

**INHIBITING PHAGOCYTOSIS WITH CD47: FROM THE EFFECTS OF RED CELL
RIGIDITY AND SHAPE TO DISPLAY ON LENTIVIRUS – IMPLICATIONS FOR AGING
AND GENE THERAPY**

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ABSTRACT

INHIBITING PHAGOCYTOSIS WITH CD47: FROM THE EFFECTS OF RED CELL RIGIDITY AND SHAPE TO DISPLAY ON LENTIVIRUS – IMPLICATIONS FOR AGING AND GENE THERAPY

Nisha G. Sosale

Dennis E. Discher

A macrophage engulfs another cell, or foreign particle, via phagocytosis, an engulfment process crucial not only to innate and adaptive immunity, but also to the maintenance of homeostasis. Phagocytosis is a receptor-mediated process that is dependent on Myosin-IIA motors, among other cytoskeletal proteins. Adhesion processes of both hematopoietic and mesenchymal derived cells can activate Myosin, and increasingly so on rigid substrates. Macrophage engulfment becomes inefficient if the macrophage also engages 'Marker of Self' CD47 that inhibits Myosin accumulation to the phagocytic synapse. CD47 is a ubiquitously expressed transmembrane cell surface protein that binds to signal regulatory protein alpha (SIRPA) that is highly expressed by macrophages. CD47's role in downregulating macrophage phagocytosis was first discovered in murine erythrocytes (RBCs), where wild-type RBCs are long-lived in circulation, while RBCs derived from a CD47 knockout mouse are rapidly cleared. More recently CD47 has been found to inhibit clearance of a variety of viable cell types including stem cells, leukocytes, platelets, and cancers. However, the limitations of CD47 as a 'marker of self' on apoptotic and experimentally oxidized cells are beginning to be realized, as CD47 surface expression does not effectively inhibit the phagocytosis of these aged cells.

Furthermore, while it is well known that macrophage-mediated clearance controls the removal of aged RBCs, that are reported to become rigid, from the bloodstream, the role that rigidification

plays in countering CD47 inhibitory signals remains to be clarified. To study the effects of RBC rigidity on the regulation of phagocytosis by CD47 expression, RBCs were controllably stiffened in different shapes without compromising CD47-SIRPA interactions. Uptake of antibody-opsonized human-RBC was accelerated, as expected, by blocking CD47, but was fastest with rigid RBC-Discocytes that mediated maximum levels of Myosin-IIA accumulation at the phagocytic synapse. Attenuation of the antibody-driven 'eat me' signal partially recovered 'Self' signaling by rigid RBC-Discocytes, and more rounded but rigid RBC-Stomatocytes also signaled 'Self' more efficiently. These results highlight the biophysical nature of the CD47-SIRPA inhibitory mechanism that can be overpowered by rigidity and can be rescued by target shape.

Resident tissue macrophages are adept in capturing non-self particles from the extracellular environment, including HIV-derived lentiviral gene therapy vectors, thus limiting efforts at therapeutic vector delivery. CD47-SIRPA inhibition has just recently been shown to inhibit *in vivo* macrophage clearance of nano-sized synthetic particles that are significantly smaller than previously studied mammalian cells. A novel lentiviral vector was engineered here to present an oriented human CD47-GFP fusion protein on the vector envelope. Using Total internal reflection microscopy (TIRFM), atomic force microscopy (AFM), and immunoblotting, we have demonstrated that engineered lentivectors display the CD47-GFP protein. In vivo results show that lentiviral vector display of CD47 increases circulation of the vector in the bloodstream, and reduces off-target transgene expression in splenic and liver macrophages. Use of this CD47-displaying lentiviral vector, further shows increased delivery to solid tumors. CD47 on the surface of lentiviral gene therapy vectors was found to attenuate off-target macrophage uptake of the vector, and thereby increase therapeutic efficiency.

The results here examine the boundaries of CD47's role as a 'marker of self'; CD47 inhibition is diminished on the surface of rigid self-cells, while an enveloped virus shows the potential to masquerade as self by taking its envelope from a cell with sufficient CD47. The observed role of red cell shape and rigidity in CD47's ability to signal self highlights the biophysical aspect of

phagocytosis with implications for CD47 inhibition of clearance of aged, diseased, and cancerous cells that differ from viable cells in their physical properties. Study of the novel CD47-lenti reveals the first evidence of CD47 being able to limit macrophage engulfment of a non-mammalian physiological entity, as current studies have as of yet been limited to mammalian cells and synthetic particles. This finding may have potential application in the understanding of disease progression and vaccine development for enveloped viruses such as HIV-1.

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CHAPTER 1: INTRODUCTION

(0) Significance & Motivation

More than 100 years ago Elie Metchnikov, while observing starfish larvae, noted that where a rose-thorn that had pierced the echinoderms translucent outer layer, the puncture was surrounded by ameboid-like cells he referred to as 'macrophage', which in greek translates to 'big eater' (Nathan 2008). These seminal studies marked the discovery of what is referred to today as the innate immune system and is made up in part by macrophages.

Macrophages carry out a diverse set of roles, key to which is phagocytosis, a cellular uptake or 'eating' process that allows for the clearance of foreign microbes, wound repair, and the bridging of innate and adaptive immune response by presenting engulfed antigens to lymphocytes (Janeway 2005, Lawrence 2011, Murray 2011). Resident macrophages are present in most tissues (Davies 2013). Understanding the mechanism and regulation of macrophage function is crucial to developing treatments towards a plethora pathological conditions caused by dysregulation of macrophage physiology, including Chrohn's disease (Baba 2013), Alzheimer's disease (Gate 2010), Asthma (Balhara 2012), and Cancer (Pollard 2009). Increased understanding is also crucial to the optimization of targeted therapeutics that must evade the immune system in order to reach target tissues (Scherphof 2006).

In the last two decades much has been discovered about the molecular mechanisms behind phagocytosis, a cytoskeleton-driven uptake process (May 2001, Swanson 2008, Flanagan 2012). Resident macrophages are adept at recognizing foreign pathogens through 'non-self' proteins on the pathogen surface (Loefering 1989), but also through the absence of 'self' proteins that include CD47. Cell-surface protein CD47 has been shown to inhibit phagocytosis of RBCs (Oldenborg 2000). SIRPA is an immunoinhibitory surface protein (Ravetch 2001), and ligand for CD47 (Jiang 1999). Signaling downstream of macrophage SIRPA affects the balance of kinase- and phosphatase-mediated phosphorylation and regulation of proteins involved in cytoskeletal

mobilization in phagocytosis. These signals can respectively activate and deactivate the macrophage cytoskeleton during phagocytosis (Tsai 2008).

CD47 inhibition has been best characterized in its inhibition of FcγR mediated phagocytosis (Oldenborg 2000), and, FcγR is the most studied phagocytic uptake mechanism (Flannagan 2012). CD47 inhibits phagocytosis of a range of viable cell types including erythrocytes (Oldenborg 2001, Tsai 2008), leukocytes (Gardai 2005), stem cells (Blazar 2001), and some cancers (Weiskopf 2013). While CD47 makes phagocytosis of viable cells inefficient, it remains to be clarified how cellular aging allows pro-phagocytic signals to dominate CD47 inhibition and thus the efficient clearance of aged cells. Details of the mechanism of uptake of aged or apoptotic cells continue to be elucidated, and if and how CD47 may regulate this uptake is of key importance to understanding both homeostasis and disease (Tada 2003, Gardai 2005, Olsson 2008). **Here we examine how rigidity of a phagocytic target affects CD47 inhibition of macrophage phagocytosis.**

For more than 3 decades erythrocytes have served as model systems for understanding macrophage phagocytosis (Flannagan 2012), including the seminal studies that identified CD47 as a marker of self (Oldenborg 2000). The finely tuned production and turnover of erythrocytes, that is in part dependent on macrophage phagocytosis, is an essential physiological process. Efficient clearance of apoptotic cells is key to tissue homeostasis and to minimizing lysis-induced inflammation (Erwig 2008). Thus, erythrocytes are a logical and relevant model for understanding CD47's role in the clearance of aged and cells that become rigidified with time in circulation (Chasis 1986). Aged, apoptotic and diseased cells differ from viable cells in many ways (DeCathelineau 2003) including cell rigidity and shape (Mohandas 2008). As cells age they become oxidized by prolonged exposure to reactive oxygen species, and this is especially relevant to erythrocytes that are responsible for oxygen transport between the lungs and tissues (Barelli 2008). This oxidation can trigger chemical changes (Lang 2014) including production of physiological aldehydes and protein cross-linking (Jain 1983) that can ultimately lead to

rigidification of the aged cell. The study of CD47 inhibition on rigidified erythrocytes may also be relevant to diseased cells that become rigidified, such as sickle cell erythrocytes (Itoh 2002) and chemotherapy-treated cancers (Lam 2007).

Studies of the CD47 inhibition mechanism have been thus far limited to mammalian cells and synthetic particles. The mechanism of phagocytosis evolved before the rise of multicellular organisms, while CD47 is restricted to higher organisms, namely mammals. This suggests that the CD47-SIRPA inhibitory signaling mechanism arose with multicellular organisms whose immune systems have a dual charge—first, protect ‘self’ and second, attack ‘non-self’. The work here extends our knowledge of the CD47 mechanism into the role of CD47 in macrophage clearance of lentiviruses. An enveloped poxvirus (Family, Poxviridae, Genus Leporipoxvirus) is reported to encode a CD47 homologue (Cameron 2005), and enhances the pathogenicity of the virus. This initial evidence led to the hypothesis driving this study: **CD47 display by enveloped lentiviral vectors may inhibit their uptake by macrophage and provide the potential for improving lentiviral vector-mediated gene therapy.**

There have been recent successful lentiviral vector clinical trials in the treatment of genetic disorders including as thalassemia and Wiskott Aldrich syndrome, that are both caused by a missing or defective gene (Aiuti 2013). However, gene-therapy treatments have been limited to ex-vivo lentiviral treatments of stem cells, followed by transplantation. This is in part because in-vivo delivery is currently challenged by efficient macrophage clearance of the therapeutic vector thus limiting delivery to target cells (Follenzi 2002, Van Til 2004). Enveloped HIV viruses (Family Retroviridae, Genus Lentivirus) derive their viral envelope from the lipid bilayer of the host mammalian cell (Desport 2010). In this work, controlled studies of a novel CD47-displaying lentiviral vector allowed for increased understanding of how viral-CD47 display affects macrophage uptake, and how this impacts in vivo clearance and gene delivery to a target cell.

This dissertation work addresses several questions regarding the regulation of phagocytosis by CD47 in two specific clinically relevant contexts: (1) rigidified

erythrocytes and (2) lentiviral vector gene therapy. The work here is motivated by the goals of furthering the basic insight into CD47-inhibition of macrophage phagocytosis, and gaining understanding of how physical parameters such as rigidity and shape affect the immuno-inhibitory mechanism of CD47. Here we aim to extend understanding of the CD47 mechanism in the context of rigidified erythrocytes and lentiviral gene therapy vectors. **The following introduction provides background on (1) Macrophage Phagocytosis, (2) CD47-SIRPA Inhibition of Phagocytosis, (3) macrophage clearance of aged erythrocytes, and (4) lentiviral vector mediated gene therapy.**

(1) Macrophage Phagocytosis

The earliest models define innate immunity as the rapid response of the immune system based on the relatively non-specific defense against non-self versus self-entities (Janeway 2005). A more recent model of immunity considers instead that the immune system distinguishes between entities that do damage versus those that do not (Matzinger 2002). This model may be relevant to the systems studied here where aged self-RBCs need to be efficiently cleared to prevent premature hemolysis and release of potentially inflammatory intracellular components (Erwig 2008). The model may also be more relevant to replication incompetent non-self lentiviral vectors that can potentially be life saving. Macrophages can recognize targets via bound molecules, including immunoglobulins, which can become deposited, and thus opsonized, both red blood cells (Franco 2013) and lentiviral vectors (DePolo 2000). Following opsonin recognition, a macrophage can engulf the recognized particle via a cellular uptake mechanism known as phagocytosis. Macrophages can distinguish self through SIRPA binding of CD47. The role of CD47-SIRPA signaling in regulating macrophage phagocytosis is the focus of this work. Specifically in the regulation of Fcγ receptor mediated macrophage phagocytosis.

FcγR signaling leads to the activation of macrophage cytoskeleton and phagocytosis. Some of the proteins involved in FcγR phagocytosis include the following: tyrosine kinases, adaptor proteins, lipid modification enzymes, Guanine nucleotide exchange factors, small GTPases, nucleation promotion factors, the Actin Nucleation Complex: Arp2/3. Myosin motors including Myosin II also become activated. Engagement of the Fcγ Receptor by IgG leads to clustering and activation of the receptors. The cytoplasmic domain of FcγR contains an ITAM (immunoreceptor tyrosine-based activating motif), a tandem YxxI/L motif. Following this clustering event, Src family kinases, known to be integral in early phagocytic events, phosphorylate ITAM tyrosine residues. Src family kinases involved include Hck, Lyn, Fgr (Ghazizadeh 1999). Src family kinase, Syk is the first kinase to become activated by FcγR ITAM. Early phagocytic events appear to be independent of Syk, but completion of phagocytosis requires Syk, as Syk^{-/-} mice are deficient in completion. Syk phosphorylation leads to recruitment of adaptor proteins involved in signaling: LAT, Grb2, Gab2, & Crk III. Lipids are critical to phagocytic signaling where changes in lipid composition are restricted to the phagocytic cup.

Macrophage pseudopods surround the target during phagocytosis (Swanson 1999). These pseudopods consist of a meshwork of filamentous actin polymers. Small GTPases are key to actin dynamics. Actin filaments are dynamic polymers formed by treadmilling, and actin-polymer formation requires actin nucleating complex Arp2/3. Arp2/3 is activated by both Nucleation promoting factors, WAVE and WASP, GTPase Cdc42, and phospholipids. In addition to filamentous actin filaments, myosin motors are also reported to be key to phagocytic uptake. Myosin II is recruited to early phagosomes where it is expected to play a role in pseudopod adhesion and closure (Vicente-Manzanares 2004). Phagocytic engulfment is proportional to myosin II activation, and this highlights the role they play in engulfment.

(2) CD47-SIRPA Inhibition of Phagocytosis

The CD47 protein consists of a single immunoglobulin superfamily domain, a hydrophobic transmembrane penta-spanning domain, and a cytoplasmic tail with several splice variants (Brown 2001, Matozaki 2008). CD47 ligands include integrins, thrombospondin-1, and SIRPA (Brown 2001). CD47 interactions with SIRPA are the focus of this work. SIRPA is mainly expressed in neurons, dendritic cells, and macrophages (Matozaki 2008). SIRPA may also be found on other cell types including neurons and epithelial cells. The extracellular portion of SIRPA consists of three Ig-domains that gives this receptor-ligand pair a unique '3+1' topology. However this can be expected to span a similar distance as canonical '2+2' Ig-domain synapse protein pairs (Greenberg 1994), such as that between phagocytic Fc γ receptors for immunoglobulin. CD47 is ubiquitously expressed on mammalian cells, and erythrocytes display 25,000 molecules per cell (Mouro-Chanteloup 2003). The major fraction of CD47 is immobile within the erythrocyte ankyrin membrane protein-complex (Dahl 2003), and is linked to the complex via an interaction with protein 4.2 (Dahl 2004). Fluorescence imaging micro-deformation (FIMD) studies indicate the minor fraction of CD47 is freely diffusible within the lipid bilayer (Dahl 2003). CD47 has been shown to be species specific (Subramanian 2006), and studies with human cells have been few thus far.

CD47 binding promotes the phosphorylation of tyrosine residues of SIRPA's cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) (Okazawa 2005, Matozaki 2008). SIRPA is among a family of ITIM-containing immune inhibitory receptors, that also includes, the B-cell inhibitory receptor PIR-B (Ravetch 2001). These serve as an important balance to signals generated from activating receptors, such as some classes Fc γ R receptors (**Figure 1**). The phosphorylated tyrosine's of SIRPA bind to, and appear to act as a substrate for, the Src homology 2 (SH2) domain-containing protein tyrosine phosphatases, SHP-1 and SHP-2, thereby activating the phosphatases (Kharitonov 1997). SHP-1 is reported to be predominantly

expressed in hematopoietic cells, and tends to be an inhibitory phosphatase, while SHP-2 is expressed in most cell types. Activated SHP-1 can deactivate Myosin-II (Baba 2003).

Macrophage interaction with CD47 displaying erythrocytes leads to decreased phosphorylation of a tyrosine residue in the myosin-II heavy chain, along with both reduced myosin-II accumulation at the phagocytic synapse and reduced phagocytic engulfment (Tsai 2008).

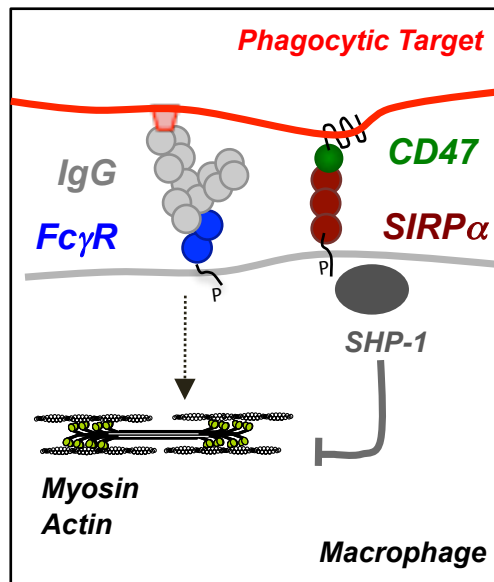


Figure 1. CD47-SIRPA Inhibition of Macrophage. CD47 binds to macrophage receptor, SIRPA, which can lead to the phosphorylation of the ITIM motif (Immunoreceptor Tyrosine-based Inhibitory Motif) located on SIRPA's cytoplasmic domain. The phosphorylated tyrosine's of SIRPA's ITIM are reported to act as a substrate for, the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-1 (Kharitononkov 1997). In parallel, Fc γ R receptors may bind to IgG that can specifically bind to the surface of a phagocytic target, and this leads to phosphorylation of the ITAM motif (Immunoreceptor Tyrosine-based Activation Motif) on the cytoplasmic region found on some classes of Fc γ R receptors. Cytoskeletal proteins, including myosin-II, become activated following Fc γ R ITAM phosphorylation. While, SHP-1 is reported to deactivate several proteins involved in phagocytic uptake, including myosin-II (Baba 2003). Thus, CD47-SIRPa signaling can inhibit myosin-driven Fc γ R mediated uptake.

The key in vivo evidence thus far for a marker-of-self role for CD47-SIRPA interactions comes from CD47-knockout mice. When red cells from these mice are injected into the circulation of control mice, the deficient cells are cleared within hours by macrophages in the spleen, whereas normal red cells circulate for weeks (Oldenborg 2000). Additional in vitro studies with both mouse and human red cells have largely confirmed that the “eat me not” signal can counter “eat me” signals on erythrocytes (Oldenborg 2001, Tsai 2008). In vitro studies of microparticles have shown that SIRPA activation increases to saturation with CD47 density indicating CD47’s real but limited ability to inhibit phagocytosis (Rodriguez 2013). In addition to erythrocytes, CD47 has been found to inhibit the uptake of leukocytes (Gardai 2005), stem cells (Blazar 2001), platelets (Olsson 2005), and cancer cells (Jaiswal 2009). However, CD47 inhibition of the uptake of apoptotic leukocytes (Gardai 2005) is limited. In apoptotic leukocytes, CD47 has been reported to localize to membrane patches distinct from those containing pro-phagocytic signals. How CD47 signaling may be affected by cellular aging is discussed in more detail in the next section.

(3) Macrophage Clearance of Aged Erythrocytes

Erythrocytes (RBCs, red cells) are enucleated cells that transport oxygen throughout the body (Bruce 2003). With up to 10^{10} red cells being produced per hour during hematopoiesis, the efficient removal of aged red cells is clearly an essential process. As erythrocytes pass through the lungs more than 1×10^5 times in their 120-day life span, they can accumulate oxidative damage (Lang 2014). Blood storage is also thought to cause oxidative stress similar to that which occurs in vivo (D’Amici 2007, Barelli 2008). Stored blood is generally cleared faster than normal (Deplaine 2011), and this contributes to clinical problems such as transfusion related acute injury (Hod 2010). As RBCs senesce, aldehydes can be produced, and experimental aldehyde rigidification increases RBC clearance from the circulation (Jain 1983). Aldehydes react primarily with amines in Lysine residues, to covalently cross-link proteins. Here, glutaraldehyde was used

to controllably cross-link and rigidify red cells, to understand the effect of cross-linking and rigidification on CD47 inhibition of macrophage phagocytosis.

Loss of CD47's protective role has long been hypothesized to impact clearance of senescent cells (Oldenborg 2000). Some reports indicate that during red cell storage, CD47 is lost from the cell surface, potentially via the release of protein containing membrane vesicles (Annis 2002, Kamel 2010, Stewart 2005, Fossati-Jimack 2002, Khandelwal 2007). However, other studies find no such loss in CD47 display with storage (Sparrow 2006, Holovati 2008). So, physical loss of CD47 does not seem to fully explain enhanced clearance of aged red cells. Oxidized and aged RBCs may differ from healthy RBCs in a number of ways besides decreased CD47 display. These can include CD47 clustering, increased IgG deposition, and changes in biophysical properties including cell rigidity and shape (Gardai 2005, Franco 2013, Mohandas 2008). The latter are the focus of this work as aged RBCs are cleared from circulation by two mechanisms that are both affected by the physical properties of the aged RBCs. These are physical exclusion from the vasculature and macrophage phagocytosis.

Healthy erythrocytes are reported to be readily deformable under applied shear stresses of that are similar in magnitude to those experienced by red cells in the circulation (Jain 1983). As they age, erythrocytes show decreased deformability relative to young cells. Deformability of red cells is crucial to their circulation through the microvasculature that can become as small as 3 μm in diameter (Deplaine 2011, Tsai 2012). Deformable discoid red cells are able to deform as they travel through small channels, and at times take on a parachute-like morphology (Skalak 1969). Aged red cells, that are more rigid than their younger counterparts, become trapped in splenic slits of the sinusoids to a greater extent than non-rigid cells (MacDonald 1987, Buffet 2011, Mebius 2008).

Macrophages are reported to contribute to the in vivo clearance of erythrocytes (Kay 1975, Oldenborg 2000). Fc γ R mediated phagocytosis is driven by the accumulation of myosin-II motors to a phagocytic synapse between a macrophage and a target cell. The motors drive actin

pseudopods to engulf the phagocytic target (Vincente Manzanares 2004). Work from our lab has shown that CD47 inhibits accumulation of myosin-II to the phagocytic synapse (Tsai 2008). While substrate rigidity drives cell spreading with assembly of stress fibers and polarization of non-muscle myosin-II for many cell types (Lo 2004, Engler 2006 Raab 2012, Oakes 2009, Shin 2014). Macrophages are mechanosensitive in adhesion and phagocytosis, and exhibit increased adhesion and cell spreading on stiffer substrates (Fereol 2006, Patel 2012, Blakney 2012). Macrophages have been shown to preferentially engulf rigid polyacrylamide beads, as compared to softer beads (Beningo 2002). Further, *in vivo* studies of controllably stiffened hydrogel particles, similar in morphology to RBC Discocytes, show that stiff gel particles are cleared more rapidly than soft particles, becoming entrapped in the lungs, among other organs. However, the aforementioned studies focus on non-physiological substrates, and thus the relevance of these findings to phagocytic uptake of cells that display CD47, is yet unknown (Merkel 2011).

Deformability also affects the shape of the target as perceived by the engulfing macrophage. For example fresh deformable erythrocytes take on a quasi-spherical shape during initial adhesion with macrophage. In contrast rigid RBCs retain the discoid shape. Target shape has been reported to be key to clearance. Spherical polystyrene particles are engulfed more readily than elliptical and cylindrical particles (Champion 2006). High resolution EM imaging shows that actin pseudopods adhere more uniformly at the synapse with spherical particles as compared to synapses with ellipsoids, in a manner dependent on the orientation of the non spherical particles. This may contribute to enhanced engulfment of spherical particles, but whether this phenomenon is relevant to CD47 inhibition of biological cells, specifically RBC Discocytes, is examined in this work.

Red cells pass through the lungs more than 10^5 times during their lifetime, causing them to be sensitive to oxidation that can lead to exposure to physiological aldehydes and subsequently cross-linking that increases cell rigidity. Rigidity may contribute to two mechanisms of clearance (1) physical entrapment in the vasculature and (2) macrophage phagocytosis (**Figure 2**). This

work focuses on the latter. Target rigidity is expected to activate macrophage Myosin-II, as observed in macrophage adhesion on rigid substrates. This counters CD47 inhibition of myosin II so that the inhibitory capacity of CD47-SIRPA to inhibit phagocytosis of rigidified cells is limited. This work thus aims to further clarify effect of target stiffness and shape on CD47 inhibition of phagocytosis.

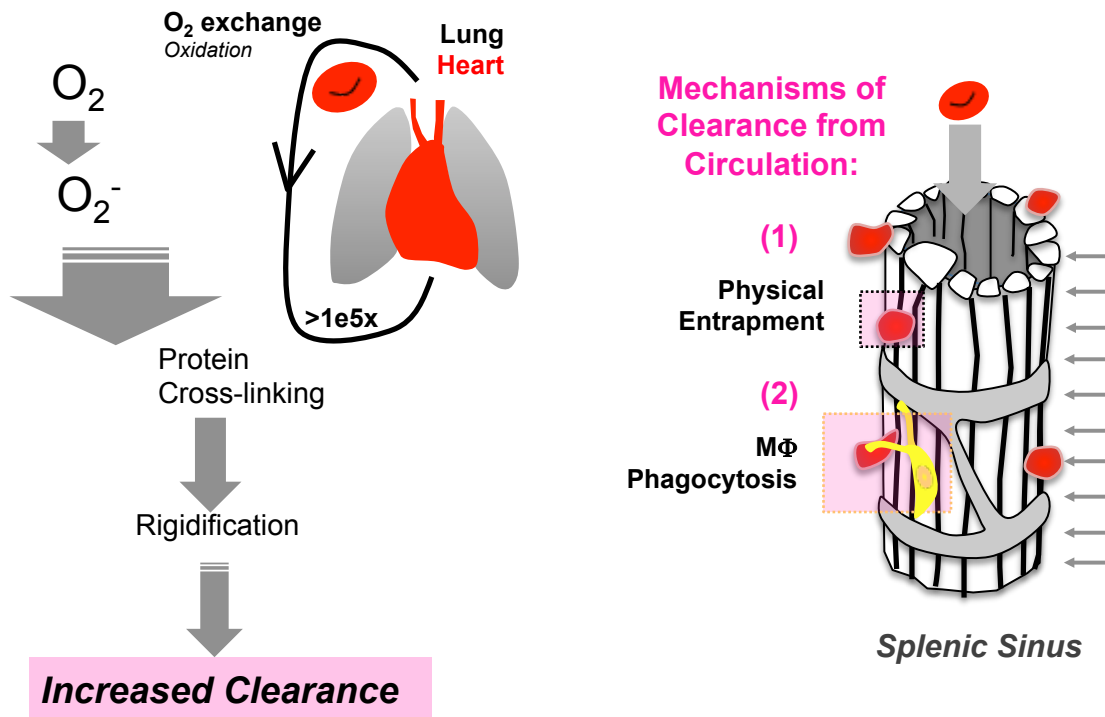


Figure 2. Mechanisms of rigidification and clearance of red blood cells. Red blood cells are pumped by the heart and passed through the lungs on the order of 1×10^5 times within their 120 day life-span. In doing so they become exposed to reactive oxygen species and aldehydes that can lead to protein cross-linking and thus rigidification of the aged cells. Aged cells can be cleared by two mechanisms throughout the body. These mechanisms are illustrated for example in a splenic sinusoid, where blood is filtered. (1) The rigidified aged cells can become physically entrapped within splenic slits as well. (2) They can also be cleared more readily by macrophage,

as macrophage phagocytosis is reported to be mechanosensitive, with more rigid particles being more readily engulfed (Beningo 2002).

