Published in final edited form as:
Biochim Biophys Acta. 2015 April ; 1853(4): 781–790. doi:10.1016/j.bbamcr.2014.09.001.

Linking Form to Function: Biophysical Aspects of Artificial Antigen Presenting Cell Design

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Artificial Antigen Presenting Cells (aAPC) are engineered platforms for T cell activation and expansion. By replacing and recapitulating the functions performed by endogenous antigen presenting cells, aAPC serve as cost-effective alternatives to cellular APC for immunotherapy and as minimalist cell-free systems for studying T cell activation.

aAPC are synthesized by coupling necessary T-cell stimulating proteins, such as co-stimulatory proteins and Major Histocompability Complex (MHC)/Human Leukocyte Antigen (HLA) loaded with antigen of interest, to the surface of an appropriate solid support. aAPC have been built upon a wide variety of biocompatible platforms, including cultured cell lines, liposomes, and biodegradable polymer particles, and have been functionalized with a variety of proteins that deliver T cell activating signals [1].

These simplified systems provide the minimum necessary signals for T cell stimulation, leading to robust T cell activation and expansion. However, as reviewed in this issue and elsewhere [2,3], it is becoming increasingly clear that T cell-APC interactions are temporally and spatially complex, with dynamic changes in the lateral organization of surface receptors on both the T cell and APC. Membrane heterogeneity, receptor clustering, and activation-induced membrane rearrangements on several scales are part of a complex molecular machine that underscores T cell activation [4].

This complexity is both a challenge and an opportunity for the biomedical engineer. On one hand, it is precisely the complex molecular mechanisms that underlie T cell receptor function and enable its precision and sensitivity. On the other hand, it is becoming clear that

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engineered platforms meant to activate immunity are capturing only the most rudimentary interactions that occur during T cell activation.

Here, we review our current understanding of the biophysical and spatial aspects of the T cell-APC interaction and its application to aAPC design. In doing so, we demonstrate how insight into the nature of T cell activation by aAPC flows in both directions. Artificial platforms for T cell activation can serve as models to better understand the endogenous system, and this knowledge can, in turn, be adapted for improved translational platforms for immunotherapy.

1. The Signal 1+2 Paradigm in aAPC Design

A general paradigm for the design of aAPC has been to mimic endogenous T cell activation by selecting T cell activating signals that lead to optimal stimulation. In the healthy host, these are provided by endogenous APC such as macrophages, B cells and dendritic cells (DCs). In aAPC design, these same signals are generated by coupling purified or recombinant proteins to an aAPC platform that can then trigger responses from receptors on the T cell membrane. Studies of T cell activation by aAPC have demonstrated that two signals, termed Signal 1 and Signal 2, are minimally necessary to trigger robust expansion of highly functional T cells (Figure 1).

1.1. Signal 1

Signal 1 is mediated by the interaction of TCR on the T cell with peptide presented by MHC on the APC. Peptide-bearing MHC preferentially interact with T cell receptors specific for one or several MHC-peptide combinations, and thus Signal 1 determines specificity of the T cell response for a given epitope. MHC-binding to TCR triggers activation of the TCR-associated CD3 signaling complex through as-of-yet incompletely understood mechanisms [5,6]. In aAPC design, Signal 1 can be provided by either MHC-peptide binding to TCR, or by engaging the CD3 complex directly with an anti-CD3 antibody (Figure 1).

Soluble Class I and Class II MHC proteins can be produced recombinantly and loaded with appropriate peptide for a variety of antigens of interest. The aAPC engineer must select an MHC allele and peptide that induce a T cell response against the antigen of interest. In humans, HLA-A2*01 has been most frequently studied, based on its high frequency among people of Northern European and American descent. In mice, K^b and D^b alleles, as well as L^d, are frequently used based on their presence in the common laboratory strains C57BL6/J and Balb/c, respectively. Following stimulation, the yield and frequency of antigen-specific cells can be monitored using soluble, multimeric MHC reagents.

