Expert Opinion

Antigen presentation on artificial acellular substrates: modular systems for flexible, adaptable immunotherapy

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Background: Recent findings on T cells and dendritic cells have elucidated principles that can be used for a bottom-up approach to engineering artificial antigen presentation on synthetic substrates. *Objective/methods*: To compare the latest artificial antigen-presenting cell (aAPC) technology, focussing on acellular systems because they offer advantages such as easy tunability and rapid point-of-care application compared with cellular systems. We review acellular aAPC performance and discuss their promise for clinical applications. *Results/conclusion*: Acellular aAPCs are a powerful alternative to natural-cell-based therapies, offering flexibility and modularity for incorporation oSf a variety of stimuli, hence increasing precision. Current technologies should adapt physiologically important signals within safe materials to more closely approximate their cellular counterparts. These constructs could be administered parenterally as APC replacements for active vaccines or used *ex vivo* for adoptive immunotherapy.

Keywords: adoptive cell transfer, artificial antigen-presenting cell, cancer, immunotherapy, vaccines

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1. Introduction

In contrast to conventional therapies such as radiation and chemotherapy, immunotherapy is highly specific on a cellular level and may achieve high potency while avoiding deleterious side effects. While passive immunotherapy with monoclonal antibodies has led to effective anti-tumor responses in a number of clinical settings [1,2], active immunotherapy, which aims to prime *in vivo* cellular responses against infectious disease or cancer antigens, has proven more challenging. Efficient stimulation of effector immune cells such as T cells is an important determinant of the success of active immunization protocols, and T cells depend on interactions with antigen-presenting cells (APCs) displaying those appropriate antigens. Immunotherapeutic responses can be elicited *in vivo* by active vaccination with antigens or by administration of antigen-specific T cells expanded *ex vivo* (adoptive immunotherapy). In both forms of therapy a desirable outcome is to induce potent cellular and humoral immune responses specific to the antigens of interest. Consistent and objective clinical response rates have yet to be universally achieved with cellular-based immunotherapies against general antigens. However, a number of clinical studies have demonstrated that this approach can lead to anti-tumor responses with meaningful clinical benefits [3-5].

1.1 Natural APCs for immunotherapy: challenges and limitations

Some of the most encouraging clinical data regarding active immunotherapy against cancer comes from studies employing adoptive transfer of tumor-reactive T cells [6,7]. This process depends on efficient antigen presentation for proper

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activation, expansion and differentiation of tumor-avid, antigen-specific T cells *ex vivo* followed by re-infusion into the host [8]. Natural antigen-presenting cells such as dendritic cells (DC) initiate the most potent immune responses, and in the last decade preventative and therapeutic applications have established proof of principle for their clinical use [9]. The use of DC for *ex vivo* or *in vivo* stimulation of an antigen-specific immune response in clinical applications is, however, still hindered by issues regarding fundamental understanding of the biology and roles of different DC subsets in immunotherapy, optimal approaches to generating the most effective DC subsets eliciting anti-tumor responses, and how the tumor microenvironment regulates DC-lymphocyte interactions [10-12]. Further research will undoubtedly establish firm principles for optimizing DC generation for cancer vaccines. However, there are also logistical issues involving the quality and quantity of isolated cells and the labor, time and cost associated with their isolation [13]. Furthermore, it is often difficult to obtain functionally fit DCs in large numbers from patients with advanced disease, and when DCs are isolated in sufficient number, their *ex vivo* culture is time consuming and expensive, requiring specialized equipment and techniques and often producing variability in quality of the resulting cells. Autologous DC application in clinical settings becomes almost personalized medicine. Custom isolation must be performed for individual patients because T cell restriction demands the use of autologous DCs (DCs isolated from the same patient) to prevent rejection of the cell product, and this limits the generalization of adoptive T cell therapy. Numerous reports have used autologous DCs or engineered cellular APCs for T cell stimulation, and these studies have been reviewed elsewhere [9-12,14-16].

1.2 Cellular versus acellular presentation

Artificial APCs were invented to overcome some of the aforementioned challenges involving the use of autologous DCs. Cell-based APC systems that use fibroblasts or insect cells transfected with ligands offer a physiological interface and antigen processing capabilities but carry the risks of infection and tumorigenicity [14,16]. The labor associated with construction and culture of different cell-based APC systems and issues related to large-scale manufacturing, transport and storage have detracted from their wide-scale use. Furthermore, it is desirable for APCs to be easily adapted to different antigens and to be able to modulate the density of antigen presentation at point-of-care settings for optimal use and ease of standardization.

For these reasons APCs based on acellular systems (aAPCs) have been proposed and tested in the expansion of T cells for the treatment of a variety of disease states [14,16]. Acellular aAPCs overcome the limitations of cell-based systems by presenting T cell stimuli on inert, cell-compatible, biocompatible platforms. This represents an off-the-shelf technology that can be easily standardized, potentially sterilized, is stable, and is ready for addition to T cell cultures. The attractiveness of this approach is its flexibility and modularity. Thus, different ligands can be added to the surface of the substrates and their densities on the surface can be tuned, facilitating consistent, reproducible results and potentially eliminating the variability observed with cell-based systems. Here we review the latest substrates used for aAPC production as well as their applications in laboratory and clinical settings.

2. Which signals are important for presentation on an artificial substrate?

Because the T cell response depends on the signals it receives from the antigen-presenting cell, control over antigen presentation translates into control over therapeutic outcomes involving T cell stimulation [16]. Efficient stimulation of antigen-specific T cells is mediated by several signals (**Figure 1**). Recognition occurs via the interaction of the T cell antigen receptor (TCR) with specific antigen in the form of a peptide/MHC complex (peptide-MHC) on the APC. In addition to this recognition signal, co-stimulation through the B7 and TNF families of receptors on the APC, which engage ligands such as CD28 on T cells, are known to amplify the antigen-specific T cell response [17]. Thus, current approaches for engineering aAPCs exploit this co-stimulatory signal as well as adhesion signals such as intercellular adhesion molecule 1 (ICAM-1) to enhance their stimulatory capability [14]. Finally, cytokines, the largest class of immunoregulatory molecules, are secreted by activated APCs and other immune cells and thus have been used as systemic agents for *in vivo* administration, supplements for stimulation in T cell cultures, and components of genetically modified cell-based immunotherapies [16].

