CD47 Enhances In Vivo Functionality of Artificial Antigen-Presenting Cells

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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. Although aAPC compare favorably with autologous dendritic cells in vitro, their effect in vivo might be diminished through rapid clearance by macrophages. Therefore, to prevent uptake and minimize in vivo functionality, we investigated the efficiency of “don’t eat me” three-signal aAPC compared with 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 vitro in human primary T-cell and macrophage cocultures. In vivo efficiency was compared in a NOD/SCID T-cell proliferation and a B16-SIY melanoma model.

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 CD47low or CD47⁻ cells are phagocytosed as part of homeostatic regulation. CD47 is upregulated on many human tumor cells and mediates tumor immune evasion by interaction with signal-regulating protein alpha (SIRPα; ref. 4). Binding to the extracellular region of SIRPα 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-SIRPα signaling in murine immunoglobulin-opsonized red blood cells (RBC) as well as in human CD47⁺-RBC and CD47⁻ 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 with nonactivated polymers (7) and coating of nanoparticles with CD47⁻RBC membranes minimized particle uptake 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; refs. 13, 14) and were successfully tested in different in vitro systems (15–17). In vivo aAPC-generated T cells inhibited tumor growth as efficiently 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).
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 signaling pathways. Artificial antigen-presenting cells (aAPC) have been described as a powerful tool for induction of tumor antigen-specific T-cell responses in vitro and in vivo. To further increase their in vivo functional half-life due to minimized phagocytic uptake by macrophages and improved antitumor efficacy, we generated CD47-positive aAPC (aAPC CD47+). These aAPC CD47+ show great potential to significantly enhance new approaches to active immunotherapy.

Micrometer-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 hypothesized 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 with nonfunctionalized 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 with aAPC, in conjunction with diverging biodistribution in different organs.

Thus, this study for the first time shows that 2-signal aAPC functionalized with an additional third signal (CD47) maintain their primary in vitro stimulatory capacity for antigen-specific T-cell activation and expansion and demonstrate enhanced in vivo efficiency.

Materials and Methods

The ethical committees of the Johns Hopkins University (Baltimore, MD) and the University of Erlangen (Erlangen, Germany) 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 matrix protein (FluM1, GILGFVFTL), and synthetic murine SIY melanoma-associated antigen (MART-1; ELAGIGILTV), in addition to influenza matrix protein (FluM1, GILGFVFTL), and synthetic murine SIY melanoma-associated antigen (MART-1; ELAGIGILTV).

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-A201 tetramer folded around MART-1 and FluM1 (Beckman Coulter). Antigen-specific T cells were stained for 30 minutes (RT) with HLA-A201 tetramers. mAb stain was performed for 15 minutes (4°C). Samples were analyzed using a Calibur flow cytometer (BD) and FlowJo software (Tree Star, Inc.).

Generation of aAPC and aAPC CD47+

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). aAPC CD47+ 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). Monocytes were isolated by plastic adherence and cultured in the presence of macrophage colony-stimulating factor (M-CSF; 50 ng/mL, R&D). Six days later, macrophages were detached with EDTA (1 mmol/L, 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, IL12, and IL10 concentrations in culture supernatants of macrophages unstimulated or stimulated with lipopolysaccharide (LPS; 100 ng/mL) were determined (18 hours) by ELISA (R&D), following the manufacturer's instructions. Results were collected using a microplate autoreader (EL309) from Bio-Tech Instruments.

Succinimidy ester labeling of aAPC/aAPC CD47+

aAPC/aAPC CD47+ were washed twice with PBS, 0.5% Tween-80, 0.2 mol/L sodium bicarbonate (pH 8.8), and resuspended in 1 mmol/L fluorescein-succinimidy-ester-Alexa-647. After 1 hour, beads were washed 3 times with PBS/0.5% Tween-80 (25).

Phagocytosis of aAPC and aAPC CD47+

Macrophages (2.5 × 105) were adhered to 24-well plates and cocultured with aAPC or aAPC CD47+ (2.5 × 105) for the time indicated (37°C). Samples were coincubated with anti-mouse-IgG-F(ab’2)-Alexa647 (1:100, Cell Signaling) for 30 minutes (4°C), macrophages washed, lysed in sterile water (30 minutes, 4°C), and the lysate analyzed by flow cytometry.

Macrophages (105) were adhered to 8-chamber slides (Nunc) cocultured with labeled aAPC/aAPC CD47+ (105) for 2 hours (37°C). Macrophages were washed 3 times with PBS, stained with anti-CD11b-FITC for 30 minutes (4°C), fixed (4% paraformaldehyde), and analyzed by confocal laser microscopy.

