CD47 Enhances \textit{In Vivo} Functionality of Artificial Antigen-Presenting Cells

Heiko Bruns\textsuperscript{1}, Catherine Bessell\textsuperscript{2}, Juan Carlos Varela\textsuperscript{3}, Carl Haupt\textsuperscript{2}, Jerry Fang\textsuperscript{2}, Shirin Pasemann\textsuperscript{1}, Andreas Mackensen\textsuperscript{1}, Mathias Oelke\textsuperscript{2}, Jonathan P. Schneck\textsuperscript{2}, and Christian Schütz\textsuperscript{2}

Abstract

\textbf{Purpose:} Artificial antigen-presenting cells, aAPC, have successfully been used to stimulate antigen-specific T-cell responses \textit{in vitro} as well as \textit{in vivo}. Although aAPC compare favorably with autologous dendritic cells \textit{in vitro}, their effect \textit{in vivo} might be diminished through rapid clearance by macrophages. Therefore, to prevent uptake and minimize clearance of aAPC by macrophages, thereby increasing \textit{in vivo} functionality, we investigated the efficiency of "don't eat me" three-signal aAPC compared with classical two-signal normal aAPC.

\textbf{Experimental Design:} To generate "don't eat me" aAPC, CD47\textsuperscript{low} or CD47\textsuperscript{hi} aAPC were analyzed in \textit{in vitro} human primary T-cell and macrophage cocultures. \textit{In vivo} efficiency was compared in a NOD/SCID T-cell proliferation and a B16-SIY melanoma model.

\textbf{Results:} This study demonstrates that aAPC\textsuperscript{CD47\textsuperscript{hi}–} in coculture with human macrophages show a CD47 concentration-dependently inhibited phagocytosis, whereas their ability to generate and expand antigen-specific T cells was not affected. Furthermore, aAPC\textsuperscript{CD47\textsuperscript{hi}–}-generated T cells displayed equivalent killing abilities and polyfunctionality when compared with aAPC-generated T cells. In addition, \textit{in vivo} studies demonstrated an enhanced stimulatory capacity and tumor inhibition of aAPC\textsuperscript{CD47\textsuperscript{hi}–} over normal aAPC in conjunction with diverging biodistribution in different organs.

\textbf{Conclusions:} Our data for the first time show that aAPC functionalized with CD47 maintain their stimulatory capacity \textit{in vitro} and demonstrate enhanced \textit{in vivo} efficiency. Thus, these next-generation aAPC\textsuperscript{CD47\textsuperscript{hi}–} have a unique potential to enhance the application of the aAPC technology for future immunotherapy applications.

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{hi} 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 (SIRP\textalpha; ref. 4). Binding to the extracellular region of SIRP\textalpha; 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\textalpha; signaling in murine immunoglobulin-opsonized 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 with nonactivated polymers (7) and coating of nanoparticles with CD47\textsuperscript{–}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 \textit{in vitro} systems (15–17). \textit{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 subsequent lead to tumor reduction in an \textit{in vivo} melanoma tumor model (19).

While these studies prove \textit{in vitro} and \textit{in vivo} functionality of our aAPC, \textit{in vivo} delivery and biodistribution is mainly determined by the size of the aAPC scaffold (10, 12, 20).

\textsuperscript{1}Department of Internal Medicine S–Hematology/Oncology, University of Erlangen, Erlangen, Germany. \textsuperscript{2}Department of Pathology, Johns Hopkins School of Medicine, Baltimore, Maryland. \textsuperscript{3}Division of Hematology, Department of Medicine, Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins Hospital, Baltimore, Maryland.

\textbf{Note:} Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

\textbf{Corresponding Author:} Christian Schütz, Department of Pathology, The Johns Hopkins Institute of Cell Engineering, Johns Hopkins School of Medicine, 733 North Broadway, MRB 632, Baltimore, MD 21205. Phone: 410-614-0642; Fax: 443-287-0993; E-mail: cschuetz4@jhmi.edu or chr.sch@hotmail.de

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T-cell generation. aAPCCD47

Materials and Methods

Micrometer-sized aAPC display limited lymphatic drainage (21) and are cleared and phagocytosed by professional phagocytes such as macrophages and immature DC (22, 23). Therefore, many efforts are made to generate optimal aAPC scaffolds that exhibit minimal systemic clearance and maximal in vivo functionality (11). Hence, we generated CD47-positive aAPC (aAPCCD47+) that exhibit enhanced stimulatory capacity and improved antitumor efficacy. These aAPCCD47+ show great potential to significantly enhance new approaches to active immunotherapy.

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 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-FTTC (Sigma), anti-IgG1-PE (Invitrogen), anti-IgG2a-FITC, and anti-CD47-FTTC (BD). Phycocerythrin (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 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 10^8 epoxy beads (Invitrogen). aAPCCD47+ additionally received 160 ng CD47-Ig/10^8 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.

