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 clearance of aAPC by macrophages, thereby increasing 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 (aAPCCD47+). aAPC and aAPCCD47+ were analyzed in vitro 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).
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 vivo. 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 vivo 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 H2Kβ-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-A0201 tetramer folded around MART-1 and FluM1 (Beckman Coulter). Antigen-specific T cells were stained for 30 minutes (RT) with HLA-A0201 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 Kβ-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-lg/10⁸ epoxy beads if not differentially indicated. The protocol followed has been previously published (13, 14).

Phagocytosis of aAPC and aAPC-CD47+

Macrophages (10⁵) were adhered to 24-well plates and cocultured with aAPC or aAPC-CD47+ (2 × 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) and 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-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 (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, TNFα, IFNγ, and MIP-1β following the protocol.

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.
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).

**51Cr release assay**

A total of \(2 \times 10^5\)/plate cognate peptide loaded (1 \(\mu\)g/10\(^6\) cells, overnight, 37°C) T2 target cells were pulsed with 200 \(\mu\)Ci \(51\text{Cr}\) 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 \(g\), 5 minutes) before incubation. Triplicate wells were averaged and percentage specific cytotoxicity was calculated as \([\text{cpm sample} - \text{cpm spontaneous release}] / 100 / (\text{cpm maximum release} - \text{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 biodistribution assay**

NOD.CB17-Prkdc<sup>scid</sup>/J (NOD/SCID) mice (male, 8 weeks, from Dr. K. Whartenby) were injected (i.v.) with \(3 \times 10^7\) aAPC or aAPCCD47<sup>+</sup>. After 1 hour, animals were sacrificed and organs harvested. Single-cell suspensions were generated using a 70-\(\mu\)m cell strainer (Fisherbrand). Beads were isolated on a magnet and washed with PBS. Recovered beads were counted and percentage calculated (% = amount organ-specific beads \(\times\)100/amount recovered beads).

**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<sup>+</sup> and 30,000 U/mouse IL2 (intraperitoneally). On day 5, mice were sacrificed and spleens analyzed for CFSE<sup>Low</sup> 2C cells. aAPC were generated with SIY<sup>Kb</sup>-Ig, anti-mouse-CD28, and rh-CD47-Ig (aAPCCD47<sup>+</sup>). 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 \(10^5\) 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<sup>+</sup> (i.v.) and 30,000 U/mouse IL2 (intraperitoneally). Tumor growth was monitored every other day using digital caliper. aAPC were generated with SIY<sup>Kb</sup>-Ig, anti-mouse-CD28, and rm-CD47-Ig (aAPCCD47<sup>+</sup>). B6, NOD/SCID, and 2C TCR Rag<sup>+</sup>/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.

**Results**

Generation of aAPC and aAPCCD47<sup>+</sup>

aAPC were generated by immobilization of HLA-A2-Ig and anti-CD28 mAb onto paramagnetic microbeads (Fig. 1A, top), and aAPCCD47<sup>+</sup> received an additional third signal,

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**Figure 1.** Schematic and characterization of an “eat me” and a “don’t eat me” aAPC. A, schematic of a 2-signal aAPC (top; aAPC) coated with HLA-A2-Ig and anti-CD28 mAb and a 3-signal aAPC (bottom; aAPC<sup>CD47+</sup>) coated with an additional rh-CD47-Ig. B, phenotypical characterization of aAPC (blue line) and aAPC<sup>CD47+</sup> (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 top left corner of each histogram represent MFI.
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 nonphagocytosed and unstained phagocytosed 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 phagocytosed aAPC, was seen up to 1 hour, and nearly 100% of all aAPC were phagocytosed after 3 hours. Therefore, we considered 3 hours as an optimal time to investigate the effect of CD47 on aAPC inhibit phagocytosis by human type 2 macrophages. A, characterization of human type 2 macrophages (M2) by flow cytometry (top) and by ELISA (bottom). 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 hours). Data depicted represent 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 cocultures without water lysis stain positive (supernatant; nongated bead population), whereas phagocytosed beads do not stain positive for anti-mouse-Alexa647 (cell lysate). In the latter sample, nonphagocytosed beads were separated from cells before water lysis and subsequent staining of phagocytosed beads. C, time course of aAPC phagocytosis by M2. aAPC and M2 were cocultured for the indicated amount of time at a 1:1 ratio. Samples were analyzed for phagocytosis as depicted in B. A sample of aAPC without M2 served as control (rightmost bar). Data represent mean (±SD) of 3 independent experiments. D, dose-dependent inhibition of aAPC phagocytosis by CD47-Ig on aAPC. aAPC^{CD47+} generated with different amounts of CD47-Ig (indicated at x-axis, per 10^8 beads) were cocultured with M2 for 3 hours at a 1:1 ratio. aAPC with 0 ng CD47-Ig served as positive control. Samples were analyzed for phagocytosis as depicted in B. Data represent mean (±SD) of 4 independent experiments. *, statistical significance P < 0.05 (Mann-Whitney test).
CD47 on aAPC phagocytosis. aAPC generated with different amounts of CD47 ranging from 0.16 to 1600 ng/10^6 aAPC, corresponding to a hypothetical 20 to 200,000 CD47 molecules/aAPC (Fig. 2D), demonstrated a dose-dependent aAPC<sub>CD47+</sub> phagocytosis inhibition of about 90%. Results were statistically significant starting from 1.6 ng/10^6 aAPC (P < 0.05; Mann–Whitney test). Analysis of aAPC<sub>CD47+</sub> revealed comparable amounts of HLA-A2-lg 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 (Supplementary Fig. S1). On the basis of these findings, we conducted all the subsequent experiments using aAPC<sub>CD47+</sub> coated with 160 ng CD47 to ensure maximal inhibition of phagocytosis and comparable aAPC functionality.