Generation of human antigen-specific cytotoxic T lymphocyte

PBMC of HLA-A02 donors were isolated by density gradient centrifugation. Subsequently, CD8+ T cells were enriched using a CD8+ T cell Isolation Kit II (Miltenyi Biotec) following the manufacturer’s protocol. Purified T cells (106) were cultured with aAPC or aAPC CD47+ as previously published (13).

Detection of intracellular cytokines in human antigen-specific cytotoxic T lymphocyte

Antigen-specific T cells were analyzed for expression of CD107a, IL2, TNF-α, IFN-γ, and MIP-1B following the protocol...
previously published (26). All possible combinations of different effector function were calculated using the Boolean gate platform of FlowJo version 9.3.1 software (Tree Star).

$^{51}$Cr release assay

A total of $2 \times 10^7$/plate cognate peptide loaded (1 $\mu$g/10$^6$ cells, overnight, 37°C) T2 target cells were pulsed with 200 $\mu$Ci $^{51}$Cr for 1 hour (37°C). T cell to T2 (E:T) ratios were calculated on 2,000 T2 cells per well. V-bottom plates were spun down (300 x g, 5 minutes) before incubation. Triplicate wells were averaged and percentage specific cytotoxicity was calculated as $\frac{\text{cpm sample} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100$. Spontaneous release: T2 cells were plated without T cells. Maximum release: T2 cells were plated in 0.15% Triton-X-100 (Sigma).

In vivo biodistribution assay

NOD.CB17-Prkdcscid/J (NOD/SCID) mice (male, 8 weeks, from Dr. K. Whartenby) were injected (i.v.) with $3 \times 10^7$ aAPC or aAPCCD47$^+$. After 1 hour, animals were sacrificed and organs harvested. Single-cell suspensions were generated using a 70-mmc cell strainer (Fisherbrand). Beads were isolated on a magnet and washed with PBS. Recovered beads were counted and percentage calculated $(\% = \frac{\text{amount organ-specific beads}}{\text{amount recovered beads}} \times 100)$. aAPC were generated with SIY-Kb-Ig, anti-mouse-CD28, and rh-CD47-Ig (aAPCCD47$^+$.)

In vivo carboxyfluorescein diacetate succinimidyl ester proliferation assay

On day 0, NOD/SCID mice were injected (i.v.) with $5 \times 10^6$ carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled activated 2C TCR transgenic T cells (2C cells) and 30,000 U/mouse IL2 (intraperitoneally). On days 1, 2, and 3, mice received (i.v.) $10^7$ aAPC or aAPCCD47$^+$ and 30,000 U/mouse IL2 (intraperitoneally). On day 5, mice were sacrificed and spleens analyzed for CFSE$^{low}$ 2C cells. aAPC were generated with SIY-Kb-Ig, anti-mouse-CD28, and rh-CD47-Ig (aAPCCD47$^+$). Data were analyzed using the proliferation function of FlowJo software (version 9.3.1).

In vivo tumor inhibition assay

On day 0, C57BL/6 (B6) mice (female, 8 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 IL2 (intraperitoneally). Mice were treated on days 6, 7, and 8 with $10^7$ aAPC or aAPCCD47$^+$ (i.v.) and 30,000 U/mouse IL2 (intraperitoneally). Tumor growth was monitored every other day using digital caliper. aAPC were generated with SIY-Kb-Ig, anti-mouse-CD28, and rm-CD47-Ig (aAPCCD47$^+$). B6, NOD/SCID, and 2C TCR Rag$^{-}$ 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 GraphPad Prism5.

Results

Generation of aAPC and aAPCCD47$^+$

aAPC were generated by immobilization of HLA-A2-Ig and anti-CD28 mAb onto paramagnetic microbeads (Fig. 1A, top), and aAPCCD47$^+$ received an additional third signal,
rh-CD47-Ig (Fig. 1A, bottom). 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 whether CD47 on aAPC can inhibit uptake 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, IL12, and IL10 after stimulation with LPS were generated (Fig. 2A). Consistent with previously published studies (28), M2 demonstrated phagocytic activity (data not shown). Because tumor-associated macrophages (TAM) 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 coculture with macrophages. Subsequent after macrophages were lysed, beads were analyzed to detect stained nonphagocyted and unstained phagocyted aAPC/aAPC-CD47+ (Fig. 2B). To allow for optimal phagocytosis, we evaluated time-dependent uptake of aAPC by macrophages (Fig. 2C). A gradual increase of phagocyted aAPC was seen up to 1 hour, and nearly 100% of all aAPC were phagocyted after 3 hours. Therefore, we considered 3 hours as an optimal time to investigate the effect of
Conducted all the subsequent experiments using aAPC CD47

Figure 3. aAPC and aAPCCD47 are protected from cleavage of HLA-A2-Ig and anti-CD28 in cocultures with human macrophages. A, confocal image of phagocytosed aAPC or aAPCCD47, after coculture (2 hours) with human macrophages. aAPC/aAPCCD47 were stained with a succinimidyl ester-Alexa647 before coculture (red), and macrophages were visualized by DAPI (blue) and anti-CD11b (green) staining. B, aAPC and aAPCCD47 were cocultured with human macrophages at a 1:1 ratio for 24 hours. 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; coculture). “Control” (white bars) represents same aAPC and aAPCCD47 coated with 160 ng CD47 to ensure maximal inhibition of (Supplementary Fig. S1). On the basis of these findings, we conducted all the subsequent experiments using aAPC coated with 160 ng CD47 to ensure maximal inhibition of phagocytosis and comparable aAPC functionality.