Clearly, an ideal aAPC should include these important physiological signals but also be amenable to adjustment of those signals to effect optimal outcomes in culture. In addition, if such systems can be rendered physiologically compatible, then application can be extended *in vivo* for active immunization. Here we compare and contrast, in the order in which they were invented, present acellular aAPC platforms, assess their capacity to incorporate the signals needed for T cell stimulation and review the results that have been achieved to date on the bench and in the clinic.

3. Particulate-based substrates and T cell signaling

While any surface presenting protein ligands for T cell stimulation can be classified as an artificial antigen-presenting substrate, we restrict this review to acellular substrates that are particulate in nature and present ligands or mediators intended to stimulate T lymphocytes. Thus, for the purpose of this review, we classify acellular aAPCs as those constructs derived from natural macromolecules such as lipids, synthetic products such as polymers (biodegradable and non-biodegradable) and magnetic materials. These constructs can perhaps be

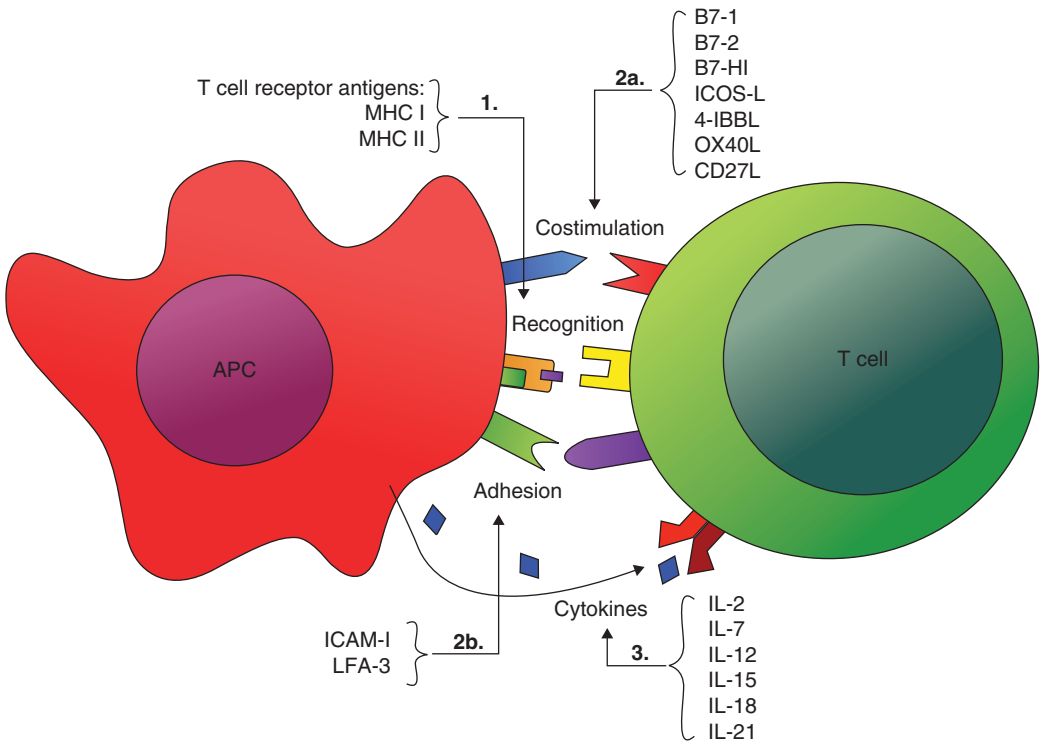

Figure 1. Schematic of signal classes presented by an antigen–presenting cell (APC). Three signals are essential for optimal T cell stimulation: **1.** Recognition signals that ligate the T cell antigen receptor, peptide/MHC complexes or antibodies cross-linking the T cell receptor (TCR), **2.** Costimulatory molecules of the B7 or TNF family and adhesive molecules that strengthen interactions between cells, 3. Cytokines secreted by APC or other immune cells that bind to receptors on the T cell surface.
ICAM-1: Intercellular adhesion molecule 1; ICOS-L: Inducible T-cell co-stimulator ligand; LFA-3: Lymphocyte function-associated antigen 3.

injected into the body for parenteral administration given an understanding of their biodistribution, pharmacokinetics and *in vivo* toxicology. Four types of materials have been used to construct the majority of particulate aAPC systems that have been evaluated thus far *in vitro* and *in vivo* (**Table 1**). These include liposomal materials, latex beads, magnetic beads, and biodegradable polymers. A schematic depicting the different configurations of these platforms is shown in **Figure 2**.

3.1 Liposomes as aAPCs

Liposomes are primarily amphiphilic phospholipids and cholesterol self-assembled into spherical vesicles with an aqueous interior (**Figure 2**). The popularity of these systems is in part due to the facile nature of their construction. They easily self-assemble in aqueous environments due to hydrophobic–hydrophilic interactions; furthermore, their sizes can be tuned from diameters ranging between 60 nm and 1 µm for unilamelar vesicles by mechanical methods. Liposomes were originally used as drug delivery vehicles [18,19]. The amenability to chemical functionalization of the phospholipid head of liposomes and the attachment of proteins using standard chemical methods allowed for tethering of T cell antigens and hence their use as artificial antigen-presenting vesicles.

Liposome-based antigen presentation was first reported in 1978 as an artificial system for stimulation of polyclonal murine lymphocytes [20]. Other applications that followed used liposomal aAPCs fabricated from different lipid preparations as well as isolated tumor cell or viral membranes [20-23]. Isolated cell membranes as liposomal components provided the advantage that relevant antigens need not be identified.

The use of liposomes as aAPCs highlighted important biophysical concepts for effective T cell stimulation. For example, levels of stimulation were shown to depend on the density of T cell antigens on the liposome surface. In addition, different ratios of recognition to adhesion molecules on the aAPC surface were shown to influence the potency of stimulation [20,22]. Fluidity of the presenting surface also seemed to play an important role. Liposomes presenting class-II-peptide-loaded MHCs upon incubation with $CD4^+$ T cells induced the formation of supramolecular interfacial clusters, the product of a capping effect, rich in the TCR-associated molecules CD3 and MHC on the liposome and that are very similar to activation clusters induced by natural APCs upon interaction with T cells [24]. Indeed, it was shown that pre-clustering of T cell antigens into microdomains on the liposomal aAPC surface further enhanced aAPC function [25,26]. A characteristic feature of those microdomains is enrichment

Table 1. Artificial antigen-presenting cell platforms reported in the literature.

