CD47 enhances in vivo functionality of artificial antigen-presenting cells.

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Running title: “don’t eat me” aAPC for enhanced in vivo function

Key words: aAPC, CD47, phagocytosis, antigen-specific T cells, in vivo

Financial support: C. Schütz is supported by a German Research Foundation (DFG) Postdoctoral Fellowship (SCHU-2681/1-1) and a HERA Women’s Cancer Foundation OSB1 Grant. This work was supported by the National Institutes of Health (P01-AI072677 and R01-CA108835), TEDCO/Maryland Innovation Initiative and Neximmune, Inc. MD Biotech Center (J.P.S.) and by the ELAN Fond of the University Hospital of Erlangen and the Roggenbuck-Stiftung Hamburg (H.B.).

Disclosure: H.B., C.B., J.C.V., C.H., J.F., S.P., A.M., C.S. have nothing to disclose. Under a licensing agreement between NexImmune and the Johns Hopkins University, J.P.S. and M.O. are entitled to shares of royalty received by the University on sales of aAPC products described in this article. They also own NexImmune stock, which is subject to certain restrictions under University policy. Dr. Schneck is a member of the company's Board of Directors and Scientific Advisory Board. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

Text word count: 4352

Number of figures: 6
Translational Relevance

CD47 is a species-specific marker of self; it delivers a negative signal to resident macrophages subsequently inhibiting the clearance of intact hematopoietic cells. In addition it binds to T cells resulting in modulation of cell signalling pathways. Artificial Antigen-Presenting Cells (aAPC) have been described as a powerful tool for induction of tumor-antigen specific T cell responses \textit{in vitro} and \textit{in vivo}. To further increase their \textit{in vivo} functional half-life due to minimized phagocytic up-take by macrophages and improved anti-tumor efficacy we generated CD47 positive aAPC (aAPC\textsuperscript{CD47+}). These aAPC\textsuperscript{CD47+} show great potential to significantly enhance new approaches to active immunotherapy.
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Abstract

Purpose: Artificial Antigen-Presenting Cells, aAPC, have successfully been used to stimulate antigen-specific T cell responses in vitro as well as in vivo. While aAPC compare favorable to autologous dendritic cells in vitro, their effect in vivo might be diminished through rapid clearance by macrophages. Therefore, to prevent uptake and minimize clearance of aAPC by macrophages, and thereby increasing in vivo functionality, we investigated the efficiency of “don’t eat me” three-signal aAPC compared to classical two-signal aAPC.

Experimental Design: To generate “don’t eat me” aAPC, CD47 was additionally immobilized onto classical aAPC (aAPC CD47+). aAPC and aAPC CD47+ were analyzed in in vitro human primary T cell and macrophage co-cultures. In vivo efficiency was compared in a NOD/SCID T cell proliferation and a B16-SIY melanoma model.

Results: This study demonstrates that aAPC CD47+ in co-culture with human macrophages show a CD47 concentration dependent inhibition of phagocytosis, while their ability to generate and expand antigen-specific T cells was not affected. Furthermore, aAPC CD47+ generated T cells displayed equivalent killing abilities and polyfunctionality when compared to aAPC generated T cells. In addition, in vivo studies demonstrated an enhanced stimulatory capacity and tumor inhibition of aAPC CD47+ over normal aAPC in conjunction with diverging bio-distribution in different organs.

Conclusion: Our data for the first time show that aAPC functionalized with CD47 maintain their stimulatory capacity in vitro and demonstrate enhanced in vivo efficiency.
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Thus this next generation aAPC^{CD47+} have a unique potential to enhance the application of the aAPC technology for future immunotherapy approaches.
Introduction

CD47 is a species-specific marker of self(1) that is ubiquitously expressed on normal cells and serves as a “don’t eat me”-signal(2). It binds to several molecules such as integrins and thrombospondin and is involved in cell migration processes, axon development and phagocytosis(3). Old and apoptotic CD47\textsuperscript{low} or CD47\textsuperscript{-} cells are phagocytosed as part of homeostatic regulation. CD47 is up-regulated on many human tumor cells and mediates tumor immune evasion by interaction with signal-regulating protein alpha (SIRP\textgreek{a})(4). Binding to the extracellular region of SIRP\textgreek{a} expressed on macrophages delivers a negative signal inhibiting myosin and phagocytic synapse formation leading to a blockade of the clearance of intact hematopoietic cells(5,6). CD47-SIRP\textgreek{a} signalling in murine immunoglobulin-opsonised red blood cells (RBC) as well as in human CD47\textsuperscript{+}-RBC and CD47\textsuperscript{-}-microparticles leads to inhibition of ingestion by macrophages(1,6). CD47-activated polyvinyl chloride (PVC) and polyurethane (PU) surfaces demonstrated reduced cell adherence and neutrophil activation when compared to non-activated polymers(7) and coating of nanoparticles with CD47\textsuperscript{+}-RBC-membranes minimized particle up-take by macrophages(8). Therefore, use of CD47 has been proposed for the development of “stealth” particles for drug delivery(9–12).

