Title: TTI-621 (SIRPαFc): A CD47-Blocking Innate Immune Checkpoint Inhibitor with Broad Anti-Tumor Activity and Minimal Erythrocyte Binding

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Translational Relevance (142 words):

SIRPαFc (TTI-621) is a novel innate immune checkpoint inhibitor designed to: 1) bind human CD47 on tumor cells and prevent it from delivering inhibitory signals to macrophages and 2) engage Fc gamma receptors (FcγR) expressed by macrophages to further enhance phagocytosis. Here we show that TTI-621 avidly binds to CD47 on a wide range of human tumor cells and selectively promotes macrophage-mediated phagocytosis of both hematologic and solid tumor cells. Furthermore, these \textit{in vitro} effects translate into significant antitumor activity in mouse models of leukemia and lymphoma. Importantly, unlike anti-CD47 antibodies, TTI-621 binds minimally to human erythrocytes, minimizing potential toxicity related to hemolytic anemia. Based on the demonstrated antitumor activity in the context of minimal erythrocyte binding, two Phase 1, multicenter studies have been initiated to evaluate TTI-621 in subjects with relapsed/refractory hematological malignancies and solid tumors (NCT02663518 and NCT02890368, respectively).
ABSTRACT (250 words):

**Purpose:** The ubiquitously expressed transmembrane glycoprotein CD47 delivers an anti-phagocytic (do not eat) signal by binding signal-regulatory protein α (SIRPα) on macrophages. CD47 is over-expressed in cancer cells and its expression is associated with poor clinical outcomes. TTI-621 (SIRPαFc) is a fully human recombinant fusion protein that blocks the CD47:SIRPα-axis by binding to human CD47 and enhancing phagocytosis of malignant cells. Blockade of this inhibitory axis using TTI-621 has emerged as a promising therapeutic strategy to promote tumor cell eradication.

**Experimental Design:** The ability of TTI-621 to promote macrophage-mediated phagocytosis of human tumor cells was assessed using both confocal microscopy and flow cytometry. *In vivo* antitumor efficacy was evaluated in xenograft and syngeneic models and the role of the Fc region in antitumor activity was evaluated using SIRPαFc constructs with different Fc tails.

**Results:** TTI-621 enhanced macrophage-mediated phagocytosis of both hematologic and solid tumor cells, while sparing normal cells. *In vivo*, TTI-621 effectively controlled the growth of aggressive AML and B lymphoma xenografts and was efficacious in a syngeneic B lymphoma model. The IgG1 Fc tail of TTI-621 plays a critical role in its anti-tumor activity, presumably by engaging activating Fcγ receptors on macrophages. Finally, TTI-621 exhibits minimal binding to human erythrocytes, thereby differentiating it from CD47 blocking antibodies.

**Conclusions:** These data indicate that TTI-621 is active across a broad range of human tumors. These results further establish CD47 as a critical regulator of innate immune
surveillance and form the basis for clinical development of TTI-621 in multiple oncology indications.
INTRODUCTION

The phagocytic activity of macrophages is regulated by both activating (“eat”) and inhibitory (“do not eat”) signals. CD47, a widely expressed transmembrane glycoprotein, serves as a critical inhibitory signal, suppressing phagocytosis by binding to signal regulatory protein alpha (SIRPα) on the surface of macrophages. Engagement by CD47 triggers tyrosine phosphorylation of the cytoplasmic tail of SIRPα, leading to recruitment of the Src homology-2 domain containing protein tyrosine phosphatases SHP-1 and SHP-2 and prevention of myosin-IIA accumulation at the phagocytic synapse (1). CD47 is believed to regulate the natural clearance of senescent erythrocytes and platelets by splenic macrophages (2, 3). In addition, the CD47-SIRPα interaction may represent an important mechanism by which malignant cells escape immune-mediated clearance.

CD47 has been shown to be over-expressed in numerous hematologic malignancies, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma, myelodysplastic syndrome (MDS), and in multiple types of non-Hodgkin lymphoma (NHL), including diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma, and marginal cell lymphoma (4-10). Similarly, elevated CD47 expression has been demonstrated on solid tumors, including bladder, brain, breast, colon, esophageal, gastric, kidney, leiomyosarcoma, liver, lung, melanoma, ovarian, pancreatic, and prostate tumors (11-15). CD47 has been found to be an adverse prognostic factor where high CD47 expression correlates with more aggressive disease and poorer clinical outcomes. For example, overall survival was significantly lower for DLBCL or mantle cell lymphoma patients who had elevated CD47
expression, and higher CD47 expression on tumor cells was associated with significantly poorer event-free survival in patients with CLL (7). Similar trends have been reported in other hematologic malignancies (5, 6, 8) and solid tumors (11, 13, 16, 17). Additionally, there is evidence to suggest that increased CD47 expression is associated with the transition from low-risk to high-risk MDS and subsequent transformation to AML (10). These findings are consistent with tumor cells exploiting the suppressive CD47-SIRPα axis to evade macrophage-mediated destruction. Blocking CD47 has thus emerged as a promising therapeutic strategy and several studies have shown that interrupting the CD47-SIRPα signaling pathway using anti-CD47 monoclonal antibodies (mAbs) promotes anti-tumor activity against human cancers both in vitro and in vivo (6-9, 18). However, the expression of CD47 on erythrocytes raises concerns about the potential for anti-CD47 mAbs to cause hemolytic anemia, as seen in preclinical studies (19). Furthermore, erythrocyte CD47 constitutes a massive antigen sink that may limit the ability of CD47-targeting agents to reach tumor cells. Finally, CD47 targeting agents bound to erythrocytes may cause interference with blood typing tests.