Platform	Diameter	Recognition ligands	Costimulatory ligands	Adhesion ligands	Exogenous cytokines	Cytokines released by aAPC	Ligand attachment	*In vitro* stimulation	*In vivo* use	Method of immunotherapy	Ref.
Liposome	100 nm – 1 μm	HLA or MHC or tumor membrane	Tumor membrane	Tumor membrane	T helper factor	None	Hydrophobic association	Murine splenocytes	None	N/A	[20-23]
Liposome	60 nm – 1 μm	Anti-CD3 or MHC II (preclustered)	Anti-CD28 (preclustered)	Anti LFA-1 (preclustered)	IL-2 & IL-15	None	Hydrophobic (neutravidin & CTB for clustering)	Human and murine $CD4^+$, Human $CD3^+$ & LN	None	N/A	[24-26]
Magnetic bead	4.5 μm	Anti-CD3 or MHC tetramer	Anti-CD28	None	IL-2	None	Adsorption	Human PBMC, Human $CD4^+$	Human trials	Adoptive	[41-44,69-73]
Magnetic bead	4.5 μm	HLA-Ig	Anti-CD28	None	TCGF (in vitro)/ IL-2 (in vivo)	None	Adsorption	Total or Antigen-specific human $CD8^+$	SCID mouse with human melanoma	Adoptive	[13,45,68]
PLGA	8 μm	Anti-CD3 or MHC-peptide	Anti-CD28	None	None	IL-2	Avidin–Biotin	Murine splenocytes	B16 murine melanoma	Active	[54]
PGA	7 μm	Anti-CD3	Anti-CD28	None	None	None	Adsorption	$CD4^+$ murine T cells	MethA murine tumor	Active	[52]
Latex bead	5 μm	MHC, tumor membranes, anti TCR	B7, tumor membrane	ICAM, tumor membrane	IL-2, IL-1, IL-12, IFN-α/β	None	Adsorption, avidin–biotin (MHC)	Murine splenocytes, $CD4^+$ & $CD8^+$, $CD44^{high}$ or^{low}	EG.7, P815, MCA-203, EL4 and RMA murine tumors, human trials	Active, Adoptive	[28-37,55-57, 66]
Latex bead	5 μm	MHC tetramer, MHC-Ig	Anti-CD28, CD83, 4-1BBL	None	IL-2 and IL-7	None	Adsorption	Murine splenocytes, Human PBCM	B16 murine melanoma	Adoptive	[39,40]

CTB: Cholera toxin B; HLA: Human leukocyte antigen; LFA: Lymphocyte function-associated antigen; LN: Lymph node; PBMC: Peripheral blood mononuclear cells; SCID: Severe combined immunodeficiency; TCGF: T cell growth factor; TCR: T cell receptor.

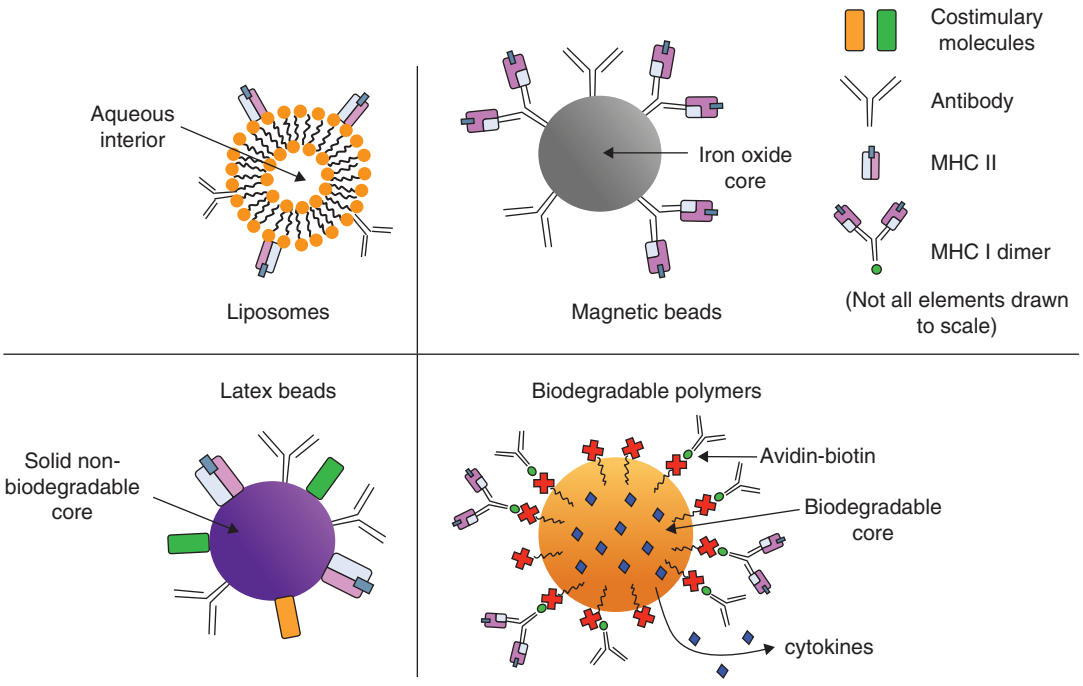

Figure 2. Schematic representations of four types of acellular aAPC. Particles can be coupled with recognition, costimulatory or adhesion molecules by different binding schemes. Examples of configurations are shown here.

of cholesterol, a major component of lipid rafts. Cholestrol in rafts interacts avidly with the beta subunit of cholera toxin which, when bioinylated, can provide an adaptor for multiple proteins through the use of neutravidin. Biotinylated MHC and anti-CD28 or biotin-anti-CD3, anti-CD28, and anti- lymphocyte function-associated antigen 1 (LFA-1) were attached to these microdomains via cholera toxin–avidin linkage, ensuring not only defined stoichiometry, but also proper orientation. The important biophysical finding here is that distribution of T cell antigens, co-stimulatory and adhesion molecules on the APC surface significantly affected the magnitude and direction of lymphocyte stimulation [25]. When used for polyclonal stimulation of human T cells, liposomal aAPCs with segregated antigen-clustered microdomains preferentially expanded $CD8^+$ cells, but maintained $CD4^+$ cells at a low level and avoided terminal differentiation of the cells *in vitro* [26]. It was also observed that a 150-fold expansion of melanoma antigen recognized by T cells (MART-1)-specific cells was possible after 2 weeks of culture with exogenous addition of IL-2 and IL-15 [26].