Previously, our group developed artificial antigen-presenting cells (aAPC) by chemically coupling a signal 1 chimeric MHC-immunoglobulin dimer (MHC-Ig) and a signal 2 anti-CD28 activating antibody onto the surface of a cell-sized iron-oxide particle. These aAPC demonstrated antigen-specific T cell generation and expansion favorable to autologous dendritic cells (DC)(13,14) and were successfully tested in different in vitro systems(15–
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In vivo aAPC-generated T cells inhibited tumor growth as efficient as DC-generated T cells(18). Furthermore, adoptively transferred low affinity T cells where efficiently activated by coadministration of aAPC and subsequently lead to tumor reduction in an in vivo melanoma tumor model (19).

While these studies prove in vitro and in vivo functionality of our aAPC, in vivo delivery and biodistribution is mainly determined by the size of the aAPC scaffold(10,12,20). Micro-meter sized aAPC display limited lymphatic drainage(21) and are cleared and phagocytosed by professional phagocytes such as macrophages and immature DC(22–24). Therefore, many efforts are made to generate optimal aAPC scaffolds that exhibit minimal systemic clearance and maximal in vivo functionality(11).

We hypothesised that aAPC additionally functionalized with CD47 (aAPC^{CD47+}) would minimize macrophage mediated phagocytic clearance without interfering with antigen-specific T cell generation. aAPC^{CD47+} compared to non functionalized aAPC demonstrated an equal ability to generate and expand functional antigen-specific T cells in vitro. Phagocytosis of aAPC^{CD47+} by human macrophages was inhibited in a CD47 concentration dependent manner. In addition, aAPC^{CD47+} demonstrated an enhanced in vivo T cell stimulatory capacity and improved tumor inhibition when compared to aAPC, in conjunction with diverging bio-distribution in different organs.

Thus, this study for the first time shows that two-signal aAPC functionalized with an additional third signal (CD47) maintain their primary in vitro stimulatory capacity for
“don’t eat me” aAPC for enhanced in vivo function antigen-specific T cell activation and expansion and demonstrate enhanced in vivo efficiency.
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Material and Methods

The ethical committees of the Johns Hopkins University and the University of Erlangen approved this study and all healthy volunteers gave written informed consent.

Peptides, antibodies and HLA-A2 tetramers

HLA-A2 and H2Kb restricted peptides (>95%) were obtained from Johns Hopkins University core facility: human modified melanoma associated antigen (MART-1; ELAGIGILTV), influenza matrix protein (FluM1, GILGFVFTL) and synthetic murine SIY peptide (SIYRYYGL). Peptides were dissolved in 10% DMSO at 1 mg/ml and sterile filtered.

The following monoclonal antibodies (mAb) were used for flow cytometric analysis of T cells and aAPC: anti-CD8-FITC (Sigma), anti-IgG1-PE (Invitrogen), anti-IgG2a-FITC and anti-CD47-FITC (BD). Phycoerythrin (PE)-conjugated HLA-A*0201 tetramer folded around MART-1 and FluM1 (Beckman Coulter). Antigen-specific T cells were stained for 30 min (RT) with HLA-A*0201 tetramers. mAb stain was performed for 15 min (4°C). Samples were analyzed using a Calibur flow cytometer (BD) and FlowJo software (Tree Star, Inc.).

Generation of aAPC and aAPCCD47+

aAPC were generated by coupling HLA-A2-Ig or Kb-Ig (5 µg) and anti-human-CD28 (clone 9.3) or anti-mouse-CD28 (5 µg) onto 10⁸ epoxy beads (Invitrogen). aAPCCD47+ additionally received 160 ng CD47-Ig/10⁸ epoxy beads if not differentially indicated. The protocol followed has been previously published(13,14).
Preparation of macrophages

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of buffy coat preparations from blood of healthy donors (DRK, Germany). Monocytes were isolated by plastic-adherence and cultured in the presence of M-CSF (50 ng/ml, R&D). 6 day later macrophages were detached with EDTA (1 mM, Sigma). Expression of surface markers CD68, HLA-DR (BD), CD163 (eBioscience), CD11b (Miltenyi Biotec) and SIRP-α (Biolegend) were evaluated by flow cytometry.