CD47 on aAPC phagocytosis. aAPC generated with different amounts of CD47 ranging from 0.16 to 1600 ng/10⁸ aAPC, corresponding to a hypothetical 20 to 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/10⁸ 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 to 160 ng CD47, whereas high amounts of CD47, 1,600 ng, results in reduced amounts of CD28 protein levels. Furthermore, mean fluorescence intensity (MFI) for HLA-A2-Ig and anti-CD28 of all positively stained aAPC and aAPC CD47⁻ were at same levels when compared with controls (Fig. 3C). Thus, these data demonstrate that internal cleavage processes clear ingested aAPC and aAPC CD47⁻, whereas nonphagocytosed aAPC and aAPC CD47⁻ remain unaltered in cocultures 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 and B). 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 and B). Slight differences were related to the antigen specificity of used T cells (Supplementary Fig. S2A) and background

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 whether aAPC and aAPC CD47⁻ phagocytosed by macrophages are still functional, we performed 24-hour cocultures to provide sufficient time to allow internal digestion processes to be executed (Fig. 3). Almost 100% of all aAPC from cocultures 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 Fig. 2D (0 and 160 ng rh-CD47-Ig, respectively). Control stains of aAPC and aAPC CD47⁻ not cocultured 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 (MFI) for HLA-A2-Ig and anti-CD28 of all positively stained aAPC and aAPC CD47⁻ were at same levels when compared with controls (Fig. 3C). Thus, these data demonstrate that internal cleavage processes clear ingested aAPC and aAPC CD47⁻, whereas nonphagocytosed aAPC and aAPC CD47⁻ remain unaltered in cocultures with macrophages and potentially execute their primary function.
nonspecific 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, IL2, TNF, and IFNγ (26). All possible combinations of different effector functions indicated comparable activation marker expression by those T cells (Fig. 5C). Thus,

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**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 3 weeks. Expansion (A) and specificity (B) data of 5 different donors and 2 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).
In vivo activity of aAPCCD47

While aAPCCD47− showed comparable in vitro functionality and reduced phagocytosis compared to aAPC, we investigated whether 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 underwent only 4 division cycles. However, about 50% of the T cells in aAPCCD47−-treated animals underwent 6 to 7 divisions at the same time. Therefore, aAPCCD47− demonstrated an enhanced in vivo capacity compared with aAPC. aAPC and aAPCCD47− did not show any stimulatory differences when tested in an in vitro proliferation assay (Supplementary Fig. S3C).

To investigate whether differences in stimulatory capacity might be due to changes in biodistribution, we injected NOD/SCID mice with either aAPC or aAPCCD47− (Fig. 6B). Most aAPCCD47− cleared the lungs (only 54% remaining) compared with aAPC (68% remaining) but also showed a tendency to increasingly accumulate in the kidneys, liver, and spleen. Thus, these data provide evidence that aAPCCD47− have an improved ability to get past the highly active alveolar macrophages leading to an enhanced peripheral function.

Finally, using an immunocompetent B16-SIY mouse melanoma model, we investigated whether better stimulatory capacity and preferential biodistribution of aAPCCD47− 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 aAPCCD47− showed reduced tumor growth with statistically significant differences seen by day 9 (Fig. 6C). By day 19, mice treated with aAPCCD47− 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 were treated with aAPC, but only 7 of 9 aAPCCD47−-treated animals developed a tumor before day 19. Together, these experiments show that aAPCCD47− exhibit an enhanced in vivo functionality and increased antitumor activity.