TTI-621 (SIRPαFc) is a novel innate immune checkpoint inhibitor that binds human CD47 and prevents it from delivering a “do not eat” signal to macrophages. It is designed to function as a decoy receptor, binding CD47 on the surface of tumor cells and blocking its anti-phagocytic “do not eat” signal, thereby allowing macrophages to phagocytose malignant cells. Moreover, the IgG1 Fc region of SIRPαFc can interact with human Fcγ receptors on macrophages to further enhance phagocytosis, tumor antigen presentation, and effective anti-tumor activity. Here, we fully characterize this novel agent and
demonstrate that TTI-621 strongly binds to a wide range of human tumors and induces potent phagocytosis of human tumor cells in vitro and in vivo while sparing most normal cells. Although the decoy receptor binds circulating platelets and leukocytes, TTI-621 shows only minimal binding to human erythrocytes, thereby mitigating concerns of anemia associated with anti-CD47 mAbs. These results further establish CD47 as a critical regulator of innate immune surveillance and form the basis for clinical development of TTI-621 in multiple oncology indications.
METHODS

SIRPαFc Proteins

TTI-621 consists of the N-terminal V domain of human SIRPα (GenBank AAH26692) fused to the human IgG1 Fc region (hinge-CH2-CH3, UniProtKB/Swiss-Prot, P01857). Variant proteins were generated in which the identical human SIRPα domain was linked to a human IgG4 Fc region (hinge-CH2-CH3, UniProtKB/Swiss-Prot, P01861) or an IgG4 Fc region which was mutated to remove residual Fc interactions (20). Both IgG4-based fusion proteins contained a hinge-stabilizing mutation that prevents the formation of intrachain disulfide bonds (21). Two mouse surrogate SIRPαFcs were constructed, one using the N-terminal V domain from NOD mouse SIRPα (22) and the second using a mutated (CV1) N-terminal V domain of human SIRPα (23). In both mouse surrogates the SIRPα domains were linked to a mouse IgG2a Fc (hinge-CH2-CH3, UniProtKB/Swiss-Prot, P01863). All constructs were generated by overlapping PCR using standard molecular biology techniques and expressed in stably transfected CHO-S cells (Invitrogen). Proteins were purified from culture supernatant using protein A and hydrophobic interaction chromatography, concentrated and residual endotoxin removed. Control human IgG1 and mouse IgG2a Fc proteins lacking the SIRPα domain were also generated and similarly purified. All proteins displayed >99% purity by HPLC and <0.4 EU/mg endotoxin.
Antibodies

The anti-CD47 antibodies BRIC126 (Serotec), 2D3 (eBioscience) and CC2C6 (BioLegend) were obtained from commercial sources. Clones B6H12.2 (ATCC® HB–9771™) and 5F9 (19) were generated internally based on publicly available sequences. Rituximab (Hoffman-La Roche Ltd, Basel, Switzerland) was obtained from the London Health Sciences Centre pharmacy (London, Ontario).

Cells

The following human tumor cell lines were used: DLBCL (HT, Ly1, Pfeiffer, NUDUL1, SUDHL4, SUDHL6, SUDHL8, SUDHL16, Toledo), multiple myeloma (MM1.s, 8226, H929, U266), non-DLBCL B-cell lymphomas (Raji, Namalwa, C1R, Ly5), AML (HL-60, KG-1, THP-1 and TF-1, AML-2), chronic myeloid leukemia (K562, KU812), acute T-cell leukemia (ENL-1, Jurkat), T-cell lymphoma (HH, H9, SUPT1, DERL7), lung cancer (A549), epidermoid cancer (A431), ovarian cancer (OVCAR-3), colon cancer (DLD-1), breast cancer (SKBR3), melanoma (A375, SK-MEL-1, G-361, WM-115, SK-MEL-28) and Merkel cell carcinoma (MCC26, MKL-1). All cell lines were obtained from American Type Culture Collection (ATCC) except MCC26 and MKL-1 (Sigma-Aldrich), AML-2 (kindly provided by Mark Minden, University Health Network, Toronto), EN-1 (kindly provided by Aaron Schimmer, University Health Network, Toronto) and Ly5 (kindly provided by David Spanner, Sunnybrook Research Institute, Toronto). The mouse A20 B lymphoma line was obtained from ATCC.
Viably frozen primary tumor cells from the peripheral blood or bone marrow of patients with B-cell ALL, T-cell ALL, MDS and AML were obtained from the University Health Network (UHN) BioBank (Toronto) according to procedures approved by the Research Ethics Board of UHN.

Human macrophages were prepared from heparinized whole blood obtained from normal healthy human donors (Biological Specialty Corporation); informed consent was obtained from all donors. Peripheral blood mononuclear cells (PBMCs) were isolated over Ficoll-Paque Plus density gradient (GE Healthcare) and CD14+ monocytes were isolated from PBMCs by positive selection using CD14 antibody-coated MicroBead separation (Miltenyi Biotec). Monocytes were differentiated into macrophages by culturing for at least 10 days in X-Vivo-15 media (Lonza) supplemented with M-CSF (PeproTech). One day prior to phagocytosis assays, the monocyte-derived macrophages were primed with IFN-γ (PeproTech) to generate M1 macrophages, or with IL-4 (Peprotech) to generate M2 macrophages. Unless otherwise specified, all phagocytosis assays were carried out using M1 macrophages. When required, macrophages were harvested using Enzyme-Free Cell Dissociation Buffer (ThermoFisher).

**Tumor Cell Binding**

Cell lines or primary patient samples were added in duplicate to 96-well plates and incubated with titrated amounts of biotinylated TTI-621 or biotinylated isotype-matched control IgG Fc, together with Near-IR LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) for 30 min on ice. Cells were washed, stained with phycoerythrin (PE)-conjugated
streptavidin (eBioscience), washed, and resuspended in Stabilizing Fixative (BD Biosciences). Flow cytometry was performed on a FACSVerse flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar Inc.). Half-maximal effective concentration (EC<sub>50</sub>) values were calculated using a sigmoidal dose-response curve in GraphPad Prism software.