Analysis of cytokines

TNF, IL-12 and IL-10 concentrations in culture supernatants of macrophages un-stimulated or stimulated with LPS (100 ng/ml) were determined (18h) by ELISA (R&D), following the manufacturer’s instructions. Results were collected utilizing a microplate autoreader (EL309) from Bio-Tech Instruments (Winooski).

Succinimidyl-ester labelling of aAPC/aAPC<sup>CD47<sup>+</sup></sup>

aAPC/aAPC<sup>CD47<sup>+</sup></sup>, were washed twice with PBS, 0.5% Tween80, 0.2 M sodium bicarbonate (pH 8.8) and resuspended in 1 mM fluorescein-succinimidyl-ester-Alexa-647. After 1h beads were washed three times with PBS/0.5% Tween80(25).

Phagocytosis of aAPC and aAPC<sup>CD47<sup>+</sup></sup>
Macrophages (2.5x10^5) were adhered to 24-well plates and co-cultured with aAPC or aAPC^{CD47+} (2.5x10^5) for the time indicated (37°C). Samples were co-incubated with anti-mouse-IgG-F(ab')_2-Alexa647 (1:100, Cell Signaling) for 30 min (4°C), macrophages washed, lysed in sterile water (30 min, 4°C) and the lysate analyzed by flow cytometry.

Macrophages (10^5) were adhered to 8-chamber slides (Nunc) and co-cultured with labelled aAPC/aAPC^{CD47+} (10^5) for 2 h (37°C). Macrophages were washed three times with PBS, stained with anti-CD11b-FITC for 30 min (4°C), fixed (4% paraformaldehyde) and analyzed by confocal laser microscopy.

**Generation of human antigen-specific CTL**

PBMC of HLA-A^*02 donors were obtained by density gradient centrifugation. Subsequently, CD8^+ T cells were enriched using a CD8^+ T cell Isolation Kit II (Miltenyi Biotec) following the manufacture’s protocol. Purified T cells (10^6) were cultured with aAPC or aAPC^{CD47+} as previously published(13).

**Detection of intracellular cytokines in human antigen-specific CTL**

Antigen-specific T cells were analyzed for expression of CD107a, IL-2, TNF-α, IFN-γ and MIP-1β following the protocol previously published(26). All possible combinations of different effector function were calculated utilizing the Boolean gate platform of FlowJo version 9.3.1 software (Tree- Star).

**51Cr release assay**
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2x10^5/plate cognate peptide loaded (1 µg/10^6 cells, overnight, 37°C) T2 target cells were pulsed with 200 µCi ⁵¹Cr for 1 h (37°C). T cell to T2 (E:T) ratios were calculated on 2000 T2 cells/well. V-bottom plates were spun down (300xg, 5 minutes) before incubation. Triplicate wells were averaged and percentage specific cytotoxicity was calculated as [(cpm sample - cpm spontaneous release) x100 / (cpm maximum release – cpm spontaneous release)]. Spontaneous release: T2 cells were plated without T cells. Maximum release: T2 cells were plated in 0.15% Triton-X-100 (Sigma).

**In vivo bio-distribution assay**

NOD.CB17-Prkdc^scid/J (NOD/SCID) mice (male, eight weeks, from Dr. K. Whartenby) were injected (i.v.) with 3x10^7 aAPC or aAPC^CD47+. After 1h animals were sacrificed and organs harvested. Single cell suspensions were generated utilizing a 70 µm cell strainer (Fisherbrand). Beads were isolated on a magnet and washed with PBS. Recovered beads were counted and percentage calculated (% = amount organ-specific beads x 100/amount recovered beads).

**In vivo CFSE proliferation assay**

On day 0 NOD/SCID mice were injected (i.v.) with 5x10^6 carboxy-succinimidyl-ester (CFSE) labelled activated 2C TCR transgenic T cells (2C cells) and 30.000 U/mouse IL-2 (i.p.). On days 1, 2 and 3 mice received (i.v.) 10^7 aAPC or aAPC^CD47+ and 30.000 U/mouse IL-2 (i.p.). On day 5 mice were sacrificed and spleens analysed for CFSE^low 2C cells.
“don’t eat me” aAPC for enhanced in vivo function were generated with \textsuperscript{SIY-K}\textsuperscript{b}-Ig, anti-mouse-CD28 and rh-CD47-Ig (aAPC\textsuperscript{CD47+}). Data was analyzed utilizing the proliferation function of FlowJo software (version 9.3.1).