(4) Lentiviral vector Mediated Gene Therapy

The therapeutic potential of Lentiviral Vectors (Lentis) is convincingly evidenced by the successful developments in ex vivo treatments of stem cells (Worsham 2006, Lee 2009) and recent clinical trials (Cartier 2009, Aiuti 2013). Lentis offer an advantage over carriers such as liposomes and other viral vectors in that they are capable of stably integrating genetic material in a wide range of cell types (Naldini 1996). Lentiviruses, unlike other retroviruses, can transduce non-replicating cells where the chromosomal DNA is not readily accessible (Naldini 1996). Lentiviral vectors are advantageous over Adenoviral vectors that have been found be very immunogenic, and now are mainly used in the context of vaccines where an adaptive immune response is the desired outcome (Varnavski 2002). The lentivector can carry a relatively large transgene and have been reported to have no upper bound in transgene size, with inserts ~18kb being packaged (Kumar 2001). There is no report of cellular transformation as a result of lentiviral transduction, which is an advantage over other retroviral vectors such as Murine Leukemia vectors (Hacein-Bey Abina 2003).

The recent clinical trials (Cartier 2009, Aiuti 2013) were limited to ex vivo lentiviral treatments of hematopoietic stem cells followed by autologous transplantation. This is, in part, because lentiviral vectors are primarily cleared by macrophages in studies of in vivo delivery. Biodistribution analyses show that the majority of the delivered transgene can be found in the spleen, liver, and bone marrow (Pan 2002, Follenzi 2002). Furthermore, Immunohistochemical analyses of the murine liver reveal that the majority of transgene expression is found within Kupffer cells, the spider-like resident macrophage of the liver (van Til 2004). Thus the in vivo clearance of Lentis is in part mediated by resident macrophage, and likely involves recognition of IgG bound to the LV surface. VSV-G pseudotyped lentiviral vectors cab become neutralized by IgGs found in naïve serum (DePolo 2000). Fetal bovine serum that is used in lentiviral vector production can also contain anti-viral IgGs (Offit 1984). Immunoblotting analyses of serum from

pre-injected and vector-injected mice suggest that mice have pre-existing anti-vector antibodies (Baeklandt 2003).

Current methods of enhancing delivery of lentiviral vectors have focused on engineering the surface of the vector to display novel envelope proteins, known to bind a specific target cell with relatively high affinity, in order to preferentially enhance gene transfer within that target cell (Bischof 2010, Frecha 2008, Di Nunzio 2007). This technique takes advantage of the fact that lentiviruses are enveloped viruses that self assemble beneath the cellular surface, taking a piece of the lipid bilayer as the viral envelope as it buds from the cell in a cytoskeleton driven manner (Gladnikoff 2009). Therefore proteins displayed on the cell surface during lentiviral transfection have the potential to also become displayed on the lentiviral vector surface.

Target cell specific promoters have also been engineered to enhance gene expression within target cell transduction (Follenzi 2004, Brown 2007). Additionally small inducer molecules have been engineered to induce regulated transgene transcription, whereby the therapeutic gene is only transcribed upon the administration of the small molecule (Vogel 2008). These current approaches enhance entry to target cells, but do not directly reduce uptake or gene expression in phagocytes. Reducing LV uptake by macrophage cells via CD47 display is optimal in that it reduces the large loss of vector to an off-target macrophage. The increased amount of LV available in circulation enhances the chance of the LV to reach a target cell type.

Lentiviruses, including HIV-1, are nanoparticles, with diameters that are reported to range between 90 and 260 nanometers (Fuller 1997, Dorfman 1994, Reicin 1996, Nakai 1996). The lentivirus assembles beneath the cell membrane inducing membrane curvature, leading to the formation of a bud. The budding process is complete when the quasi-spherical virion buds from the cell, enveloped in the plasma membrane and associated integral membrane proteins (Barerra 2008). The curvature may in part be induced by capsid protein (Lanman 2004). HIV-1 contains an inner core composed mainly of three key structural proteins matrix, nucleocapsid, capsid (p24), with the latter assembling as hexamers and pentamers into a 'fullerene cone' lattice (Zhao 2013).

The nucleocapsid is associated with the viral RNA while matrix is reported to play a number of roles including linking the viral envelope with the capsid (Bukrinskaya 2007).

The lentiviral vector is an engineered form of HIV. The genes encoding the structural elements, envelope material, and the therapeutic transgene have been engineered to three distinct plasmids (Dull 1998). Following the 5' long terminal repeat region of the lentivector's therapeutic gene sequence there is a highly structured RNA sequence, known as the packaging signal (Ψ) that has a strong affinity for the viral nucleocapsid protein (Russel 2003), and thus is the only genetic material that becomes encapsidated. This renders the viral vector unable to replicate since the genes for structural proteins, are not encapsidated. VSV-G, the vesicular stomatitis virus envelope glycoprotein, is commonly used lentiviral vector envelope protein (Naldini 1996). VSV-G mediates efficient gene transfer in a wide range of cell types (Farley 2007) and remains functional upon exposure to the force imposed by ultracentrifugation-based purification and concentration processes (Reiser 2000). Reverse transcriptase and integrase, virally encoded enzymes, are encapsidated within the vector and mediate reverse transcription and integration of the transgene into the host genome. The lentiviral vector is thus an efficient non-replicating gene transfer vector.

Synthetic particles that were engineered to display a recombinant CD47 protein show prolonged in vivo circulation and enhanced tumor delivery (Rodriguez 2013). While phagocytosis is typically defined to be limited to the engulfment of micron-sized particles, studies of nanobeads and viruses show that their uptake is enhanced by IgG interactions (Rodriguez 2013, Takeda 1990). Further the reduced uptake of CD47-nanoparticles appears to be dependent on the SIRPA inhibition pathway, as uptake is increased to levels compared to a control nano-bead by both SIRPA blocking (anti-mSIRPA) and a pharmacological SHP-1 inhibitor (NCS-87877). The hypothesis driving this work is that LVs can be engineered to display CD47 on their surface, and this CD47 display will reduce macrophage uptake thus enhancing vivo circulation and delivery to target tissues.

CHAPTER 2: CELL RIGIDITY AND SHAPE OVERRIDE CD47's 'SELF' SIGNALING IN PHAGOCYTOSIS BY HYPERACTIVATING MYOSIN-II

Abstract

A macrophage engulfs another cell or foreign particle in an adhesive process that often activates Myosin-II, unless the macrophage also engages 'Marker of Self' CD47 that inhibits Myosin. Adhesion processes of many cell types also activate Myosin and increasingly so on rigid substrates. Here we demonstrate that the rigidity of a phagocytosed RBC hyperactivates Myosin to overwhelm 'Self' signaling to macrophages. RBC stiffness is one among many factors, including shape, that change in senescence, some anemias, and diseases such as malaria. Controlled stiffening of normal human RBCs in different shapes did not compromise interactions of CD47 on RBCs with the macrophage 'Self'-recognition receptor, SIRPA. Uptake of antibody-opsonized RBC was always fastest with rigid RBC-Discocytes, which also showed maximal active Myosin at the phagocytic synapse and which biophysically out-competed 'Self' signaling by CD47. Rigid but more rounded RBC-Stomatocytes signaled 'Self' more so than rigid RBC-Discocytes, highlighting the effects of shape. Physical properties of phagocytic targets can thus modulate 'Self' signaling as seems relevant to splenic clearance of rigid red cells after storage or clearance of rigid pathological cells such as sickle and thalassemic red cells.

Introduction

Factors that promote the cytoskeleton-intensive process of phagocytosis (**Fig. 1A, left**) are opposed by several inhibitory factors (Ravetch 2000) that ultimately dictate whether a macrophage engulfs a target cell or particle. Immunoglobulin-G (IgG) bound to a target engages Fc γ Receptor on a macrophage, for example, and coordinates the assembly of numerous

phagocytic synapse proteins (Vincente-Manzanares 2004, Swanson 2008, Flannagan 2012) including non-muscle myosin-II motors that help drive uptake (Olazabal 2002, Araki 2002, Tsai 2008). If CD47 is displayed in parallel on a target, it binds the macrophage's inhibitory receptor SIRPA (Jiang 1999), which activates the immunomodulatory phosphatase SHP-1 (Veillette 1998) that regulates multiple proteins (Okazawa 2005), including myosin-IIA (Baba 2003). Myosin-IIA inhibition at the phagocytic synapse could explain various observations that 'Marker of Self' CD47 partially inhibits phagocytosis of mouse-RBCs (Oldenborg 2001) as well as normal white blood cells (Gardai 2005), stem cells (Blazar 2001), and cancer cells (Jaiswal 2009, Weiskopf 2013). Macrophage uptake of opsonized RBCs is also reported to contribute to clearance of RBCs in senescence (Kay 1975, Turrini 1991, Oldenborg 2000, Lutz 2004, Bosman 2008, Hod 2010) and in various diseases, including inherited anemias including sickle cell and thalassemia (Mohandas 2008, Reliene 2002). Aged and diseased RBCs differ from normal ones in physical properties such as rigidity (Chasis 1986, Mohandas 1994, Raat 2007); and stiffness also changes in cancer cells (Bercoff 2014, Lam 2007, Cross 2007), which could be important to broad anti-cancer efforts exploiting CD47-SIRPA interactions (Rodriguez 2013, Weiskopf 2013)^{12,17}. Particle studies indeed show that stiff gel particles are engulfed in greater numbers than soft particles (Beningo 2002), but relevance to cells with or without 'self' is untested. Normal human RBCs are controllably stiffened here in order to assess phagocytosis of rigid 'self' cells (**Fig. 1A, right**).

As RBCs senesce, aldehydes are produced, which greatly accelerates RBC clearance from the circulation (Jain 1983) (e.g. **Fig. S1A**). While aldehydes react primarily with amines in Lys residues, which only occur in CD47 distal to its binding site with SIRPA (PDB: 2JJS), aldehydes can sometimes react with Arg (Salem 2010), which CD47 has in its binding site (Arg¹⁰³) so that 'Marker of Self' interactions might be inhibited. It is very clear that aldehyde-mediated cross-linking of various RBC membrane proteins stiffens the cells (Fig. S1A). Rigid RBCs in healthy or disease states become stuck in narrow capillaries throughout the body (Ballas 1996, Tsai 2012),

especially splenic slits that impede rigid RBCs (Deplaine 2011), which could facilitate probing and clearance by splenic macrophages (Mebius 2005). Synthetic polymer 'RBC mimics' that lack any CD47 or other RBC proteins are also removed from circulating blood more rapidly when stiff compared to soft (Merkel 2011).

Distinguishing physical enlodgement within blood vessels from enhanced phagocytosis has rarely been clarified *in vivo*, and a reasonable hypothesis examined here is that cell rigidity promotes phagocytic uptake by overwhelming CD47's signaling of 'Self'. Mechanistically, in the adhesion of most cell types to a planar substrate, a stiffer substrate activates myosin-II moreso than a soft substrate; and such activation drives polarization in hematopoietic stem cells (Shin 2014), cell spreading of neutrophils (Oakes 2009), and actomyosin stress fiber assembly in various fibroblastic cell types (Engler 2006). Myosin-II is shown here to be hyperactivated by RBC rigidity and to oppose CD47's 'Self' signaling, thereby increasing phagocytosis. The findings are broadly relevant to accelerated senescence and clearance after blood storage (Hod 2011), to anemias that affect red cell shape and rigidity, to phagocytosis of cancer cells, and even to synthetic particles used *in vivo*.

Materials and Methods

Reagents, cell lines, and standard methods are described in supplemental Methods. All blood was collected after informed consent with IRB approval at the University of Pennsylvania. This study was conducted in accordance with the Declaration of Helsinki.

SIRPA Binding Assay Soluble SIRPA-Glutathione S-Transferase (GST) fusion protein was produced by transfection in COS cells. For some experiments SIRPA protein was cysteine linked to fluorophore. Red blood cells were isolated as in phagocytosis assays above. SIRPA was pre-complexed with anti-GST, and incubated with RBC (30 min RT). Cells were pelleted and re-suspended in 5% FBS/PBS, and analyzed with a BD LSRII flow cytometer.

Preparation of RBCs RBCs were isolated into 50 mM EDTA, plasma and buffy coat were removed, RBCs were washed with PBS, incubated with 0-50 mM Glutaraldehyde (GA) for 1 min at RT, and extensively washed. Prior to GA rigidification, GA-stomatocytes, were incubated with 200 mOsm PBS (30 min) then treated with GA (50 mM, 200 mOsm, 1 min, RT). For Antiserum and IgG opsonization, $\leq 10 \mu\text{L}$ packed RBC were incubated with 1-10 μL opsonin and CD47-blocked with 1-3 μL anti-CD47. RBCs were incubated shaking (Argos RotoFlex, 45 min, RT). RBCs were pelleted, and incubated with PKH26 (RT, 30 min). RBCs were counted by hemocytometer.

Phagocytosis Assay (Microscopy) THP-1s were treated with 100 ng/mL phorbol-myristate-acetate (PMA), for 2 days. Blebbistatin pre-incubation used 20 μM Blebbistatin at 37°C for 1 hour prior to RBC addition. RBCs were fed to macrophage at a ratio of 20:1, then incubated for 45 min at 37°C. Then cells were rinsed with PBS and fixed with 4% Formaldehyde. Non-ingested RBCs were differentiated by bound anti-rabbit-AF488 antibody. The phagocytosis index was calculated by counting the number of phagocytosed RBCs ≥ 200 macrophages, and expressed as the number of engulfed RBC per macrophage.

Phagocytosis Assay (Flow Cytometry) THP-1 macrophages and opsonized RBCs incubation were performed as in 'Phagocytosis Assay (Microscopy)'. Macrophages were isolated for flow cytometry analysis: Wash 2x w. PBS, Add 0.5 mL H₂O for 1 min, Trypsin Rinse followed by Trypsin incubation (5 min, 37°C) quenched with RPMI. Macrophage were isolated, centrifuged (3000G, 5 min), and resuspended with Hoechst 33342 (10 min), centrifuged and resuspended in 5% FBS/PBS and analyzed by a BD LSRII cytometer.

Immunofluorescence of the Phagocytic Synapse THP-1 Macrophage were cultured on Nunc LabTek chambers, co-incubated with opsonized RBCs as in '*Phagocytosis Assay (Microscopy)*', washed and fixed with 4% Formaldehyde, treated with Phalloidin-TRITC, Hoechst, anti-Myosin IIA, and anti-rabbit-AF488. Images were acquired with an inverted microscope (IX71; Olympus) with a 60x (oil, 1.4 NA) objective using a cascade CCD camera (Photometrics). Image acquisition was performed with ImagePro (Media Cybernetics, Inc.). Intensity analysis of the phagocytic synapse was performed using imageJ with a 38 x 1 pixel box, where the synapse was aligned at the box center. Fluorescence intensity was normalized to the minimum signal, and averaged over ≥ 3 randomly selected synapses.

Competitive in vivo Phagocytosis Assay RBCs were rigidified and opsonized as in vitro phagocytosis assays above. CD47⁺ and CD47-blocked RBCs were pre-treated with distinct lipophilic-dyes (PKH27 or DiR), mixed 1:1, and 2×10^7 RBCs were injected via tail vein to NOD/SCID/IL2rg^{-/-} mice (NSG), following a protocol approved by the IACUC at the University of Pennsylvania. 15 minutes post-injection spleens were isolated, washed, treated with RBC-Lysis-Bufer (Sigma 10min RT) and analyzed for IR intensity with a LI-COR Odyssey (LI-COR). Flow cytometry differentiated splenic macrophage from splenocytes via Cd11b expression and each population was analyzed for RBC signal.

Statistical Analysis All statistical analyses were performed using GraphPad Prism 4. Unless otherwise noted, all statistical comparisons were made by unpaired two-tailed Student *t* test and were considered significant if $P < 0.05$.

Results

Rigid human RBCs bind SIRPA but are rapidly engulfed

Normal human RBCs that are chemically modified with brief treatments of glutaraldehyde (GA) display functional CD47, as demonstrated by binding of both soluble SIRPA and a blocking anti-CD47, B6H12 (**Fig. 1B**). Flow cytometry was used to measure binding and to also show that binding of soluble SIRPA to RBCs could be inhibited by pre-treatment of cells with B6H12 (**Fig. 1B, left inset**). The recombinant GST-SIRPA used in these studies was detected either by pre-complexing with fluorescent anti-GST⁴⁴ or by direct fluorescence labeling of the reactive cysteines in GST, with the latter showing CD47 clustering (**Fig. 1B, right inset**) consistent with lateral mobility of a minor fraction of CD47 on human RBCs⁴⁵.

Figure 1. SIRPA binds CD47 on both rigid and Flexible RBC

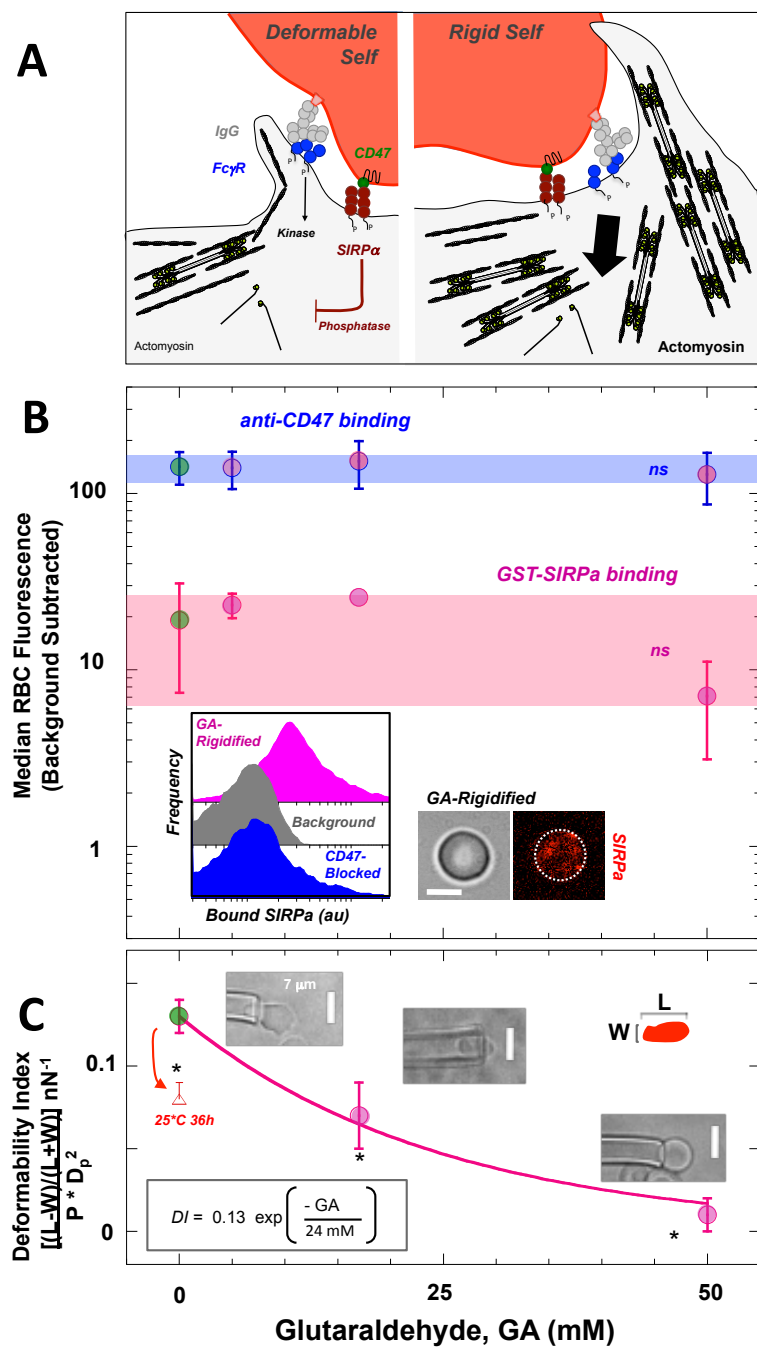


Figure 1. SIRPA binds CD47 on both rigid and native RBC. (A). Downstream of $Fc\gamma R$ binding of IgG, kinases phosphorylate multiple cytoskeletal proteins, including myosin-II, which drive assembly of the phagocytic cup and promote uptake. CD47-SIRPA signaling leads to activation of SHP-1 phosphatase that can deactivate Myosin-II. Since, substrate rigidity initiates assembly and polarization of Myosin-II in many cell types, phagocytic target rigidity is expected to counterbalance CD47-mediated inhibition of the motor. Our working hypothesis is that with flexible self cells (left), CD47 initiated inhibition can overcome myosin-II activation, whereas with rigid self cells (right), the myosin-II driven cytoskeleton is not diminished by CD47-SIRPA 'self' signals. (B) Binding of saturating concentrations of soluble human-SIRPA (GST conjugate) to human RBC treated with Glutaraldehyde (GA, 1 min at RT) shows no significant difference as compared to native RBC (green). High affinity mAb anti-CD47 (B6H12) also binds RBCs independent of GA concentration. Flow cytometry histograms (B, left inset) show SIRPA binds rigidified RBCs unless blocked by pre-treating RBCs with anti-CD47, indicating that SIRPA binds to CD47 (ns = not significant). SIRPA that is covalently labeled with fluorophore binds to rigidified cells (B, Inset images); the clustering suggests that CD47 is mobile (Scalebar: 5 μm). (C) Aspiration of RBCs into micropipettes similar in diameter to phagocytic cups and in vivo capillaries shows GA-treatment rigidifies cells and so does RT storage. The maximal RBC length and width under aspiration were quantified by image analysis and normalized by pressure and pipette cross-section (Native (green): n = 9, 17 mM-GA-Discocyte: n = 23, 50 mM GA-Discocyte: n = 2, error bar = SD). (* $p \leq 0.05$ compare to Native, trendline $R^2 = 0.99$).

For rabbit-RBC suspensions in fluid shear, the mean deformability of cells decreases exponentially with aldehyde concentration (Jain 1983) (**Fig. S1A**). To assess cell-to-cell variability after GA treatment and on a scale similar to a phagocytic cup that forms in RBC engulfment, individual human-RBCs were aspirated here into micropipettes of slightly smaller diameter than the cells. All native RBCs were rapidly aspirated and highly distorted, whereas GA-RBC always entered more slowly, deformed less, and with extreme GA treatment became stuck at the micropipette entrance (**Fig. 1C, S1B**). The exponential decrease in single cell deformability index with GA is consistent with past bulk results for aldehyde-treated RBC in shear (Jain 1983), and the reasonably small cell-to-cell variations indicate uniformity of the reaction. In addition, RBC kept at room temperature for 1.5 days led to cell rigidification similar to a 17 mM GA treatment (Fig 1B). Such a treatment is not as extreme as time-and-temperature treatments that are already known to drive rapid clearance by the spleen (i.e. refrigerating at 4°C for >1 mo or heating of RBCs to 50°C for 20 min (Deplaine 2011)).

Fresh RBC-discocytes normally possess a highly flexible membrane that extends easily under forces that even a few myosin motors might apply (~10 pico-Newtons (Mohandas 1994, Li 2005)). Time-lapse imaging of human-RBCs opsonized with anti-human-RBC anti-serum while being engulfed by human-derived THP1 macrophages showed that CD47-blocked RBCs undergo 'classical' phagocytosis, with engulfment complete within five minutes after initial contact (**Fig. 2A**). Regardless of whether CD47 is blocked or not, erythrophagocytosis begins with the macrophage pinching the RBC membrane into a semi-conical nascent synapse and causing the rest of the discocyte to become more spherical (**Fig. 2A,B, S2A**). Pinching of membranes is also evident at the beginning of phagocytosis of giant lipid vesicles that are subsequently ruptured (**Fig. S2B**). With RBCs, macrophage pseudopods then zipper along the membrane, constricting the erythrocytes in a manner expected to pressurize the hemoglobin-filled cytoplasm. Myosin-IIA contributes to cytoskeletal dynamics beneath the phagocytic cup (Araki 2002), and confocal

imaging confirms that an engulfed RBC is quasi-spherical with a diameter of $\sim 6 \mu\text{m}$, consistent with a sphere of conserved cytoplasmic volume (Fig. 2A, Fig. S2). When CD47 is not blocked as on native RBCs, such engulfment to spherical completion is infrequent ($\leq 25\%$). What is most often seen for native RBC is that macrophage-imposed deformations begin similarly, but are relatively larger and more sustained as CD47 signals 'Self' (Fig. 2B). GA-discocytes are not deformable and are very rapidly engulfed (**Fig. 2C**).

Regardless of self-signaling, extension of native RBCs that are being phagocytosed can provide estimates of the forces that macrophages exert in engulfment (**Fig. S2C**). Time-lapse images were quantified most simply in terms of the RBC's projected length (L_p) along the phagocytosis axis and perpendicular to the synapse. L_p is initially $\sim 8 \mu\text{m}$, the diameter of a human-RBC discocyte (**Fig 2D**). When CD47 is functionally signaling 'Self', L_p increases the most up to $\sim 12 \mu\text{m}$, thus stretching the RBC by $\sim 50\%$. Pseudopods extend from the macrophage but do not surround the RBC at the distal end; the RBC deformation process seems similar to that reported for macrophages pre-treated with a myosin-light chain kinase inhibitor (Araki 2002) – which will prove to be no coincidence. With GA-rigidified RBC, the RBC is often flipped up and is then very rapidly engulfed, suggestive of rigidity-enhanced phagocytosis seen previously with polymer beads (Beningo 2002). The GA-RBCs contact the macrophage *en face* and seem to strongly adhere, but consistent with these GA-discocytes being rigid they do not become fully spheroid in the phagosome as occurs with native RBC. The frequency of 'classical' phagocytosis events in which engulfment vectors inward and the RBC does not greatly stretch is 100% for GA-discocytes and 75% for CD47-blocked native RBCs but only 25% for native RBCs signaling 'Self', and frequency versus engulfment time are linearly correlated (**Fig. 2E**). Importantly, when native RBCs are encountered, the process is most often 'non-classical' with the large distensions persisting ~ 2 -fold longer than the 'classical' trend and ~ 5 -fold longer than uptake of rigid RBCs.