Alternatively, Signal 1 can be provided by an antibody against the CD3 signaling complex. A variety of activating CD3 antibodies are available, including the OTK3 clone in humans and 145-2C11 in mice. Importantly, activation via CD3 triggers non-specific expansion of *all* T cells, including regulatory T cells and cells reactive against irrelevant antigens; over time, this can result in preferential expansion of irrelevant cells and reduced activity against the target. Thus, for most applications, a source of T cells enriched for activity against the antigens of interest is desired. In cancer immunotherapy, tumor infiltrating lymphocytes can

provide such a source of anti-tumor activity [7], or antigen-specific cells can be purified from Peripheral Blood Mononuclear Cells or other polyclonal sources by HLA-tetramer-based enrichment prior to polyclonal expansion [8]. Unfortunately, anti-CD3/anti-CD28 aAPC have only shown the ability to expand and sustain CD4 [9] but not CD8 T cell cultures [10,11] without additional feeder cell support, making them an appropriate choice only when CD4 cells are required, or when the additional cost and labor associated with culturing autologous feeder cells can be tolerated.

1.2 Signal 2

Signal 2, the co-stimulatory signal, is a series of interactions between receptors on the APC and T cell surface that modify TCR signaling by providing both activating and/or inhibitory signals. The prototypical interaction, between B7.1/B7.2 on the APC and CD28 on the T cell surface, leads to optimal T cell expansion. If Signal 1 is engaged in the absence of Signal 2 *in vitro*, CD4+ T cells enter a state of *anergy*, in which T cell proliferation and effector function after re-stimulation are limited [12]. Ineffective Signal 2 stimulation can also lead to the development of suppressive T cells, and the balance between anergy and regulatory development is an area of active study.

Several activating Signal 2 interactions have been identified, including the B7 family proteins B7.1 and B7.2, and their cognate T cell receptor CD28; and the tumor necrosis factor receptor (TNFR) family ligands OX-40L, CD70, 4-1BBL, which interact with OX-40, CD27, and 4-1BB on the T cell, respectively. Certain Signal 2 interactions can also be inhibitory to T cell expansion and effector function, such as the interaction of CTLA-4 with B7.1 or PD-1 with PD-L1 (B7.H1) or PD-L2 (B7-DC)[13].

In aAPC design, Signal 2 can be provided by coupling one or more of the aforementioned APC receptors to the surface of an aAPC platform (Figure 1). In practice, activating antibodies against co-stimulatory T cell receptors, such as the anti-mouse CD28 antibody 37.51, have been shown to be an effective replacement for more costly recombinant APC proteins [14]. While the downstream signaling mechanisms of each co-stimulatory receptor vary, engagement by certain “activating” antibody clones appears to be a near-universal mechanism for triggering co-stimulatory receptor activation.

Minimally, the addition of anti-CD28 antibody is required to design effective aAPC that induce robust T cell proliferation [15,16] and maintain the full complement of T cell effector functions [16,17]. Under certain conditions of extremely strong Signal 1 activation, robust expansion can be observed with Signal 1 alone [18], but the precise phenotypic characteristics of such T cells and their effectiveness in immunotherapy have not been described. On the other hand, many platforms instead rely on multiple co-stimulatory signals delivered simultaneously [19].

The precise signals delivered by each Signal 2 are likely to differ, and thus the choice of Signal 2 protein may be an important parameter in optimizing aAPC for a given application. For example, 4-1BBL may be more effective than anti-CD28 as an activating signal for memory CD8 T cell expansion [20,21], or even synergize with simultaneously presented anti-CD28 [22]. The role of inhibitory signals has been explored to a lesser extent; the

addition of a PD-L1 to aAPC does not appear to decrease T cell proliferation [23], but may have a role in shaping the subsequent response.

1.3 Signal 3

In addition to the classic description of Signal 1+2, Signal 3 has been coined as a catch-all term for a variety of soluble signals released by APC that influence T cell activation and development. These include lymphotrophic cytokines such as IL-2, IL-7, and IL-15; inflammatory signals such as TNF-α; and cytokines which modulate T cell development such as TGF-β, IL-12, IL-4, and IL-5. The precise effect of each of these signals is beyond the scope of this review, but the cytokine milieu released by APC before and during T cell activation is a critical determinant of T cell development after activation [24–26].

