Fig. 1A, structure). We designed by simulation a 21–amino acid SeSelf peptide around this sequence with the aims of minimizing species specificity (13) and eliminating glycosylation of CD47, which impedes binding (23). Biotinylation on an amino-terminal PEG linker provided a means of attachment to streptavidin beads for in vivo studies. Relative to control nanobeads, the SeSelf peptide increased the persistence of beads in the circulation with a mean doubling time, T, of 20 ± 3 min, whereas Scrambled peptide (see supplementary materials) had little impact on circulation (Fig. 1C). An apparent difference in persistence in the circulation between SeSelf peptide and hCD47 is not significant (P = 0.18).
Prolonged circulation of hCD47 beads and Self beads is based on a delay of phagocytic clearance by the spleen and perhaps liver, but nanobeads localize nonetheless to these organs in whole-body imaging of near-infrared fluorescent (NIRF) beads (Fig. 2A and fig. S3, A to D) by interactions that are likely similar to those that promote RBC adhesion in spleen and greatly increase splenic hematocrit (24). Recombinant mCD47 also enhanced persistence in circulation and again showed moderate but suppressed splenic localization (fig. S3B). For additional insight into persistent circulation and potential therapeutic application of Self-nanoparticles, human-derived A549 lung adenocarcinoma epithelial cells were grafted into the flanks of NSG mice. NIRF nanobeads were injected into the tail veins weeks later, and the tumors were imaged both in vivo and ex vivo to quantify accumulated signal. As early as 10 min post injection, hCD47 and Self nanobeads gave mean tumor intensities twice those of noninjected mice, whereas control beads gave background-level signal (Fig. 2B). With hCD47 and Self nanobeads, the fluorescence at every time point is statistically similar but significantly higher than that of control beads (P < 0.05), and the increase fits first-order kinetics (τ = 52 min), consistent with enhanced perfusion and progressive clearance. At 40 min, both hCD47 and Self nanobeads give higher signals than controls, ~10 to 20 times as much, and a second injection of hCD47 nanobeads after 2 hours showed a similar signal increase (fig. S4, A and B). Tumor accumulation fits a first-order process (with τ = 52 min giving T = τ ln 2 = 36 min) that is much faster than control beads (T = 210 min), and both time scales are similar to those obtained for persistent circulation (Fig. 1C), consistent with the hypothesis that enhanced tumor signal results from persistent circulation.

After in situ imaging, tumors and other major organs were subsequently excised and imaged ex vivo (fig. S4C). The Self beads and hCD47 beads show at least 16- to 22-fold enhancement of the very low signals obtained with control beads either with or without Scrambled peptide, with no statistical difference between Self peptide and hCD47. The fraction of nanobeads in the blood within the tumor is small (fig. S4C, inset), and so the majority of signal derives from beads that have accumulated in the tumor, most likely by enhanced permeation and retention (EPR) through the leaky vasculature that is characteristic of many solid tumors (25). On the basis of these results, the hydrophobic anticancer drug paclitaxel (Tax) was loaded into the Self nanobeads, as well as into beads with PEG and/or antibody against hCD47 (fig. S5, A and B). The latter antibodies have been used therapeutically to mask self on cancer cells (26), but targeting antibodies are a double-edged sword when attached to beads, because they also promote clearance (fig. S1D). Tax-loaded beads that displayed either recombinant hCD47 (fig. S5C) or Self-peptide plus PEG and hCD47-targeting antibody (fig. S5D) consistently shrink tumors more than beads lacking Self. The Self beads also did as well or better than the standard paclitaxel.

Fig. 3. Persistence of hCD47 and Self nanobeads depends on hCD47 density, consistent with low-affinity binding to NSG mSIRPα relative to hSIRPα variants. (A) The number of hCD47 molecules on the 160-nm beads 35 min after injection was either measured in two-color experiments (solid symbols; n = 7 mice) or single-color experiments (open symbols; n = 6 mice), with an average of 55% protein lost in circulation (inset). Self peptide levels are estimated to have a similar loss (n = 4 mice). Fluorescent nanobeads (PKH26 in flow cytometry, top) were confirmed by forward and/or side scatter, and fluorescent hCD47-specific antibody measured hCD47 levels (left, control nanobead sample; right, hCD47 nanobead sample). The inhibition curve gives Kd = 110 molecules/nanobead. (B) Affinity of soluble hCD47 for NSG neutrophils and monocytes, from flow cytometry analysis of Cy5-biotin–specific antibody. Lymphocytes are negative for SIRPα and do not bind soluble hCD47. (Inset) Preinjection of blocking anti-mSIRPα eliminates the enhanced circulation of Self nanobeads of Fig. 1C. After 35 min, most splenic macrophages (F4/80+) that displayed either recombinant hCD47 (fig. S5C) or Self peptide (attached to biotin via either PEG or C6, 6-aminohexanoic acid) significantly inhibits phagocytic uptake (P < 0.05 different from control). All data are means ± SEM.