Figure 2. Phagocytic uptake of opsonized RBC is faster with CD47 inhibition but fastest for rigid RBC.

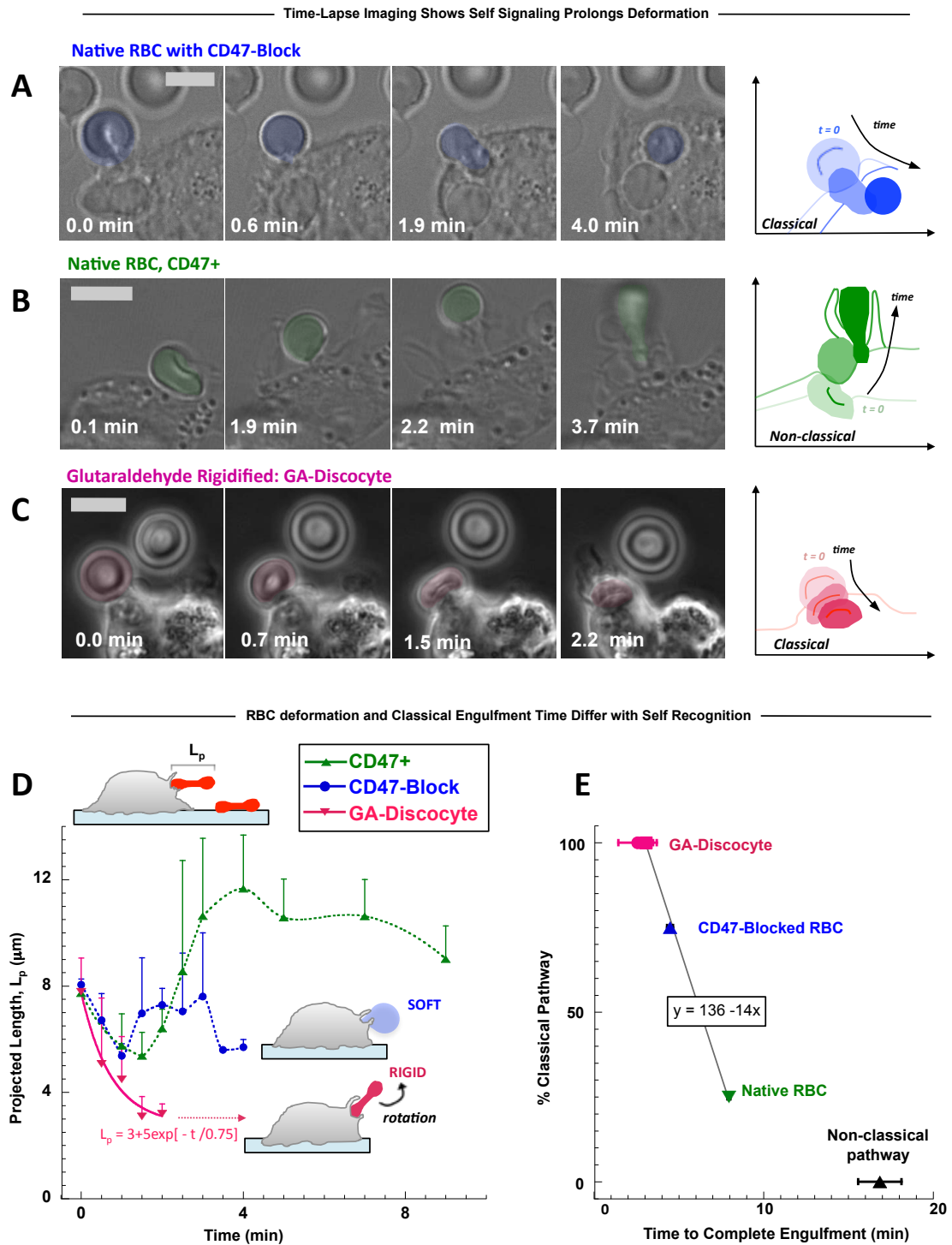


Figure 2. Phagocytic uptake of opsonized RBC is faster with CD47 inhibition but fastest for rigid RBC. Human-derived THP-1 macrophages were incubated with human-RBCs that were opsonized with anti-hRBC antiserum and also either: **(A)** blocked with anti-CD47, **(B)** Native RBC with active CD47+, or else **(C)** rigidified as GA-Discocytes. Time-lapse imaging in DIC and phase contrast begins with initial adhesion between macrophage and RBC targets, and ends upon complete engulfment. The RBC of interest in each frame is colored blue, green, or pink. Scale bars: 8 μm . At the right of each of the time-lapse series, silhouettes of the target RBC clarify the changes in RBC morphology and position relative to the initial macrophage boundary, as indicated by the gray line, throughout engulfment. **(D)** The projected length (L_p) of the engulfed RBC along the phagocytosis axis shows that phagocytic deformation is fast and ‘classically’ vectored inward for rigid RBCs and for CD47-blocked RBCs compared to native RBCs ($n \geq 3 \pm \text{SD}$). When CD47 can signal ‘Self’, phagocytosis is much slower and L_p often increases (up to 1.5-fold L_{p0}). **(E)** The percentage of classical uptake events for each RBC treatment is plotted versus the time required to complete engulfment, with the frequency of classical uptake showing a negative linear correlation with engulfment time (Line fit: $R^2 = 1.0$). Non-classical uptake is most frequently observed with Native RBCs that signal ‘Self’ and deviates from the classical trend by ≥ 2 -fold.

Myosin-II localization to the Phagocytic Synapse is promoted by RBC Rigidity

Adhesion to a rigid (*not* soft) substrate for many cell types drives cell spreading with assembly of stress fibers and polarization of non-muscle myosin-II (Shin 2014, Oakes 2009, Lo 2004, Raab 2012); and Macrophages are certainly mechanosensitive in adhesion (Fereol 2006, Patel 2012, Blakney 2012) and phagocytosis (Beningo 2002). Target rigidity can therefore contribute to the generation of contractile forces during the phagocytosis of foreign cells. Accumulation of myosin-IIA at the phagocytic synapse between THP1 macrophages and opsonized human-RBC is largely inhibited by CD47 (Tsai 2008), as reproduced in a comparison of CD47-blocked native hRBC and native hRBC (**Fig. 3A,B**). Rigid GA-discocytes, in comparison, show significantly greater accumulation of myosin-IIA within the macrophage distal to the human-human phagocytic synapse, and this apparent hyperactivation of contractility is completely inhibited by the myosin-II ATPase inhibitor blebbistatin (Fig. 3A,B).

Actomyosin stress fibers are common in stromal cells such as fibroblasts while adhering to rigid plastic, but stress fibers are rare in macrophages. Nonetheless, addition of the opsonized and rigid GA-discocytes caused a fraction of cells to assemble stress fibers that were not observed when native hRBC were added in identical numbers (**Fig. 3C,D**). Compared to native hRBCs that were CD47-blocked, rigid GA-discocytes induced the formation of straight and tensed (Tanner 2010) stress fibers in 4-fold more macrophages, while addition of blebbistatin produced 'relaxed' arcs as seen in similarly treated myocytes (Sen 2011). Stress fibers were also induced in macrophages when CD47 was blocked.

We had shown previously (Tsai 2008) that phosphorylation of tyrosines in myosin-IIA's head and tail activate the motor, increasing its accumulation at the phagocytic synapse and increasing the efficiency of phagocytic uptake, while CD47-SIRPA's activation of the tyrosine phosphatase SHP-1 *de*-activates myosin-IIA (**Fig. 3E**). Indeed, an inhibitor of SHP-1 enhanced uptake of GA-discocytes to levels similar to that of an anti-SIRPA antibody (**Fig. S3A**). Rigidity of a phagocytic

target thus tends to hyperactivate myosin-IIA in macrophages, and immunoblots against the myosin-IIA heavy chain in whole macrophage lysates after phagocytosis indeed show a high molecular weight form consistent with greater assembly of myofibers for rigid RBC than native RBC. Other sites of myosin-IIA phosphorylation include a serine residue (S9143) in the tail that limits filament assembly (Dulyaninova 2007) and reduces contractility in cells on soft substrates (Raab 2012). The high molecular weight myosin-IIA bands show no detectable phospho-S1943 that is otherwise prominent in lower bands (**Fig. S3B**), which reinforces the conclusion that RBC rigidity promotes myosin-IIA assembly.

Figure 3. Myosin II Accumulation at Phagocytic Synapse is strongly promoted by target rigidity and more weakly inhibited by CD47

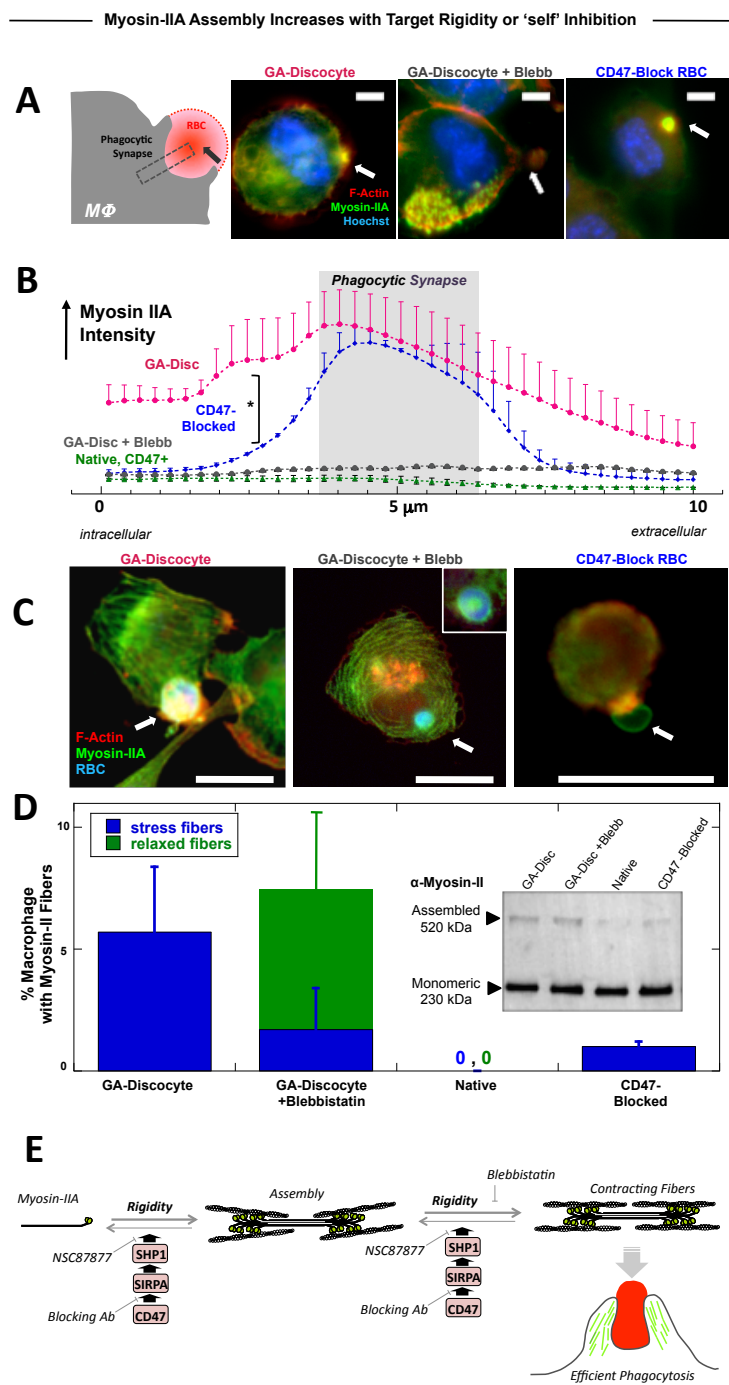


Figure 3. Myosin II Accumulation at the Phagocytic Synapse is strongly promoted by target rigidity and more weakly inhibited by CD47. (A) The schematic (left) illustrates the intensity analysis that was performed on immunofluorescence images of the macrophage phagocytic synapse, where THP-1 were either pre-treated or not with the myosin-II inhibitor Blebbistatin (20 μ M), followed by incubation with various anti-serum opsonized RBCs for 45 min at 37°C, then fixed and immunostained for Myosin-IIA (green), F-actin (red), and DNA (blue) (Scale bar 10 μ m). (B) Accumulation of Myosin II at the phagocytic synapse was quantified ($n \geq 3 \pm$ SD), proving highest for rigid GA-Discocytes and, secondarily, for CD47-blocked RBC (* $p < 0.05$). Blebbistatin suppresses Myosin-IIA accumulation to levels similar to Native RBC, denoted CD47+. (C) Fluorescence images of F-actin and Myosin-IIA localization in macrophage fed either GA-Discs with or without pre-treatment with Blebbistatin (20 μ M), or CD47-blocked RBC (Scale bar 30 μ m). (D) Actomyosin fiber formation was quantified in macrophages, showing that GA-Discocytes caused the highest frequency of cells with stress fibers. Blebbistatin pre-treated macrophages showed curved and relaxed fibers, whereas macrophages cultured with native RBCs do not show fibers. (D, inset) Western blot for non-muscle myosin IIA heavy chain, of macrophage lysates following phagocytosis, shows the presence of 230 kDa and 520 kDa bands, with the high molecular weight band consistent with stable myosin assembly. (E) Schematic of Myosin-II assembly in phagocytosis in which Myosin-II dimers assemble and contract actin filaments in response to rigidity of human RBCs, while becoming more disorganized in response to CD47-SIRPA mediated activation of immunoinhibitory phosphatase, SHP-1. Blebbistatin blocks the activity of myosin II ATPase, and thus the ability of the head to generate contractile forces, relaxing the stress fibers. The SHP-1 inhibitor NSC87877 reduces inhibition by CD47 and thus increases RBC uptake (Fig. S3A).

Rigid Discocytes are phagocytosed in greater numbers than flexible Discocytes

Canine RBCs are opsonized by autologous IgG (Christian 1993) that increases 7-fold towards the end of the cell's life span *in vivo* (Rettig 2014), with similar evidence for human RBCs that have a similar life span (Turrini 1991, Galili 1986). Aged human-RBCs lack other 'eat me' signals such as exposed phosphatidylserine (Franco 2012). Engulfment of native human-RBCs by THP1 macrophages increases here with pre-opsonization of the hRBCs by an anti-hRBC antiserum, saturating at >10-fold higher levels than unopsonized hRBC (**Fig. 4A**). IgG concentration in serum is ~100 μ M (Turrini 1993) and is 20% of total serum protein, but the highest IgG concentration used here is only ~1 μ M with a fraction specific for human RBCs. Direct imaging of engulfed RBCs per macrophage at the end of the 45 min *in vitro* assay was done by scoring at least 100 randomly chosen macrophages, and for higher throughput, a flow cytometry assay was adapted (see Methods), and yielded the same relative phagocytic Index as microscopy, with normalization to uptake of native RBCs. Blocking hCD47 nearly doubles the uptake of native cells that are highly opsonized, but blebbistatin inhibition of myosin-II always produces uptake levels statistically similar to native hRBCs (**Fig. 4B, left**). Rigidified GA-discocytes are engulfed at similar levels as CD47-blocked native RBCs (**Fig. 4B, right**), while decreasing the GA treatment decreases phagocytosis (**Fig. S3C**) and follows an exponential trend consistent with the aldehyde-dependence of rigidification (Fig. 1C) and *in vivo* clearance (Fig. S1A). Remarkably, blocking CD47 on the rigid GA-discocytes shows no effect on engulfment (Fig. 4B, right). In contrast, blebbistatin always inhibits uptake of GA-Discocytes (Fig. 4B, far right), with phagocytosis is reduced to levels similar to native RBCs that signal 'Self'. The results thus indicate that increased uptake of GA-Discocytes and CD47-Blocked RBCs depends largely on active myosin-II.

At high opsonization, increasing amounts of anti-CD47 blocking antibody B6H12 promotes engulfment of native RBCs, saturating near the B6H12-independent engulfment of GA-discocytes (**Fig. 4C**). At low opsonization, the same increases in this anti-CD47 have no significant effect on the minimal engulfment of native RBCs (**Fig. 4D**, lower curve), which indicates that the B6H12 antibody is not sufficiently abundant on hRBCs to contribute significantly to opsonization. On the other hand, the same low opsonization of GA-discocytes led to greater engulfment with increasing anti-CD47 (Fig. 4D, upper curve), which indicates that hCD47 is functional on these GA-treated cells, consistent with binding to soluble SIRPA (Fig. 1A). Because we had previously shown hCD47 normally decreases splenic clearance in the NOD/SCID/Il2rg^{-/-} (NSG) mice¹², we injected stiff, highly opsonized hRBC into these mice to examine splenic clearance. The presence or not of anti-hCD47 blocking antibody B6H12 had no effect on splenic macrophage uptake (**Fig. 4E, S3E**), consistent with *in vitro* results (Fig. 4C).

Figure 4. Rigid RBCs & CD47-Blocked RBCs both promote Opsonization-Driven Phagocytosis unless Myosin-II is directly inhibited

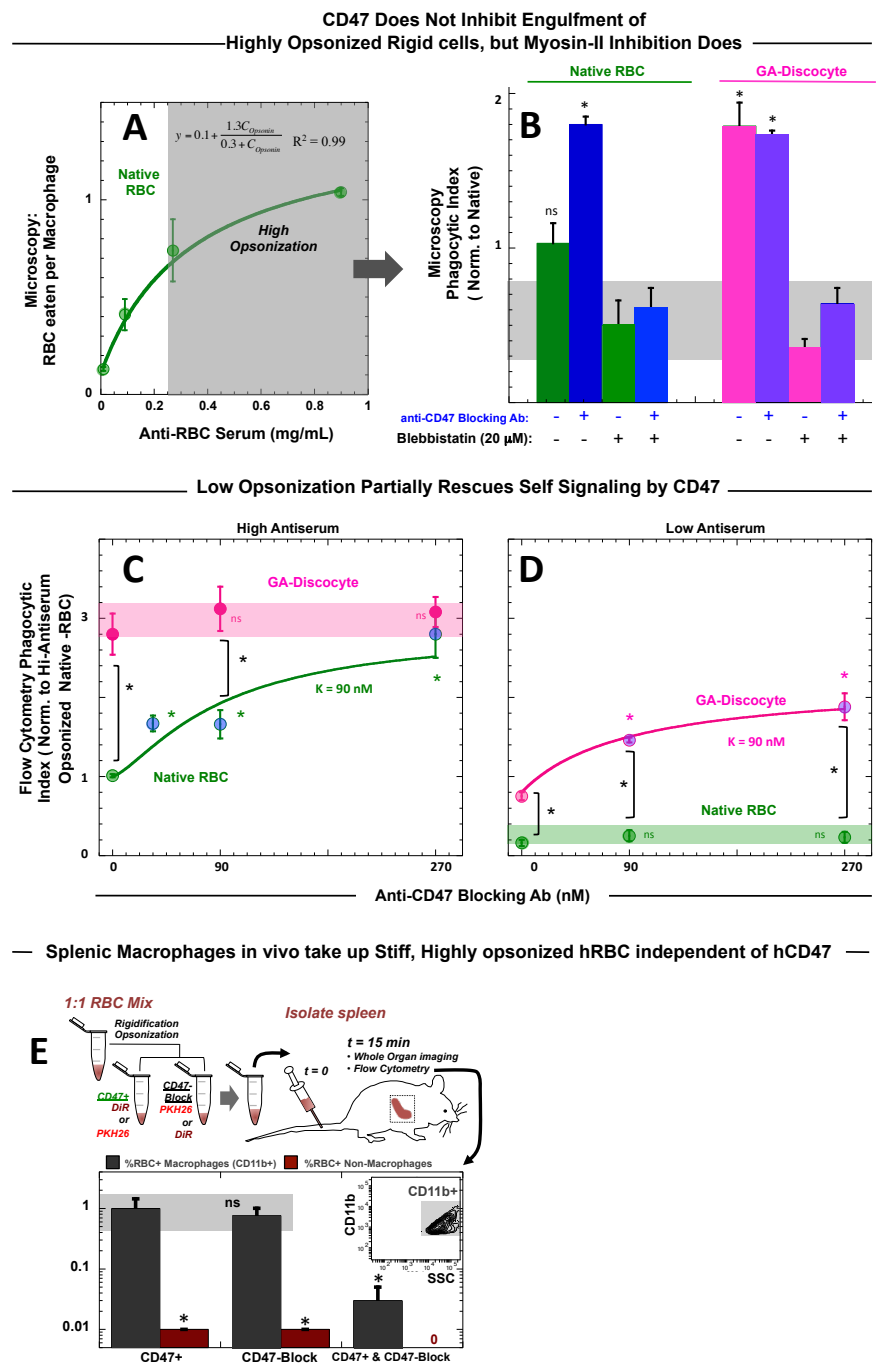


Figure 4. Rigid RBCs and CD47-Blocked RBCs both promote Opsonization-Driven Phagocytosis unless Myosin II is directly inhibited. (A) Phagocytic uptake shows an increasing and saturating response to anti-serum concentration. Here “high opsonization” is defined as a dose of opsonin greater than that required to result in half of the saturation level of engulfment. (B) All results were obtained for highly opsonized RBCs incubated with THP1s for 45 min at 37°C. A microscopy based phagocytosis assay indicates that native RBC were engulfed more so when they were CD47-blocked, unless THP1 were also pre-treated with blebbistatin (20 μ M). Rigidified GA-Discocyte engulfment proved independent of CD47 blocking. However, engulfment of rigidified RBC was inhibited by pre-treating THP1s with blebbistatin. The gray bar highlights a baseline level of phagocytosis that is significantly higher than baseline when RBC targets are either CD47-blocked or GA-rigidified, where blebbistatin pre-treatment of both native-blocked and rigid cells keeps uptake at this baseline ($*p < 0.05$). (C) At high opsonization, phagocytosis of GA-Discocytes is unaffected by blocking of CD47 (ns), while (D) at low opsonization blocking of CD47 on GA-Discocytes does increase phagocytosis. In contrast, for (C) deformable ‘Native RBCs’ at high opsonization, phagocytosis increases with blocking of CD47, whereas at (D) low opsonization, phagocytosis of native cells is insignificant regardless of blocking CD47 (ns). The latter finding indicates that the anti-CD47 blocking treatment alone is not sufficient to drive engulfment of Native RBC. For all experiments ($*p < 0.05$; $n \geq 3 \pm \text{SEM}$). (E) *In vivo* phagocytosis by splenic macrophages was assayed by injecting into NSG mice human-RBCs that were highly opsonized as in (C), rigidified (17 mM GA per Fig. 1C), and either CD47-Blocked or not (CD47+) as distinguishable after mixing by also labeling with spectrally distinct lipophilic dyes, DiR or PKH26 (swapped between mice, $n=4$). Spleens were isolated 15 min post-injection and imaged, showing similar splenic numbers of DiR-labeled hRBCs for both CD47-Blocked and CD47+ hRBCs (Fig. S3E). After dissociation of spleens, splenic macrophages were distinguished from splenocytes using Cd11b expression. Whereas 1% of splenic

macrophage were positive for CD47-Blocked and for CD47+ hRBCs, positive macrophages with both hRBCs were rare (~0.01%) as were hRBCs in splenocytes (CD11b-).

The anti-CD47 dependencies of engulfment for highly opsonized native hRBCs and for low opsonized rigid RBCs fit to the same saturable binding curves ($K = 90$ nM and ~2.5-fold increase from baseline to saturation in Fig. 4C,D). Similar mechanisms of anti-CD47 activity are thus implied. In the absence of any exogenous opsonization, engulfment is always low as expected, but (i) rigid GA-discocytes are engulfed more than native RBC and (ii) blocking with anti-CD47 – which impedes weakly adhesive interactions with macrophage SIRPA (Tsai 2008) – *decreases* engulfment of rigid GA-discocytes without affecting engulfment of native RBC (**Fig. S3D**). The results provide further evidence that CD47 is functional on GA-RBC, and seem consistent with CD47 acting as an adhesive ligand for cell tethering of apoptotic and/or damaged cells (Tada 2003). However, whether cell stiffness changed in the cited studies and had any effect as seen here is unclear.

RBC shape also modulates engulfment

Changes in RBC shape during phagocytosis clearly decrease with RBC stiffness (Fig. 2), but whether the initial shape of the RBC influences engulfment and CD47 signaling is unclear and is certainly relevant to altered RBC shape in hereditary anemias (e.g. hereditary spherocytosis and sickle cells (Chasis 1986); and senescence (Bosman 2008). Moreover, since rigid polystyrene spheres displaying CD47 can signal ‘Self’ and inhibit phagocytosis (Tsai 2008, Rodriguez 2013), we generated and assayed more rounded and rigid RBCs. By treating RBCs with a mild hypotonic buffer prior to GA crosslinking, cells take on a morphology similar to that seen in hereditary stomatocytosis (Mohandas 2008, Da Costa 2013) (**Fig. 5A inset, S4A**). In patients, stomatocyte-spleen interactions were abnormally high but also reduced by a drug that decreases production of TNF- α (Smith 1997), which is often associated with upregulation of adhesion molecules on vascular endothelium (Janeway 2005). Rigid GA-Stomatocytes bind soluble SIRPA (**Fig. S4B**) similarly to GA-Discocytes (Fig. 1B), but the former signal ‘Self’ more effectively than

the latter. This is based on the observation that engulfment numbers increase with anti-CD47 blocking at high opsonization using both anti-hRBC serum (**Fig. 5A**) and purified anti-hRBC IgG (**Fig. 5B**). As with native RBC, immuno-blocking of CD47 on GA-stomatocytes increases the rate of engulfment as measured by time-lapse imaging (**Fig. S4C**). While inhibitory signaling is less effective than that seen with native cells, the GA-Stomatocyte results suggest a shift in the balance towards increased CD47 inhibition and away from rigidity induced myosin-II activation.

Intriguingly, with anti-CD47, GA-Discocytes are engulfed similarly to GA-Stomatocytes with anti-RBC antiserum (Fig 5A), while GA-Discocytes are engulfed less with purified anti-hRBC (Fig 5B). The latter result is consistent with IgG-opsonized “spheres” being phagocytosed more readily than “non-spheres” in studies of particles (Champion 2006).