**Erythrocyte Binding**

Erythrocytes were isolated from sodium heparinized whole blood from healthy human donors (Biological Specialty Corporation) by centrifugation followed by several washes with PBS. The resulting packed erythrocytes were diluted in PBS and added in duplicate to 96-well plates. Binding was performed by incubating erythrocytes with titrated amounts of TTI-621, anti-CD47 monoclonal antibodies (BRIC126 (Serotec), 2D3 (eBioscience), CC2C6 (BioLegend), B6H12 (in-house), 5F9 (in-house)). Cells were washed and subsequently stained with biotin-conjugated anti-human IgG Fc PAN (Hybridoma Reagent Laboratory), followed by detection with PE-conjugated streptavidin (eBioscience). Flow cytometry was performed on a FACSVerse flow cytometer (BD Biosciences).

**Hemagglutination Assays**

Titrated amounts of TTI-621 or anti-CD47 mAbs (up to 3 μM) were added to wells containing erythrocytes diluted in PBS, and the plates were incubated overnight at 37°C in 5% CO<sub>2</sub>. The extent of hemagglutination was assessed by scoring each well on a scale
of 1 to 6, with 1 representing the absence of hemagglutination and 6 representing complete hemagglutination.

**Phagocytosis Assays**

Confocal-based phagocytosis assay: Tumor cells were labeled with CellTrace CFSE (Life Technologies) and added to primed macrophages in 24-well plates at a 1:5 effector:target ratio. Macrophages and tumor cells were co-cultured for 2 hours at 37°C in 5% CO₂ in the presence of TTI-621 or control Fc protein and subsequently stained with Alexa Fluor 555-conjugated Wheat Germ Agglutinin (Invitrogen). Phagocytosis was assessed by confocal microscopy on a Quorum Wave FX-X1 Spinning Disc Confocal System and images were analyzed using Velocity software (PerkinElmer). A phagocytosis index was calculated as: (number of tumor cells inside macrophages/number of macrophages) x 100; counting at least 200 macrophages per sample. All tumor cells counted were confirmed to be internalized using z-stack images. Statistical significance was calculated by unpaired t-test vs isotype control using GraphPad Prism software.

Flow cytometry-based phagocytosis assay: Tumor cells were labeled with Violet Proliferation Dye 450 (BD Biosciences) and added to primed macrophages in 96-well plates at a 1:5 effector:target ratio. Macrophages and tumor cells were co-cultured for 2 hours at 37°C in 5% CO₂ in the presence of TTI-621 or control Fc protein and subsequently stained with Near-IR LIVE/DEAD Fixable Dead Cell Stain (Invitrogen), APC-conjugated anti-human CD14 (61D3, eBioscience) and PE-conjugated anti-human CD11b (ICRF44, eBioscience), washed and resuspended in Stabilizing Fixative (BD
Cells were acquired on a FACSVerse flow cytometer, and data were analyzed using FlowJo software (Treestar Inc.). Macrophages were identified as live, single, CD14+CD11b+ cells. Doublets were excluded by SSC-W and SSC-H discrimination. Percent phagocytosis was assessed as the percent of macrophages that were VPD450+. The gating strategy and representative dot plots are shown in Supplementary Figure 1. Statistical significance was calculated by unpaired t-test vs isotype control using GraphPad Prism software.

**AML Xenografts**

AML xenografts were performed in 10 week-old female NOD.SCID mice bred and maintained in the Barrier Unit at the UHN Animal Facility (Toronto, Canada). One day prior to transplantation, mice were sublethally irradiated (275 cGy) and pretreated with anti-CD122 antibody (0.2 mg/mouse) to deplete residual host NK cells. On the day of transplantation, viably frozen mononuclear cells collected from AML patients 90543 and 90191 were thawed, counted, and transplanted intra-femorally into the preconditioned mice at a dose of 5×10⁶ cells/mouse in a total volume of 30 µl. Twenty-one days after engraftment, mice were dosed with TTI-621 (8 mg/kg) or equimolar amount of control human IgG1 Fc (5.4 mg/kg) at 0.3 mL/mouse, 3 times/week for 4 weeks. Upon euthanization, bone marrow from injected and non-injected bones was collected and stained with mouse anti-human antibodies including CD47-FITC, CD33-PE, CD19-PC5, CD45-APC, CD34-APCCy7, CD38-PECy7. After staining, washed cells were run on an LSRII flow cytometer (BD). 10,000 to 20,000 events were collected for each sample.
Collected data were analyzed by FlowJo software to assess AML engraftment levels in the injected femur, non-injected bones, and in the spleen as determined by the percentage of human CD45⁺CD33⁺ cells.

**B Cell Lymphoma Xenografts**

Lymphoma xenografts were performed in 6 to 7 week-old female NOD.Cg-PrkdcscidHhr/NCrHsd (SHrN) mice, a hairless severe combined immunodeficient strain, obtained from Harlan Laboratories (Montreal, Canada) and maintained at the Victoria Research Laboratories Vivarium (London Health Sciences Centre). Raji and Namalwa cells (1 x 10⁶ per injection) were injected subcutaneously into each flank of SHrN mice in a volume of 0.1 mL PBS (i.e., 2 tumor injection sites per mouse). Toledo cells (1 x 10⁷ per injection) were injected in 50% Matrigel (ECM gel, Sigma-Aldrich, St. Louis, MO) in a volume of 0.1 mL into the left flank for SHrN mice. Mice were kept under isoflurane-mediated anesthesia during the injections. Three days after Raji and Namalwa tumor cell implantation and 10 days after Toledo tumor cell implantation, animals received either 10 mg/kg of mouse SIRPαFc (NOD SIRPα) or 6.75 mg/kg control IgG2a Fc, daily 5 times per week, for 3 weeks by intraperitoneal injection. Tumor volumes were estimated twice weekly by standard caliper measurements of length and width then calculated as follow: \( \pi \times \frac{1}{6} \times (\text{longest diameter}) \times (\text{shortest diameter})^2 \). Tumor volumes were monitored until they reached the maximum volume of approximately 1500 mm³ or maximum permissible markers of discomfort in the mice were reached (i.e., mouse discomfort or body weight loss reached maximum allowable levels), at which time the
mice were sacrificed. All studies were conducted according to the animal care guidelines described by the Canadian Council on Animal Care (CCAC) and monitored by The Western University Animal Use Subcommittee. Statistical significance was calculated by two-way ANOVA using GraphPad Prism software.