Succinimidyl ester labeling of aAPC/aAPCCD47+

aAPC/aAPCCD47+ 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-succinimidyl-ester-Alexa-647. After 1 hour, beads were washed 3 times with PBS/0.5% Tween-80 (25).

Phagocytosis of aAPC and aAPCCD47+

Macrophages (2.5 × 10^5) were adhered to 24-well plates and cocultured with aAPC or aAPCCD47+ (2.5 × 10^5) 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 (10^5) were adhered to 8-chamber slides (Nunc) and cocultured with labeled aAPC/aAPCCD47+ (10^5) 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 (10^6) were cultured with aAPC or aAPCCD47+ 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
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).

**S^1Cr release assay**
A total of 2 × 10^7/plate cognate peptide loaded (1 µg/10^6 cells, overnight, 37°C) T2 target cells were pulsed with 200 µCi S^1Cr 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 [(cpm sample – cpm spontaneous release) × 100/(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 biodistribution assay**
NOD.CB17-Prkdc^scid/J (NOD/SCID) mice (male, 8 weeks, from Dr. K. Whartenby) were injected (i.v.) with 3 × 10^7 aAPC or aAPCCD47^+. After 1 hour, animals were sacrificed and organs harvested. Single-cell suspensions were generated using 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 × 100/amount recovered beads).

**In vivo carboxyfluorescein diacetate succinimidyl ester proliferation assay**
On day 0, NOD/SCID mice were injected (i.v.) with 5 × 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 CFSElow 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 × 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\(^{\text{CD47}^+}\) displayed comparable amounts of HLA-A2-Ig and anti-CD28 mAb, whereas staining with anti-CD47 demonstrated that only aAPC\(^{\text{CD47}^+}\) 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-\(\alpha\), 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 nonphagocytoed and unstained phagocytoed aAPC/aAPC\(^{\text{CD47}^+}\) (Fig. 2B). To allow for optimal phagocytosis, we evaluated time-dependent uptake of aAPC by macrophages (Fig. 2C). A gradual increase of phagocytoed aAPC, was seen up to 1 hour, and nearly 100% of all aAPC were phagocytoed 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 whereas in “lysis control” (gray bars), aAPC and aAPCCD47 respectively (black bars; coculture). “Control” (white bars) represents same aAPC and aAPCCD47 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, aAPC positive for both molecules. C, aAPC and aAPCCD47 were statistically signifi
cant starting from 1.6 ng/10^8 aAPC and aAPCCD47 phagocytosis and comparable aAPC functionality.

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 CD47+ coated with 160 ng CD47 to ensure maximal inhibition of phagocytosis and comparable aAPC functionality.

aAPC and aAPCCD47+ 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 aAPCCD47 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 aAPCCD47+ stained positive for HLA-A2-Ig and anti-CD28 (Fig. 3B). These results correlated with the actual amount of phagocytosed aAPC and aAPCCD47+ as shown in Fig. 2D (0 and 160 ng rh-CD47-Ig, respectively). Control stains of aAPC and aAPCCD47+ 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 aAPCCD47+ were at same levels when compared with controls (Fig. 3C). Thus, these data demonstrate that internal cleavage processes clear ingested aAPC and aAPCCD47+, whereas nonphagocytosed aAPC and aAPCCD47+ remain unaltered in cocultures with macrophages and potentially execute their primary function.

**Comparative in vitro function of aAPC and aAPCCD47**

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 aAPCCD47+ with that of aAPC. To this end, both aAPCCD47+ 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 aAPCCD47- showed no statistically significant differences during 3 weeks of T-cell culture (Fig. 4A and B). Furthermore, aAPC- and aAPCCD47+-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<sup>CD47</sup>⁺-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,
aAPC and aAPCCD47 demonstrated equal in vitro induction, expansion, and functionality of antigen-specific T cells over a course of 3-week stimulation.

**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 adaptively 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 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.
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 about 3.5 CD47-molecules/μm² 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/μm² (6). Furthermore, the theoretical CD47 molecule density on aAPC^CD47+ (160 ng CD47-Ig/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^CD47+ from macrophage cocultures revealed that 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 dependent on CD47 expression and effector function. In vivo with human CD47 which renders these animals a perfect tool function (9, 36). We demonstrated that aAPC^CD47+ molecules/dependent inhibition of ingestion starting at 1.6 ng CD47-Ig/10^8 experiments with macrophages demonstrated a concentration-dependent killing efficiency of aAPC and 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) are 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. Macksen is a consultant/advisory board member for NexeImmune, Inc. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**
Conception and design: H. Bruns, A. Macksen, 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. Schnick, C. Schütz
Writing, review, and/or revision of the manuscript: H. Bruns, C. Bessell, J.C. Varela, A. Macksen, M. Oelke, J.P. Schnick, 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. Schnick, C. Schütz

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