During *in vitro* T cell stimulation by aAPC, the addition of exogenous IL-2 is required for robust T cell expansion [27–29]. Alternatively, proliferation can be supported by a variety of common gamma chain cytokines, such as IL-7, IL-15, and IL-21, which may have the further benefit of maintaining T cell replicative potential and inducing memory formation compared to IL-2 [30,31]. These cytokines have been added to aAPC-stimulated cultures to generate cells more amenable to tumor immunotherapy [32]. IL-12 and Type 1 interferons added to culture can support proliferation at low to intermediate antigen doses, and the development of full effector function at any antigen dose [33–35].

There may be a further benefit to delivery of Signal 3 directly from aAPC rather than addition to culture, which could increase local cytokine concentration at the T cell-aAPC interface, spatially co-localize all 3 T cell stimulatory signals, and deliver cytokines to the appropriate site after *in vivo* administration. Thus far, application of Signal 3 in aAPC design has been limited by the capabilities of aAPC platforms. However, recent developments in design of aAPC based on biodegradable polymers, which can release encapsulated proteins in a spatially localized manner during hydrolytic degradation, have shown that IL-2 delivery from aAPC can significantly enhance T cell proliferation *in vitro* [36–38]. This “paracrine” delivery was ten-fold more effective in inducing T cell expansion than the same overall dose of IL-2 in the culture media [37].

2. Fixing Signals to a Solid Substrate

aAPC are constructed by coupling T cell activating proteins described in the previous section to a suitable, biocompatible platform. While multimerized, soluble MHC can induce some degree of T cell activation [39–41], large numbers of MHC molecules are required per T cell [42–45]. In contrast, as few as 10 cognate MHC presented on an APC cell surface can be sufficient to induce activation [46,47]. Similarly, fixing MHC-peptide (pMHC) or anti-CD3 to a physical substrate, such as a latex bead, liposome, or microplate surface, significantly enhances the strength of T cell stimulation [40,48–52].

This solid substrate enhancement points to fundamental questions about the mechanism by which TCR is triggered by MHC, which despite recent advances remains an open area of study. Two models, here termed “mechanical triggering” and “rapid re-binding,” may

explain this phenomenon. A third complementary model, spatial segregation, will be discussed in detail in subsequent sections.

During T cell activation, fluctuations of the APC membrane can generate a pico- to femtonewton force on the T cell membrane [53]. These forces can also be triggered by a timed series of active pushing and pulling processes on the T cell [54]. Mechanical forces in this range induced by micromanipulation of TCR by pMHC or clonotypic antibodies are sufficient to trigger downstream signaling responses in T cells [55,56], and mechanical signals between T cells and APC enhanced by shear flow triggered stronger T cell stimulation *in vitro* [57]. Thus, “mechanical triggering” of the TCR has been proposed as a model of TCR activation by MHC, and may play a significant role in signal transmission.

The fixing of soluble MHC to a solid substrate allows the transmission of mechanical forces from a cell or microparticle surface. This is reflected in the dependence of T cell activation on substrate rigidity, with the strongest activation of mouse T cells triggered by ligands presented on polymer substrates with elastic moduli greater than 10–200 kPa [58]. Interestingly, with human T cells, it was noted that elastic moduli significantly above this range impeded stimulation [59], a finding attributed to decreased bond lifetimes under greater loading force [60]. While TCR interactions with anti-CD3 antibody generated the primary traction forces mediated by T cells, engagement of CD28 enhances the TCR associated force via an intracellular signaling pathway mediated by PI3K [61].