**Syngeneic B Cell Lymphoma Model**

Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the University of Toronto animal facility (Mississauga, ON). A20 cells (2x10⁶) were injected subcutaneously into the right hind flank of 8-week-old BALB/c female mice in a volume of 0.1mL. When the tumors were palpable (approximately 60 mm³), they were randomized and injected intratumorally with 200 μg (10 mg/kg) of a mouse SIRPαFc surrogate (CV1 SIRPα) fusion protein in a 50 μL volume of PBS. Control groups were injected with vehicle alone in a 50 μL volume. Animals were dosed twice weekly for a total of five doses. Tumors were monitored three times a week and tumor volume was calculated as ½ length×width². Tumor volumes were monitored until one or more tumor dimensions reaches the maximum permissible measure (15 mm), or when maximum permissible markers of discomfort were observed, at which time the mice were sacrificed. All animal procedures were approved by the animal care committee of the University of Toronto in accordance with the CCAC. Statistical significance was calculated by two-way ANOVA using GraphPad Prism software.

**RESULTS**
Structure of TTI-621

TTI-621 (SIRPαFc) was generated by directly linking the sequences encoding the N-terminal CD47 binding domain of human SIRPα with the Fc domain of human IgG1 (Figure 1). The SIRPα region interacts with CD47, while the Fc region binds to Fcγ receptors. TTI-621 is secreted by a genetically-engineered Chinese hamster ovary (CHO) cell line as a 77 kDa disulfide-linked, N-glycosylated homodimer consisting of two identical 345 amino acid chains.

TTI-621 Binds to CD47 and Enhances Macrophage Phagocytosis of Tumor Cells In Vitro

The binding of TTI-621 to CD47 on malignant human cells was assessed by flow cytometry. TTI-621 was found to bind strongly to a panel of 19 tumor cell lines derived from patients representing a wide range of both hematologic and solid tumors (Supplementary Table 1). TTI-621 also exhibited strong binding to primary tumor samples obtained from the blood of patients with B cell acute lymphoblastic leukemia (B-ALL), T-ALL and AML, and bone marrow samples from patients with MDS, with an average binding EC₅₀ value of 197 ± 182 nM (Supplementary Table 2). CD47 is widely expressed on normal cells, and TTI-621 also demonstrated binding to human CD4⁺ T cells, CD8⁺ T cells, B cells, platelets, natural killer (NK) cells, granulocytes, monocytes and NK T cells from the peripheral blood of healthy donors (Supplementary Table 3).
The ability of TTI-621 to promote macrophage-mediated phagocytosis of human tumor cells was assessed using both confocal microscopy and flow cytometry. Monocyte-derived macrophages were co-cultured with tumor cells for two hours, and in cultures left untreated or treated with a control Fc fragment, macrophages exhibited a low level of phagocytosis, consistent with CD47-mediated suppression. In contrast, blockade of CD47 on the target cells using TTI-621 significantly increased macrophage phagocytosis of tumor cells (Figure 2A). Compared to a control Fc protein, TTI-621 promoted macrophage phagocytosis of 77% (23/30) of tumor cell lines established from patients with hematological malignancies and 67% (8/12) of human solid cancer cell lines (Figure 2B). A marked pro-phagocytic effect of TTI-621 was also observed on primary samples from patients with AML, MDS, multiple myeloma, B-ALL and T-ALL (Figure 2C). TTI-621 enhanced macrophage-mediated killing of 97% (32/33) of primary blood cancer samples tested. Drug activity was further characterized by titrating TTI-621 on selected human tumor cell lines (n=13) and primary tumor samples (n=4) (representative data in Figure 2D). As summarized in Supplementary Table 4, TTI-621 treatment resulted in a saturable, dose-dependent phagocytic response with an average EC$_{50}$ of 10 ± 14 nM.

We then assessed the effect of TTI-621 on macrophage-mediated phagocytosis of normal cells in vitro. As shown in Figure 2E, TTI-621 potently increased phagocytosis of primary AML tumor cells, while sparing normal peripheral blood monocytes, indicating that TTI-621-enhanced phagocytosis is tumor cell-specific.
Collectively, these *in vitro* data demonstrate that TTI-621 induces potent, tumor-specific macrophage phagocytosis across a broad range of hematological and solid tumors. In fact, we have not observed a tumor type that is refractory to TTI-621 treatment, consistent with prior data demonstrating that the CD47 immune checkpoint is widely used by malignant cells to escape immune surveillance (5, 24).

**TTI-621 and Mouse Surrogate SIRPαFc have Potent Anti-Tumor Activity *In Vivo***

To determine whether the potent effects of TTI-621 *in vitro* translated into *in vivo* anti-tumor activity, we employed an AML xenograft model using primary patient samples. Engrafted mice were treated with TTI-621 or an Fc fragment control three times/week for four weeks. Although control-treated animals exhibited significant engraftment, particularly in the injected bone marrow, TTI-621 treatment significantly reduced the tumor burden in bone marrow and spleen (Figure 3A and B). In fact, tumor cells were undetectable in most animals following TTI-621 therapy.