**In vivo tumor inhibition assay**

On day 0 C57BL/6 (B6) mice (female, eight weeks, Jackson Laboratories) were injected (s.c.) with \(10^6\) B16-SIY melanoma cells into the right flank. Five days later all tumor-bearing mice received \(5 \times 10^6\) activated 2C cells (i.v.) and 30,000 U/mouse IL-2 (i.p.). Mice were treated on days 6, 7 and 8 with \(10^7\) aAPC or aAPC\textsuperscript{CD47+} (i.v.) and 30,000 U/mouse IL-2 (i.p.). Tumor growth was monitored every other day utilizing digital caliper. aAPC were generated with \textsuperscript{SIY-K}\textsuperscript{b}-Ig, anti-mouse-CD28 and rm-CD47-Ig (aAPC\textsuperscript{CD47+}). B6, NOD/SCID and 2C TCR Rag\textsuperscript{−/−} transgenic mice were maintained in the Johns Hopkins animal facilities and procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies. Statistical analysis was performed in GrapPad Prism5.
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Results

**Generation of aAPC and aAPC^{CD47+}**

aAPC were generated by immobilization of HLA-A2-Ig and anti-CD28 mAb onto paramagnetic microbeads (Fig. 1A, upper panel), and aAPC^{CD47+} received an additional third signal, rh-CD47-Ig (Fig. 1A, lower panel). Both aAPC and aAPC^{CD47+} displayed comparable amounts of HLA-A2-Ig and anti-CD28 mAb whereas staining with anti-CD47 demonstrated that only aAPC^{CD47+} showed high amounts of immobilized CD47-molecules (Fig. 1B).

**CD47 on aAPC inhibit phagocytosis by human macrophages**

To investigate if CD47 on aAPC can inhibit up-take by macrophages we used a well characterized human macrophage model(27,28). Type 2 macrophages (M2) expressing high levels of CD68, CD11b, HLA-DR, SIRP-α and CD163 and secreting elevated amounts of TNF, IL-12 and IL-10 after stimulation with lipopolysaccharide (LPS), were generated (Fig. 2A). Consistent with previously published studies(28), M2 demonstrated phagocytic activity (data not shown). Since tumor-associated macrophages (TAMs) have generally been shown to display a M2-like phenotype(29), all phagocytosis assays were performed with M2.

To analyze phagocytosis, aAPC were stained with anti-mouse-IgG-Alexa647 after co-culture with macrophages. Subsequent after macrophages were lysed beads were analyzed to detect stained non-phagocytosed and un-stained phagocytosed
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aAPC/aAPC\(^{CD47^+}\) (Fig. 2B). To allow for optimal phagocytosis we evaluated time-dependent up-take of aAPC by macrophages (Fig. 2C). A gradual increase of phagocytosed aAPC, was seen up to 1 h, and nearly 100 % of all aAPC were phagocytosed after 3 h. Therefore we considered 3 h as an optimal time to investigate the effect of CD47 on aAPC phagocytosis. aAPC generated with different amounts of CD47 ranging from 0.16 - 1600 ng/10\(^8\) aAPC, corresponding to a hypothetical 20 – 200.000 CD47-molecules/aAPC (Fig. 2D), demonstrated a dose dependent aAPC\(^{CD47^+}\) phagocytosis inhibition of about 90%. Results were statistically significant starting from 1.6 ng/10\(^8\) aAPC (P<0.05, Mann Whitney test). Analysis of aAPC\(^{CD47^+}\) revealed comparable amounts of HLA-A2-Ig and anti-CD28 mAb when coated with 0.16 – 160 ng CD47, while high amounts of CD47, 1600 ng, results in reduced amounts (Supplementary Fig. S1). Based on these findings we conducted all the subsequent experiments utilizing aAPC\(^{CD47^+}\) coated with 160 ng CD47 to ensure maximal inhibition of phagocytosis and comparable aAPC functionality.