Secondly, fixation of Signal 1 to a solid support constrains the receptor-ligand interaction to a planar cell-surface interface and allows for rapid re-binding of cell surface receptors. During physiological T cell/APC interactions, receptors are confined to the two-dimensional axis of cell-cell contact. In contrast, biophysical measurements of TCR-MHC interactions have traditionally been made by characterizing binding of soluble MHC dimers or antibodies measured either by flow cytometry or surface plasmon resonance, which involve a third degree of freedom. “Two dimensional” measurements that more closely re-capitulate TCR-MHC interactions at the cell-cell membrane can be made by assessing interactions of a T cell with an MHC-coated particle [62,63], similar in design to an aAPC. 2D affinity analysis has resulted in the identification of a wider range of cognate T cell ligands [64,65]. Measurements in two dimensions broadly agree with kinetic binding parameters measured *in situ*, which show large increases in both association (100-fold) and disassociation (4–12 fold) compared to measurements in solution, ultimately resulting in increased overall affinity [66].

This enhanced 2D affinity, and particularly enhanced on-rate from constrained MHC, has led to a “rapid re-binding” model, wherein strong pMHC agonists confined to the surface efficiently rebind the same or neighboring TCR within individual receptor nanoclusters [63,65–68]. Compared to on-rates driven primarily by MHC diffusion, re-binding that takes place in a constrained two-dimensional membrane is significantly enhanced. In this model, the assembly of downstream signaling ligands can tolerate brief loss of contact between TCR and MHC as long as the receptor is efficiently rebound. Thus, the ability of cognate MHC-peptide to trigger TCR activation depends not only on the kinetic off-rate, as had been

proposed by earlier models [69], but also the on-rate-the ability of MHC to re-bind the same or neighboring [68,70] TCR.

MHC can be fixed to a number of different platforms, from easily cultured cell lines to biodegradable polymer scaffolds. While different platforms possess different mechanical and surface properties, the most commonly used substrates all share in common biocompatibility and ease of synthesis. The earliest platforms were APC-like cells based on cultured cell lines [71,72], particularly the K562 human erythromyeloid line [19,20,73–76] and the murine NIH/3T3 fibroblast line [77–79], engineered to express Signal 1 and Signal 2. Among the earliest cell-free platforms used for T cell activation were liposomes, spherical vesicles with an aqueous interior generated by self-assembly of amphiphilic phospholipids and cholesterol, also modified to express the relevant T cell-activating signals [50,51,80–83].

Synthetic substrates have the additional benefit of having tunable mechanical, geometrical, and biodegradable properties, either by adjusting the synthesis method or base material. Sepharose [84,85] and latex (polystyrene) beads [10,28,86–89] were the first synthetic bead-based platforms used in aAPC design, and have been instrumental as reductionist systems for studying basic aspects of T cell biology. Iron-dextran microparticles have also been extensively characterized as aAPC, both for translational applications of tumor-specific T cell expansion [15,90] and as a tool for the study of basic aspects of T cell antigen recognition and development [91–94]. Additionally, microparticles can be synthesized from a variety of biodegradable polymers, such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their co-polymer, poly (lactic-co-glycolic acid) (PLGA) and used for antigen-specific T cell activation [38,95], with significant biocompatibility advantages over non-degradable platforms such as polystyrene.

3. Microscale T Cell-aAPC Interactions

Thus far, the primary focus of aAPC design has been the selection of T cell stimulating signals and platforms to which they are coupled. However, it has become evident that receptor organization between the T cell and APC plays an additional important role in endogenous activation. Recapitulating and even enhancing this aspect of T cell activation represents a new frontier in optimal aAPC design. Here, we will briefly review relevant spatial considerations of natural T cell-APC interactions.