The presence of CD47 on non-tumor tissue has the potential to bind SIRPαFc and remove it from circulation, potentially resulting in a significant antigen sink effect. Since TTI-621 does not bind to mouse CD47 (data not shown), TTI-621 treatment of xenograft recipient does not model this antigen sink effect. To overcome this limitation, mouse surrogate fusion proteins (mSIRPαFc) were constructed using the mouse IgG2a Fc region, allowing for full effector function, analogous to the human IgG1 Fc region in TTI-621. Treatment of mice with mSIRPαFc may thus more closely mimic the anticipated pharmacokinetic and Fc effector activity profile of TTI-621 in human subjects. The *in vivo* efficacy of
mSIRPαFc was assessed in three aggressive B cell lymphoma xenograft models: Namalwa and Raji (Burkitt lymphomas) and Toledo (DLBCL). Hairless NOD.SCID (SHrN™) mice were implanted subcutaneously with tumor cells and treated with mSIRPαFc five times/week for three weeks starting either three days after engraftment (Namalwa and Raji) or 10 days after engraftment (Toledo). mSIRPαFc treatment markedly reduced the growth of Raji tumors (Figure 3C) and completely ablated Namalwa and Toledo tumors (Figure 3D and E); in the latter two models, most mice remained tumor-free 60 days after inoculation. Moreover, mSIRPαFc was superior to Rituximab therapy in both Namalwa and Toledo xenografts.

To overcome the limitations inherent with xenograft models, we also assessed whether mSIRPαFc could reduce tumor burden in an immune-competent syngeneic system. BALB/c mice were subcutaneously inoculated with A20 B cell lymphoma cells, and mSIRPαFc was administered by intratumoral injection twice weekly starting seven days post-engraftment. As shown in Figure 3F, mSIRPαFc treatment significantly reduced the growth of A20 tumors, confirming that CD47 blockade with mSIRPαFc is also efficacious in animals with an intact immune system.

Collectively, these in vivo data suggest that blockade of the CD47:SIRPα axis using SIRPαFc has broad applicability across a variety of malignancies.

**Blockade of CD47 using SIRPαFc Requires an IgG1 Fc Tail for Maximum Potency**
Engagement of Fcγ receptors (FcγRs) on macrophages by SIRPαFc may deliver a pro-phagocytic signal that could augment the effect of CD47 blockade. TTI-621 possesses an IgG1 Fc tail, allowing for binding to the high-affinity receptor FcγRI (CD64) as well as to the low-affinity receptors FcγRII (CD32) and FcγRIII (CD16). To determine whether the IgG1 Fc tail is required for maximum potency, we compared the *in vitro* activity of TTI-621 with a variant SIRPαFc in which the IgG1 Fc region of TTI-621 was replaced with an IgG4 Fc tail. IgG4 Fc regions bind well to CD64 but have weaker interactions than IgG1 with CD32 and CD16 (25). We compared the pro-phagocytic activity of both SIRPαFc s using classically activated (M1) and alternatively activated (M2) macrophages. We have previously shown that M1 macrophages are CD32*hi* CD64*hi* *in vitro*, whereas M2 macrophages are CD32*hi* CD64*lo* (26). TTI-621 enhanced phagocytosis by both macrophage subsets equally well. In contrast, SIRPαFc with an IgG4 tail induced significantly less phagocytosis by M2 macrophages (Figure 4A). These data suggest that an IgG1 tail is necessary for SIRPαFc’s enhancement of phagocytosis by both M1 and M2 macrophages.

We next compared the *in vivo* activity of TTI-621 and the variant IgG4-containing SIRPαFc in the AML xenograft model. We also tested a SIRPαFc with a mutated IgG4 Fc region that is completely devoid of Fc effector functions. As shown in Figure 4B, treatment with all three SIRPαFcs reduced tumors to undetectable levels in the spleen. In the injected femur and non-injected bone marrow, TTI-621 treatment completely ablated tumor growth in all but one mouse. SIRPαFc with an IgG4 tail reduced tumor burden in the non-injected bone marrow, but not in the injected femur.
compared to controls, whereas the mutated IgG4 fusion protein was unable to control tumor burden in either bone marrow compartment (Figure 4B).

The contribution of the Fc region raises the question of whether TTI-621 activity requires neutralization of the CD47 “do not eat” signal, or whether it simply opsonizes CD47-expressing cells for Fc receptor-mediated destruction, similar to antibodies that trigger classical antibody-dependent cellular phagocytosis (ADCP). To address this, we compared the in vitro activity of TTI-621 to two isotype-matched (mouse IgG1) anti-CD47 antibodies: clone B6H12, which blocks the CD47-SIRPα interaction and the non-neutralizing clone 2D3. As shown in Figure 4C, 2D3 induces a low level of phagocytosis, attributable to opsonization of CD47 without blockade of the CD47-SIRPα interaction. B6H12 is more effective than 2D3 at inducing the phagocytosis of a human B lymphoma target, indicating that efficient phagocytosis in this system requires blockade of the CD47-SIRPα interaction. TTI-621 exhibits even greater activity than B6H12, which presumably reflects the combined effect of CD47 blockade and more effective Fc receptor engagement by the TTI-621 human IgG1 Fc region.

Collectively, these data show that SIRPαFc with an IgG1 tail (TTI-621) is significantly more potent at promoting phagocytosis in vitro and controlling tumor burden in vivo, and that both CD47 blockade and Fc-mediated effector functions contribute to the mechanism of action of TTI-621.
TTI-621 Induces Anemia in Non-Human Primates But Binds Minimally to Human Erythrocytes

A significant concern with CD47-blocking agents is related to the high expression of CD47 on human erythrocytes and the potential for such agents to cause anemia, as seen in preclinical studies (19). To assess the risk of anemia and other adverse events, primate repeat-dose toxicology studies of TTI-621 were conducted in non-human primates. Cynomolgus monkeys were selected as relevant species based on the high CD47 sequence homology (97.6% identity to human CD47) and cross-reactivity studies.