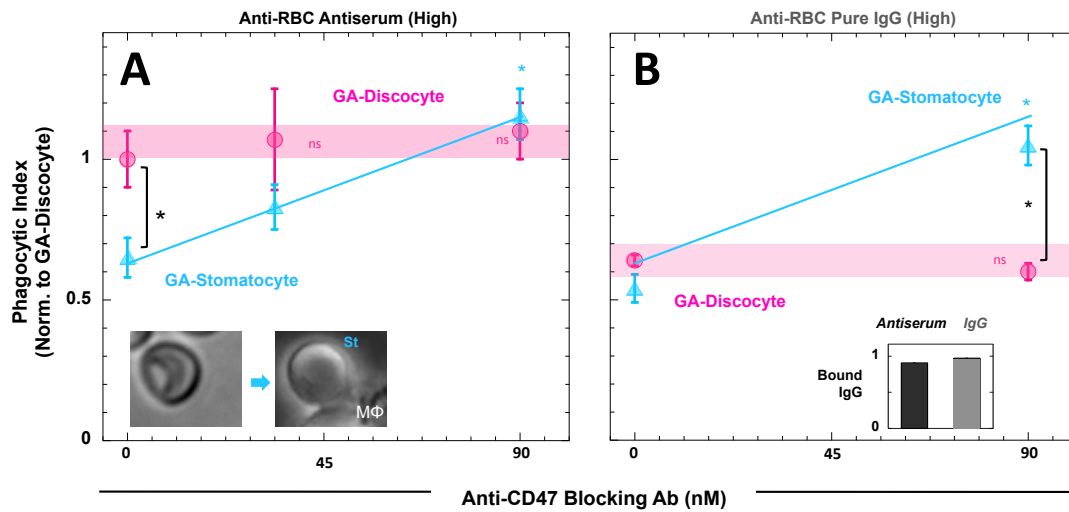


Figure 5. Shape of Rigid RBC Modulates CD47's "Don't Eat Me" Signal. (A) RBCs treated with a hypotonic buffer followed by GA reaction generated rigid GA-Stomatocytes (inset image, left). Imaging of the macrophage phagocytic synapse with the stomatocyte shows pseudopods closely apposed to the rounded cell (inset image, right). With high opsonization by anti-serum, flow cytometry based phagocytosis assays of the more rounded GA-Stomatocytes showed less engulfment than GA-Discocytes in the absence of anti-CD47 blocking antibody, while blocking CD47 equalized uptake. (B) Anti-RBC IgG purified from anti-serum was used at similarly high opsonization for the RBC targets (inset D), but uptake of GA-Discocytes was low as compared to antiserum opsonized targets. Blocking of CD47, in this case, led to significantly greater engulfment of the more rounded but rigid GA-Stomatocytes compared to the GA-Discocytes. For all experiments (* $p < 0.05$; $n \geq 5000$ macrophage in duplicate, $\pm$ SD).

Native cells are also taken up more so with antiserum opsonization as compared to purified IgG opsonization (**Fig. S5**). Antiserum contains additional opsonizing factors, particularly C3b (Janeway 2005), that induce complement-receptor mediated phagocytosis characterized by larger phagosomes in which the macrophage membrane loosely apposes the target (DeCathelineau 2003, Erwig 2008) with adhesion foci rather than dense adhesions per IgG-driven phagocytosis (Allen 1996). The complicated biconcave contour of a rigid GA-discocyte, and the resulting non-uniform membrane contact with a macrophage could make complement-driven uptake more efficient.

Discussion

CD47-SIRPA interactions at the macrophage surface somehow activate within the macrophage the tyrosine phosphatase SHP-1 (Jiang 1999) that targets multiple proteins including head and tail tyrosines in myosin-IIA's heavy chain, making phagocytosis inefficient (Tsai 2008). Myosin-II in a broad range of adhesive cell types is also *de*-activated and stress fiber assembly suppressed by the softness of planar substrates (Shin 2014, Engler 2006, Lo 2004, Rehfeldt 2012). Macrophages certainly exhibit similar mechanosensitive adhesion (Fereol 2006, Patel 2012, Blakney 2012) as well as reduced phagocytosis of soft synthetic spheres (Beningo 2002). The balance studied here between CD47-SIRPA signaling against phagocytosis *versus* rigidity and shape of human RBC promoting phagocytosis is summarized in a structure-function heatmap of normalized results (**Fig. 6A**). The few differences in uptake evident for native RBC *versus* rigid GA-Discocytes when myosin-II is inhibited most simply reveal 'classical' uptake of GA-RBC without red cell deformation and also a tendency of GA-Discocytes to stimulate stress fiber assembly even though myosin-IIA's ATPase is inhibited. At the opposite end of the heatmap, blocking of CD47 has little effect on uptake of GA-Discocytes (compare 'Blocked' to CD47+), with

a slight attenuation of the uptake phenotype for 'Blocked' cells perhaps reflecting the fact that blocking impedes the weakly adhesive interactions of CD47 with macrophage SIRPA (Tsai 2008). Native CD47-blocked RBC show an intermediate heatmap profile but group more with the rigid GA-Discocytes, consistent with strong myosin-II activation.

For all of the various RBC targets, IgG on the RBC is likely recognized by the macrophage receptor FcγRIIA, which triggers phagocytic cup formation independent of Myosin-II (**Fig. 6B**). While subsequent internalization depends on myosin-II (Olazabal 2002), the flexible native RBC show that CD47-SIRPA inhibition dominates the opsonization signaling that otherwise activates myosin-II. Blocking CD47 on soft RBCs leads to the characteristic hour-glass deformations seen when discocytes from different species are engulfed⁶⁹, consistent with CD47-SIRPA interactions being species specific (Tsai 2008, Subramanian 2006, Takenaka 2007).

Macrophages cannot deform GA-rigidified discocytes, which induces Myosin-II activation, assembly, and accumulation at the phagocytic synapse, contributing to rapid rotation of the target in 'en face' ingestion. Loss of RBC deformability seems to contribute to rapid clearance of RBCs from the circulation (Deplaine 2011)), consistent with rapid removal of relatively rigid apoptotic bodies (DeCathelineau 2003, Erwig 2008), but the relative contributions of cell stiffness, opsonin density, and even the roles of macrophages are rarely clear. Rapid uptake can limit signaling from other receptor-ligand interactions as can a rigid discocyte's concave shape, which can also limit contact between the macrophage's nascent phagosome membrane and a target membrane (Fig. S4D). A more spherical RBC shape indeed rescues CD47-SIRPA mediated signaling consistent with the more tightly apposed myosin-rich projections seen with rounded GA-stomatocytes (Fig. S4D). Rigid polystyrene spheres with CD47 attached likewise signal 'Self', minimizing macrophage uptake *in vitro* and *in vivo* while also suppressing myosin-IIA localization to the phagocytic synapse (Rodriguez 2013).

Loss of deformability of aged erythrocytes has long been thought to contribute to their clearance from circulation, with additional determinants possibly including partial loss of CD47 (6-50%(Annis 2002, Kamel 2010)) and also oxidation of CD47 (Olsson 2008, Burger 2012). The results here clarify the complementary role that target deformability plays in clearance by tissue macrophages, with findings relevant perhaps to chemotherapy-rigidified leukemias, rigid-walled microbes (yeast and bacteria), and particles used in gene and drug delivery as well as the well-known rigid red cells that result from blood storage and that occur in common diseases such as sickle cell and thalassemia.

Figure 6. Myosin II Activity at the Phagocytic Synapse with Opsonized Target is Inhibited by CD47 but enhanced by Target Rigidity

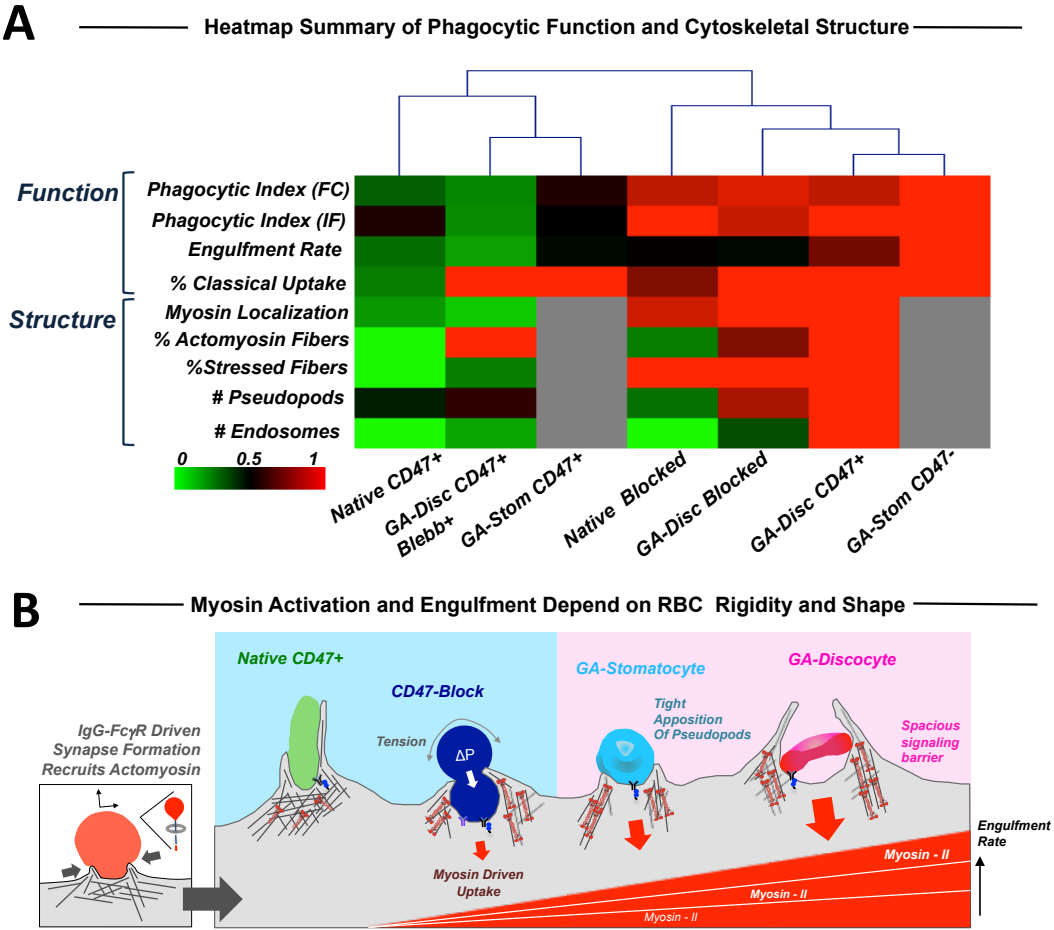


Figure 6. Myosin II Activity at the Phagocytic Synapse with Opsonized RBC Targets is inhibited by CD47, while Enhanced by Target Rigidity. (A) Heatmap summary of normalized phagocytosis and immunofluorescence results (Multi-Experiment Viewer Software). The dendrogram of hierarchical clustering using the Euclidian distance metric indicates that macrophage response to rigid GA-discocytes is distinct from that of native RBC targets and that blocking CD47 on deformable RBCs (but not on GA-Discocytes) reduces the difference as does blebbistatin pre-treatment of macrophages encountering GA-Discocytes (GA-Disc CD47+ Blebb+). CD47 inhibition is partially rescued with rigid but rounded GA-stomatocytes (GA-Stom CD47+), unless CD47-Blocked (GA-Stom CD47-). **(B)** Macrophage recognition of IgG opsonin on the surface of RBC by Fc γ R phagocytic receptors activates cytoskeletal proteins including Myosin-II. Signaling via SIRPA to phosphatases does occur with CD47 on either flexible 'Self' cells or sufficiently rounded but rigid 'Self' cells. RBC rigidity has the effect of rapidly and strongly activating adhesion and myosin-II contractions. Rigid discocytes maintain their shape throughout engulfment, which limits contact between macrophage receptors and ligands, particularly SIRPA and CD47 on the RBC.

Supplementary Materials and Methods

Cells, Reagents, and Antibodies THP-1 macrophage (American Type Culture Collection and European Collection of Cell Cultures) were cultured in RPMI (Invitrogen) supplemented with 10% FBS (Sigma). Differentiation of THP-1 cells was achieved in 100 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) for 2 d and confirmed by attachment of cells to culture vessel. For microscopy based phagocytosis assays macrophage were cultured in 4-cm² Lab-Tek chambered coverglass (Nunc International), and for flow based phagocytosis assays macrophage were cultured in 6-well plastic dishes (Corning). Dulbecco's phosphate-buffered saline (DPBS) without Ca²⁺ or Mg²⁺ (Invitrogen) was supplemented or not with BSA (Sigma-Aldrich). PKH26 (Sigma-Aldrich) was used for erythrocyte cell labeling. RBC opsonins included Anti-human RBC antibody (rabbit, Rockland Pharmaceuticals) and Anti-human RBC IgG (rabbit, Rockland Pharmaceuticals). Anti-CD47 mAb clone B6H12 (BD Biosciences) was used to block CD47. Secondary antibodies used to detect bound opsonins included Donkey Anti-rabbit IgG conjugated with AlexaFluor 488 or AlexaFluor647 was used to detect opsonin. Anti-Myosin IIa was used in IF of the Phagocytic synapse (mouse Abcam). Anti-Myosin IIa (rabbit Sigma) was used in actomyosin fiber analyses and western blotting. Hoechst 33342 (Invitrogen) was used to identify the macrophage nucleus. Reagents used to detect F-actin included Phalloidin conjugated with AlexaFluor 488 (Invitrogen) or TRITC (Sigma). Racemic Blebbistatin was used in phagocytosis assays (EMD Biosciences).

Micropipette Aspiration Capillary tubes were pulled into micropipettes and trimmed by microforge (Vibratome) to mean diameter ~6 μ m. Micropipettes were attached to a dual-stage water manometer with adjustable height reservoirs. Suction was applied by syringe, and pressure measured by transducer (Validyne). Pipettes pre-rinsed with 3% BSA were used to aspirate

RBCs (200 Pa), imaged (Nikon TE300) with 40x objective, captured with Cascade CCD camera (Roper Scientific), and analyzed with ImageJ.

Time Lapse Microscopy Phase-contrast imaging was performed in a humidified chamber at 37°C and 5% CO₂ using an inverted microscope (Olympus IX-71) with a 40x objective (NA 0.6) and high-resolution CCD camera (CoolSNAP HQ; Photometrics). softWoRx [DeltaVision] was used for image-capture. DIC imaging was performed in a temperature-controlled chamber with THP-1 cultured in HEPES buffered RPMI, using an inverted microscope (Leica TCS SP5) with a 63x water-immersion objective (NA 1.2). Time-lapse imaging was initiated when a RBC adhered to a macrophage. ImageJ was used to analyze dimensions of RBC during engulfment.

Confocal Microscopy Macrophage and RBC were co-cultured in 4-cm² Lab-Tek chambered coverglass (Nunc International) were imaged using an inverted microscope (Leica TCS SP5) with a 63x water-immersion objective (NA 1.2). Anti-Rabbit IgG conjugated to AlexaFluor647 was excited with a He Ne laser at 633 nm, and emission was collected in the range of 650-720. Phalloidin-AlexaFluor488 was excited with an argon laser at 488 nm, and emission was collected within 500-550 nm. DiIC18 was excited with DPSS laser at 561 nm, and emission was collected within 600-650 nm, and DAPI was excited with the multiphoton laser at 730 nm, and emission was collected between 400-460 nm. Bidirectional scanning with a correction factor of -27 was used with a line average and frame average of 3.

Western Blotting THP-1 macrophages were plated at 2E5 cells per well of a 6 well plate (Corning), and fed RBC at a ratio of 1 macrophage to 20 RBC. RBC conditions were, native, native-CD47-block, GA-Discocyte, and GA-Discocyte with Blebbistatin (20 uM). Cells were lysed

with ice-cold RIPA buffer supplemented with 1% protease inhibitor, 1% phosphatase inhibitor, and 1% Vanadate. For every 100 μ l of lysates, 30 μ l of LDS, and 5 μ l of Beta-ME were added prior to heating on heating block for 10 min at 70°C in water. 1 x 6 well plate was lysed for each condition, and equal volume of lysates was loaded in each lane of 3-8% Tris Acetate gel. Proteins were transferred to PVDF membranes, and blocked with 5% milk in TBS (m/v). Membranes were blotted for pS1943 (rabbit, Cell Signaling) and Nonmuscle Myosin-IIA (rabbit pAb, Sigma), and probed with anti-rabbit-HRP secondary, followed by chromosensor detection.

Vesicle Preparation Giant vesicles composed of 1-palmitoyl-2-oleoyl-Glycero-3-phosphocholine (POPC) (Avanti) with <1% DiIc18 were prepared by electroformation. 5 μ L of POPC/DiIc18 stock (10 mg/mL) was transferred to cleaned ITO glass slides with a Hamiltonian syringe, spread over the glass with the syringe tip, dried in fume hood for 2h. ITO glass slides and spacer were assembled and fill with an osmotically adjusted sucrose solution (340 mOsm). The apparatus was then treated with a sinus wave with voltage of 1.4V (RMS) and frequency of 10 Hz for 1.5 hours. Vesicles were equilibrated to ambient conditions 1h before transferring to glass vial. For phagocytosis assays, GUVs were pre-incubated with 10 μ l anti-RBC antiserum (30 min, RT).

Supplemental Figures

Figure S1. Rigid Cells show reduced in vivo circulation and reduced in vitro aspiration speed

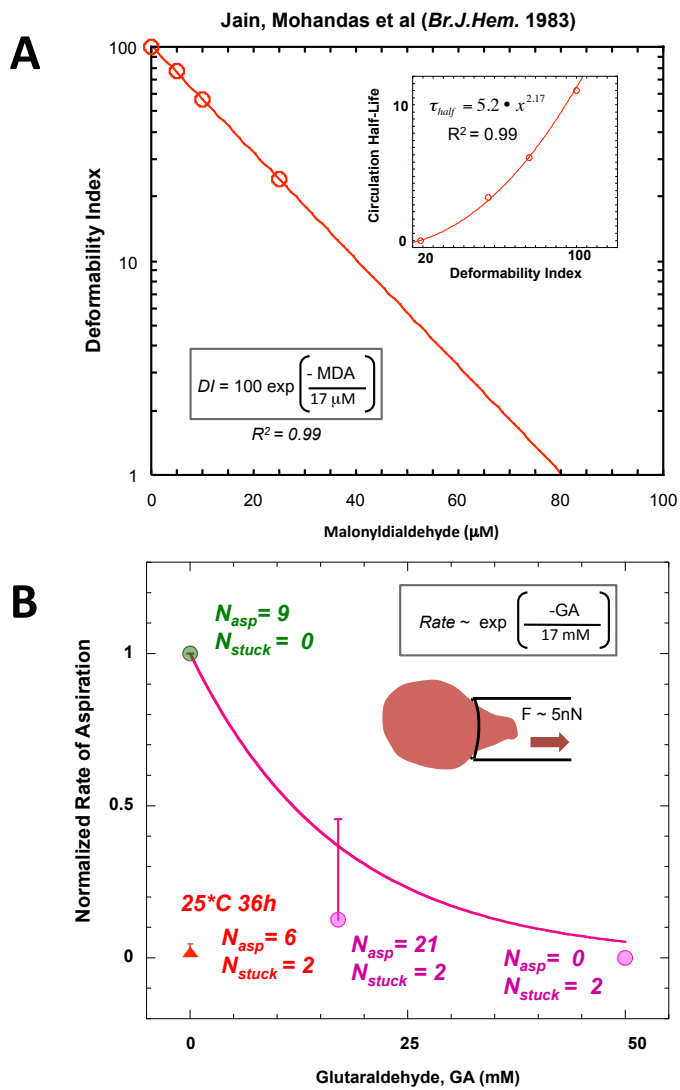
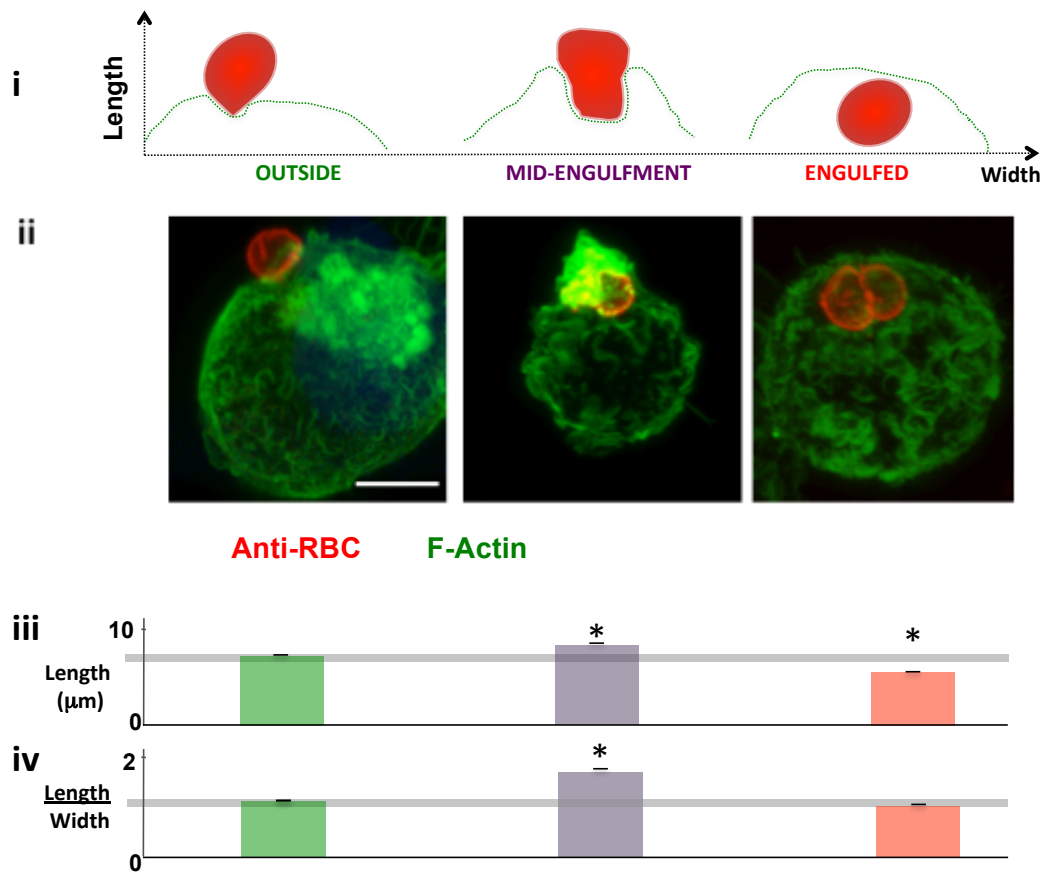


Figure S1. Rigid Cells show reduced in vivo circulation and reduced in vitro aspiration speed. (A) In studies of Jain *et al.* (1983) rabbit RBC were treated with 0-80 μM Malondialdehyde (MDA) for 1 hour at 37°C, and measurements of RBC deformability in bulk shear by ektacytometry demonstrated an exponential decay with MDA, Deformability Index (DI) = $101 \cdot \exp(-[\text{MDA}]/17.5 \mu\text{M})$, $R^2=0.999$. (inset) The in-vivo circulation half-life positively correlates with DI (Jain 1983), fitting to $t_{\text{half}} = 5.2 DI^2$. (B) GA treated RBCs were aspirated in micropipettes similar in size to phagocytic cups. Aspiration rate was quantified and normalized relative to the rate of native cell aspiration. The number of cells that were fully aspirated versus the number that became stuck at the pipette entrance was quantified and annotates each data point.