*aAPC and aAPC\(^{CD47^+}\) clearance by macrophages after internalization*

Ingestion of aAPC by macrophages results in clearance from the circulation and in reduced probability of aAPC/T cell interaction (Fig. 3A). While addition of CD47 onto the surface of aAPC efficiently inhibited phagocytosis around 20% were still ingested (Fig. 2D, 160 ng rh-CD47-Ig). To test if aAPC and aAPC\(^{CD47^+}\) phagocytosed by macrophages are still functional we performed 24 h co-cultures to provide sufficient time to allow internal digestion processes to be executed (Fig. 3). Almost 100 % of all aAPC from co-cultures
“don’t eat me” aAPC for enhanced *in vivo* function with macrophages did not stain positive for either of the tested molecules whereas 70% of all aAPC$^{CD47^+}$ stained positive for HLA-A2-Ig and anti-CD28 (Fig. 3B). These results correlated with the actual amount of phagocytosed aAPC and aAPC$^{CD47^+}$ as shown in figure 2D (0 ng and 160 ng rh-CD47-Ig respectively). Control stains of aAPC and aAPC$^{CD47^+}$ not co-cultured with macrophages and either lysed (lysis control) or untreated (control) did not show any significant changes of HLA-A2-Ig and anti-CD28 protein levels. Furthermore, mean fluorescence intensity for HLA-A2-Ig and anti-CD28 of all positively stained aAPC and aAPC$^{CD47^+}$ were at same levels when compared to controls (Fig. 3C). Thus, this data demonstrates that internal cleavage processes clear ingested aAPC and aAPC$^{CD47^+}$, whereas non-phagocytosed aAPC and aAPC$^{CD47^+}$ remain unaltered in co-cultures with macrophages and potentially execute their primary function.

**Comparable in vitro function of aAPC and aAPC$^{CD47^+}$**

The primary function of aAPC is effective stimulation and subsequent expansion of antigen-specific T cells. One important goal of our study was to compare the functionality of aAPC$^{CD47^+}$ with that of aAPC. To this end, both aAPC$^{CD47^+}$ and aAPC were loaded with either low-affinity modified MART-1 peptide, which is derived from a melanocyte self-antigen, or high-affinity viral FluM1 peptide derived from influenza matrix protein 1. Loaded aAPC$^{CD47^+}$ and aAPC were used to stimulate and expand antigen-specific T cells from HLA-A$^*$02:01 healthy donors (Supplementary Fig. S2B). Both antigen-specificity and expansion of T cells stimulated with aAPC and aAPC$^{CD47^+}$ showed no statistically significant differences during 3 weeks of T cell culture (Fig. 4A,B).
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Furthermore, aAPC and aAPC^{CD47^+}-generated MART-1- and FluM1-specific T cells exhibited comparable amounts of cognate peptide pulsed T2 target cell killing (Fig. 5A,B). Slight differences were related to the antigen-specificity of utilized T cells (Supplementary Fig. S2A) and background non-specific killing did not exceed moderate levels (10-30%). Finally we determined polyfunctionality of aAPC and aAPC^{CD47^+}-generated T cells by intracellular staining for CD107a, MIP-1β, IL-2, TNF and IFN-γ(26). All possible combinations of different effector functions indicated comparable activation marker expression by those T cells (Fig. 5C). Thus, aAPC and aAPC^{CD47^+} demonstrated equal in vitro induction, expansion and functionality of antigen-specific T cells over a course of three weeks stimulation.

**In vivo activity of aAPC^{CD47^+}**

While aAPC^{CD47^+} showed comparable in vitro functionality and reduced phagocytosis compared to aAPC, we investigated if these characteristics resulted in a better in vivo activity. Nonobese diabetic/ severe-combined-immunodeficient (NOD/SCID) mice were adoptively transferred with CFSE labeled 2C T cells and treated with cognate aAPC either with or without rh-CD47-Ig (Supplementary Fig. S3A). Mice were sacrificed and spleens analyzed for CD8 expression and CFSE dilution in 10 generations of divided T cells (Fig. 6A). The majority of T cells (~30 %) in aAPC treated mice under went only 4 division cycles. However, about 50% of the T cells in aAPC^{CD47^+} treated animals underwent 6 – 7 divisions at the same time. Therefore, aAPC^{CD47^+} demonstrated an enhanced in vivo
“don’t eat me” aAPC for enhanced in vivo function capacity compared to aAPC. aAPC and aAPC^{CD47^+} did not show any stimulatory differences when tested in an in vitro proliferation assay (Supplementary Fig. S3C).

To investigate if differences in stimulatory capacity might be due to changes in biodistribution, we injected NOD/SCID mice with either aAPC or aAPC^{CD47^+} (Fig. 6B). Most aAPC^{CD47^+} cleared the lungs (only 54% remaining) compared to aAPC (68% remaining) but also showed a tendency to increasingly accumulate in the kidneys, liver and spleen. Thus, this data provides evidence that aAPC^{CD47^+} have an improved ability to get pass the highly active alveolar macrophages leading to an enhanced peripheral function.