Instability of liposomes in culture and during storage, however, has detracted from their widespread use as APC surrogates [27]. Thus other non-degradable aAPCs such as those fabricated from polymeric substrates have been investigated.

3.2 Latex beads as aAPCs

Unlike the fluid membranes and aqueous interiors of liposomes, latex polystyrene beads are solid spheres that can be coated by nonspecific interactions with different ligands to function as aAPCs. Despite the non-fluidic interface and their non-physiological nature, latex beads have proven invaluable in revealing aspects of aAPC–T cell signaling important for constructing optimal aAPCs [28-37]. These constructs were first used to demonstrate the importance of aAPC size for T cell stimulation. Latex microspheres coated with MHC molecules or tumor cell membranes activated splenocytes depending on their size, with 4 – 5 µm diameter aAPCs producing the strongest responses [38]. It was proposed that a large, continuous area of contact between a T cell and an aAPC was necessary because a greater number of smaller beads (1 – 3 µm diameter) could not achieve responses equivalent to their larger counterparts [38]. Additionally, the presence of co-stimulatory (B7.1) and adhesion ligands (ICAM-1) on the aAPC surface was shown to enhance T cell stimulation [35]. Importantly, it was the higher density of costimulatory ligands per bead, and not a greater number of beads presenting at lower densities that was crucial for greater responses [35]. Activated T cells acquired effector function after 3 days of stimulation but ceased proliferation at this time unless exogenous cytokines such as IL-2 were added [36], highlighting the necessity of cytokine delivery for long-term stimulation of lymphocytes in culture.

The simplicity of preparation of aAPC with latex beads facilitated other interesting investigations examining effects of specific signals on the phenotype, function, and numbers of T cells produced. These included: the difference in response between naive and memory cells to aAPCs presenting MHC and anti-CD28 [30], the importance of different

inflammatory and cytokine signals for the activation of $CD4^+$ and $CD8^+$ T cells [28,31-34], and characterization of non-responsive states resulting from delivery of specific signals [32,34,36]. Recent work with latex beads also showed that stimulation of naive antigen-specific murine $CD8^+$ T cells by aAPCs bearing MHC class I and B7.1 in the presence of IL-2 and IL-21 created a unique effector phenotype [28]. The resulting cells display activation markers such as CD44 and cytolytic activity, but are unable to secreteIFN-γ [28].

From the standpoint of clinically relevant CD8-restricted targets, recent studies used the melanoma antigen tryosinase-related protein 2 (TRP2) and a glioma epitope (interleukin–13 receptor α2) with latex bead-based aAPCs. PBMC from healthy donors were cultured with aAPCs presenting human MHC and the costimulatory molecules anti-CD28 and CD83 in the presence of IL-2 and IL-7 [39]. The number of glioma antigen-specific cells increased 50 – 60-fold over 4 weeks, comprising 14.7% of the total population [39]. Subsequent studies by the same group demonstrated an increase in the frequency of melanoma-specific T cells from 0.13% to 23.5% after 4 weeks of stimulation by latex aAPCs displaying TRP2 peptide, anti-CD28, CD83, and the additional costimulatory ligand 4-1BBL again in the presence of IL-2 and IL-7 [40]. The clinical relevance of this work is the important validation that significant populations of antigen-specific cells can be expanded from unsorted populations with negligible antigen specificity.

3.3 Magnetic beads as aAPCs

Separation of large diameter (5 µm or larger) artificial non-biodegradable constructs from cultured T cells is clearly important to avoid *in vivo* complications such as embolism. Magnetic bead aAPCs are thus attractive for this particular reason. In addition to easy coupling of T cell ligands via established chemistries, these substrates enable an efficient method of bead removal from cell suspensions by application of simple magnets.

Magnetic beads as artificial substrates for ligand immobilization have been proposed and utilized primarily due to their ease of separation from expanded cells and because of their commercial availability. Proteins can be coupled to the surface of these beads noncovalently by simple non-specific adsorption or through covalent coupling chemistries such as tosyl or tresyl activation, which introduce sulfonate groups on the bead for coupling to free amines on proteins. Magnetic beads coated with anti-CD3 and anti-CD28 have been used in this manner to stimulate both $CD4^+$ and $CD8^+$ cells [41,42]. This effect was not observed when non-immobilized anti-CD28 was used [43].

Subsequent studies used magnetic beads in an antigen-specific format by coupling MHC tetramers loaded with influenza peptide through MHC antibodies that were first immobilized to the surface [44]. Another configuration used MHC dimers directly coupled to the beads [13,45]. Tetramers coupled to beads were able to maintain the antigen specificity of sorted influenza-specific $CD4^+$ T cells over 9 weeks of *in vitro* culture [44]. MHC dimers were also used to confer antigen-specificity to magnetic beads [13,45]. MHC dimers produced by genetically fusing MHC molecules to an immunoglobulin constant region confer flexibility to the construct, which increases the T cell receptor binding affinity [13,45]. This approach in combination with T cell growth factors was used to generate up to 10^9 MART1-specific cells in less than 2 months [13]. We reiterate that a particular advantage of acellular systems is their modularity, allowing for easy and flexible addition or subtraction of signals that affect T cell function. This modularity needs to incorporate cytokine delivery. Cytokines or T cell growth factors are critically needed during culture of T cells for robust stimulation and are often injected *in vivo* at high doses (up to 720,000 U/kg IL-2 intravenously every 8 h) for adoptive cell transfer therapy [46]. Although high dose IL-2 therapy can effectively lead to anti-tumor responses in patients with metastatic melanoma and renal cell cancer [47,48], patients receiving this therapy can undergo significant toxicities including hypotension requiring vasopressor support. Clearly, a means to provide all signals (including cytokine delivery) in a format that is biodegradable, physiologically compatible and localized to target T cells would be an attractive alternative.