The principal dose-limiting toxicity observed in cynomolgus monkeys was anemia, which occurred at repeat doses of 3 mg/kg or greater. In addition to anemia, other cytopenias, including thrombocytopenia, lymphopenia, neutropenia and monocytopenia were observed, although these were reversible and without clinical sequelae (see Supplementary Figure 2 for representative hematology values). The bone marrow exhibited evidence of regenerative responses, notably erythropoiesis. No effects were observed on neurologic, cardiovascular or other systems.

Despite the strong binding of TTI-621 to monkey erythrocytes, we observed only minimal binding to human erythrocytes (Figure 5A), which may be due to species-specific differences in the mobility of CD47 in erythrocyte membranes (data not shown). Importantly, the low binding profile of TTI-621 to human erythrocytes is in contrast to the strong binding demonstrated by five different anti-CD47 antibody clones (Figure 5A). Minimal binding of TTI-621 was observed on erythrocytes from all 43 healthy donors.
tested regardless of gender, ABO blood group or rhesus antigen status (Figure 5B). Consistent with these binding data, TTI-621 did not induce hemagglutination of human erythrocytes \textit{in vitro} (Figure 5C). The lack of significant binding of TTI-621 to human erythrocytes thus offers a significant advantage over CD47-blocking mAbs.
DISCUSSION

Approved immune checkpoint inhibitors have extended the survival of multiple subgroups of cancer patients and thus transformed modern oncology. Although the focus thus far has been on blockade of checkpoints that suppress T cell responses (e.g., PD-1 or PD-L1), there is growing recognition that the innate immune system plays an important role in the initiation and propagation of enduring anti-tumor responses and CD47 has recently emerged as a key checkpoint of innate immunity. Our findings demonstrate that SIRPαFc (TTI-621) is an effective decoy receptor that enhances macrophage-mediated phagocytosis in a broad spectrum of human hematologic and solid tumors, both in vitro and in xenograft models. More than 97% of primary patient samples tested were sensitive to the antitumor effects of TTI-621, suggesting that this therapeutic approach will have broad applicability in human cancer.

Importantly, blockade of CD47 by TTI-621 selectively induced phagocytosis of malignant cells over normal cells, providing a therapeutic window for treatment of patients in the clinic. Preferential macrophage phagocytosis of AML cells over normal cord blood/bone marrow cells has also been reported for an IgG4-based SIRPαFc fusion protein, even when non-malignant cells outnumbered the AML cells by a 2:1 ratio (27). In addition, a mouse anti-human CD47 neutralizing antibody did not induce phagocytosis of normal peripheral blood B cells (7) or normal human pancreatic ductal epithelial cells and pancreatic stellate cells (15).
The specificity for tumor cells is thought to result from the expression of pro-phagocytic signals such as calreticulin on malignant cells but not on normal cells. Calreticulin is known to trigger macrophage-mediated phagocytosis, and the phagocytosis of cancer cells induced by CD47 blockade can be completely inhibited by antagonizing the interaction between calreticulin and its receptor (28). It is hypothesized that tumor cells evade phagocytosis because the inhibitory CD47 pathway counterbalances the pro-phagocytic calreticulin signal. Selectively targeting CD47 with TTI-621 promotes killing of tumor cells while sparing low calreticulin-expressing normal cells. There are likely to be other as yet unidentified pro-phagocytic signals on tumor cells that may vary depending on the tissue type from which the tumor is derived. The broad efficacy of TTI-621 across tumor types suggests that targeting the CD47-SIRPα axis exploits the reliance of tumor cells on CD47-mediated suppression of phagocytosis regardless of their specific underlying pro-phagocytic signals.

The potent in vivo effects of TTI-621 were attenuated when the IgG1 Fc tail of the fusion protein was substituted by an IgG4 tail with reduced Fc-mediated effector function, or with an inert mutated IgG4 tail, indicating that Fc effector function is critical for achieving maximum potency of SIRPαFc. These observations are consistent with a prior report demonstrating that engineered high affinity SIRPα monomers that bind strongly to CD47 but lack an Fc region are inactive on their own (29) and suggest that maximal antitumor activity is obtained through blockade of the CD47 “do not eat” signal and simultaneous delivery of a pro-phagocytic (“eat”) signal through macrophage FcγRs. In line with this, our data suggest that TTI-621 does not work simply by opsonization of
CD47 and ADCP, but triggers phagocytosis through CD47 blockade as well as simultaneous activation through FcγRs.

Although CD47 has recently emerged as a promising immuno-oncology target, concerns have been raised regarding the potential for anemia and an erythrocyte antigen sink, due to the expression of high levels of CD47 on human red blood cells (30, 31). In this regard, TTI-621 exhibits an advantage over anti-CD47 antibodies, in that it binds only minimally to human red blood cells. A similar observation has recently been reported by an independent group (32). The minimal binding of TTI-621 to human erythrocytes may be due to the association of CD47 with the erythrocyte spectrin cytoskeleton (30), which results in reduced membrane mobility (33) and a consequent failure to cluster CD47 effectively. Consistent with this theory, we have previously shown strong binding of TTI-621 to human erythrocytes when CD47 is first pre-clustered using a non-blocking CD47 antibody (34).