Figure S2A. During classical phagocytosis, the hRBC is first pinched, then elongated, and finally spherical after engulfment



S2B. Phagocytic Deformations quickly Rupture Lipid Vesicles

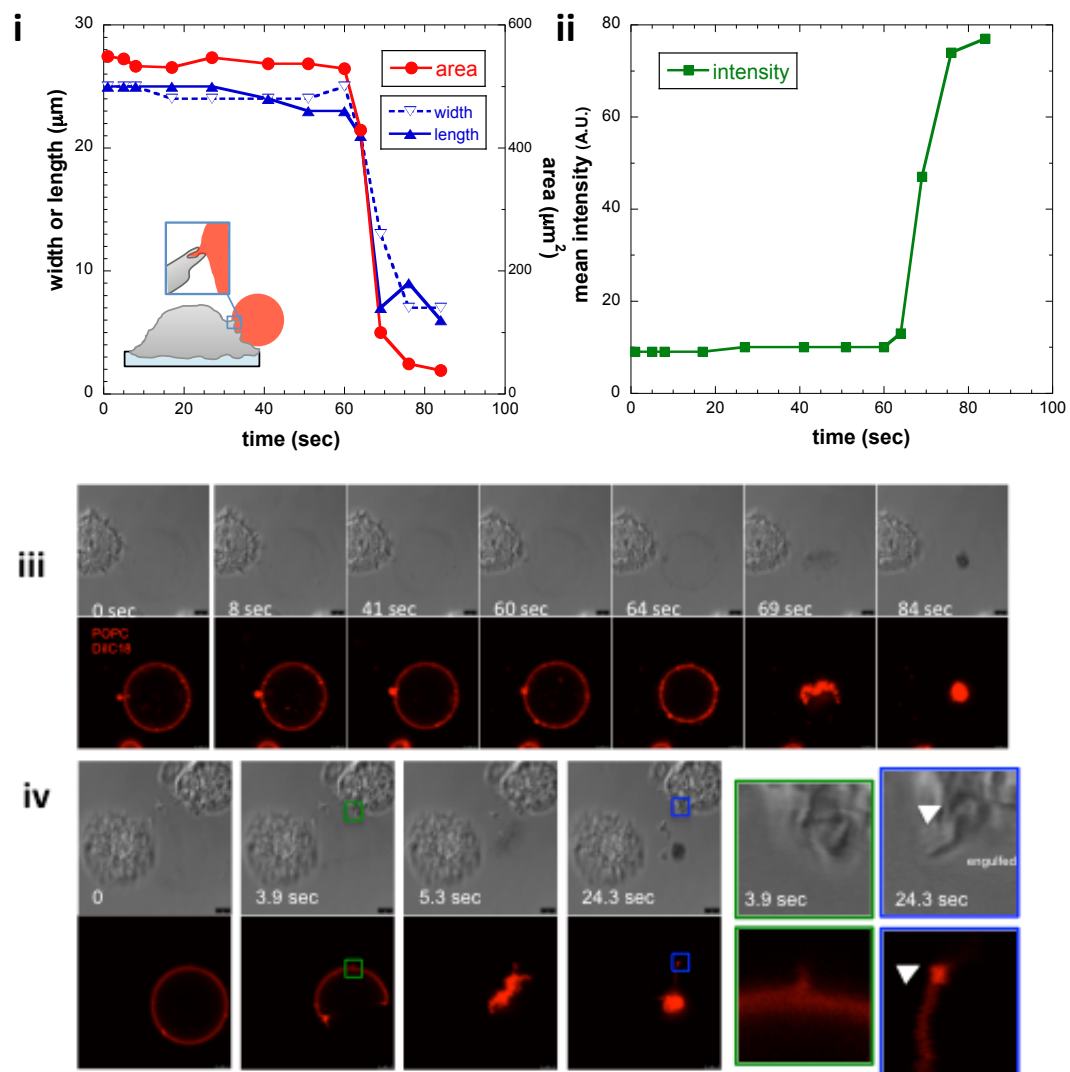


Figure S2.C Deformations of Native RBCs indicate the forces that Macrophages exert on RBC during engulfment

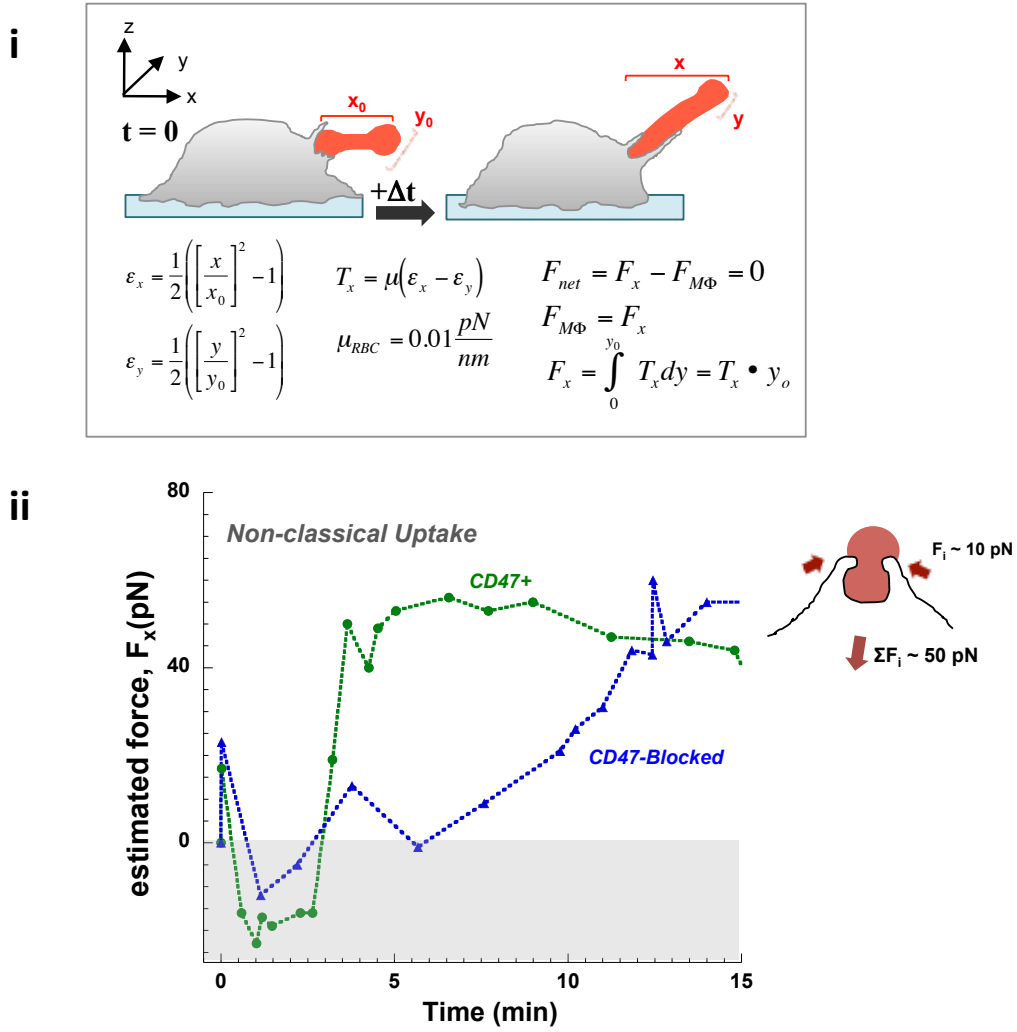


Figure S2. During classical Phagocytosis the hRBC is first pinched, then elongated, and finally spherical after engulfment. (A) (i) Schematic of classical deformations. (ii) Confocal images of macrophage cytoskeleton (Phalloidin-AF488) and RBC (anti-RBC) during the initiation, midpoint, and completion of phagocytosis were quantified for length (iii). The length to width ratio (iv) indicates that the RBC becomes circular during initial engulfment, and following engulfment. (B) Kinetics of deformation during lipid vesicle phagocytosis and rupture. The deformation along the length and width of the vesicle (i) as well as the corresponding change in mean intensity of lipid vesicle (ii) indicate in quantitative terms that the vesicle shrinks after rupture followed by rapid aggregation of the lipid vesicle in the surrounding aqueous cell culture medium. Schematic inset in (i) indicates lipid vesicle deformation. (iii) Time-lapse imaging shows phagocytosis and rupture of antiserum-opsonized POPC-DiIc18 lipid vesicle where the macrophage pseudopods can be seen co-localizing with the lipid vesicle and the lipid vesicle membrane increases in intensity suggestive of aggregation or pinching of the lipid vesicle membrane. At 64 seconds post-initial contact, the lipid vesicle decreases in size, and finally aggregates before leaving the plane of focus. (iv) In a second time-series, a lipid vesicle was contacted by pseudopods of two different macrophages. The green box at 3.9 sec highlights where the macrophage has deformed the lipid vesicle and the blue box at 24.3 sec highlights where a macrophage appears to have engulfed a portion of the ruptured vesicle and a piece of stretched membrane can be seen adjacent to the engulfed clump. Zoomed in versions of the green and blue boxes are to the right of (iv). (C) The Deformation of Native and CD47-blocked RBCs imposed by the macrophage cytoskeleton during phagocytic engulfment were quantified. (i) The x-axis is defined to be in the direction of movement of RBC towards M. Deformations are typically 50% in the x-direction so Green's strain was used to describe strain, which acts across the length of the red cell to give the resultant force in the x-direction. (ii) The force imposed by macrophage, F_{MP} , which is defined here as equal to the resultant deformation force in the x-direction, F_x , was quantified throughout the engulfment process, and was found to have a maximum from 60-120 pN. This non-classical F_{MP} is

expected to be relatively myosin-II independent, and the force is generated instead by other components of the cytoskeleton such as F-actin.

Figure S3 Further Exploring the Mechanism by which Rigidity Affects CD47-SIRPA Mediated Inhibition.

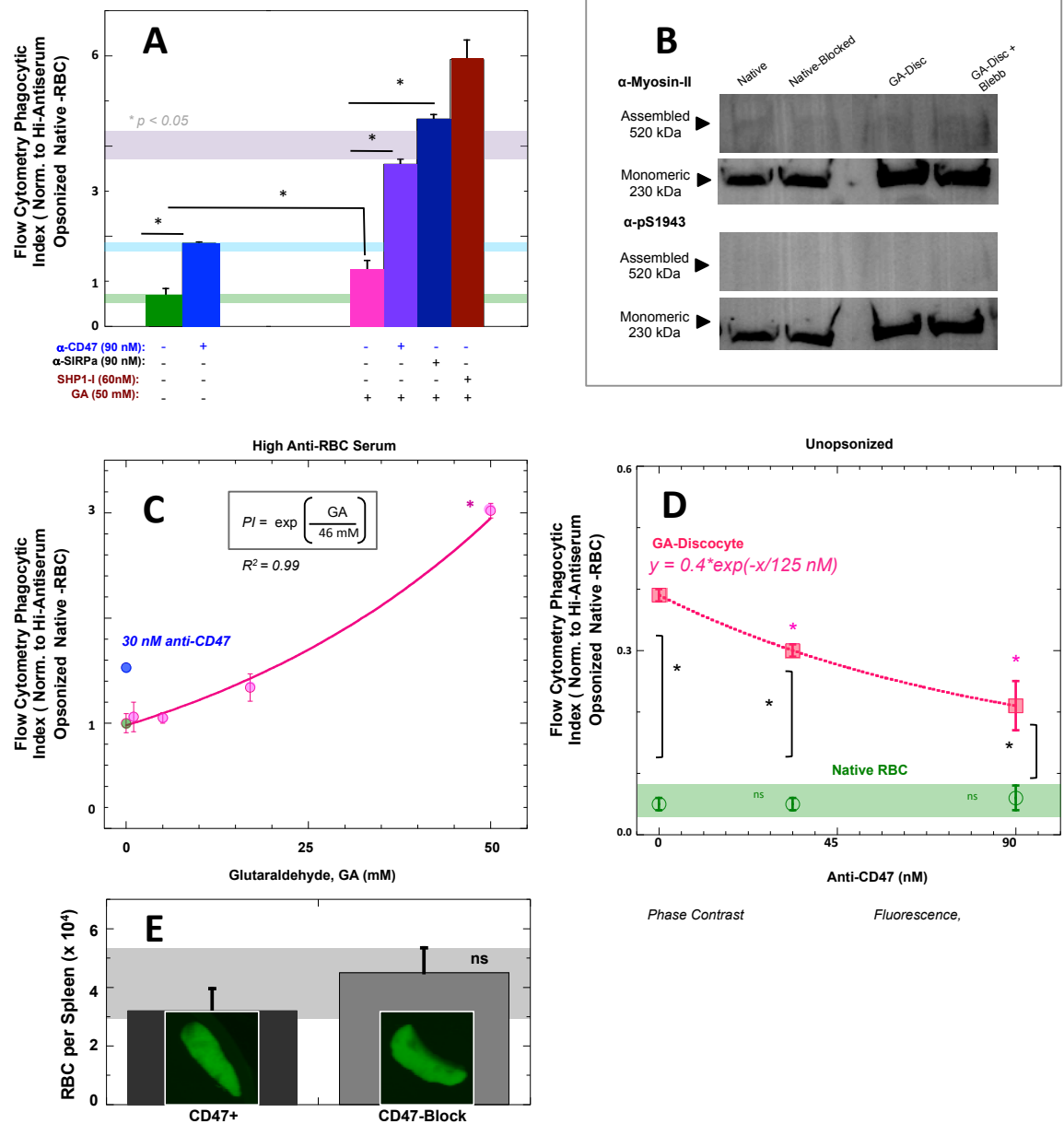


Figure S3. Further Exploring the mechanism by which rigidity affects CD47-SIRPA mediated Inhibition. (A). At intermediate levels of antiserum, CD47 can inhibit uptake of aldehyde rigidified RBC, as treatment with a CD47-blocking antibody increases uptake. This is similar to the result of CD47-blocking GA-discocytes at low antiserum (Fig 4D). Pre-treating macrophage with anti-SIRPA also increases uptake to magnitude similar to anti-CD47. SHP-1 inhibitor (60 nM) also increases uptake. (B) Western Blots for Myosin-IIA and Phosphorylation Myosin II Serine Residue 1943 were performed. (C) A flow cytometry based phagocytosis assay was performed on high antiserum opsonized GA-discs treated with a range of GA concentrations, and indicates that phagocytosis increases exponentially with GA concentration. (D) A flow cytometry based phagocytosis assay of unopsonized native and GA-Discocytes indicate blocking CD47 inhibits uptake, consistent with CD47 acting as an adhesive tether, and further that CD47 is functional in support of SIRPA binding studies (F1A). (E) A competitive in vivo phagocytosis assay was performed where highly opsonized and rigidified GA-discocytes (17 mM) that were either CD47-blocked or not were co-injected and differentiated by spectrally-distinct lipid dyes. Spleens that were injected either CD47+ DiR RBC with CD47-blocked PKH26 RBC or CD47-Blocked DiR RBC with CD47+ PKH26 RBC were analyzed for IR intensity by LI-COR (excitation 800 nm). The number of engulfed RBCs was calibrated from the LI-COR measured IR intensity of a dilutions series of pre-injected blood. CD47- blocking did not affect the RBC localization to the spleen with CD47+ and CD47-blocked RBC injected spleens, and calibration gives an estimated mean of $\sim 4 \times 10^4$ DiR-huRBCs per spleen. Combined with results in the bar graph of Fig.4E, $\sim 4 \times 10^4$ RBC were engulfed by 2% of total splenic macrophages, predicting that the murine liver contains $\sim 2 \times 10^6$ macrophage. This macrophage number seems consistent with literature that indicates typical murine *liver* contains $\sim 10^7$ macrophage, and total liver versus spleen macrophages are in a ratio of 3.2, so that murine spleen has $\sim 3 \times 10^6$ macrophages.

Figure S4. Characterization of GA-Stomatocyte

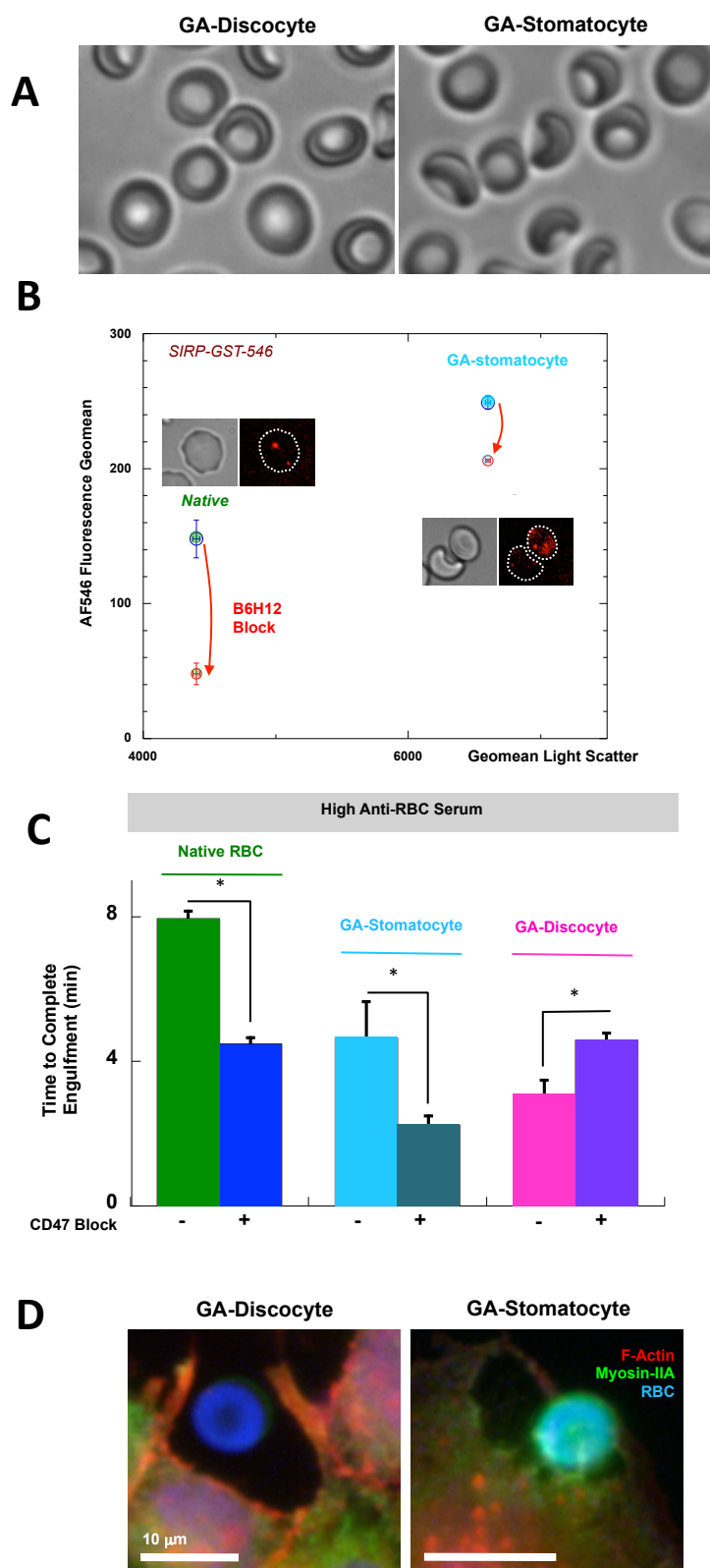


Figure S4. Characterization of GA-Stomatocyte (A) Phase contrast imaging of GA-stomatocytes and GA-Discocytes. GA-stomatocytes are more rounded than GA-discocytes. (B) SIRPA binding to GA-stomatocyte is similar to native, and can be inhibited by pre-treatment with B6H12. (C) CD47 blocking increases the rate of phagocytosis of native cells that become rounded during engulfment, and of rounded GA-stomatocytes. In contrast, CD47 blocking increases the rate of engulfment of GA-discocytes. (D) Fluorescence imaging of the macrophage synapse with GA-discocyte shows that the macrophage pseudopods can be loosely apposed to the GA discocyte surface while the synapse with GA-stomatocyte shows tightly apposed myosin-rich pseudopods.

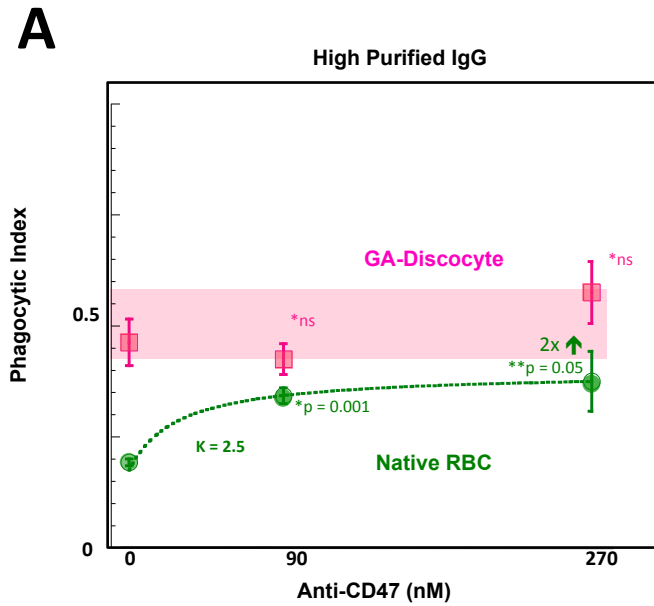


Figure S5. Uptake of IgG Opsonized Native RBC. A flow cytometry based phagocytosis assay of purified IgG-opsonized native RBC relative to purified IgG opsonized GA-discocytes (as in Fig 5B) was performed. Similarly to GA-Discocytes, and in contrast to GA-stomatocytes, uptake of purified IgG opsonized native-RBC is reduced relative to anti-serum opsonized native RBC.

CHAPTER 3: LENTIVIRAL DISPLAY OF 'MARKER OF SELF' CD47 DECREASES MACROPHAGE UPTAKE OF VIRUS AND INCREASES CIRCULATION FOR DELIVERY TO TUMORS

Abstract

Lentiviruses infect many cell types with high efficiency and are now widely used for gene delivery *in vitro*, but *in vivo* uptake of these foreign vectors by macrophages limits broader application. Lentiviruses (Lentis) are generated here from packaging cells that overexpress CD47, which partially inhibits phagocytosis of multiple human and mouse cell types when the cells also present pro-phagocytic factors. Single particle analyses show that 'hCD47-Lenti' display properly oriented human-CD47 for interactions with the phagocyte inhibitory receptor SIRPA. Macrophages derived from human and NOD/SCID/IL2rg^{-/-} (NSG) mice show a SIRPA-dependent decrease in transduction by hCD47-Lenti compared to Control Lenti, whereas human lung epithelial cells are efficiently transduced by hCD47-Lenti. Macrophage transduction by the Control Lenti is also decreased by pharmacological inhibition of Myosin-II to the same levels as hCD47-Lenti, consistent with 'Self' signaling pathways established previously for phagocytosis of cells. Systemic injection of hCD47-Lenti into NSG mice shows hCD47 prolongs circulation, unless a blocking anti-SIRPA is pre-injected, and *in vivo* transduction of spleen and liver macrophages is also decreased whereas transduction of xenografted tumor cells increases. Display of hCD47 could prove useful on other viruses when macrophage uptake is limiting.

Materials and Methods

Reagents, DNA constructs, cell lines, and standard methods are described in supplemental Methods.

Generation of HEK-CD47-GFP Producer Cell Line HEK 293T cells were transduced with a lentiviral vector encoding CD47-GFP and puromycin resistance transgenes. Lentiviral transduction was followed by puromycin selection for 3 weeks. To produce the CD47-Lenti, the HEK CD47-GFP cells were transfected using standard methods.

In Vitro Transductions Cells were plated at 1×10^4 cell/cm². Lentivectors were added at an MOI from 10-2000. Where MOI (multiplicity of infection) is the ratio of the number of vectors to number of cells. Macrophages and lentivector were co-incubated for 1 hour. 3 days post-transduction, cells were assayed for reporter gene expression by fluorescence microscopy. Total cell number was determined by DNA stain (Hoechst). Where indicated, cells were pre-treated with anti-human SIRPa (clone SE7C2) or with anti-mouse SIRPa (clone P84) 15 minutes prior to transduction. Where indicated, cells were pre-treated with Blebbistatin (5-50 μ M) for one hour prior to transduction. Anti-SIRPA and Blebbistatin incubations continued throughout the duration of the 1 hour lentivector-cell incubation.

In Vivo Injections and Sampling 3×10^8 to 1×10^9 lentivirus were injected via tail vein of NSG mice. Blood was sampled via retro-orbital bleed at 10 min, 45 min, and 24 hours post-injection and analyzed by functional titer (Supplementary Methods). 4-5 days post-injection spleen, liver, and flank tumors were collected for immunohistochemistry analysis, or flow cytometry analysis of reporter gene expression (DsRed.MST). 100 μ g of anti-mSIRPA (P84) was pre-injected 30 minutes prior to injection of lentivector for in vivo SIRP blocking studies.

Introduction

Viral delivery of genes can be extremely efficient *in vitro* (Naldini 1996), and many therapies are now emerging with Lentiviral vectors (Lentis) (Consiglio 2004, Worsham 2006, Lee 2009) including recent clinical trials (Cartier 2009, Frecha 2012, Aiuti 2013). However, *in vivo* therapies with Lenti are challenged by clearance into macrophages, which take up all types of foreign particles (Scherphof 2006, Rodriguez 2013). As a consequence, the vast majority of a Lenti-delivered transgene is expressed in these phagocytic cells of spleen, liver, and bone marrow (Pan 2002, Follenzi 2002). Immunohistochemical analysis of livers of Lenti-injected mice has also confirmed expression in the Kupffer cells, which are the dominant liver macrophages (van Til 2004) that derive from bone marrow (Klein 2007). Uptake by macrophages is generally enhanced by opsonins such as IgGs (Montalvao 2013, Takeda 1990) that can physisorb to virus during production in serum and/or post-injection (DePolo 2000), and uptake is also enhanced by the commonly used Lenti envelope protein, VSV-G (Farley 2007). Acute or chronic inflammatory responses initiated by vector components can occur after macrophage uptake (Brown 2007, Rossetti 2011). Reducing viral clearance by the innate immune system has been attempted by modification with polymers (Croyle 2004) analogous to the so-called 'stealth' coatings that delay opsonizing deposition of serum proteins and subsequent clearance of liposomes (eg. Semple 2000). However, such polymer brush coatings will tend to obscure VSV-G and other envelope proteins that seem useful for targeting viruses to non-phagocytic cell types (Yang 2008, Padmashali 2011, Hwang 2013, Verhoeven 2004, Pariente 2007).

CD47 is an integral membrane protein found on all human and mouse cells, and its immunoglobulin-like N-terminal domain is a ligand for an immunoinhibitory receptor SIRPA that is abundant on macrophages (Jiang 1999). SIRPA activates the phosphatase SHP-1 (Veillette

1998), which represses the otherwise efficient phagocytosis of cells and large particles when opsonized with IgG and complement among other antagonistic factors (Anderson 1990, Tsai 2008). By this pathway, CD47 acts as a 'Marker of Self' as originally described when CD47-deficient red cells were injected into control mice and found to be rapidly cleared by splenic macrophages (Oldenborg 2000). As the capsid of an enveloped-type virus buds through a host cell membrane, the virus membrane can in principle incorporate various integral membrane proteins and maintain protein orientation (McLellan 2011). We hypothesized that full-length hCD47 could be displayed sufficiently on the envelopes of Lenti to specifically signal against macrophage uptake of these complex particles. For *in vivo* studies, NOD/SCID/Il2rg^{-/-} (NSG) mice are used because these mice express a unique mouse variant of mSIRPA that binds human-CD47 (Takanaka 2007), and we show that CD47-Lenti exhibit enhanced circulation and gene delivery to a model disease site in these mice. The findings illustrate an approach with highly specific '*anti-targeting*' to avoid innate immune macrophages.

Results

Virus Display of 'Marker of Self' CD47

Human-derived HEK 293T cells are widely used to package lentiviral vectors (Naldini 1996), and transduction of this epithelial cell line with a human CD47-GFP construct shows the GFP signal is predominantly at the plasma membrane (**Fig. 1A**). Immunostaining of fixed but non-permeabilized cells with an antibody to hCD47's extracellular Ig domain further shows a maximum intensity at cell-cell junctions where the antibody can in principle cross-bridge hCD47 in *trans* between two membranes. CD47-GFP on epithelial cells is already known to be highly mobile (Subramanian 2007), which should be conducive to integration into viral envelopes. Flow cytometry measurements of anti-CD47 intensity on the transduced cells indicated >10-fold higher CD47 density than that of control cells (**Fig. S1A**), and mass spectrometry analyses of the 'HEK CD47+'

cells also detected three peptides from the human Ig domain with a mean ion current that was again >10-fold that of control cells (**Fig. S1B**).

Since red cell circulation relies on CD47 (Oldenburg 2000), a direct comparison with HEK producer cells was sought. The much smaller human red cell has ~25,000 molecules of hCD47 per cell (Mouro-Chanteloup 2003), and from image-based estimates of mean surface areas of RBC (Fung 1993) and HEK cells (Sommerhage 2008), calculated densities of hCD47 on RBCs and HEK CD47+ cells prove similar (**Fig. S1C**). Control HEK cell membranes not only display much lower hCD47 density, but their hCD47 density is below the levels needed for inhibition of macrophage uptake based on studies of recombinant hCD47 Ig domain on opsonized beads (Tsai 2010, Rodriguez 2013). Control Lentis that bud from conventional HEK cells are therefore unlikely to signal 'Self' with hCD47, but viruses that bud through the HEK CD47+ membranes can in principle display abundant hCD47 (**Fig. 1B**)

Lentiviral supernatants made by transfecting HEK-CD47 cells with standard plasmids (Methods) were concentrated by ultracentrifugation and then immobilized on coverslips pre-coated with anti-CD47 in order to image GFP containing particles. High-resolution Total Internal Reflection Fluorescence Microscopy (TIRFM) revealed mostly nano-sized fluorescent particles (**Fig. 1C, S2A**), and control particles were not fluorescent. Distributions of particle intensities were dominated by a single population (**Fig. S2B, C**) as were particle height distributions determined by atomic force microscopy, which gave a ~120 nm peak (**Fig. S2D, E**) that is consistent with dimensions of standard lentivirus (Koi 2007, Fuller 1997, Dorfman 1994, Reicin 1996, Nakai 1996). Photobleaching of GFP occurred over minutes and showed no evidence of single molecule step-wise bleaching (**Fig. S2F**), which indicates at least a dozen or more CD47-GFP molecules per particle and seems consistent with 90-260 nm particles having CD47 densities similar to the producer cells (~7-60 molecules per spherical virus). Importantly, ~70% of attached GFP particles were also double-positive for acridine orange, which permeates membranes and

fluoresces red when bound to single stranded RNA or DNA (**Fig. 1C**) – as expected for the ssRNA genome of lentivirus (Desport 2010). Immobilization of the intact CD47-lenti to glass coverslips was also observed only with antibody to hCD47's Ig domain and binding occurred in minutes, whereas anti-GFP and non-specific antibody showed little to no immobilization of particles even after incubations of hours (**Fig. 1D**). The lack of binding of anti-GFP suggests this C-terminal epitope is within the lentiviral envelope as expected, whereas CD47's Ig domain is outward oriented on the virus and thus available for binding SIRPA.