3.4 Biodegradable particles as aAPCs

Biodegradable polymers are well-suited to fabrication of vehicles because they can be made as particulate solid-phase supports in a variety of sizes and are capable of encapsulating cytokines that can be released at predictable rates using the appropriate polymers. This platform, however, presents a unique challenge to ligand presentation. While the biodegradable nature of these platforms is advantageous for safe *in vivo* use and controlled release of soluble mediators, bulk and surface erosion of the particles compromises long-term ligand presentation. Thus, ligands that are attached covalently or are nonspecifically adsorbed to the surface are too short-lived for sustained interactions with cells, which need to last from days to weeks.

There have been a variety of materials used to engineer solid biodegradable particles with and without surface functionality [49-51]. Perhaps the most widely used are the aliphatic polyesters, specifically the hydrophobic poly(lactic acid) (PLA), the more hydrophilic poly(glycolic acid) (PGA) and their copolymers, poly(lactide-co-glycolide) (PLGA). The degradation rate of these polymers, and often the corresponding release rate of encapsulated product, can vary from days (PGA) to months (PLA) and is easily manipulated by varying the ratio of PLA to PGA. In 2004 Shalaby, et al. described the use of 7 µm jet-milled aAPCs fabricated from PGA and coated with murine or human anti-CD3 and anti-CD28 through irreversible adsorption. Stimulation with these aAPCs resulted in significant proliferation by day 3 of both mouse and human cells [52]. Levels of proliferation exceeded those observed for plate-bound antibodies, but fell

short of the level obtained by antibody-coated magnetic beads [52]. IFN-γ secretion could be detected in 58% of cells stimulated with antibody-coated PGA particles, while no cytokine secretion or proliferation occurred in response to uncoated particles [52].

In a second aAPC system, PLGA particles 6 – 10 μm in diameter were fabricated using single and double emulsion techniques. Ligand presentation in this system was achieved via a unique method that incorporated an avidin–palmatic acid conjugate into the surface during particle fabrication. Palmatic acid preferentially interacts with the PLGA core while avidin partitions to the interface resulting in sustained and high density presentation of the avidin [53]. This technology achieved durable coating of particles with biotinylated ligands as the avidin–fatty acid conjugate is maintained on the particle surface over time because of preferential non-covalent interaction with the polymer core [53]. Stable presentation of biotinylated ligands was observed for more than 20 days in solution [53]. To construct aAPCs from avidin-coated particles, biotinylated ligands were stably bound to the avidin-coated surface for presentation to T cells [54]. Because the procedure is a one-pot process that mixes fatty acid-avidin, which partitions to the surface, with cytokines that become entrapped in the PLGA matrix, the fabrication procedure yields particles that present ligands and encapsulating cytokines that release over a period of weeks while maintaining antigen presentation [54].

Unsorted murine splenocytes, upon stimulation with PLGA particles (10 μg aAPCs per 10^5 cells) presenting anti-CD3 and anti-CD28 and releasing IL-2, showed a pronounced skewing by day 4 to nearly 100% $CD8^+$ T cells [54]. This population expanded 45-fold over one week and expressed levels of the IL-2 receptor alpha subunit, CD25 approximately twice as high as other methods [54]. These effects were not observed in the absence of encapsulated IL-2. Compared with soluble antibodies or magnetic beads in cultures supplemented with exogenous IL-2, this system stimulated cells significantly better even in the absence of encapsulated cytokine [54]. In the presence of encapsulated IL-2, paracrine delivery compared with exogenous addition increased the magnitude of expansion and skewed expansion to the CD8 phenotype at significantly lower doses of the cytokine.

4. Therapeutic and clinical use of aAPCs

Few studies have used acellular APC platforms *in vivo* as APC substitutes. Here we describe the findings from these studies regarding the efficacy of acellular platforms in induction of *in vivo* therapeutic immune responses. Interestingly, most *in vivo* studies have involved latex, magnetic, and biodegradable polymer aAPCs, while very little has been accomplished with liposomal vehicles for *in vivo* antigen presentation.

4.1 *In vivo* application of acellular APCs for active immunotherapy

Stimulation of primary T cell responses by *in vivo* injection of an acellular aAPCs was conducted as early as 1992 [55]. In this work 5 μm diameter silica microspheres bearing MHC class I moleculesisolated from tumor cells or purified tumor cell membranes were injected into mice [55]. Murine tumor cell lines included P815, a mastocytoma, EL4, a thymoma, and 2 lymphomas, RDM4 and BCLI [55]. These aAPC systems, termed large multivalent immunogen (LMI), decreased tumor growth in the mouse models [55]. Seven- to twelve-day-established tumors, however, were not reduced by tumor membrane-coated LMI alone but were successfully treated with LMI in combination with cyclophosphamide, an established alkylating chemotherapeutic drug [56].

This early work with LMI for active immunotherapy in mice prompted human Phase I trials in patients with advanced melanoma. Fifteen patients with stage IV melanoma received intradermal and subcutaneous injections, over a 3-month period, of LMI aAPCs coated with melanoma cell lysates [57]. The researchers monitored patients using two readouts: delayed-type hypersensitivity (DTH), which indicates the formation of T cell memory, and lytic activity of PBMC, indicating the formation of antigen-specific T cells against the M-1 melanoma cell line [57]. Although DTH responses were not observed in any of the participants, an anecdotal increase in the frequency of PBMCs capable of mediating specific lysis of tumor cells *in vitro* was observed (but not statistically significant) [57]. As recommended by these studies, future trials should use LMI in conjunction with adjuvant or chemotherapy to achieve outcomes similar to those observed in animal studies.

Other *in vivo* active immunotherapy studies used PGA particle-based aAPCs displaying anti-CD3 and anti-CD28 or PGA particles releasing surface-adsorbed GM-CSF in mouse models of methA fibrosarcoma. These were co-injected with tumor cells or injected directly into established tumors [52]. GM-CSF particles prevented tumor growths in 75% of the mice, while particles with anti-CD3 and anti-CD28 prevented growth in 68%; the combination treatment involving both particle types prevented growth in 100% of mice [52]. Combination treatment also resulted in regression of tumor mass in a treatment study. Here 57% of animals showed regression of established 2 – 4 mm diameter tumors [52].