The idea that spatial organization of the TCR and accessory signaling proteins plays a critical role in T cell activation gained support with the discovery of the immune synapse [96], a micro-scale cell-cell interaction structure formed during activation of T cells by APC. During immune synapse formation, adhesion molecules such as LFA-1 migrate to the periphery of the T cell contact site, the peripheral supramolecular activation cluster (pSMAC), whereas TCR, CD3, and other signaling proteins are found in the central SMAC (cSMAC) [97] (Figure 2). TCR nanoclusters form at the periphery of contact and traverse toward the cSMAC [98] where they are subsequently degraded. However, the discovery of the synapse was soon followed by studies showing that synapse formation, which occurs minutes after T cell-APC contact, was preceded by strong TCR signaling from TCR

nanoclusters and was thus only a piece of the T cell activation puzzle [99]. Today, it is appreciated that the nature of the synapse varies with the strength and duration of stimulus, the activation state of the T cell, and the nature of the APC [100]. Additionally, the synapse can perform numerous functions, from turning off signaling [101] to providing a cell-cell contact zone for exchange of cytokines and cytotoxic granules [102].

Fundamental insights into the nature of the synapse have been made using planar lipid bilayers, acellular T cell activation platforms similar in concept to aAPC (reviewed in [103]). Planar synthetic surfaces can be designed using numerous techniques, most commonly by inserting freely diffusible, GPI-anchored T cell activating proteins into lipid membranes coated onto glass slides. The flat contact surface allows the use of imaging techniques with high spatial and/or temporal resolution such as total internal reflection microscopy [104], as well as control over activation parameters such as ligand strength and density.

New surface fabrication techniques allow T cell activating ligands to be patterned in precisely controlled ways on planar bilayers, providing a tool to study the effect of microscale APC membrane organization on T cell activation. For example, the presence of anti-CD28 at the periphery of the T cell contact site on planar arrays containing anti-CD3, CD28, and ICAM-1 significantly enhanced IL-2 secretion and downstream signaling by mouse CD4 T cells [105]. Similarly, mechanically trapping TCR nanoclusters at the peripheral regions of a forming synapse significantly prolonged and strengthened signaling from TCR nanoclusters [106]. However, separation of anti-CD3 and anti-CD28 contact sites by several microns in human T cells curtailed co-stimulatory activity [107]. Thus, while existing aAPC fabrication techniques generally rely on randomly distributed ligands, these findings suggest it may be possible to fine-tune T cell activation from aAPC by patterning activating ligands in ways that mimic T cell-APC interaction. However, this will require the development of technologies for heterogeneous ligand distribution on particle surfaces [108,109] (Figure 3A).

More effective aAPC mediated stimulation may also be possible by mimicking the micro-scale, cytoskeletal membrane rearrangements that occur at the T cell-APC interface. T cells have a sensitive leading edge that makes primary contact with APC, triggering a cell-cell interaction program [110] that leads to cellular flattening and an augmented area of surface contact [111]. Simultaneously, cytoskeletal rearrangements in the APC drive membrane polarization [112] and increased surface contact.

In contrast, most synthetic aAPC platforms are spherical, a shape which is easily synthesized using standard chemical synthesis procedures such as double-emulsion of PLGA [113]. This geometry not only fails to recapitulate APC membrane dynamics observed during activation, but also minimizes the surface area and maximizes the curvature at the T cell-aAPC interface. Recently, we utilized a novel particle fabrication method to control microparticle geometry with defined MHC dose and density [114]. Compared to spherical aAPC, ellipsoidal aAPC with increased aspect ratio and surface area preferentially engaged cognate T cells, with T cells observed to favor engaging aAPC along their long axis (Figure 3B). This interaction was reflected in enhanced T cell expansion and *in vivo* tumor killing activity

after stimulation by ellipsoidal aAPC and highlights the importance of geometry considerations in aAPC design. Similar synthesis techniques could be used to mimic additional membrane geometries relevant to T cell-APC interactions, such as membrane protrusions/lamellopodia that enhance close membrane apposition [115].

Particle geometry also influences uptake by the reticuloendothelial system, an important consideration for aAPC that are directly administered *in vivo*. Ellipsoidal particles with a long characteristic axis show reduced phagocytosis [116–119]. Interestingly, receptor-mediated internalization of antibody coated non-spherical particles is significantly enhanced, suggesting the interaction of geometry and uptake may be pathway dependent [120]. These contrasting properties of identically shaped aAPC may be used to their advantage to prolong circulation time where receptor-mediated internalization is preferred, such as in drug delivery applications. In either case, *in vitro* uptake studies may not mimic *in vivo* behavior; adsorption of complement and other serum proteins significantly alter trafficking and clearance characteristics of *in vivo* administered aAPC.