Finally, utilizing an immune competent B16-SIY mouse melanoma model we investigated if better stimulatory capacity and preferential biodistribution of aAPC^{CD47^+} directly translates into improved T cell functionality. To this purpose, we adoptively transferred tumor-bearing B6 mice with 2C T cells and treated those animals with cognate aAPC either with or without rm-CD47-Ig (Supplementary Fig. S3B). Mice that received aAPC^{CD47^+} showed reduced tumor growth with statistically significant differences seen by day 9 (Fig. 6C). By day 19, mice treated with aAPC^{CD47^+} had the smallest tumor burden, with an average tumor size of 67 mm², compared to 122 mm² for mice treated with aAPC. Furthermore, all animals treated with aAPC but only 7 out of 9 aAPC^{CD47^+} treated animals developed a tumor before day 19. Together, these experiments show that aAPC^{CD47^+} exhibit an enhanced in vivo functionality and increased anti-tumor activity.
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Discussion

Generation and expansion of tumor-specific T cells for adoptive immunotherapy is an ongoing and challenging task. Many groups have developed non-cellular aAPC with in vivo applicability to establish a cost-, labour- and time-saving technology(20,30,31). Classical non-cellular aAPC feature two signals, an antigen-specific and a co-stimulatory signal. Most of the aAPC have been generated utilizing only one co-stimulatory signal but some groups successfully developed aAPC with combinations of those molecules such as anti-CD28/anti-LFA1(32) and anti-CD28/anti-4-1BB(33). However, to date no aAPC have been generated with more than the two classical signals. Thus, to our knowledge, this study demonstrates for the first time that aAPC displaying an additional “don’t eat me” signal (i.e. CD47) show favourable anti-phagocytic activities. All the while maintaining their primary function, generation and expansion of antigen-specific T cells in vitro and displaying enhanced in vivo functionality.

We generated “don’t eat me” aAPC (aAPC$^{CD47^+}$) by addition of CD47-Ig onto the surface of classical aAPC. Co-culture experiments with macrophages demonstrated a concentration dependent inhibition of ingestion starting at 1.6 ng CD47-Ig/10$^8$ beads. This resembles a theoretical density of ~3.5 CD47-molecules/µm$^2$ and results in up-take of only 40-60 % of aAPC$^{CD47^+}$. These results are in line with data published by Tsai et al. that showed that opsonised particles inhibit phagocytosis with an effective $K_i=20$ molecules/µm$^2$(6). Furthermore, the theoretical CD47-molecule density on aAPC$^{CD47^+}$
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(160 ng CD47-Ig/10^8 beads) was in the same order of magnitude (~350 molecules/µm^2) to reported physiological conditions on human RBC (~250 molecules/µm^2)(34).

Staining of aAPC and aAPC^{CD47+} from macrophage co-cultures revealed that all non-phagocytosed aAPC/aAPC^{CD47+} displayed the same amount of immobilized molecules when analyzed for MFI indicating that phagocytosed aAPC, independent of their CD47 status, were cleared by internal digestion processes. Thus, the favorable effect of CD47 is exclusively related to inhibition of phagocytic processes rather than to cellular digestion processes which is in line with previously published data(1,9,35).

We demonstrated efficient expansion of antigen-specific T cells with no significant differences between aAPC and aAPC^{CD47+}. Furthermore, utilizing either the low-affinity modified MART-1 or the high-affinity viral FluM1 peptide sequences for antigen-specific T cell generation we did not observe any significant differences in T cell stimulation and expansion establishing that aAPC and aAPC^{CD47+} display a comparable functionality regardless of TCR affinity and function. Additionally, aAPC/aAPC^{CD47+}-generated T cells demonstrated comparable killing efficiencies and polyfunctionality. Thus aAPC and aAPC^{CD47+} demonstrated comparable in vitro functional stimulation in an antigen-specific T cell population. Together these data provide significant evidence suggesting that a third “don’t eat me” signal did not interfere with overall T cell expansion and effector function.
NOD/SCID mice express a SIRP-α variant that cross-interacts with human CD47 which renders these animals a perfect tool for in vivo investigations of human aAPC and aAPC$^{CD47^+}$ function(9,36). We demonstrated that aAPC$^{CD47^+}$ stimulated 2C T cells underwent more proliferation cycles in vivo than aAPC stimulated 2C T cells. Experiments with CD47-functionalized microparticles have been reported demonstrating reduced phagocytosis by macrophages in vitro and in vivo(6,9,34). Stacheleke et al. showed that CD47 functionalized surfaces can resist inflammatory cell interaction both in vitro and in vivo(7) and anti-CD47 mAb can inhibit migration of several cell types(5). Therefore, it was likely that aAPC$^{CD47^+}$ would display a different biodistribution pattern than aAPC. We demonstrated that the amounts of aAPC and aAPC$^{CD47^+}$ in the lungs were significantly different. The presence of CD47 enabled more aAPC$^{CD47^+}$ to pass the pulmonary alveolus than aAPC. Finally, tumor growth was significantly inhibited when animals were treated with aAPC$^{CD47^+}$ compared to aAPC suggesting an enhanced in vivo functionality of aAPC$^{CD47^+}$.