While it is acknowledged that TTI-621 binds to human platelets and leukocytes (and thus may be associated with the development of thrombocytopenia and/or leukopenia), the extremely low erythrocyte-binding profile of TTI-621 offers several potential advantages over anti-CD47 mAbs that strongly bind to erythrocytes. First, treatment with TTI-621 is less likely to result in anemia. CD47 is thought to protect erythrocytes from macrophage-mediated clearance (2), and CD47 blocking antibodies are known to trigger anemia in non-human primates, a finding that may limit their clinical utility despite the employment of a priming strategy (19). Second, minimal erythrocyte binding permits the use of an
IgG1-based fusion protein, and thus maximizes macrophage phagocytosis of tumor cells, without concern for opsonizing red blood cells and targeting them for destruction. Third, CD47-targeting agents that bind erythrocytes may interfere with transfusion typing and cross-matching tests, as seen with other agents that bind erythrocytes (35, 36). Finally, TTI-621 is likely to have a superior pharmacokinetic profile compared to anti-CD47 mAbs by avoiding the significant antigen sink created by dense cell surface expression of CD47 on erythrocytes, enabling more comprehensive engagement of tumor-expressed CD47.

We demonstrated that CD47 blockade with SIRPαFc is efficacious in AML and B lymphoma xenograft models, as well as in a B lymphoma syngeneic model. Macrophages, in addition to their direct tumoricidal properties, function as antigen presenting cells, and thus it is possible that enhancement of phagocytosis by TTI-621 treatment may also result in an enhanced adaptive immune response. In support of this, CD47 antibody blockade has been shown to augment tumor antigen presentation and priming of an anti-tumor cytotoxic CD8+ T-cell response in immune-competent mice (37). In addition, CD47 blockade using a high-affinity SIRPα-variant-human Ig fusion protein has also been shown to promote tumor-specific CD8+ T-cell responses through a dendritic cell-based mechanism (38). These studies provide compelling evidence to support the hypothesis that TTI-621 has the potential to generate an enduring anti-tumor response by acting at the nexus of the innate and adaptive immune systems. We propose a mechanism in which TTI-621 blocks the CD47 “do not eat” signal on tumor cells while simultaneously delivering pro-phagocytic signals to macrophages through FcγRs, leading
to tumor cell phagocytosis, enhanced antigen presentation and stimulation of a tumor antigen-specific T cell response (Figure 6).

In summary, these data affirm CD47 as a critical regulator of immune surveillance and provide a strong rationale for therapeutic targeting of CD47. Simultaneous blockade of the inhibitory signal of CD47 with an associated engagement of FcγR on macrophages form the basis for clinical development of TTI-621. Two Phase 1, open label, multicenter studies are currently ongoing to evaluate TTI-621 in patients with relapsed/refractory hematological malignancies (NCT02663518) and solid tumors (NCT02890368).

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REFERENCES


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FIGURE LEGENDS

Figure 1: Structure of TTI-621

TTI-621 consists of the N-terminal domain of human SIRPα (shown in red) linked to a human IgG1 Fc region (shown in blue). The hinge and inter-chain disulfide bonds are shown as black lines.

Figure 2: TTI-621 promotes macrophage-mediated phagocytosis of human tumor cells in vitro. (A) Representative scanning confocal microscopy images after macrophages were co-cultured with a primary AML patient sample for 2 hours in the presence of 10 μM TTI-621 or control IgG1 Fc protein. Tumor cells and macrophages are stained green and red, respectively. (B) Macrophage-mediated phagocytosis of established human tumor cell lines from patients with B-cell malignancies (n=17), myeloid malignancies (n=7), T-cell malignancies (n=6), skin cancers (n=7) and other solid cancers (n=5) in the presence of 1 μM TTI-621 (black bars) or control IgG1 Fc protein (white bars). Phagocytosis was quantified by determining a phagocytosis index (number of engulfed tumor cells per 100 macrophages) using confocal microscopy or measuring percentage phagocytosis by flow cytometry, as described in the Materials and Methods section. (C) Macrophage-mediated phagocytosis of primary human tumor samples from patients with hematological malignancies (n=33) in the presence of 1 μM TTI-621 (black circles) or control IgG1 Fc protein (white circles). (D) Representative titration of TTI-621 (black circles) on a primary AML patient sample. Control Fc protein (white circle) was tested at 1 μM. (E) Macrophage-mediated phagocytosis of primary
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AML tumor sample or normal monocytes was assessed by confocal microscopy in the presence of 1 μM TTI-621 or control IgG1 Fc. Statistical significance was assessed by unpaired t-test vs Fc control (*p<0.05, **p<0.01, ***p<0.001, NS=not significant).

Figure 3: TTI-621 and its mouse surrogate are efficacious in vivo. (A-B) NOD.SCID mice were preconditioned with sublethal irradiation and anti-CD122 antibody (to deplete residual NK cells) and then transplanted with AML cells from patient #0905443 (A) or patient #090191 (B) by intrafemoral injection. Treatment with TTI-621 (8 mg/kg IP 3x/week for 4 weeks) or control IgG1 Fc protein was initiated 21 days post-transplantation. The % AML engraftment (% cells expressing human CD45 and CD33 markers) was assessed by flow cytometry. Each symbol represents one mouse, bars indicate mean values. P-values were determined by t-test vs Fc control protein. Data shown are representative of 9 separate AML patient xenografts. (C-E) SHrN™ mice (n=5 per group) received subcutaneously implanted Raji (C), Namalwa (D) or Toledo (E) cells. Three days after implantation (Namalwa and Raji) or 10 days after implantation (Toledo), mice were dosed IP with either a mouse surrogate SIRPαFc (10 mg/kg), control mouse IgG2a Fc (6.67 mg/kg) or rituximab (8 mg/kg) five times a week for three weeks (indicated by the arrow heads). Tumor volumes were estimated by caliper measurement from both flanks and the means for those measurements were calculated in mm³. Mice were sacrificed when tumor volumes exceeded 1500 mm³ or when there was extensive ulceration. Mean tumor volumes are recorded only for time points in which ≤ 1 mouse per group was sacrificed. Data shown are representative of 4 (Raji), 2 (Namalwa) and 2
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(Toledo) independent experiments. Mice were terminated at a tumor volume of 1500 mm\(^3\) \(*\) 2x10\(^6\) A20 cells were implanted subcutaneously into the right flank of Balb/c mice on day 0. Mice were randomized (n=9-10 mice per treatment arm) when the mean tumor size was palpable at which time the tumors were approximately 60 mm\(^3\) in volume. A mouse surrogate SIRPαFc (10 mg/kg) or vehicle was given bi-weekly by intratumoral administration. Mice were sacrificed when at least one tumor dimension exceeded 15 mm. Mean tumor volumes ± standard error of the mean were recorded only for time points in which ≤ 2 mice per group were sacrificed. Data shown is representative of 2 independent experiments. Statistical significance was assessed by two-way ANOVA (***p<0.001).