Figure 1. Virus Display of ‘Marker of Self’ CD47

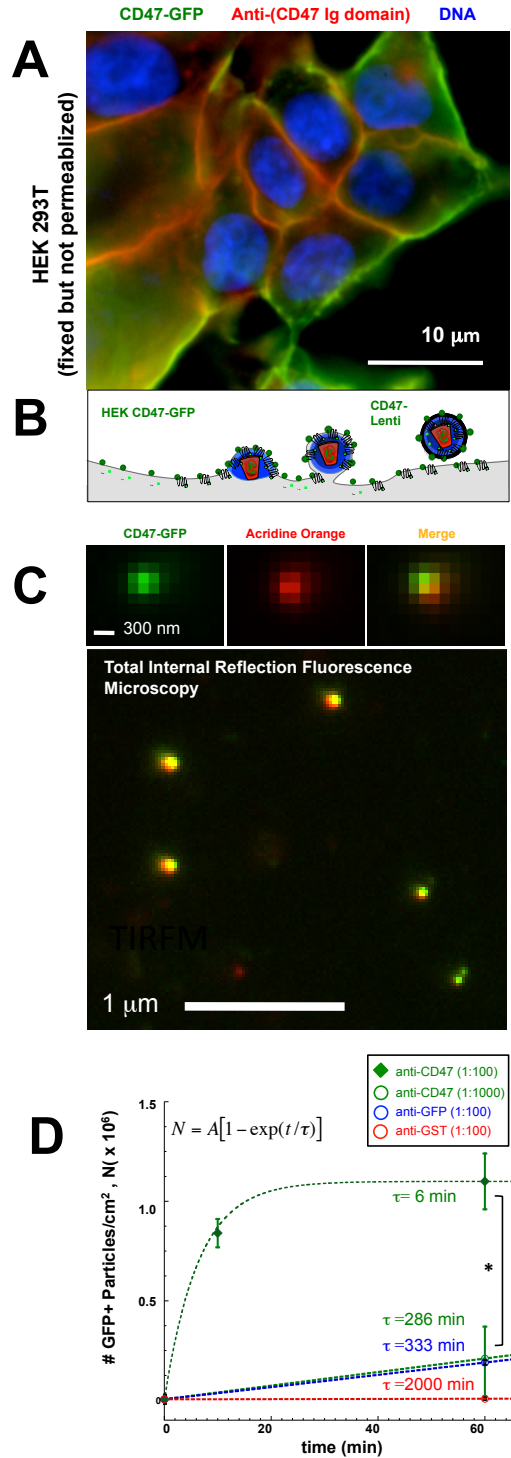


Figure 1. Novel Lentivector Displays CD47-GFP (A) In HEK-CD47-GFP Producer cells the CD47-GFP (green) and anti-CD47 (red) signals are localized to the cell periphery, distinct from the nucleus (Hoechst, blue). CD47-GFP signal colocalizes with anti-CD47 immunostaining of the HEK-CD47-GFP cell membrane. (B) Lentiviruses are enveloped viruses, where the viral capsid and genome self-assemble beneath the cell membrane and the assembled lentivirus then buds taking a piece of the cell membrane as the viral envelope. (C) HEK-CD47 lentiviral transfection supernatants were incubated with anti-CD47 coated coverslips, and adherent material was stained for acridine orange, which co-localized with the CD47-GFP fusion protein. (D) CD47-lenti binds IgG coated glass in an anti-CD47-dependent and anti-GFP independent manner ($n \geq 3$, $p \leq 0.05$).

Equilibrium density gradient ultracentrifugation of CD47-Lenti supernatants should separate the relatively dense capsid-containing virions from lighter cell debris, vesicles, and media components (Dettenhoffer 1999). Such a separation was confirmed by assays for functional titer of infectious virus in HEK cells (**Fig. S3A-C**); the virally delivered transgene in these and all studies below is a CMV-promoter driven RFP variant DsRed.MST (Vinterson 2004, Bevis 2002), which allows quantitation of fluorescent cells. Coomassie-stained SDS-PAGE separations of ultracentrifuged fractions suggested that functional virus contained both CD47-GFP and the p24 protein that makes up the viral capsid (Zhao 2013), with CD47-GFP being confirmed by anti-GFP immunoblotting (**Fig. S3D,E**).

CD47 inhibition of Macrophage Transduction & Enhancement of A549 Transduction

Macrophage transduction by CD47-Lenti versus control Lenti was assessed first with human-derived, PMA-differentiated THP1 cells at a vector to cell ratio (i.e. multiplicity of infection, MOI) that ranged from 10 to 2000 (**Fig. 2A**). At intermediate MOI, CD47-Lenti transduced ~3-fold fewer macrophages than control lenti; and across the entire range of MOI's, standard hyperbolic dose-response curves fit very well, giving half-max transduction constants (K) of 3700 versus 1024 MOI units respectively for CD47-Lenti *versus* control Lenti. Transduction of the human-derived lung cancer epithelial line (A549) cell line showed that the CD47-Lenti can mediate high gene transfer (**Fig. 2B**). Indeed, transgene expression efficiency in A549 cells was 3-fold *greater* for CD47-Lenti *versus* control Lenti, with respective half-max transduction constants (K) of 270 versus 760 MOI units. Note that the control Lenti K's for A549 cells and THP1 macrophages differ by only ~25% (760 versus 1024), consistent with standard Lentivirus being broadly infectious across cell types (Farley 2007).

Figure 2. CD47 inhibition of Macrophage Transduction & Enhancement of A549 Transduction

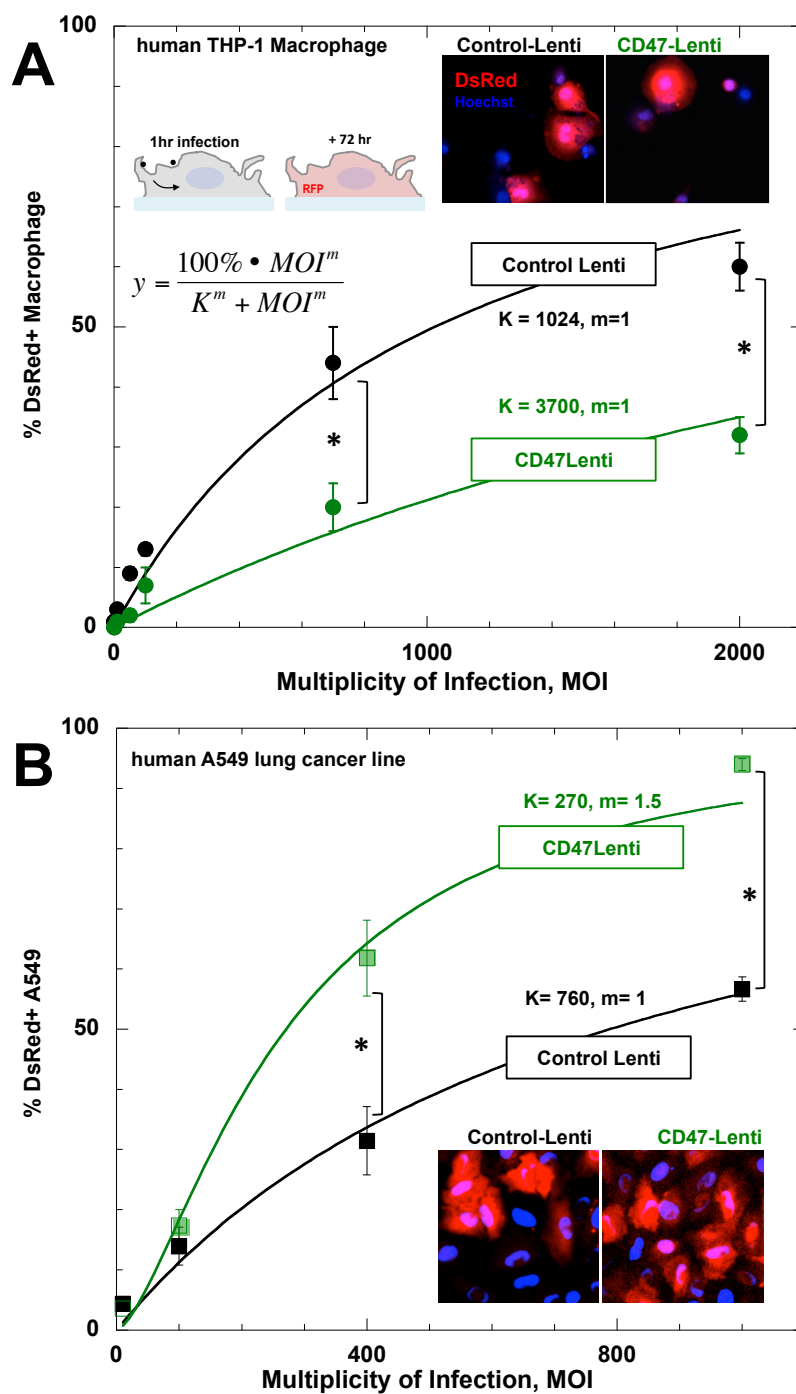


Figure 2. CD47 inhibition of Macrophage Transduction & Enhancement of A549 Transduction. CD47 and Control-Lentis encode the reporter transgene RFP variant DsRed.MST under the control of a CMV promoter. Either control or CD47 displaying Lenti were added to cells *in vitro* at a vector to cell ratio (VCR) from 10 to 2000. Lentivectors were co-incubated with cells for 1 hour followed by washing. 72 hours later the percentage of transduced THP-1 macrophages **(A)** and A549 cancer epithelial cells **(B)** was determined by the RFP and Hoechst fluorescence quantitation. Both cell types showed a saturating response to VCR, and the transduction response was fit with Michaelis-Menten kinetics (K and m inset) ($n \geq 3$, $p \leq 0.05$, $R^2 > 0.95$).

Mechanistic reasons for the transduction differences with CD47-Lenti were first explored by pre-blocking SIRPA on both cell types. While SIRPA acts as an inhibitory receptor for uptake by macrophages, it is not reported to have such a function on other cell types such as lung epithelial cells perhaps because the downstream phosphatase SHP1 is relatively restricted to hematopoietic cells (**Fig. 3A**). In anticipation of *in vivo* studies below, primary NSG-derived splenic macrophages were used *in lieu* of the human macrophage THP-1 line, and we first confirmed that CD47-Lenti transduced NSG-macrophages about 3-fold less effectively than control Lenti at the same MOI (**Fig 3B, left gray bar**). Importantly, pre-blocking the NSG macrophages with anti-(mouse-SIRPA) (Fukunaga 2004) eliminated the difference in transduction between CD47-Lenti and control Lenti, which we plot simply as a 'Transduction Ratio' that becomes ~1 upon SIRPA blocking. Parallel experiments with the A549 cells used anti-(human-SIRPA) and showed that the higher transduction of these epithelial cells by CD47-Lenti was eliminated by this function blocking antibody (**Fig 3B, right red bar**). The noted increase in transduction of A549 cells by CD47-Lenti versus Control-Lenti also depends on SIRPA, as an anti-SIRPA antibody decreases the Transduction Ratio to ~1 (**Fig. 3B, right bars**). Thus with respect to CD47-Lenti interactions, SIRPA in these epithelial cells acts opposite to SIRPA in macrophages.

CD47-SIRPA activation of the immuno-inhibitory phosphatase SHP-1 inactivates several proteins that make engulfment efficient, particularly nonmuscle Myosin-IIA motors that help pull targets in during phagocytosis by macrophages (Tsai 2008). The Myosin-II ATPase inhibitor blebbistatin should therefore inhibit uptake similar to the effect of displaying CD47 (Fig. 3A). THP-1 macrophages were pre-treated for 1 hr with the Myosin II inhibitor blebbistatin (5-50 μ M) and then control Lenti or CD47-Lenti were added. While the effect of blebbistatin on macrophage transduction by CD47-lenti was insignificant (**Fig. 3C**), which is consistent with redundant pathways, transduction by the control Lenti decreased with high blebbistatin to that of CD47-

Lenti. The half-max inhibition constant of $\sim 5 \mu\text{M}$ is also consistent with inhibition of the Myosin-IIA ATPase (Allingham 2005). These results support the conclusions that Myosin-IIA is specifically involved in the efficient uptake of Lentis by macrophages and that CD47 reduces macrophage uptake of Lentis by inhibiting Myosin-IIA motor activity. Phagocytosis of IgG-opsonized red blood cells is also made efficient by Myosin-IIA that the CD47 on the red cell inhibits to suppress engulfment (Tsai 2008). The comparison begins to suggest that lentiviruses are strongly opsonized to drive clearance, perhaps with IgG, which is consistent with serum opsonization of lentivirus (Jolly 1989a, Jolly 1989b, DePolo 2000).

Figure 3. Mechanism of Inhibition & Enhancement

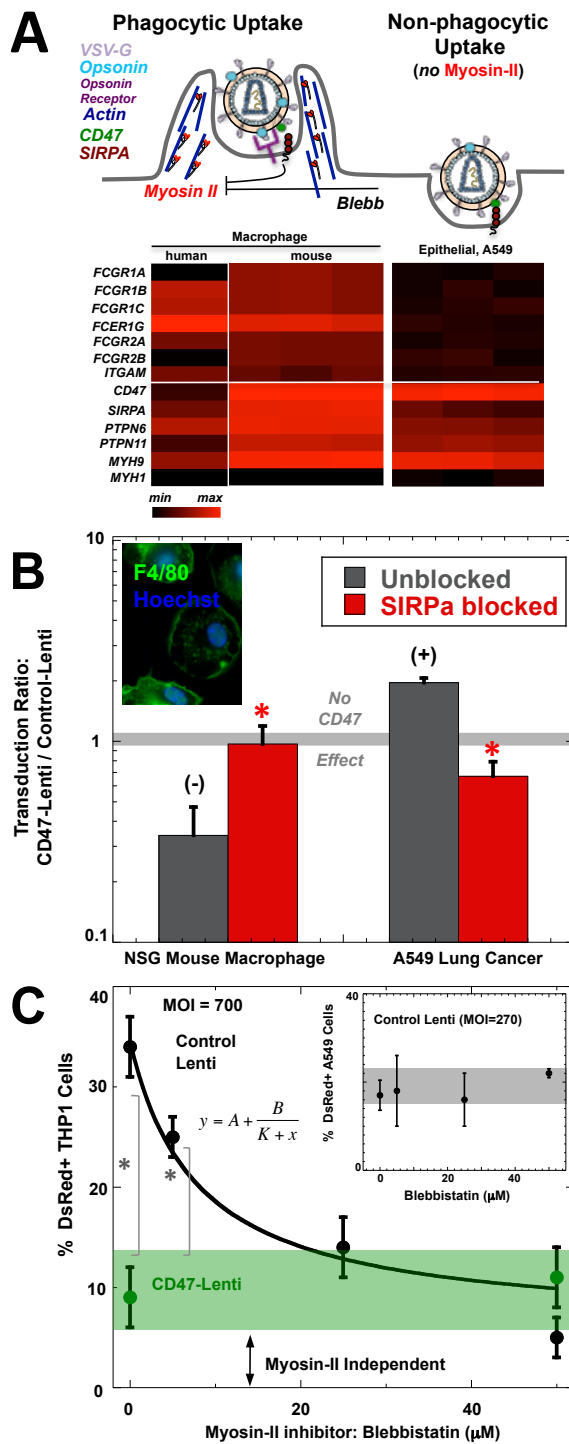


Figure 3. Mechanism of Inhibition & Enhancement. (A) Lenti CD47 display is expected to inhibit uptake by macrophage via binding to SIRPa, which leads to activation of the SHP-1 phosphatase that deactivates proteins involved in macrophage uptake including FcR mediated activation of Myosin-II motor. Myosin-II can also be inhibited by Blebbistatin. Epithelial derived A549 cells express SIRPA but, in contrast to macrophage, lack FcRs. Heatmaps quantify mRNA levels of relevant genes where black indicates minimum value and red the maximum value {(min, max): human THP-1 (2.4, 11.5), mouse spleen (5.2, 12.0), human A549 (1.9, 3.6)}. (B) Splenic macrophage and A549 cells were pre-treated with an anti-SIRPa IgG prior to transduction or not. The percentage of transduced cells was quantified by fluorescence microscopy detection of DsRed and Hoechst signals. The ratio of CD47-Lenti transduction to control lenti transduction was calculated for each condition. A ratio of 1 indicates equal transduction by the CD47- and control Lentis ($n \geq 3$, $p \leq 0.05$) (C) Macrophages were pre-treated with blebbistatin (5-50 μM) and then transduced with either control or CD47-Lenti ($n \geq 3$, $p \leq 0.05$). The control lenti macrophage transduction response to blebbistatin concentration fit well to a standard inhibition curve, ($y = A + B/(K+X)$, $R^2 > 0.98$), with $A = 6\%$, $B = 225\% \mu\text{M}$, and, $K = 7.8 \mu\text{M}$. (C, inset) A549 cells were pre-treated with Blebbistatin for 1 hour prior to transduction with control lenti. A549 transduction shows no response to blebbistatin.

CD47-Lenti Enhances *in vivo* Circulation Unless SIRPA is Blocked

To determine the impact of CD47 display on *in vivo* circulation of lentivirus, either a control or CD47-lenti were injected into the tail veins of NSG mice, and the blood was periodically sampled for a functional titer in HEK 293T cultures (**Fig. 4A**). Both control and CD47-Lenti were progressively cleared from the bloodstream, but the control-Lenti was cleared more rapidly so that the ratio (circulating CD47-lenti) / (Control-lenti) increased with time (**Fig. 4B**). At 10 min post-injection, this ratio favored CD47-lenti by just 3-fold, but by 45 min the advantage of CD47-lenti increased to nearly 10-fold relative to control vectors. This ratio remains high up to 24 hrs, but decreasing titers add uncertainty to long-term measurements. Fitting just the initial 45 min of ratio data with an exponential not only provides a good fit but also yields a fitting constant $T = 16$ min for the time at which CD47-displaying Lenti are 2-fold more abundant than control Lenti (**Fig. S4A**). This persistence ratio doubling time agrees well with the $T \approx 20 - 30$ min obtained for the CD47-conferred advantages in circulation of highly opsonized mouse red cells and also highly opsonized opsonized nanobeads (Rodriguez 2013). Circulation results for *unopsonized* mouse and human red cells (Strowig 2011, Oldenborg 2000) yield in similar analyses $T \sim 10$ hrs (**Fig. S4A**), which means the advantage of CD47 is slow to manifest for unopsonized entities in circulation. The comparison again suggests that lentiviruses are strongly opsonized to drive clearance, consistent with serum opsonization of lentivirus (Jolly 1989a, Jolly 1989b, DePolo 2000), while also indicating that CD47 delays opsonization-driven clearance *in vivo*.

The lentivector titer in circulation 45 min after injection relative to that at 10 min defines for each mouse a viral kinetics ratio (VKR) that succinctly characterizes the effect of blocking *in vivo* with anti-mSIRPA. Anti-mSIRPA blocks macrophage interactions with CD47 not only *in vitro* (**Fig. 3B**) but also *in vivo* where it binds NSG macrophages (Rodriguez 2013). Pre-injecting this antibody in NSG mice indeed decreases the VKR significantly for CD47-Lenti (**Fig. 4C**). CD47-Lenti's antibody-blocked VKR is also statistically similar to that of control Lenti, which shows a VKR that is statistically independent of whether anti-mSIRPA is pre-injected or not. The enhanced *in vivo* circulation of CD47-Lenti is therefore due to a CD47-SIRPA interaction.

Figure 4. CD47 Enhances in Vivo Circulation

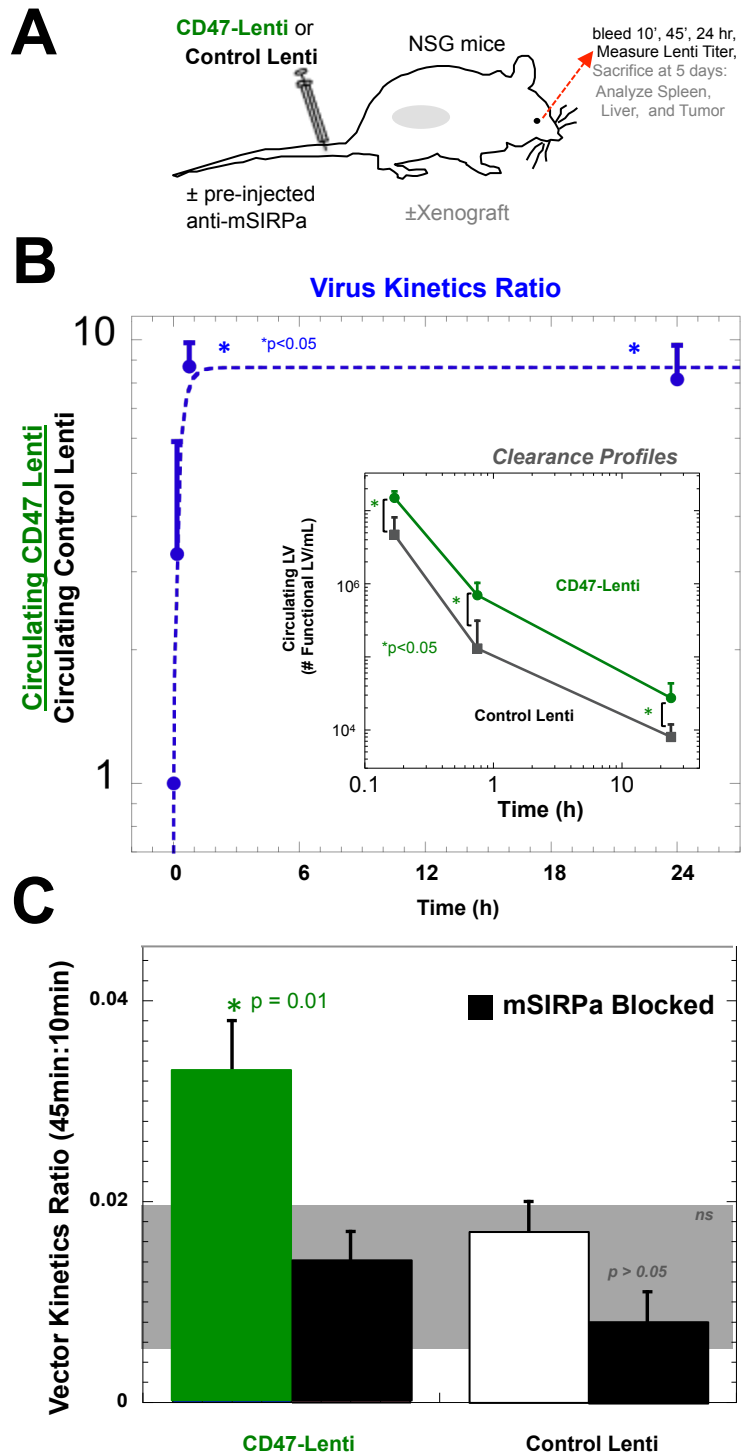


Figure 4. CD47 Enhances In Vivo Circulation. (A) CD47- or Control Lentis were injected via tail vein to NSG mice. A 10 min 45 min, and 24 hour orbital bleed were analyzed via functional titer for Lenti concentration. (B) Either control or CD47 lenti (1×10^9) were injected via tail vein to NSG mice. The circulation ratio, the relative concentration of CD47-Lenti relative to control-Lenti, was quantified ($n = 13$, $p \leq 0.05$). Both control and CD47-Lenti were cleared from the blood stream, with the control-lenti being cleared more rapidly (**inset** $p \leq 0.05$) and consistent with VKR (Fig. 4A). The circulation advantage ratio, R , is the ratio of CD47-Lenti to control Lenti, remaining in circulation as determined by a functional titer assay of blood samples. By 45 minutes post-injection, CD47-Lentis are nearly 10-fold more abundant in circulation than control-Lenti ($n=13$ $p \leq 0.05$). The time constant T is where $R=2$). The time constant, τ , for Lentivectors is $\tau=0.35h$ (**blue**). (C) The Virus Kinetics Ratio (VKR), or the ratio of virus remaining in circulation at 45 minutes relative to that remaining at 10 minutes, was quantified and gives a measure of the persistence in circulation of the lentivector. Further, SIRPA was blocked in vivo with an anti-SIRPA mAb against mouse SIRPA, or not, prior to injection of lentivirus ($n \geq 3$, $p \leq 0.05$)

***In vivo* Transgene Expression: decreased in Macrophages, increased in Tumors**

CD47-inhibited clearance of cells has primarily been attributed to impeding uptake by splenic red pulp macrophages (Oldenborg 2000). Spleens of NSG mice here were therefore isolated 5 days after injection in order to allow ample time for expression of the transgene, and then the spleens were dissociated into single cell suspensions for identification of transduced macrophages (positive for F4/80, also known as *Emr1*). Mice injected with CD47-Lenti showed nearly two-fold fewer DsRed⁺ splenic macrophages relative to mice injected with control Lenti (**Fig. 5A**). This seems quantitatively consistent with the 2-3 fold enhancement of circulating CD47-Lenti (Fig. 4B,C).

NSG mouse livers were also removed and sectioned, with confocal imaging of immunofluorescence showing F4/80⁺ cells with a reticulated morphology consistent with Kupffer cells (**Fig. S5A**) and also an abundance of mouse-CD47⁺ cells with round morphologies consistent with hepatocytes (**Fig. S5B**). Both control-Lenti and CD47-Lenti transduced Kupffer cells and hepatocytes (**Fig. S5C, D**), but quantitation indicated two-fold fewer DsRed⁺ Kupffer cells for CD47-Lenti (**Fig. 5B**). To our knowledge, CD47-inhibited clearance has not been reported for liver macrophages; but these cells do derive from bone marrow (Klein 2007), and phagocytosis of bone marrow macrophages is inhibited by the CD47 pathway (Oldenborg 2000). Expression profiles in the liver were therefore analyzed and provide evidence of *Emr1*-expressing cells with reasonably abundant *SIRPA* and *SHP1* (*Ptpn6*) (**Fig. S5E**). Moreover, in light of the similar two-fold decrease in Kupffer cell and splenic macrophage transduction with CD47-Lenti here, a similar inhibitory pathway seems likely.