To our knowledge, this is the first time that functionalized particles initially developed for immunotherapeutic approaches where additionally functionalized with a third signal, CD47. aAPC$^{CD47^+}$ showed comparable in vitro performance but favorable in vivo behavior when compared to aAPC. However, there are a couple of critical questions that need to be addressed before this new aAPC approach can be tested in clinical studies: (i) which processes other than phagocytosis are involved, (ii) are the in vivo effects solely dependent on T cell numbers or (iii) do aAPC$^{CD47^+}$ change the micro milieu generated by
“don’t eat me” aAPC for enhanced in vivo function macrophages leading to different T cell priming in vivo, (iv) can aAPC$^{CD47^+}$ induce endogenous anti-tumor responses and (v) how do aAPC$^{CD47^+}$ compare to other tumor treatments such as adoptive T cell transfer or vaccination. Although further experiments have to be conducted to gain more detailed insights into aAPC$^{CD47^+}$ in vivo function the results shown here prove that aAPC$^{CD47^+}$ greatly increases the clinical applicability of such platform technologies and justifies the further investigation as a novel treatment option for a wide array of diseases.
Acknowledgements

C. Schütz is supported by a German Research Foundation (DFG) Postdoctoral Fellowship (SCHU-2681/1-1) and a HERA Women’s Cancer Foundation OSB1 Grant. This work was supported by the National Institutes of Health (P01-Al072677 and R01-CA108835), TEDCO/Maryland Innovation Initiative and Neximmune, Inc. MD Biotech Center (J.P.S.) and by the ELAN Fond of the University Hospital of Erlangen and the Roggenbuck-Stiftung Hamburg (H.B.). The authors wish to thank Dr. K. Whartenby, Department of Neurology, Johns Hopkins School of Medicine to generously provide the NOD/SCID mice.
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Figure Legends

Figure 1: Schematic and characterization of an “eat me” and a “don’t eat me” aAPC.

(A) Schematic of a two-signal aAPC (upper panel; aAPC) coated with HLA-A2-Ig and anti-CD28 mAb and a three-signal aAPC (lower panel; aAPC\(^{CD47^+}\)) coated with an additional rh-CD47-Ig. (B) Phenotypically characterization of aAPC (blue line) and aAPC\(^{CD47^+}\) (red line). Both aAPC were stained with anti-IgG1 and anti-IgG2a to detect the HLA-A2-Ig and the anti-CD28 mAb respectively. rhCD47-Ig expression was determined by anti-CD47 staining. Control (black filled) indicates staining of empty beads and numbers in the upper left corner of each histogram represents mean fluorescence intensity (MFI).

Figure 2: CD47-Ig on aAPC inhibits phagocytosis by human type 2 macrophages. (A) Characterization of human type 2 macrophages (M2) by flow cytometry (upper panel) and by ELISA (lower panel). Antibody stains (filled gray) and isotype stains (solid black) are displayed in overlay histograms. For detection of cytokines, macrophages were stimulated with LPS (100 ng/ml, 48 h). Data depicted represents mean (+/- SD) generated out of triplicate values. (B) Evaluation of phagocytosis assay. Gated unstained beads indicate the amount of phagocytosed beads (sample). Beads in supernatant of co-cultures without water lysis stain positive (supernatant; non gated bead population) whereas phagocytosed beads do not stain positive for anti-mouse-Alexa647 (cell lysate). In the later sample non-phagocytosed beads were separated from cells prior to water lysis and subsequent staining of phagocytosed beads. (C) Time course of aAPC
phagocytosis by M2. aAPC and M2 were co-cultured for the indicated amount of time at a 1:1 ratio. Samples were analysed for phagocytosis as depicted in B. A sample of aAPC without M2 served as control (most right bar). Data represents mean (+/-SD) of three independent experiments. (D) Dose dependent inhibition of aAPC phagocytosis by CD47-Ig on aAPC. aAPC\textsuperscript{CD47+} generated with different amounts of CD47-Ig (indicated at x-axis, per 10\(^8\) beads) were co-cultured with M2 for 3 h at a 1:1 ratio. aAPC with 0 ng CD47-Ig served as positive control. Samples were analysed for phagocytosis as depicted in B. Data represents mean (+/-SD) of four independent experiments. * indicates statistical significance \(P<0.05\) (Mann Whitney test).