Discussion

Generation and expansion of tumor-specific T cells for adoptive immunotherapy is an ongoing and challenging task. Many groups have developed noncellular aAPC with in vitro applicability to establish a cost-, labor-, and time-saving technology (20, 30, 31). Classical noncellular aAPC feature 2 signals, an antigen-specific and a costimulatory signal. Most of the aAPC have been generated using only one costimulatory 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 2 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 favorable antiphagocytic 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 (aAPCCD47+) by addition of CD47-Ig onto the surface of classical aAPC. Coculture...
experiments with macrophages demonstrated a concentration-dependent inhibition of ingestion starting at 1.6 ng CD47-lg/10^8 beads. This resembles a theoretical density of about 3.5 CD47-molecules/μm² and results in uptake of only 40% to 60% of aAPC<sub>CD47</sub>-<sup>+</sup>. These results are in line with data published by Tsai and colleagues that showed that opsonized particles inhibit phagocytosis with an effective K<sub>i</sub> = 20 molecules/μm² (6). Furthermore, the theoretical CD47 molecule density on aAPC<sub>CD47</sub>-<sup>+</sup> (160 ng CD47-lg/10^8 beads) was in the same order of magnitude (∼350 molecules/μm²) to reported physiologic conditions on human RBC (∼250 molecules/μm²; ref. 34).

Staining of aAPC and aAPC<sub>CD47</sub>-<sup>+</sup> from macrophage cocultures revealed that all nonphagocytosed aAPC/aAPC<sub>CD47</sub>-<sup>+</sup> 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<sub>CD47</sub>-<sup>+</sup>. Furthermore, using 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<sub>CD47</sub>-<sup>+</sup> display a comparable functionality regardless of T-cell receptor (TCR) affinity and function. In addition, aAPC/aAPC<sub>CD47</sub>-<sup>+</sup>-generated T cells demonstrated comparable killing efficiencies and polyfunctionality. Thus, aAPC and aAPC<sub>CD47</sub>-<sup>+</sup> demonstrated comparable <i>in vitro</i> 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 <i>in vivo</i> investigations of human aAPC and aAPC<sub>CD47</sub>-<sup>+</sup> function (9, 36). We demonstrated that aAPC<sub>CD47</sub>-<sup>+</sup>-stimulated 2C T cells underwent more proliferation cycles in <i>in vivo</i> than aAPC-stimulated 2C T cells. Experiments with CD47-functionalized microparticles have been reported demonstrating reduced phagocytosis by macrophages in <i>vitro</i> and in <i>in vivo</i> (6, 9, 34). Stachelek and colleagues showed that CD47 functionalized surfaces can resist inflammatory cell interaction both in <i>in vitro</i> and in <i>in vivo</i> (7), and anti-CD47 mAb can inhibit migration of several cell types (5). Therefore, it was likely that aAPC<sub>CD47</sub>-<sup>+</sup> would display a different biodistribution pattern than aAPC. We demonstrated that the amounts of aAPC and aAPC<sub>CD47</sub>-<sup>+</sup> in the lungs were significantly different. The presence of CD47 enabled more aAPC<sub>CD47</sub>-<sup>+</sup> to pass the pulmonary alveolus than aAPC. Finally, tumor growth was significantly inhibited when animals were treated with aAPC<sub>CD47</sub>-<sup>+</sup> compared with aAPC suggesting an enhanced <i>in vivo</i> functionality of aAPC<sub>CD47</sub>-<sup>+</sup>.

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<sub>CD47</sub>-<sup>+</sup> showed comparable <i>in vitro</i> performance but favorable <i>in vivo</i> 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 <i>in vivo</i> effects solely dependent on T-cell numbers, (iii) do aAPC<sub>CD47</sub>-<sup>+</sup> change the micro milieu generated by macrophages leading to different T-cell priming in <i>in vivo</i>, (iv) can aAPC<sub>CD47</sub>-<sup>+</sup> induce endogenous antitumor responses, and (v) how do aAPC<sub>CD47</sub>-<sup>+</sup> compare with 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<sub>CD47</sub>-<sup>+</sup> <i>in vivo</i> function, the results shown here prove that aAPC<sub>CD47</sub>-<sup>+</sup> 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.

Disclosure of Potential Conflicts of Interest
A. Mackensen is a consultant/advisory board member for NexImmune, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: H. Bruns, A. Mackensen, M. Oelke, C. Schütz. Development of methodology: H. Bruns, C. Bessell, M. Oelke, C. Schütz. Acquisition of data (provided animals, collected samples, performed experiments, provided facilities, etc.): C. Bessell, J.C. Varela, C. Haupt, J. Fang, C. Schütz. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Bruns, C. Bessell, J.C. Varela, J.P. Schneck, C. Schütz. Writing, review, and/or revision of the manuscript: H. Bruns, C. Bessell, J.C. Varela, A. Mackensen, M. Oelke, J.P. Schneck, C. Schütz. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Haupt, J. Fang, S. Pasemann. Study supervision: J.P. Schneck, C. Schütz.

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References


20. Sunshine JC, Green JF. Nanoengineering approaches to the design of artificial antigen-presenting cells. Nanomedicine (Lond) 2013;8:1173–89.


"Don't Eat Me" aAPC for Enhanced In Vivo Function
CD47 Enhances In Vivo Functionality of Artificial Antigen-Presenting Cells

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