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To compare the effective affinity of NSG mouse to human SIRPα (hSIRPα), 10 human polymorphic variants of hSIRPα (14) were expressed on Chinese hamster ovary (CHO) cell membranes (24). Saturation binding of soluble hCD47 to each variant yielded a 60-fold range of affinities with $K_d = 0.08$ to 5 μM (Fig. 3C and fig. S7), even though all of the amino acid differences in the variants are outside of the binding interface (22). When plotted against the allele frequency of hSIRPα variants of intermediate affinity (e.g., v1 and v2) are most common, with affinities similar to that for soluble SIRPα(v1) binding to CHO-displayed hCD47 (23). hCD47's affinity for NSG-SIRPα phagocytes is within the range of reported hSIRPα variants and so is the affinity of SIRPα for the most common SIRPα variant. Synthesis and simulations of additional peptides (Fig. 3D and fig. S8) reveal a sensitivity of binding to conformation, as well as sequence, and show that a lack of affinity for hSIRPα is predictive of a failure to inhibit in vitro phagocytosis.

Whether phagocytosis of nanoparticles—including viruses—involves mechanisms similar to those for larger particles remains an open question (28). Tissue sections show that nanobeads colocalize with macrophages (Fig. 4A, i). In cultures of human-derived monocytic cell line THP1 macrophages, nanoparticles are not dense enough to settle and contact cells, but opsonized nanobeads that are added at the same total surface area as microbeads (fig. S9A) are taken up with equal efficiency (Fig. 4A, ii, a). Myosin-II accumulates at the phagocytic synapse formed with opsonized beads except when hCD47 is attached (Fig. 4A, ii, a and b). Microparticles and microbes (i.e., bacteria) give similar images (fig. S9B), and inhibition of phagocytic uptake by hCD47 is indeed independent of particle size from at least 100 nm to 10 μm (Fig. 4A, ii, c; and fig. S9C). Similar inhibition of nanobead uptake was found with biotinylated Self peptides, whereas both Scrambled peptide and disulfide-bridged peptide (Self-SS) showed no significant inhibition of uptake (Fig. 3D, i and ii). The potency of hCD47 is remarkable with $K_{in vitro} = 1.0 ± 0.3$ molecule per 45,000 nm². Equivalently, a nanobead of 60-nm radius requires only one CD47 molecule to inhibit uptake. $K_{in vitro}$ is the same as the lowest densities reported for hRBCs (12) and is far smaller than PEG densities needed to enhance nanoparticle circulation through delayed opsonization (e.g., fig. S2A: >1 PEG per 20 nm²).

IgG-driven uptake is linear in myosin-II activity (16). With the nanobeads described here, both drug inhibition of myosin-II and hCD47 inhibit uptake by up to 80% (Fig. 4A, ii, c). When CD47 binds SIRPα, SIRPα's cytoplasmic tail is hyperphosphorylated to activate SHP1 phosphatase (29), which dephosphorylates multiple proteins, including myosin-II (16). Inhibition of SHP1 produces the expected increase in phagocytosis of hCD47 beads (Fig. 4A, ii, c; and fig. S9D), and binding of hCD47 and Self peptide indeed increases phospho-SIRPα (Fig. 4B). Consistent with a common mechanism in vitro and in vivo, uptake of the various nanoparticles by THP1 cells correlates inversely with persistence in NSG mice (Fig. 4C).

Phagocytes pervade all tissues and disease sites, with key roles in recognition and clearance, as well as contributions to pro- and anti-inflammatory responses with cytokine release and oxidative burst. Whether synthetic Self peptides, CD47, or its homologs are displayed on particles, viruses (30), or artificial surfaces (31), “active stealth” signaling across length scales (fig. S10) offers additional opportunities in application as well as understanding. In particular, the SIRPα polymorphism results suggest that an intermediate affinity for Self is optimal (Fig. 3C) as a trade-off between adhesion that is not too strong (“must let go”) and signaling that is not too weak (“don’t eat me”). Additional homeostatic self factors seem likely and might similarly be used to further avoid phagocytes and thereby enhance delivery of therapeutics and imaging agents.

References and Notes
Caspase-11 Protects Against Bacteria That Escape the Vacuole

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Caspases are either apoptotic or inflammatory. Among inflammatory caspases, caspase-1 and -11 trigger pyroptosis, a form of programmed cell death. Whereas both can be detrimental in inflammatory disease, only caspase-1 has an established protective role during infection. Here, we report that caspase-11 is required for innate immunity to cytosolic, but not vacuolar, bacteria. Although Salmonella typhimurium and Legionella pneumophila normally reside in the vacuole, specific mutants (sifA and sifA, respectively) aberrantly enter the cytosol. These mutants triggered caspase-11, which enhanced clearance of S. typhimurium sifA in vivo. This response did not require NLRP3, NLRC4, or ASC inflammasome pathways. Burkholderia species that naturally invade the cytosol also triggered caspase-11, which protected mice from lethal challenge with B. thailandensis and B. pseudomallei. Thus, caspase-11 is critical for surviving exposure to ubiquitous environmental pathogens.

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Editor's Summary

Stealth Delivery

Delivery of therapeutics and imaging agents is hampered by the ability of the innate immune system to recognize and clear foreign particles. "Self" cells are protected from phagocytic clearance by the membrane protein CD47 that interacts with signal regulatory protein-α (SIRPα) on macrophages. Taking advantage of this protective strategy, Rodríguez et al. (p. 971) labeled nanoparticles with computationally designed minimal human-CD47–based peptide (hCD47). When injected into a strain of mice in which macrophages expressed a SIRP α that cross-reacts with hCD47, the peptide prevented clearance of nanoparticles and enhanced drug delivery to tumors.