Our group has investigated the efficacy of aAPC treatment with particles releasing IL-2 in an established murine melanoma model (**Figure 3**). Animals were engrafted with B16 tumors expressing luciferase, enabling bioluminescent imaging to ensure uniformity of tumor size prior to treatment (**Figure 3C**). Mice received intratumoral injections of aAPCs consisting of PLGA particles encapsulating IL-2 and displaying anti-CD3 and anti-CD28 on their surface. Compared with particles without cytokine, paracrine delivery of IL-2 from aAPCs significantly delayed tumor growth kinetics with a single injection at day 10 after tumor implantation (**Figure 3D**). Current studies are focusing on boosting this effect with multiple administrations

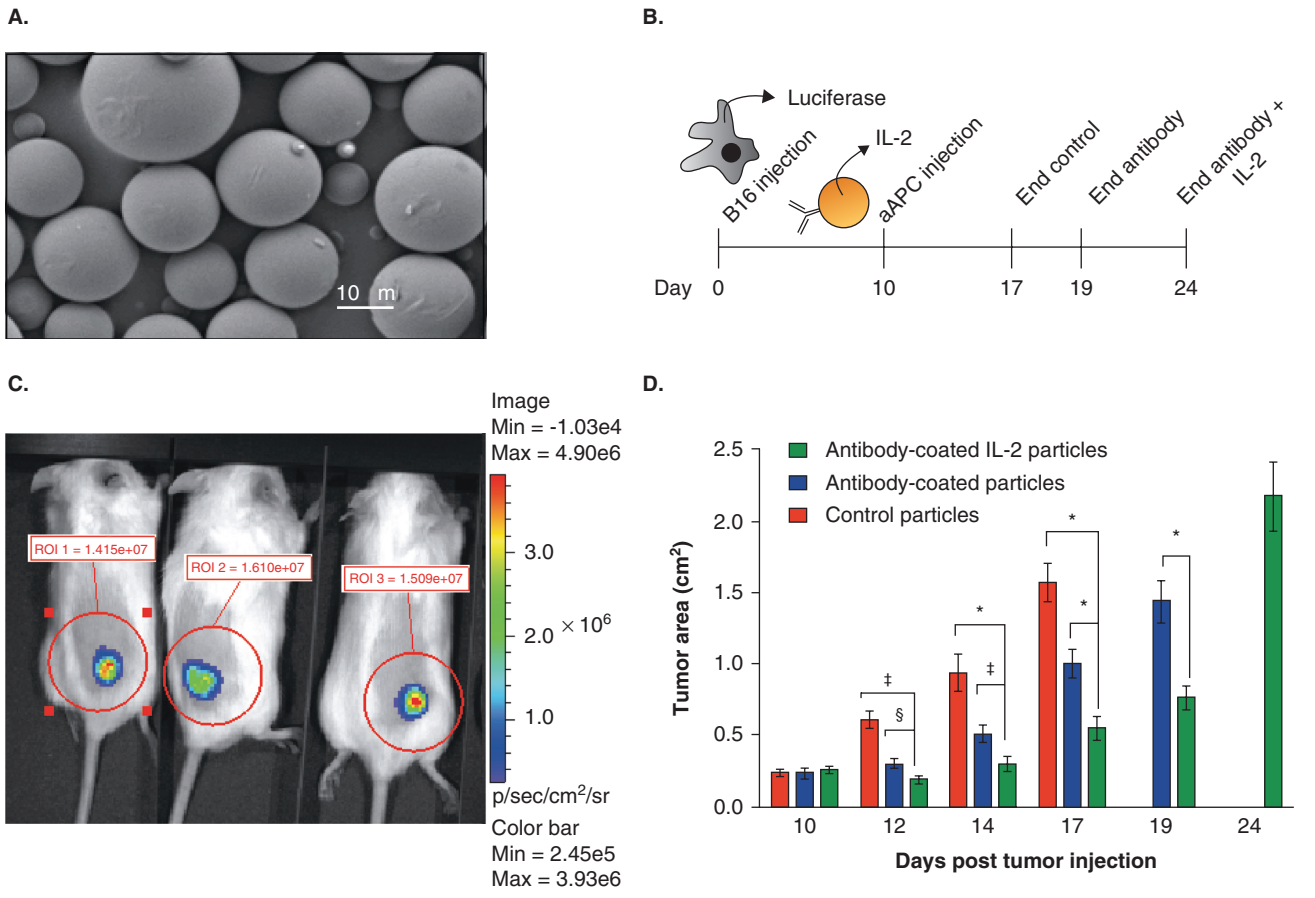

Figure 3. Delayed B16 tumor kinetics in animals intratumorally injected with biodegradable particles encapsulating IL-2. **A.** poly(lactide-co-glycolide) (PLGA) particles surface modified with anti-CD3 and anti-CD28 and encapsulating IL-2 (described previously in [54]). **B.** Experimental timeline. Mice received injections of 1×10^5 B16-luciferase cells (Caliper Life Sciences, Hopkinton, MA) subcutaneously on day 0, treated with PLGA microparticles on day 10, and were killed when tumors reached 2 cm^2. **C.** Day 10 tumors imaged using Xenogen IVIS-200 (Caliper Life Sciences) following injection of D-Luciferin. Bioluminescence from the tumors (flux) expressed as photons/second/cm^2/steridian (photons/sec/cm^2/ster). ROI: region of interest, quantified using Living Image Software (Xenogen, Alameda, CA) and analyzed using Igor Pro Image Software (Wavemetrics, Portland, OR). **D.** Day 10 tumors were treated with a single intratumoral injection of 2 mg of 8 ± 2 µm PLGA particles. Tumor areas were calculated by taking the product of the cross perpendicular diameters which were obtained using tumor calipers. Control particles did not display antibodies or release cytokine. n = 3 (*, ‡, § indicate a 2-way ANOVA with p values of < 0.001, < 0.01, < 0.05, respectively). Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

of aAPCs and on elucidating the mechanism of this enhanced delay as it may be tied to local delivery of IL-2 affecting $CD8^+$ T cell responses at the tumor site [54].

Active immunotherapy studies involving acellular platforms thus far point to the feasibility and promise of this approach as an APC replacement for *in vivo* induction of therapeutic responses to tumors. A few unanswered questions remain, however, especially given the artificial nature of these platforms. Issues related to the biodistribution of the particles in the tumor site or in the body after parenteral administration need to be addressed adequately as these are foreign objects, and their toxicology is not completely understood. The magnitude and spectrum of immune responses after *in vivo* administration is also not fully characterized, and these will probably be critical factors for more rigorous studies. For these reasons alternative therapeutic modalities, such as adoptive immunotherapy, that focus on the benefits of the aAPC approach for *ex vivo* stimulation and expansion of T cells without necessitating their use *in vivo* have advanced more quickly in clinical trials and these studies have met with great promise [58].