A more persistent circulation and reduced uptake by macrophages with the CD47-Lenti suggests that the greater abundance of these novel vectors in blood should better infect other accessible cells relative to control Lenti. Solid tumors tend to be leaky to many types of circulating particles (Dvorak 1988, O'Connor 1984), and so A549 xenografts in NSG mice were established and tail-vein injected with the Lentis. As with the macrophage studies, 5 days was allowed for transgene

expression, and then the xenografted tumors were removed, disaggregated, and cells were analyzed by flow cytometry (**Fig. S5F**). The A549-identified population showed on average ~7-fold more transgene-positive cells in mice injected with CD47-lenti *versus* control-Lenti (**Fig. 5C**). With the same xenograft model, CD47-nanobeads loaded with near-infrared dyes localized to the tumors 3-5-fold more so than nanobeads without CD47 (Rodriguez 2013), and the result here is statistically similar. Only ~5% of A549 cells appeared transduced by CD47-lenti, but this seems reasonable because particles as large as lentiviruses (~100 nm) which leave the vasculature and enter tumors rarely permeate very deeply into tumors (Wong 2011). The CD47-Lenti results *in vivo* are nonetheless consistent with increased circulation, decreased uptake by macrophages, and increased delivery to other accessible cells.

Figure 5. CD47 inhibits DsRed expression by Macrophages and enhances DsRed expression in Human Tumor Cells

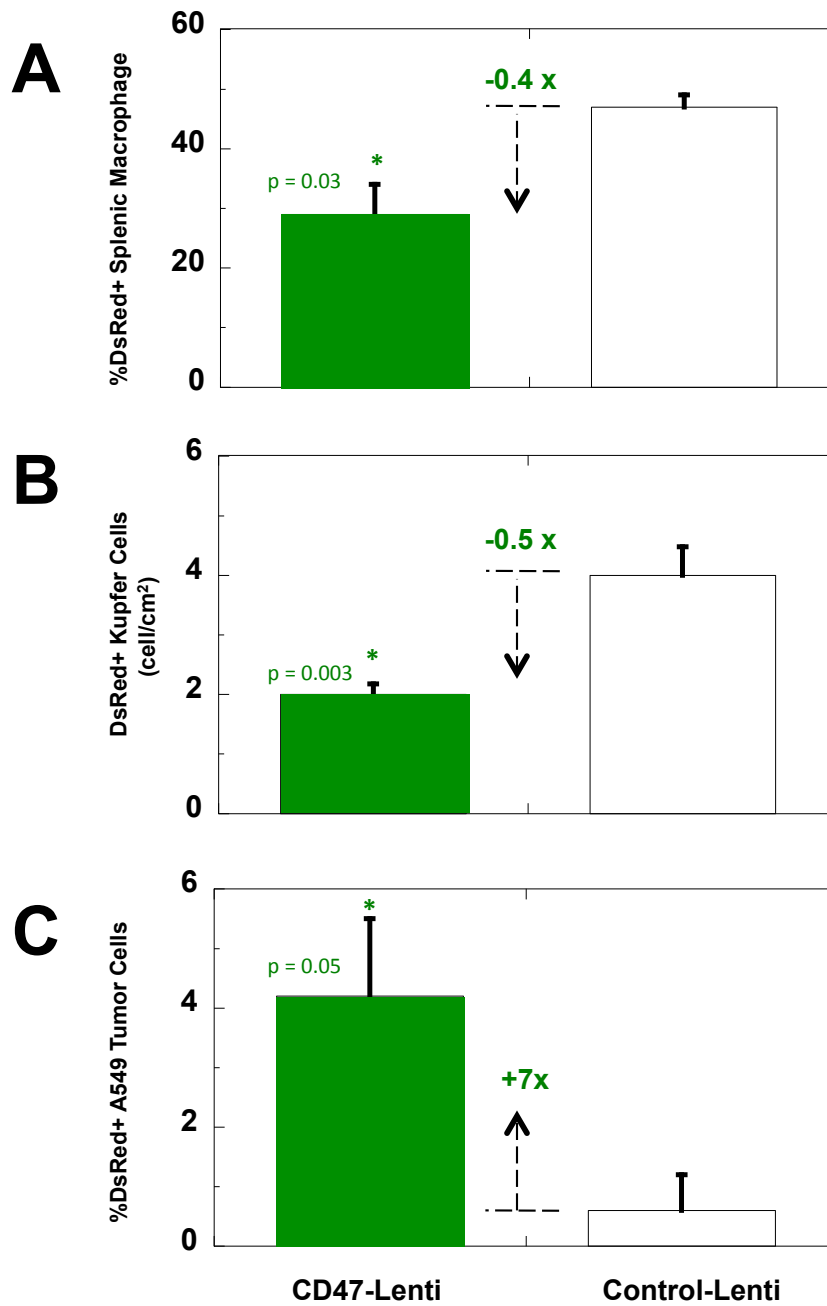


Figure 5. CD47 Reduces Transgene expression in Spleen and Liver Macrophage while Enhancing Transgene expression in Tumor Cells. (A). 1×10^9 CD47- or Control-Lenti were injected via tail-vein. 5 days post-injection spleens were mechanically dissociated. Splenic macrophage were identified by F4/80 immunostaining, and analyzed for RFP transgene expression by flow cytometry. CD47-Lenti injected spleens showed decreased splenic macrophage transduction ($n=3$, $p=0.03$). (B) 1×10^9 CD47- or Control-Lenti were injected via tail-vein. 5 days post-injection livers were isolated. Tissue sections were analyzed by IHC and imaged for RFP expression by confocal microscopy. Cells that were consistent with Kupffer cell morphology (Fig S5A-D) and positive for RFP expression were quantified ($n>6$, $p=0.003$). (C) CD47- or Control-Lenti were injected via tail-vein to A549 xenograft-tumor bearing NSG mice, and 5 days post-injection tumors were isolated and analyzed for RFP transgene expression where CD47-lentivector mediated significantly higher transgene expression ($n \geq 4$, $p < 0.05$).

Discussion

Attachment of recombinant human-CD47's immunoglobulin domain to polystyrene nano-beads that were IgG-opsonized was found in our recent studies to be sufficient to repress macrophage uptake *in vitro* and *in vivo* (Rodriguez 2013). However, lentiviruses are far more complex than polystyrene beads. The HIV-1 derived vector (Dull 1998) has an inner core composed of three key structural proteins matrix, nucleocapsid, capsid (p24), with the latter assembling as hexamers and pentamers into a 'fullerene cone' lattice (Zhao 2013). Since the lipid-based envelope derives from host cells, current methods of enhancing delivery of lentiviral vectors have rightly focused on display of envelope proteins that can enhance uptake (Yang 2008, Padmashali 2011, Hwang 2013, Bischof 2010) and gene expression within specific target cells (Follenzi 2004). Biodistribution studies show that lentiviruses are nonetheless taken up into macrophage rich tissues (Pan 2000) with gene expression preferentially found in macrophages (Van til 2004). Such uptake could in principle promote inflammation and challenge long-term transgene expression that is sought for treatment of genetic disorders.

Molecular mechanisms of macrophage uptake of viruses are likely to be diverse and of course distinct for these professional phagocytes from virus internalization processes in other cell types. Antibody dependent enhancement of HIV infection has been demonstrated *in vitro* (Joualt 1989, Jolly 1989, Takeda 1990), and Fab'2 fragments that lack the Fc domain of intact antibodies are insufficient to enhance uptake while blocking Fc receptors does inhibit infection (Takeda 1990). Relevant studies of VSV-G pseudotyped lentiviral vectors have shown in naïve serum that there are pre-existing IgGs that neutralize virus (DePolo 2000, Thiry 1978, Beebe 1981), and fetal bovine serum that is commonly used in lentiviral vector preparations can also contain virus-specific IgGs (Offit 1984). Immunoblotting analyses of serum from pre-injected and vector-injected mice likewise indicate that mice have pre-existing antibody that increases with time post-injection (Baeklandt 2003). Importantly, both liver and spleen macrophages express FcγR

receptors that bind IgG, and both liver and spleen uptake of red cells is also clearly enhanced by IgG opsonization on red cells (Loefering 1989). Intravital imaging of liver further shows the Fc γ R dependent uptake of IgG opsonized cells into Kupffer cells (F4/80+, Cd11b^{hi}) (Montalvao 2013). Likewise in spleen, red pulp and marginal zone macrophages express functional Fc γ R, as detected by IgG binding (Denham 1990). Splenic macrophages as well as bone marrow derived macrophages are clearly inhibited by CD47 displayed on cell membranes (Oldenborg 2000, 2001, Gardai 2005, Tsai 2008), and the findings here (Fig. 5A,B) suggest that Lentis which bud through cell membranes rich in hCD47 also have the ability to inhibit uptake by liver as well as spleen macrophages.

CD47 is expressed on all cell types, including leukocytes (Brown 2001) that are HIV-susceptible. HIV particles that bud from such cells might therefore display some CD47 on their envelope and thereby impact infection. Although results here suggest that HEK producer cells would normally need to overexpress CD47 (Fig. 1), while leukocyte progenitors can increase their CD47 levels in a manner that is correlated with internal tandem duplications of the receptor tyrosine kinase, CD135 (Majeti 2009). Moreover, a poxvirus encodes what has been claimed to be a CD47 ortholog that causes a more severe pathology than virus lacking the protein (Cameron 2005), although claims of relevance to CD47 remain controversial (Hatherley 2006). Decreased macrophage uptake has the potential to reduce immune responses downstream of myeloid cell uptake of Lentis (Brown 2007, Rossetti 2011), and CD47 seems to modulate inflammatory response and induction of adaptive immunity (Demeure 2000, Latour 2001, Baba JEM 2013, Tseng 2013). While phagosome processing can affect the extent of antigen presentation (Blander 2006), any effect of CD47 on antigen processing and presentation remains unclear.

CD47-Lentis as generated here from an overexpressing HEK line appears effective in enhancing gene delivery *via* reduced macrophage uptake and increased circulation. CD47-Lentis may therefore be useful for gene therapy in this receptor-specific 'anti-targeting' approach. We find

that CD47 is comparable in abundance on the Lenti surface (7-75 molecules) to gp120 on physiological HIV-1 virions (7-70 molecules) as estimated by EM and biochemical studies (Bachrach 2005). The density of both Env and CD47, here on the novel vector, are lower than that reported for VSV-G on wild-type VSV (~ 600 molecules/ μm^2) (Bunocore 2002, Cureton 2010). CD47 display may be beneficially combined with receptor-specific targeting to diseased cells. Our findings might be relevant to understanding the pathology of some enveloped viruses such as HIV-1 that are recalcitrant to vaccine development. While past studies of CD47 have largely focused on interactions involving animal cells much larger than virus, findings here deepen the current understanding of macrophage uptake processes and CD47-SIRPA inhibition at a very small scale.

Supplementary Materials and Methods

Materials Cells were washed with PBS without Ca²⁺ or Mg²⁺ (Invitrogen). For cell culture, RPMI-1640, DMEM, F12 medium, Penicillin-streptomycin, FBS, trypsin EDTA, and acridine orange were all purchased from Invitrogen. (±)-Blebbistatin was obtained from EMD Biosciences.

Antibodies Primary antibodies used for flow cytometry, western blotting, and immunofluorescence include anti-human CD47 (B6H12, BD Biosciences), anti-mouse CD47 (MIAP301, BD Biosciences), anti-GFP (Ab290, Abcam), anti-GST (Invitrogen), anti-mouse SIRPA (P84, BD Biosciences), anti-human SIRPA (SE7C2, BD Biosciences), anti-F4/80 (AbD Serotec). Secondary antibodies include donkey anti-mouse AlexaFluor 647 (Invitrogen), donkey anti-mouse-PE (Sigma), donkey anti-rabbit AlexaFluor 488 (Invitrogen), anti-rat-FITC (Sigma), anti-rabbit HRP-conjugated IgG (GE Healthcare).

Cell Culture HEK 293T, A549, and THP-1 cells (ATCC) were respectively maintained in DMEM (high glucose, phenol red-), F12, and RPMI-1640 media. THP-1 cells were maintained in RPMI-1640 supplemented with FBS and antibiotics. All media were supplemented with FBS and 1% antibiotics. THP-1 cells were differentiated in maintenance medium supplemented with 100 ng/mL phorbol myristate acetate (PMA) (Sigma) for two days and differentiation was confirmed by adherence to a solid substrate (cell culture plastic or glass).

Plasmids The Δ8.91 lentivector plasmid, VSV-G envelope plasmid under control of a CMV promoter, and lentiviral transfer plasmids containing human-CD47-EGFP, and DsRed.MST under the control of a CMV promoter were kindly provided by Dr. Philip Zoltick.

Lentivector Transfection and Concentration. Lentis were generated by transient transfection of Control HEK 293T or HEK-CD47 293T cells with the packaging, envelope, and transfer plasmids with transfection reagent (Mirus). At 1 and 2 days post transfection, viral supernatants were collected and stored at 4°C. Combined supernatants were filtered through a 0.45 µm sterile filter, then pelleted at 25,000 RPM on SW32 rotor for 1.5-2 hours. Pellets were resuspended in 100 µL PBS and stored at -80°C until use. Lentivector titer (LV/mL) was determined either by a functional titer assay or by p24 antigen ELISA (Center for AIDS Research, The University of Pennsylvania, Philadelphia, PA, USA).

Equilibrium Sedimentation of Lentiviral Vector Supernatants Concentrated lentivector supernatants were overlayed on to a continuous sucrose gradient (20-60%), ultracentrifuged at 50,000g for 18 hrs, and layers were carefully collected top to bottom by micropipette.

Widefield Fluorescence Microscopy Images were acquired with an inverted microscope (IX71; Olympus) with a 60x (oil, 1.4NA) objective using a Cascade CCD camera (Photometrics). Image acquisition was performed with Image Pro software (Media Cybernetics Inc.).

Sample Preparation for TIRF AFM Glass coverslips were cleaned with KOH followed by rinsing with sterile H₂O and drying. Where indicated cleaned coverslips were further coated with antibody (1:1000 to 1:100 dilution in PBS) for ≥ 1hr then rinsed by gentle submersion in PBS. Lentivector samples were incubated with coverslips for up to 1hr at room temperature then rinsed by gentle submersion in PBS.

Coupled Total Internal Reflection/ Atomic Force Microscopy Images were acquired with an inverted microscope (Nikon Eclipse) with a 100x (oil, 1.4NA) objective using a Cascade II EMCCD camera with liquid cooling. Samples were illuminated with Blue (Coherent Sapphire, 488 nm, 75 mW), and/or Green (Crystalaser CL, 532 nm, 100 mW) lasers. The angle of the laser

beam was adjusted to maximize GFP signal relative to background signal. A Veeco AFM was mounted on top of the inverted microscope and imaging was performed in tapping mode on areas of sample that were confirmed via TIRFM to contain GFP-positive particles.

Functional Titer of Lentiviral Stocks HEK 293T cells were plated at 1.2×10^5 cells/well in 12 well cell culture dishes (3.8 cm^2). 24 hours post cell plating, serial dilutions of lentiviral vector stocks were prepared and added to HEK293T cells pre-treated with DMEM supplemented with Polybrene (5ug/mL) (Sigma) for 1 hour. Exactly 72 hours post addition of lenti, DsRed+ cells were quantified by fluorescence microscopy. The concentration of functional lentiviral vector stocks was determined from the number of RFP+ HEK cells obtained from a given volume of lentiviral vector stock.

Flow Cytometry of HEK Producer Cells and hRBC Cells were briefly trypsinized and quenched with DMEM supplemented with 10% FBS followed by immunostaining and analysis of B6H12-PE and GFP fluorescence with a BD FACS Calibur.

Immunofluorescence Analysis of HEK-CD47-GFP HEK 293T cells were cultured on LabTek culture dishes, fixed with 3.6% Formaldehyde, and immunostained with anti-human CD47, followed by anti-mouse-IgG-AlexaFluor 647 secondary antibody, and Hoechst 3342.

Western Blotting Cells and lentivector supernatants were lysed with RIPA buffer supplemented with a mammalian protease inhibitor cocktail (Sigma). The lysate protein concentration was analyzed by a BCA assay (Pierce). Equal masses of whole lysates were separated on 4-12% Bis/Tris SDS-PAGE gels (Invitrogen) then protein was transferred to a polyvinylidene fluoride (PVDF) membrane with iBlot Gel Transfer Device (Invitrogen), followed by blocking with 5% nonfat dry milk solution for one hour. Incubation with primary antibody was done at 4°C overnight, washed 3x with TTBS. These were then incubated with secondary anti-Rabbit

HRP for 1-3 hours at room temperature. The HRP substrate, Chromosensor TMB substrate (Genscript), was applied for 5 min, followed by washing 3x with TTBS. Blot Images were analyzed with ImageJ.

Ex Vivo Spleen Culture Spleens were isolated from NSG mice, mechanically dissociated and treated with 1 mL Red Blood Cell Lysis buffer (Sigma) for 1 minute at room temperature. The cell mixture was then diluted in 20 mL RPMI-1640, centrifuged for 300 x g for 5 minutes, cells were further washed with RPMI-1640 >3 times. Cells were then plated at 300 μ L of a 1×10^7 cells/mL suspension to each chamber of an 8-chambered glass-bottomed dish (Nunc Lab Tek dishes) in RPMI-1640. 24 hours post-plating cells were washed 3x to remove nonadherent cells. Adherent cells were immunostained with anti-F4/80 to confirm F4/80 expression of adherent cells.

Tumor Xenografts and Flow Cytometry Analysis of Dissociated Tumors 1×10^6 A549 cells were suspended in Matrigel and injected to each flank of NSG mouse. 5 weeks post A549 injection mice were injected via tail vein with 3×10^8 Lentivectors. Tumors were isolated and minced with dissection scissors. Dissociated tissue was transferred to trypsin, incubated for 3 minutes, followed by quenching with 2% FBS/PBS. Dissociated cells were pelleted, and resuspended in warm Dispase (5 mg/mL Stem Cell Technologies) supplemented with Collagenase I (Sigma, 3 mg/mL) and DNAase (Calbiochem, 1mg/mL).

Immunohistochemistry of Tissue Sections Organs were soaked in 4% formaldehyde for 4 hours at 4°C then transferred to 4% FA, 20% sucrose in PBS for 48-72h. Tissue was cut into 2-5 mm cubes and transferred to OCT and snap frozen. OCT blocks were sectioned by microtome, rinsed with PBS, blocked with 10% FBS/PBS, stained with primary and secondary antibodies in 10% FBS/PBS. Sections were extensively rinsed between steps by submerging in PBS.

Supplementary Figures

Figure S1. Quantification of CD47 on HEK Producer Cells

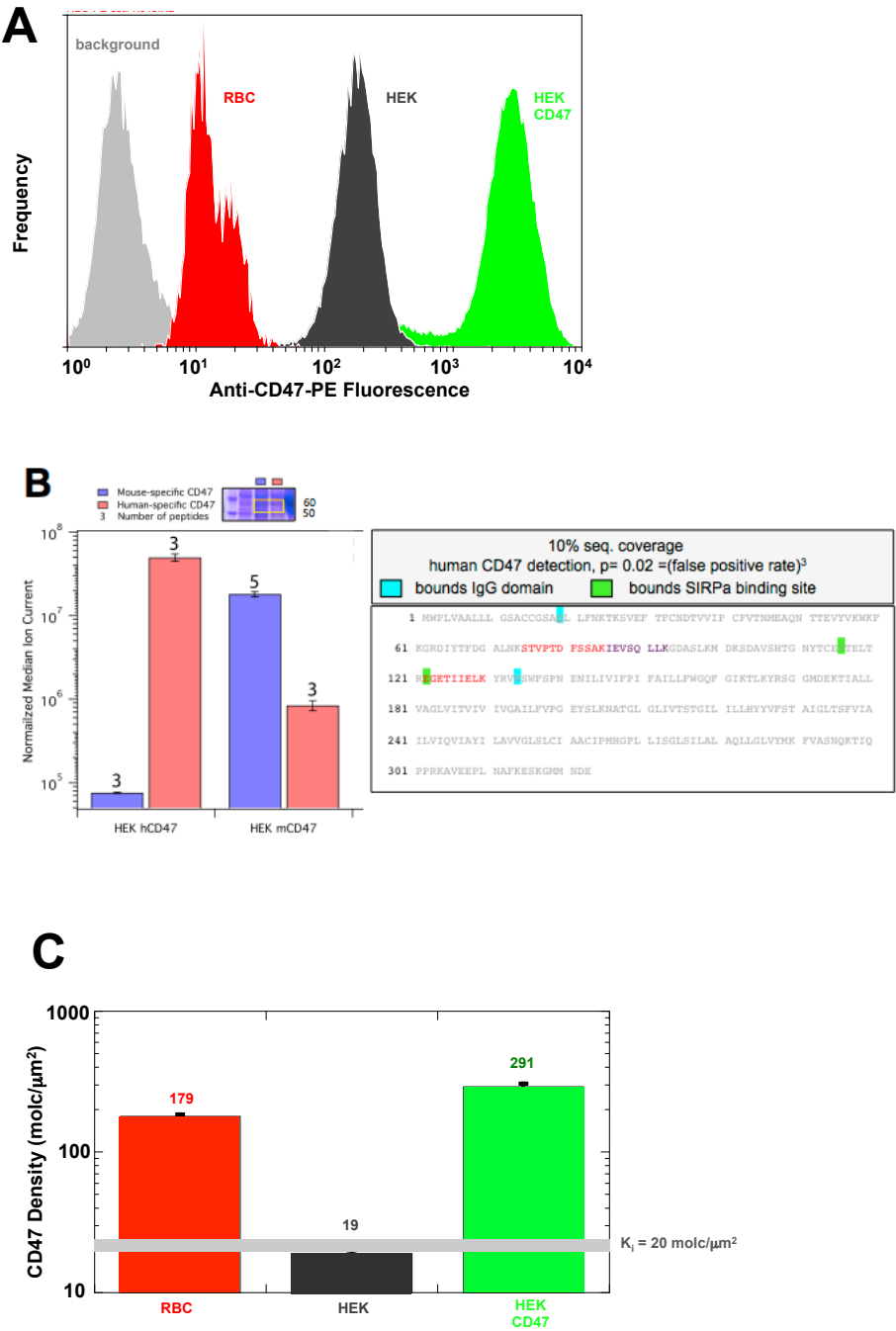


Figure S1. Quantification of CD47 on HEK Producer Cells. (A) CD47 display by HEK and HEK-CD47-GFP producer cells and human RBCs was quantified by anti-CD47 detection via flow cytometry. (B) Lysates of HEK-CD47, a similarly engineered HEK-murineCD47 cell line, and hCD47-Lenti were separated by SDS_PAGE gel, and proteins within $50 < MW < 60$ were isolated and analyzed by Mass-Spectrometry for CD47-derived peptides. (C) The density of CD47 was estimated on each cell type, by determining the number of CD47 molecules per cell by correlation with the anti-CD47 fluorescence geomean of RBC which are reported to display ~25,000 molecules of CD47 (Mouro-Chanteloup 2003). The density on the cell surface was estimated by taking the ratio of the calculated number of CD47 molecules and the reported surface area of HEK cells of 2591 um^2 (Sommerhage 2008) or RBCs of 140 um^2 (Hategan 2003).

Figure S2. TIRF Microscopy and AFM of CD47-GFP Displaying Lentivirus

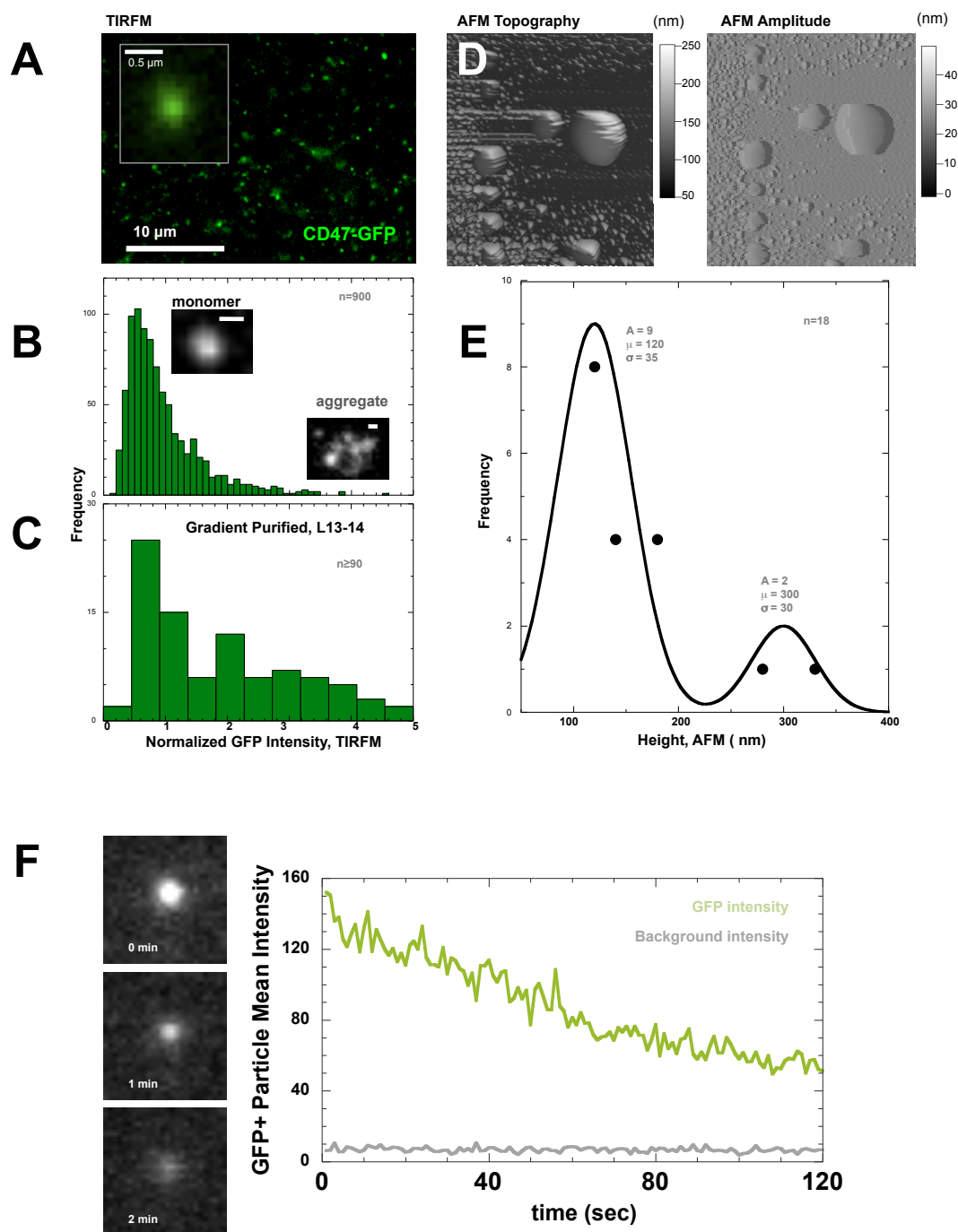


Figure S2. TIRF Microscopy and AFM of CD47-GFP Displaying Lentivirus. (A) Lentivirus supernatants were incubated with clean uncoated-glass coverslips and then imaged via TIRF microscopy for CD47-GFP intensity. (B) The GFP intensity for individual particles in whole lentivector supernatants was quantified. (C) The GFP intensity for individual particles in layers 13 and 14 of a sucrose gradient purified supernatants was quantified (Fig S3). (D) AFM imaging in tapping mode reveals pleomorphic quasi-spherical lentivectors. 3D pseudo-image of height (left) and cantilever amplitude (right). (E) The height of the lentiviral particles has a bimodal distribution, with the data fitting to a double Gaussian and fitting parameters A , μ , and σ are inset. (F) Lentiviral vectors were exposed to the TIRFM blue laser (~ 480 nm) for at least 2 minutes, and the change in CD47-GFP intensity of particulates was quantified.