Figure 4: SIRPαFc with an IgG1 Fc tail has potent anti-tumor efficacy. (A) M1 and M2 monocyte-derived macrophages were generated by priming for 24 hours with IFN-γ or IL-4, respectively. Macrophage phagocytosis of a DLBCL cell line (Toledo) was assessed by flow cytometry (% Phagocytosis) in the presence of SIRPαFc with an IgG1 Fc tail (TTI-621) or an IgG4 Fc tail (both at 1 μM concentration). Data shown represent n=5 donors. (B) NOD.SCID mice were preconditioned with sublethal irradiation and anti-CD122 antibody (to deplete residual NK cells) and then transplanted with AML cells from patient #090191 by intrafemoral injection. Treatment with SIRPαFc (8 mg/kg IP 3x/week for 4 weeks) or control IgG1 Fc protein was initiated 21 days post-transplantation. The % AML engraftment (% cells expressing human CD45 and CD33 markers) was assessed by flow cytometry. Each symbol represents one mouse, bars
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indicate mean values. P-values were determined by one-way ANOVA. Data shown are representative of 2 independent experiments. (C) Monocyte-derived macrophages were generated as described and primed for 24 hours with IFN-γ. Macrophage phagocytosis of a DLBCL cell line (Toledo) was assessed by flow cytometry (% Phagocytosis) in the presence of TTI-621, anti-CD47 mAbs B6H12 or 2D3, or isotype-matched controls (all at 1 μM).

**Figure 5: TTI-621 exhibits minimal binding to human erythrocytes.** (A) Human erythrocytes were stained with saturating concentrations of TTI-621 or CD47-specific antibodies (clones BRIC126, 2D3, CC2C6, B6H12 or 5F9) and analyzed by flow cytometry. Representative histograms are shown, with specific staining shown in black and isotype control staining in grey. (B) Summary data showing the mean fluorescence intensity for 43 erythrocyte donors. (C) Hemagglutination assays were conducted with human erythrocytes and titrated amounts of TTI-621 or CD47-specific antibodies. The extent of hemagglutination was assessed by blinded scoring on a scale of 1 to 6, with 1 representing the absence of hemagglutination and 6 representing complete hemagglutination.

**Figure 6: Proposed mechanism of action of TTI-621-mediated CD47 anti-tumor activity.** (A) CD47 sends an inhibitory signal to macrophages by binding to SIRPα. (B) TTI-621 binds to CD47 on tumor cells and blocks this interaction, (C) while engaging FcγR on macrophages, (D) leading to macrophage-mediated phagocytosis of tumor cells.
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(E) Macrophages that have phagocytosed target cells can present tumor peptides in the context of MHC to tumor-specific CD8$^+$ T cells, (F) activating the adaptive immune response and leading to destruction of tumor cells by cytotoxic CD8$^+$ T cells.
Figure 2

A

No Treatment  Control Fc (10 μM)  SIRPaFc (10 μM)

B

B Cell Malignancies

Myeloid Malignancies

T Cell Malignancies

Solid Tumors

Skin Cancer

C

Hematological Tumors

D

Phagocytosis of Primary AML

E

Phagocytosis Index

AML cells  Normal Monocytes
Figure 3

A

B

C

D

E

F

- Injected Bone Marrow
- Non-Injected Bone Marrow
- Spleen

- Injected Bone Marrow
- Non-Injected Bone Marrow
- Spleen

- Burkitt Lymphoma (Raji)
- Burkitt Lymphoma (Namalwa)

- Diffuse Large B Cell Lymphoma (Toledo)
- B Lymphoma (A20)

- Tumor Volume (mm³)
- Dosing Window
- Day post-Engraftment

- Control Fc
- SIRPα Fc
- Rituximab

- Control Fc
- SIRPα Fc
- Rituximab

- Control Fc
- SIRPα Fc
- Vehicle

- p = 0.008
- p = 0.003
- p = 0.004

- p = 0.003
- p = 0.003
- p = 0.002

- p = 0.0004
- p = 0.0003
- p = 0.0003

- p = 0.003
- p = 0.003
- p = 0.003

- p = 0.0004
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- p = 0.003
- p = 0.003
- p = 0.003

- p = 0.0004
- p = 0.0004
- p = 0.0004

- TTI-621
- Toledo
- (Raji)
- (Namalwa)
- (Toll)
Figure 3

A

Injected Bone Marrow

Non-Injected Bone Marrow

Spleen

B

Injected Bone Marrow

Non-Injected Bone Marrow

Spleen

C

Burkitt Lymphoma (Raji)

D

Burkitt Lymphoma (Namalwa)

E

Diffuse Large B Cell Lymphoma (Toledo)

F

B Lymphoma (A20)
Figure 4

A

B

C

Blockade of the CD47-SIRPα interaction: No Yes Yes

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Figure 5
TTI-621 (SIRPαFc): A CD47-Blocking Innate Immune Checkpoint Inhibitor with Broad Anti-Tumor Activity and Minimal Erythrocyte Binding

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