4.2 *Ex vivo* application of acellular APCs for adoptive immunotherapy

This therapeutic method involves the *ex vivo* expansion of T cells, which are infused back into patients to bolster the natural immune response. Such an approach was first studied

in humans using cytomegalovirus (CMV)-specific T cells that were cloned *in vitro* and administered to immunodeficient bone marrow recipients [59,60]. Although susceptible to infection, the patients did not develop CMV disease or viremia after adoptive T cell transfer, suggesting that their immunity against CMV had been recovered. Following this success, researchers demonstrated that the adoptive transfer of autologous antigen-specific T cells was a viable therapeutic approach to the Epstein–Barr virus (EBV) [61] as well as HIV-related infection [62,63]. Moreover, expanded tumor-specific T cells have been shown to strengthen patients' immune responses to melanoma by infiltrating the tumor site and inducing tumor regression in approximately 50% of patients [58,64,65]. Thus, adoptive T cell transfer has potential applications in the treatment of both infectious diseases and cancer.

In the studies noted above, cells were expanded *ex vivo* using various combinations of irradiated feeder cells, the human anti-CD3 antibody OKT3, and high concentrations of IL-2 (6000 IU/ml) [58,64,65]. The use of acellular formats such as latex- and magnetic-bead-based aAPCs began with studies conducted in a variety of murine models. The first of such studies showed altered trafficking of adoptively transfered T cells in the presence of LMI. In this work, Goldeberg, et al. used 5 µm latex beads coated with avidin to bind MHC molecules loaded with a peptide derived from ovalbumin, a model antigen. These were capable of stimulating $CD8^+$ ova-specific OT-I T cells. OT-I cells were isolated from lymph nodes, adherence depleted, and intravenously injected directly into B6 recipients [66]. Two to three days later, mice were challenged by intraperitoneal injection of EG.7 cells, a thymoma tumor that expresses ovalbumin, with or without intraperitoneal or intravenous injection of aAPCs [66]. Adoptively transfered cells migrated to the spleen and lymph node by day 1 and were found in the peritoneal cavity on day 4 only in mice that received aAPCs [66]. Mice receiving aAPCs also showed reduced tumor burdens and harbored significantly higher numbers of OT-I cells [66].

A second adoptive transfer study involving latex-bead-based aAPCs was performed using the B16 murine melanoma model. Pulmonary tumors were treated by intravenous administration on day 3 of 10^7 T cells specific for the TRP2 melanoma antigen [40]. Cells were generated by *in vitro* culture of wild-type splenocytes with aAPCs (10 cells:1 aAPC) presenting TRP2-specific MHC dimer and co-stimulatory molecules and were sorted for antigen specificity prior to injection [40]. Subcutaneous tumors were treated on day 5 in the same manner. Neither case employed exogenous cytokine addition. Mice displayed significantly fewer pulmonary tumors on day 20 when treated with aAPC-expanded cells (compared with transfer of naive $CD8^+$ cells) [40]. In the subcutaneous tumor experiments, mice receiving aAPC-expanded cells experienced 100% survival at day 60 with negligible tumor burden, while mice receiving naive $CD8^+$ cells as a negative control did not survive more than 27 days [40]. Despite the long culture periods required (28 days followed by cell selection for antigen specificity) and the large number of cells required (10^7 cells per mouse), this study demonstrated remarkable efficacy regarding the utility of adoptive cell transfer therapy. We note that in other B16 treatment studies similar efficacies were observed in mice with 10^6 or fewer cells cultured over a period of 7 days when preparative lymphodepletion was employed [67].

The use of magnetic-bead-based aAPCs was investigated in a severe combined immunodeficiency (SCID) mice bearing human melanoma tumors [68]. These mice received MART-1 specific T cells generated from human PBMC after 1 month of *in vitro* culture with magnetic beads presenting MART-1-loaded human MHC dimers and anti-CD28 (1:1 ratio of aAPCs to cells) [68]. Mice bearing 2-week-old tumors received an intravenous injection of 3×10^6 MART-1 specific cells and IL-2 injections (2×10^5 IU/mouse) on days 0 and 2 following cell transfer [68]. Magnetic-bead-expanded cells were compared with cells stimulated by monocyte-derived DC. Tumors displayed significantly delayed growth kinetics in mice receiving aAPC-expanded cells (compared with untreated tumors) and these cells performed on par with cells expanded by DCs.

Magnetic-bead-based aAPCs have also been used in human adoptive cell transfer trials in HIV infection [69,70] or in conjunction with hematopoietic stem cells transplants for hematological malignancies [71-73]. In these cases $CD4^+$ T cells were stimulated *ex vivo* with magnetic beads displaying human anti-CD3 and anti-CD28, and the resulting populations (following aAPC removal) were adoptively transfered to patients to repopulate the lymphocyte compartment. In the case of HIV infection, classical methods of T cell activation, such as phytohemagglutinin and IL-2, also activate viral replication, making T cell expansion futile [43]. Activation with anti-CD3 and anti-CD28 coated magnetic bead aAPCs, however, expanded $CD4^+$ T cells without viral activation and decreased surface expression of CCR5, a critical co-receptor for viral entry, on T cells [74].

When eight patients with established HIV infection received three infusions of aAPC-expanded $CD4^+$ T cells, a decrease in CCR5 expression was observed at 158 days, suggesting resistance to HIV [69]. In addition, sustained increases in $CD4^+$ T cell counts were achieved [69]. In a subsequent study by the same group, the ability of the same aAPCs to expand $CD4^+$ T cells containing lentiviral genetic modifications conferring HIV resistance was investigated. T cells were transduced with vectors to produce an RNA antisense sequence that prevents productive replication of the HIV-1 virus [75,76]. Five patients received a single infusion of these genetically modified lymphocytes resulting in increased cellular responses, as assessed by antigen-specific IFN-γ secretion, in four patients [70]. Similarly, treatment of patients with a variety of hematological malignancies by adoptive transfer of T cells stimulated *ex vivo* by magnetic-bead-based aAPCs presenting anti-CD3 and anti-CD28 resulted in notable responses in clinical settings [71-73] (For review see [77]).