**aAPC and aAPC<sub>CD47+</sub> 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-lg). To test whether aAPC and aAPC<sub>CD47+</sub> 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<sub>CD47+</sub> stained positive for HLA-A2-lg and anti-CD28 (Fig. 3B). These results correlated with the actual amount of phagocytosed aAPC and aAPC<sub>CD47+</sub> as shown in Fig. 2D (0 and 160 ng rh-CD47-lg, respectively). Control stains of aAPC and aAPC<sub>CD47+</sub> not cocultured with macrophages and either lysed (lysis control) or untreated (control) did not show any significant changes of HLA-A2-lg and anti-CD28 protein levels. Furthermore, mean fluorescence intensity (MFI) for HLA-A2-lg and anti-CD28 of all positively stained aAPC and aAPC<sub>CD47+</sub> were at same levels when compared with controls (Fig. 3C). Thus, these data demonstrate that internal cleavage processes clear ingested aAPC and aAPC<sub>CD47+</sub> whereas nonphagocytosed aAPC and aAPC<sub>CD47+</sub> remain unfettered in cocultures with macrophages and potentially execute their primary function.

**Comparable in vitro function of aAPC and aAPC<sub>CD47+</sub>**

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<sub>CD47+</sub> with that of aAPC. To this end, both aAPC<sub>CD47+</sub> and aAPC were loaded with either low-affinity modified MART-1 peptide, derived from a melanocyte self-antigen, or high-affinity viral FluM1 peptide derived from influenza matrix protein 1. Loaded aAPC<sub>CD47+</sub> and aAPC were used to stimulate and expand antigen-specific T cells from HLA-A<sup>02:01</sup> healthy donors (Supplementary Fig. S2B). Both antigen specificity and expansion of T cells stimulated with aAPC and aAPC<sub>CD47+</sub> showed no statistically significant differences during 3 weeks of T-cell culture (Fig. 4A and B). Furthermore, aAPC- and aAPC<sub>CD47+</sub>-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
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-1\(\beta\), IL2, TNF, and IFN\(\gamma\) (26). All possible combinations of different effector functions indicated comparable activation marker expression by those T cells (Fig. 5C). Thus,
"Don't Eat Me" aAPC for Enhanced In Vivo Function

Figure 6. aAPCCD47+ demonstrate enhanced in vivo functions over classical 2-signal aAPC. A, cell generation analysis of CFSE-labeled adoptively transferred 2C cells from mice treated with aAPC or aAPCCD47+. 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 aAPCCD47+ on days 1, 2, and 3. CFSE analysis was performed on day 4 by flow cytometry. B, biodistribution of aAPC and aAPCCD47+ in NOD/SCID mice. C, B16-SIY tumor-bearing B6 mice were adoptively transferred with activated 2C cells and treated with either aAPC or aAPCCD47+. Tumor size was measured by caliper on every other day. * (P < 0.05) and ** (P < 0.005) indicate statistical significance (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 pass 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 vivo 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/μm and results in uptake of only 40% to 60% of aAPC^{CD47}. These results are in line with data published by Tsai and colleagues that showed that opsonized particles inhibit phagocytosis with an effective K_i = 20 molecules/μm^2 (6). Furthermore, the theoretical CD47 molecule density on aAPC^{CD47} (160 ng CD47-lg/10^8 beads) was in the same order of magnitude (~350 molecules/μm^2) to reported physiologic conditions on human RBC (~250 molecules/μm^2; ref. 34).

Staining of aAPC and aAPC^{CD47} from macrophage cocultures revealed that all nonphagocytosed 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 digestive 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, 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^{CD47} display a comparable functionality regardless of T-cell receptor (TCR) affinity and function. In addition, 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 and colleagues 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 with 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) the in vivo effects solely dependent on T-cell numbers, (iii) do aAPC^{CD47} change the micro milieu generated by macrophages leading to different T-cell priming in vivo, (iv) can aAPC^{CD47} induce endogenous antitumor responses, and (v) how do aAPC^{CD47} 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^{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.

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, acquired and managed patients, 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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