Figure S3. Immunoblotting of Sedimentation-Equilibrium Purified CD47-Lenti

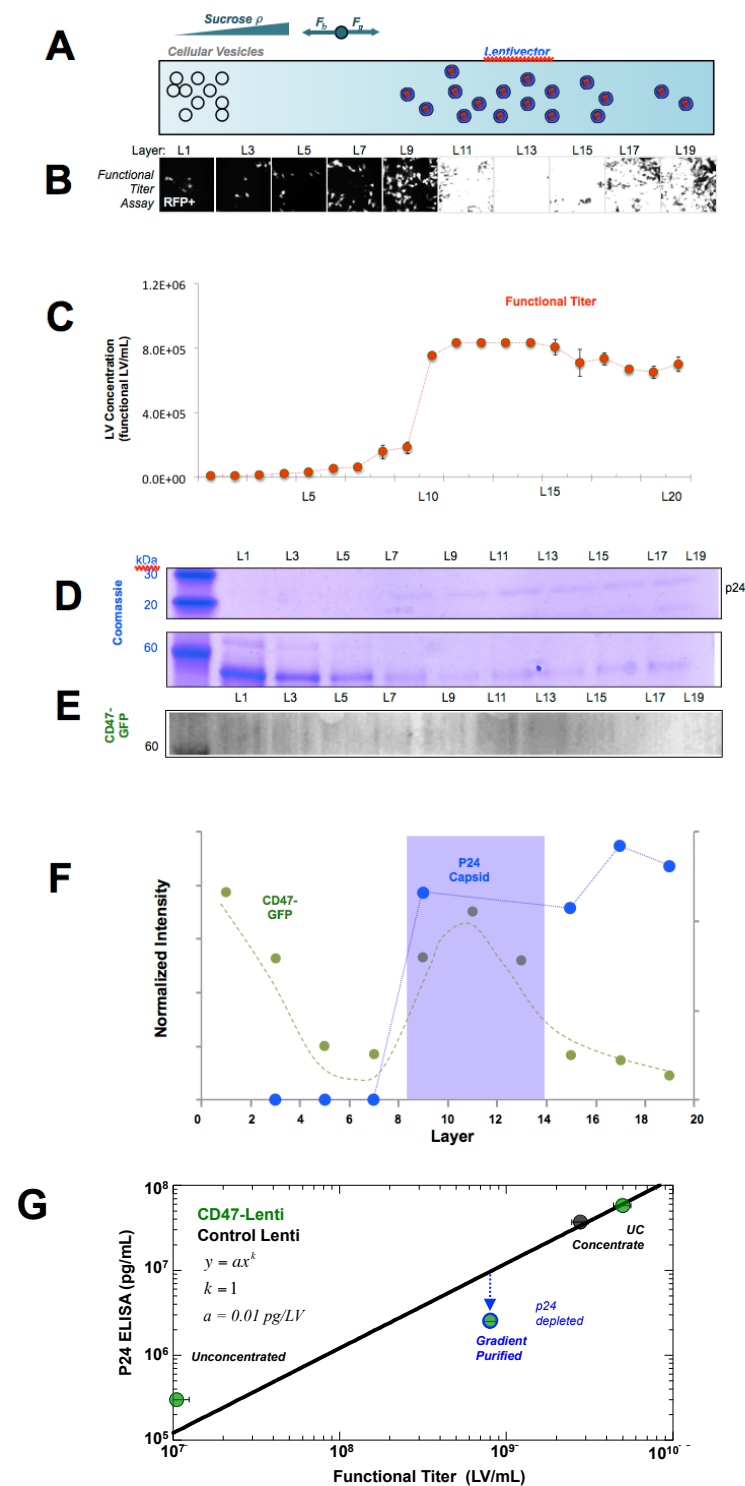


Figure S3. Immunoblotting of Sedimentation-Equilibrium Purified CD47-Lenti. CD47-Lenti supernatants were purified over a continuous sucrose density gradient, and then carefully aspirated top to bottom with a high-gauge needle into 20 x 50 μ L aliquots. **(A)** Particles are expected to travel through the gradient until reaching an isopycnic region. Capsid containing Lentis should migrate farther through the gradient than less dense cellular vesicles. Each of the 20 layers was analyzed for **(B, C)** functional titer, **(D)** Coomassie stain for total protein, and **(E)** western blotting for GFP. The anti-GFP immunoblot indicated a 60 kDa band that is consistent with the CD47-GFP fusion protein. **(F)** Densitometry of the coomassie and western blot was performed. **(G)** p24 ELISA and functional titer measurements are compared for unconcentrated, sucrose gradient purified, and concentrated lentiviral vector preparations. P24 is related to functional titer by $y=a*x^k$ with $a=0.01$ pg/LV and $k=1$ ($R^2=0.99$).

Figure S4. In Vivo Circulation Analyses and Quantitative Analysis of Published Data

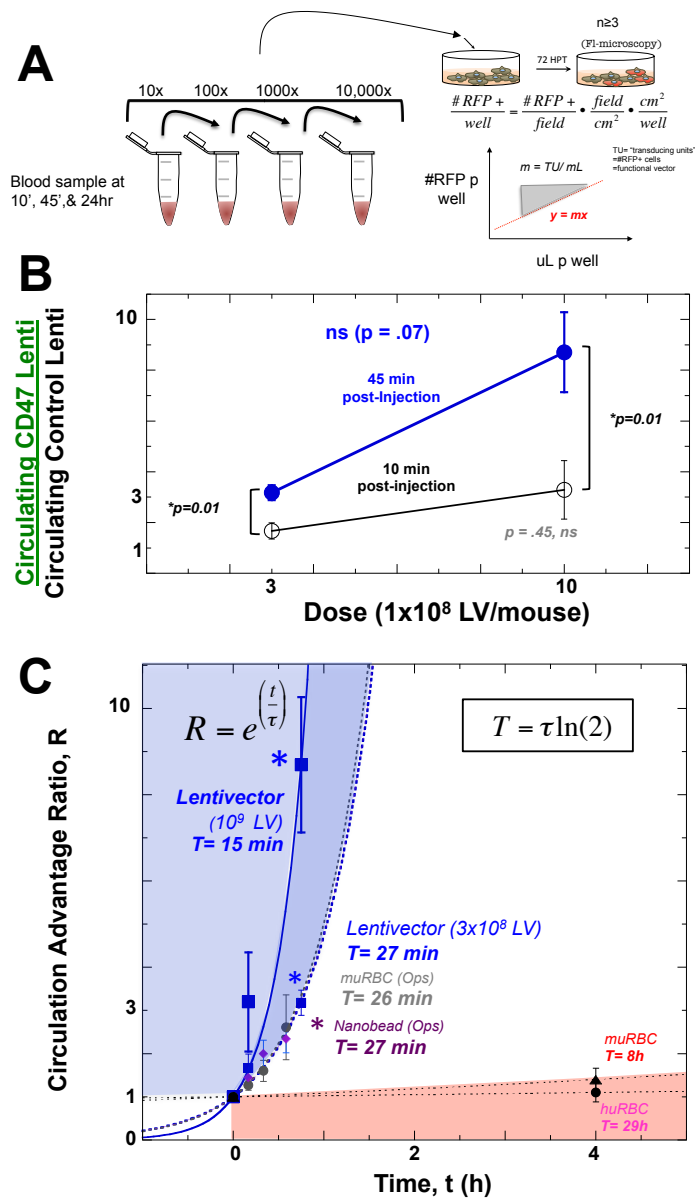


Figure S4. In Vivo Circulation Analyses and Quantitative Analysis of Published Data. (A)

Schematic of functional titer of in vivo blood samples that was used to quantify lentiviral vectors remaining in circulation. **(B)** The circulation ratio is plotted for in vivo dosage from 3×10^8 LV/mouse to 10×10^8 LV/mouse. **(C)** The time constant, T , is equal to the product of $\ln(2)$ and τ . The time constant for Lentivectors is **$T=0.15\text{h}$ (blue)**. The time constants for other relevant particles are as follows: opsonized muRBC $T= 0.26\text{ h}$ (Rodriguez 2013, **grey**), opsonized polystyrene nanoparticles $T = 0.27\text{ h}$ (Rodriguez 2013, **light blue**), unopsonized hRBC in NSG mouse versus unopsonized muRBC in SIRPA^{-/-} mouse $T= 29\text{h}$ (Strowig 2011, **pink**) and unopsonized muRBC in C57BL/8 mice $T=8\text{h}$ (Oldenberg 2000, **red**) and are plotted for reference.

Figure S5. Immunohistochemistry and Flow cytometric analyses of Liver and Xenograft Tumors

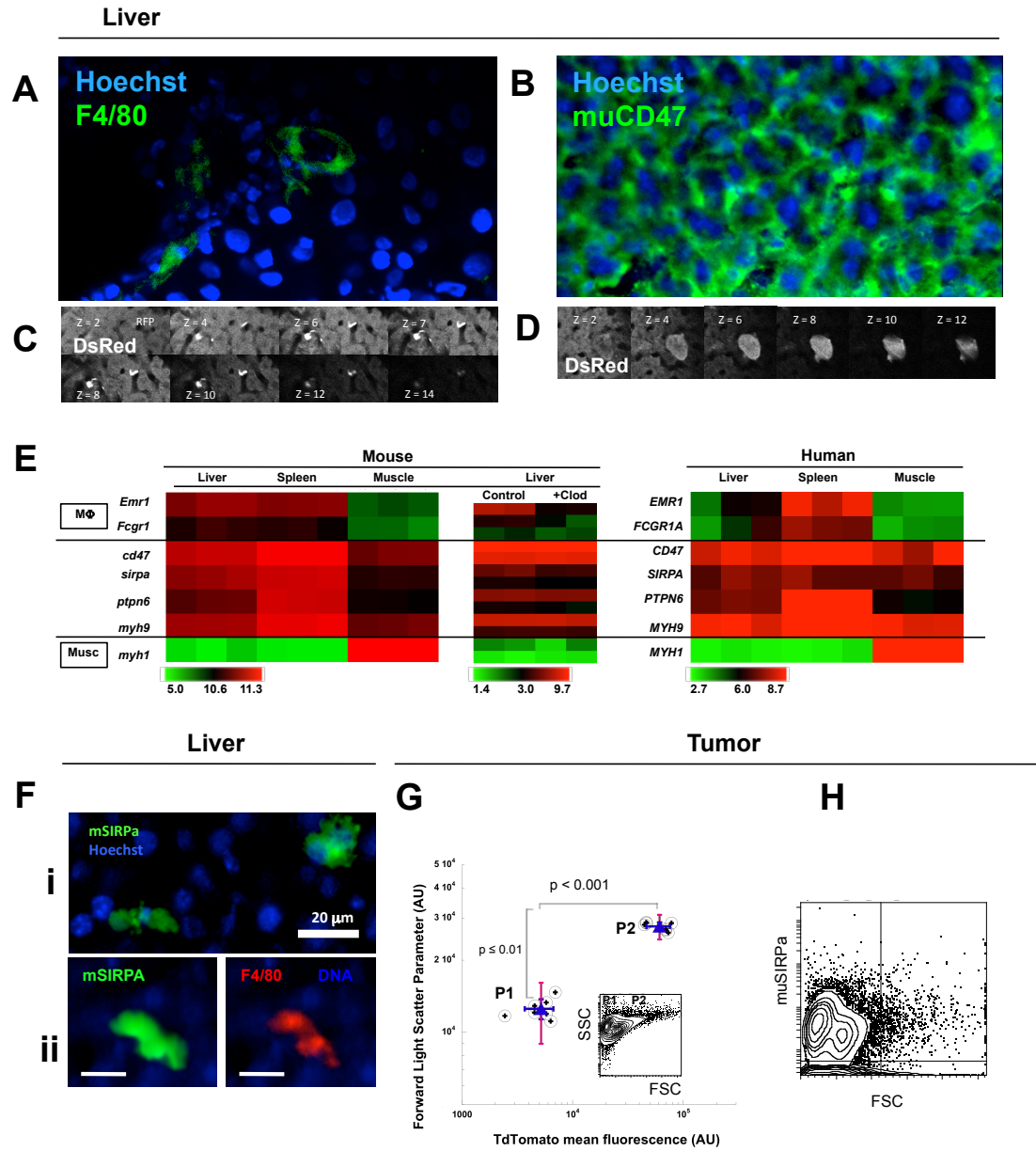


Figure S5. Immunohistochemistry and flow cytometric analyses of liver and xenograft

tumors. NSG mice were injected via tail vein with either a control or CD47-Lenti (1×10^9). Mice were sacrificed and *in vivo* perfused with PBS and 4% formaldehyde. Livers were isolated and incubated in a formaldehyde-sucrose solution for 48 hours prior to cryosectioning and immunostaining for **(A)** anti-F4/80 and **(C)** anti-mouse CD47. Liver sections were immunostained for RFP and imaged by confocal microscopy **(B & D)**. **(E)** Microarray data indicate the transcription of key genes within spleen, liver, and muscle tissues of man and mouse; these are compared by heatmap analysis. As shown in color bars below maps, green indicates low and red high gene transcription. **(F)** Liver sections were immunostained for mSIRPA and nuclei were identified by Hoechst staining (i). A F4/80 immunostain colocalized with mSIRPA expression (ii). **(G)** TdTomato A549 tumors were isolated, dissociated and analyzed for TdTomato Fluorescence and forward and side light scatter. Forward and side scatter contour plots identify two distinct populations **(G, Inset)**, P1 and P2. The forward-scatter and Tdtomato fluorescence was quantified for each population. Error bars indicate ± 1 SD (blue) and ± 3 SD (pink) ($n=3$, $p \leq 0.01$). **(G)** A549 tumors were isolated and dissociated into single cell suspension for analyzed by flow cytometry for anti-mSIRPA.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTION

The goal of this work was to improve our understanding of CD47's role in regulating phagocytosis in an effort to fill critical gaps in two distinct clinically relevant fields of research: aged red blood cell clearance and lentiviral vector mediated gene therapy.

(1) Phagocytic Clearance of Rigid Red Blood Cells

In the RBC field, it was known that phagocytosis of viable IgG opsonized cells can be inhibited by CD47 display, but not known how rigidification, as occurs in cell aging, affects CD47's ability to inhibit uptake. The work here shows that rigidification of red cells overrides CD47 by hyperactivating myosin IIA at the phagocytic synapse. Fluorescence imaging of the phagocytic synapse revealed that at the synapse with rigid cells, macrophages had accumulated significantly higher levels of myosin-IIA. Further rigidification correlated with enhanced phagocytosis, and no effective result of a CD47 blocking antibody on phagocytosis. Previous studies showed red pulp splenic macrophage to be sensitive to CD47 display, showing a significant increase engulfment of RBCs derived from a CD47 knockout mouse (CD47 $-/-$) relative to control RBCs (CD47 $+/+$) (Oldenborg 2000). Work here indicates that blocking CD47 has no effect on splenic macrophage engulfment of rigidified cells, suggesting that rigidification effectively overrides CD47 inhibition of engulfment in vivo.

Work from our lab has previously identified Myosin-II as a downstream target of CD47-SIRPa signaling (Tsai 2008), and separately studied the broad roles of matrix rigidity in regulation of the cytoskeleton in cellular functions such as adhesion. Here, Substrate rigidity was found to enhance activation of Myosin-II in mesenchymal and hematopoietic stem cells (Rehfeldt 2012, Shin 2014). These two lines of study intersect in this work that examines the effect of target cell rigidity on myosin accumulation at the phagocytic synapse, and how this affects CD47 inhibition

of myosin-II accumulation. Macrophage phagocytosis has previously been proven to be mechanosensitive, with macrophages being more efficient in the engulfment of rigid cells (Beningo 2002). Where rigidity enhances myosin II accumulation at the synapse, CD47 decreases myosin II accumulation at the synapse. As a result CD47 inhibition is less efficient in the phagocytosis of rigid cells. We expand our understanding of CD47 as a marker of self that prevents phagocytosis of viable cells, into understanding how the effects of CD47 may be modulated as cells age. Cellular rigidity is reported to increase with age (Mohandas 2008), and we find here that rigidification overrides the self-signal allowing cells that have sufficiently aged to be efficiently engulfed by macrophage. This was not previously known and this work helps us better understand the role of rigidity in CD47 signaling. We now know that among the changes that occur with erythrocyte aging, rigidity itself does play a role in affecting CD47's real but limited ability to signal self. Thus, the ability of cellular rigidity to override CD47 signaling offers insight into how aged and rigidified self-cells that still express high levels of CD47 can become cleared as needed.

While this work offers novel insight into how aged cells are cleared, there are other biophysical and biochemical factors besides stiffness that can be considered to affect CD47 inhibition. Changes in biophysical properties, cell rigidity and shape, was the focus of this thesis work, but increased IgG deposition on aged cells may be an additional complementary factor that enhanced uptake of rigid aged cells. Furthermore, aldehydes are produced as a result of oxidation (Barelli 2008), and can cross-link amino groups. Analyses of the membrane of the proteome of stored red cells that are exposed to oxidative stress have demonstrated dramatic alteration and cleavage of several RBC proteins including integral membrane protein, Band 3 (D'Amici 2007). Cross-linking of erythrocyte Band 3 has been shown to lead to IgG-opsonization and subsequently complement deposition (Franco 2013) that allows macrophages to recognize and clear these aged-erythrocytes (Kay 1975).

Previous work from our lab found that display of recombinant CD47 prolongs *in vivo* circulation and targeted delivery of polystyrene nanoparticles (Rodriguez 2013). Similarly, CD47 mediated inhibition was also observed in macrophage engulfment of polystyrene microparticles that are similar in size to erythrocytes (Tsai 2008). Polystyrene is significantly more rigid than erythrocytes. A previous study indicated that macrophage phagocytosis of synthetic particles is mechanosensitive, and the results from this thesis work that indicate that CD47 signaling is overridden with rigid erythrocytes. Thus CD47 inhibition of uptake of rigid polystyrene particles could be expected to be limited as well. However, it is clear that CD47 densities comparable, or even less than, those found on healthy erythrocytes is sufficient to inhibit uptake of these rigid spheres. While the polystyrene particles are more rigid than erythrocytes, they are also spherical in contrast to rigid erythrocytes that have a non-deformable discoid shape. To understand how this difference in shape could affect the ability of CD47 to functionally signal self on rigid spheres, we also studied the effect of CD47-inhibition of engulfment of hypotonically rounded erythrocytes. Rounding the erythrocyte rescued some of CD47's ability to signal self, indicating that not only rigidity, but also shape can regulate CD47 inhibition.

(2) The Novel CD47-Lenti Provides Increased Therapeutic Potential

In vivo delivered lentivectors are efficiently cleared by macrophage, with the majority of the delivered transgene found in the macrophage-rich spleen and liver (Pan 2002). Within the liver the majority of the delivered gene is expressed in resident liver macrophage (van Til 2004). Macrophage clearance has thus been a roadblock to the development of the therapeutic. The work here shows that a novel lentiviral vector that displays CD47 on the envelope shows reduced macrophage transduction, which correlates with enhanced gene delivery to xenograft tumors. CD47 offers the potential to limit clearance by macrophages in a fashion that complements current targeting techniques. Importantly, the engineering of the novel CD47 over-expressing

HEK 293T producer cell will allow this technique can be readily implemented with minimal perturbation to current transfection protocols.

Lentiviral vectors are mainly cleared and engulfed by resident tissue macrophage in the liver and spleen. This makes therapy inefficient and may contribute to inflammation that further reduces therapeutic potential. Engineering vectors to display proteins with high affinity for a targeted cell type has been one technique already developed to enhance delivery. However macrophages are ubiquitous and specialized in clearance of small particles, so that it is likely that a lentivector, even one engineered with a targeted envelope protein, may be engulfed by a macrophage before reaching the target cell. CD47 on the surface of the vector can bind to SIRPA and trigger downstream inhibition of the myosin-II motor. Macrophage uptake has been shown to be FcR and IgG dependent, and this type of uptake mechanism is known to be inhibited by CD47-SIRPA signaling. Thus CD47 is an efficient anti-targeting mechanism in which presentation of the CD47 molecule allows the lenti to escape macrophage in the spleen and the liver, and potentially other resident tissue macrophage, that are sensitive to CD47 inhibition.

Thus CD47 display was found to inhibit macrophage uptake of lentiviral vectors in vivo in a manner that is dependent on SIRPA. This extends the current understanding of CD47 ability to act as a 'marker of self' on the surface of mammalian cells and synthetic particles towards understanding the potential for human pathogens, such as the lentivirus, to utilize the 'marker of self' to avoid macrophage clearance. This perhaps has implications for viral infection. HIV-1 infects macrophage, where IgG opsonization and display of a functional Fc receptor increases macrophage infection (Takeda 1990). This suggests that IgG can enhance uptake into macrophage in a manner that does not necessarily lead to destruction of the virus. Phagocytic clearance of a pathogen can affect the macrophage's inflammatory state and lysosomal maturation (Janeway 2005). The implications of CD47 display for inflammation and antigen processing are beginning to be understood (Baba 2013, Tseng 2013). An enveloped poxvirus

(Family, Poxviridae, Genus Leporipoxvirus) is reported to encode a CD47 homologue (Cameron 2005), and enhances the pathogenicity of the virus, potentially because reduced uptake into macrophage reduces the host's ability to mount a sufficient immune response. Based on the results here with CD47 displaying lentiviral vectors, it seems reasonable to hypothesize that CD47-SIRPA interactions can affect pathogenicity of an enveloped virus such as HIV-1, that typically buds from leukocytes that are known to display CD47 (Brown 2001).

(3) Future Direction

Taken together the work here underscores contributions of biophysical properties of cellular targets to CD47 inhibition. Specifically, the rigidity and shape of phagocytic targets were identified here to affect CD47's ability to signal self and thus inhibit macrophage phagocytosis. Importantly, the work highlights a novel, powerful, and simple method of improving lentiviral vector mediated gene therapy, a promising therapeutic treatment. The next steps would be to apply both of these findings towards the improvement of current therapies. A proposed therapeutic to enhance anti-cancer gene therapy, that is based on the novel findings of this work, is discussed in more detail below.

The current work with lentiviral vectors showed that reduced macrophage uptake correlated with enhanced delivery to a xenograft tumor. The work utilized CD47-Lenti that encoded an RFP reporter gene that allowed for efficient detection of transduced cells. Expanding on this work, it would be useful to look at delivery of a transgene that limits growth and enhances cytotoxicity for cancer cells. It is expected, that CD47-Lenti display will enhance circulation and tumor delivery in a manner that is independent of the encoding transgene such that transgenes with therapeutic efficacy can be better delivered via the CD47-Lenti as compared to a Control Lenti.

CD47 is expressed on all cells, and so there are broader implications of its functions beyond its role in erythrocytes. CD47 was initially identified as a surface protein that was over-expressed on breast cancer (Campbell 1992). Much of the recent work on the role of CD47 in cancer has shown that CD47 overexpression in various types of tumors allows the cancerous cells to avoid macrophage clearance (Majeti 2009). This suggests that cancer cells have overtaken CD47 signaling to promote their growth. Thus there is great interest in CD47 as an anti-cancer target. Current efforts to combat CD47 expression have focused on small molecule therapeutics that block CD47 as well as siRNAs that downregulate CD47 expression. The work here with rigid red blood cells highlights that cellular rigidification is another route to overcome CD47 inhibition. Rigidification may inhibit CD47 signaling in tumor cells, and thus enhance their clearance in vivo. When leukemia is exposed to therapeutic agents such as daunorubicin, the cellular rigidity increases two-fold and leads to increased physical entrapment in the vasculature (Lam 2007). The effect that this rigidification has on macrophage clearance, and particularly on CD47 inhibition of macrophage phagocytosis of leukemia, remains to be determined.

As demonstrated here for controllably rigidified erythrocytes, the increased rigidity of chemotherapy-treated cancer cells may contribute to enhanced macrophage clearance by increasing myosin-II activation in a manner that overrides CD47 'self' signaling. As identified in this work, targeted rigidification may be a complementary method to counter CD47-signaling. Aldehyde rigidification occurs also in the course of normal ageing. The most aggressive cancers may be able to subvert this rigidification mechanism, potentially enhancing their ability to persist. ALDHs are a group of enzymes that are reported to deactivate physiological aldehydes. Aldehyde dehydrogenase (ALDH) is overexpressed in certain aggressive forms of cancer (Ginestier 2007). Aldehydes are capable of covalently cross-linking amino groups, and in vivo aldehyde treatments lead to cellular rigidification. Thus ALDH may allow cells, and especially cancer cells that over-express the enzyme, to escape this rigidification process. Although shRNA, RNAi, and small

molecule treatments against ALDH have been explored in cancer cells (Raha 2014), and found to mediate cytotoxicity, their effects on CD47 inhibition remain to be determined.

The conclusions from the two main branches of this thesis, considered together, suggest that (1) CD47 on vector surfaces can reduce phagocytic clearance and thereby enhance delivery to target cells, including tumors, and (2) that rigidification is a means to override CD47 signaling. Rigidification as a means for counteracting CD47 inhibition may have therapeutic efficacy in overcoming CD47 overexpression and avoidance of immunosurveillance in cancer. For instance, an important contribution to the existing literature may come from determining whether or not the CD47-Lenti can mediate a therapeutic effect on cancer cells, via delivery of an ALDH shRNA, with the goal of cancer cell rigidification in an effort to diminish CD47 inhibition of immune clearance of cancer cells.

The results here indicate that CD47 display by lentiviral vector can increase circulation of lentiviral vectors, and increase vector delivery to xenograft tumors. CD47-display, may be enhanced by currently utilized targeting mechanisms, including the display of cancer specific receptors under control of a cancer specific promoter (Pariente 2007). Lenti CD47-display is complementary to already developed targeting mechanisms such as pseudotyping and promoter engineering. While these methods enhance delivery to a target cell, CD47 avoids clearance by macrophage and thus the combination is expected to be synergistic, making lentiviral gene therapy even more effective. While studies have shown that ALDH inhibition is effective anti-cancer target, as ALDH counteracts negative effects of reactive oxygen species (Raha 2014), whether delivery of an ALDH shRNA, by a CD47-displaying lentivector has increased efficacy remains to be determined. Further whether or not ALDH silencing results in increased rigidification in a manner that may counteract CD47 inhibition also remains to be examined. Thus the two arms of this thesis identify potential improvement to existing therapies. First, rigidification may be a parameter that can be manipulated in order to overcome CD47 overexpression in cancer cells. Second, CD47 display by

lentiviral vectors can increase circulation and thus delivery to target tissues of lentiviral gene therapy vectors.

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