Many aspects of the aforementioned adoptive transfer studies are encouraging. Cells generated from unselected precursors acquire antigen specificity and demonstrate activity *in vivo* with clinically relevant antigens. Several studies, especially those performed in mice, involve tumors with antigens that are well characterized and expressed in a relatively uniform manner. Other studies use mice lacking a functional immune system, which is often exploited by the tumors for protection through the use of myeloid derived suppressor cells and indeed can thwart immunotherapy [78]. The translation of the results from these studies using immunodeficient mice to clinical applications will need to be made with caution as immunodeficient mice may not sufficiently mimic the immunological state of cancer patients, who have an immune system, albeit attenuated. More significant is the issue of persistence of adoptively transferred cells. Cells expanded *ex vivo* often have limited persistence *in vivo*, necessitating frequent injections. Thus, the use of exogenous cytokines such as IL-2, IL-7, IL-15 and IL-21 provides survival signals for activated effector cells, suggesting their use *ex vivo* or following infusion of transferred cells is critical for the success of adoptive therapy. However, high systemic doses of these cytokines can be quite toxic, limiting therapeutic efficacy. Further studies are needed to determine optimal conditions for *ex vivo* stimulation and *in vivo* survival that yield persistent and viable cells.

5. Expert opinion

Artificial antigen-presentation on synthetic substrates is an attractive strategy for stimulation and expansion of T cells primarily because it offers the flexibility over assembly of different combinations and ratios of ligands enabling the investigation of a wide range of activation conditions. These conditions can affect the quantity and quality of expanded cells. Additionally, because such systems are not subject to genetic variability of ligand expression or culture conditions that may alter their function, they offer savings in time and labor. Some aAPC formulations, such as liposomal or biodegradable polymer systems, already consist of materials that have a long history of use in the pharmaceutical industry and can be produced under good manufacturing practices (GMP) conditions. Thus for *ex vivo* use these systems offer attractive advantages over live cells.

The *in vivo* applications of such systems are promising as well. An artificial APC displaying tumor-associated antigens obviates the concern over how tumors affect DC function, phenotype or activation state. Cancer is associated with an environment that disfavors effective antigen presentation by professional APCs with the production of suppressor cells and tumor-associated macrophages that hinder DC function [11]. Artificial APCs, when homed to tumor targets, are not susceptible to dysfunction because of their synthetic nature.

Despite these positive aspects some challenges remain with current aAPC technology. One of the main hurdles to effective *in vivo* use of aAPCs is the issue of size. Particles between 5 and 10 μm in diameter are most efficient at T cell stimulation; however, particles of this size pose a significant risk if systemically administered. Non-deformable particles composed of latex, magnetic materials, and biodegradable polymers may lodge in capillary beds and lead to embolism. Thus, it will be necessary to create nanoscale aAPCs to enable efficient transport through the body. Ultimately, a balance between nanosizing of such systems for circulation requirements and induction of immunity will need be achieved to realize therapeutic efficacy.

Artificial particles that are injected systemically need to avoid clearance by the reticuloendothelial system (RES) for proper homing in to targets. The most widely used surface coupling group for this purpose is PEG because this group creates a hydrophilic surface that facilitates long circulation of the particles. This strategy has been used successfully in making 'stealth' liposomes with affinity towards target cells [79-81]. Functionality could also be introduced by incorporating PEG with functional endgroups for coupling to target ligands.

Passive delivery of nanoscaled aAPCs may also be harnessed to target tumors for antigen presentation at the tumor site. Aggressive tumors inherently develop leaky vasculature with 100 – 800 nm pores due to rapid formation of vessels that must serve the fast-growing tumor. This defect in vasculature coupled with poor lymphatic drainage serves to enhance the permeation and retention of nanoparticles within the tumor region. Often called the EPR effect [82,83], this phenomenon is a form of passive targeting. The basis for increased tumor specificity is the differential accumulation of particles in tumor tissue versus normal cells, which results from particle size rather than binding. Normal tissues contain capillaries with tight junctions that are less permeable to particulates. Passive targeting can therefore result in increases in particle concentrations in solid tumors of several-fold relative to free drug molecules. While this effect has been observed for drug delivery, it is unclear if it can be exploited for antigen-presentation at the tumor site and expansion of antigen-specific tumor infiltrating lymphocytes using nanoparticles surface engineered with specific T cell antigens [84].

Acellular aAPCs engineered to encapsulate soluble mediators overcome another limitation. The majority of studies discussed in this review involved the addition of exogenous cytokines. Natural aAPCs are known to secrete cytokines in a paracrine fashion to lymphocytes [16]. Indeed, exogenous additions of this third signal are needed for efficient stimulation of T cells *ex vivo* and their systemic injection is needed for persistence and viability of expanded subsets in many cases. Exogenous addition of cytokines *in vivo* can cause significant toxicity, as in the case of IL-2 [85]. Thus, the ability to deliver cytokines locally in a paracrine fashion from aAPC constructs may not only enhance the persistence of targeted cells but may alleviate the high doses needed for *in vivo* efficacy.

An important limitation of artificial systems that are injected into the body is an incomplete understanding of

how these constructs biodistribute in various organs and their pharmacokinetics in disease models. No current animal model has proved to be a reliable predictor of clinical results nor is any model widely accepted as a gold standard for testing novel immunotherapies. When considering the available models in which to test aAPCs and the clinical data regarding immunotherapy, these constructs are subject to many of the same barriers that thwart more conventional tumor therapies. Importantly, the persistence of T cells *in vivo* is an issue of paramount importance for adoptive immunotherapy. In order to achieve a durable response, immunological memory is required. Recent work has shown that the phenotype of *ex vivo* expanded cells can dictate the survival potential of transferred cells *in vivo* [86]. In addition, treatment of animals or patients with lymphodepleting regimens prior to adoptive cell transfer therapy can result in homeostatic proliferation of the transferred cells, leading to greater persistence [87].

The most promising use of aAPCs at present is their *ex vivo* application, which allows for control over the number and phenotype of T cells expanded. For *in vivo* applications, a greater understanding of their biodistribution and immune response is needed prior to realizing their full potential. With this understanding and the novel biologically inspired strategies incorporated within these platforms there is no doubt that these hurdles will be crossed for practical application in clinical settings.

Declaration of interest

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