**Figure 3:** aAPC\textsuperscript{CD47+} are protected from cleavage of HLA-A2-Ig and anti-CD28 in co-cultures with human macrophages. (A) Confocal image of phagocytosed aAPC or aAPC\textsuperscript{CD47+} after co-culture (2 h) with human macrophages. aAPC/aAPC\textsuperscript{CD47+} were stained with a succinimidylester-Alexa647 prior to co-culture (red) and macrophages were visualized by DAPI (blue) and anti-CD11b (green) staining. (B) aAPC and aAPC\textsuperscript{CD47+} were co-cultured with human macrophages at a 1:1 ratio for 24 h. Samples were lysed with water, beads magnetically separated and stained with anti-IgG1 and anti-IgG2a for detection of HLA-A2-Ig and anti-CD28 respectively (black bars; co-culture). “Control” (white bars) represents same aAPC and aAPC\textsuperscript{CD47+} batches not co-cultured with human macrophages, whereas in “lysis control” (grey bars) aAPC and aAPC\textsuperscript{CD47+} were treated with water prior to staining. Data displayed represents average (+/-SD) amount of aAPC positive for both molecules. (C) aAPC and aAPC\textsuperscript{CD47+} were gated for HLA-A2 and anti-
“don’t eat me” aAPC for enhanced *in vivo* function

CD28 and expression level of both molecules compared. Data displayed represents mean (+/- SD) MFI of HLA-A2-Ig and anti-CD28. Data in B and C generated from 6 independent experiments.

**Figure 4:** aAPC^{CD47^+} demonstrated same *in vitro* efficacy in generation and expansion of antigen-specific human T cells. Enriched T cells were stimulated with either aAPC or aAPC^{CD47^+} for a total of three weeks. Expansion (**A**) and specificity (**B**) data of 5 different donors and two different antigens are displayed. There was no significant difference between T cells stimulated with aAPC or aAPC^{CD47^+} when tested for each antigen and day (Mann-Whitney test).

**Figure 5:** Functionality of aAPC^{CD47^+} generated antigen-specific T cells is equal to aAPC-generated antigen-specific T cell functionality. FluM1 specific (solid line) and MART-1 specific (dotted line) T cells were generated with either aAPC (filled dots) or aAPC^{CD47^+} (filled squares). On day 14, 51Cr release assay was performed to analyze antigen-specific lysis of T2 cells pulsed with FluM1 (**A**) or MART-1 (**B**) peptide. Data generated out of triplicate values from one representative experiment out of three independent experiments. (**C**) Intra cellular detection of CD107a, MIP1-β, TNF, IFN-gamma and IL-2 production after stimulation of aAPC or aAPC^{CD47^+} generated T cells with cognate peptide loaded T2 cells. Peptide names FluM1 (left column) and MART-1 (right column) indicate loading of aAPC and aAPC^{CD47^+} utilized for antigen-specific T cell generation over 14 days. “function” indicates T cells that express the same number of any investigated
“don’t eat me” aAPC for enhanced in vivo function polyfunctionality marker in any possible combination. Data generated from triplicates of one out of three independent experiment.

**Figure 6: aAPC$^{CD47^+}$ demonstrate enhanced in vivo functions over classical two-signal aAPC.** (A) Cell generation analysis of CFSE labeled adoptively transferred 2C cells from mice treated with aAPC or aAPC$^{CD47^+}$. Increasing # correlated with dilution of CFSE and proliferation of 2C cells. Mice were adoptively transferred with CFSE$^+$ 2C cells on day 0 and treated with aAPC or aAPC$^{CD47^+}$ on day 1, 2 and 3. CFSE analysis was performed on day 4 by flow cytometry. (B) Bio-distribution of aAPC and aAPC$^{CD47^+}$ in NOD/SCID mice. (C) B16-SIY tumor bearing B6 mice were adoptively transfered with activated 2C cells and treated with either aAPC or aAPC$^{CD47^+}$. Tumor size was measured by caliper on every other day. * (P<0.05) and ** (P<0.005) indicates statistical significance (Mann Whitney test).
Fig. 4

A

Day 7

Day 14

Day 21

fold expansion

aAPC aAPC^{CD47^+}
aAPC aAPC^{CD47^+}
aAPC aAPC^{CD47^+}

MART-1 FluM1

B

Day 7

Day 14

Day 21

% specific CTL

aAPC aAPC^{CD47^+}
aAPC aAPC^{CD47^+}
aAPC aAPC^{CD47^+}

MART-1 FluM1

Author Manuscript Published OnlineFirst on January 15, 2015; DOI: 10.1158/1078-0432.CCR-14-2696
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
CD47 enhances in vivo functionality of artificial antigen-presenting cells.

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Clin Cancer Res  Published OnlineFirst January 15, 2015.