4. Nanoscale Clustering and Nanoscale aAPC

Upon engagement with cognate antigen, TCR microclusters, estimated to be 35–70 nm in diameter and containing 7–20 TCR [121], form at the periphery of the T cell-APC contact site (first described in [122] and reviewed in [2]). These clusters are enriched for signaling molecules such as Lck, Zap-70, Lat, and SLP76, suggesting that they are directly involved in T cell signaling. Microcluster formation precedes but is associated with synapse formation, as both involve a cortical F-actin flow that leads to inward migration of TCR.

Spatial heterogeneity in membrane organization and TCR clustering can be detected even in the absence of stimulation by cognate antigen. Using immunoprecipitation and immunoblotting techniques, Fernandez-Miguel et al. demonstrated that the αβ T cell receptor can exist as a multivalent structure on naive T cells, composed of at least two TCR and a higher order CD3 stoichiometry [123]. TCR likely exist in several distinct monovalent and multivalent forms prior to antigen engagement [124], with the number of TCR in a cluster prior to activation ranging from a single receptor to 20 [125] within clusters that are 15–30 nm in diameter [126]. Although both TCR clusters formed prior to and after antigen engagement are generally less than 100 nm in diameter, the former have been termed “nanoclusters,” and the later, “microclusters.” The precise relationship between these structures has not been fully elucidated; however, it is possible that TCR nanoclusters concatenate to form the larger activation microclusters that are observed by TIRF [121]. Thus, receptor organization at both micro- and nanoscales play a central role in T cell activation (Figure 2).

In parallel, spatial heterogeneity of MHC on the APC surface mirrors the T cell membrane. Near-field scanning techniques have resolved protein-rich “patches” of MHC with radii between 70 and 600 nm on the surface of resting APC [127], containing approximately 25–125 MHC each. During antigen processing, MHC bearing peptides derived from a given pathogen are deposited as a cluster on the APC membrane [128–130], generating peptide-specific clusters that facilitate interaction with peptide-specific TCR. Subsequently, during T

cell activation, MHC move in concert with their binding partners to form clusters in the cSMAC zone of the APC, with adhesion molecules such as ICAM-1 migrating to the pSMAC. These pre-formed clusters and subsequent movements are controlled by the intricate interactions of lipid and protein domains, such as lipid rafts and tetraspanin domains in the membrane [131], as well as protein interactions with the actin cytoskeleton [131].

Spatial rearrangements may serve as a critical piece of the mechanism behind T cell triggering after engagement by cognate MHC [132]. A common element in spatial activation models is kinase concentration and phosphatase exclusion – phosphorylating proteins are concentrated in the region that contains their substrate, whereas dephosphorylating proteins are excluded, tipping the local balance in favor of signal transduction. Consistent with this hypothesis, it was recently shown that close apposition between the T cell and APC membrane drove exclusion of proteins such as the phosphatase CD45, whereas the binding energy between TCR and pMHC was sufficient to keep these proteins within the central contact area [5]. This mechanism was sufficient for T cell signaling in a reconstituted system. Furthermore, new high-resolution imaging techniques have demonstrated nanoscale co-localization of TCR and downstream signaling proteins, which is enhanced after cognate pMHC binding [121]. Furthermore, spatial co-localization may play a complementary role with other activation models, including induction of conformational changes in the TCR-CD3 complex that initiate downstream activation [133].

Receptor clustering prior to ligand engagement can be a higher order mechanism which enhances binding and sensitivity. For example, clustering of chemo-receptors on *E coli.* significantly lowers the threshold and enhances the dynamic range of chemotactic responses [134]. Preliminary evidence suggests that such clustering may be partially responsible for the high degree of T cell sensitivity to antigen; for example, only multivalent but not monovalent TCR/CD3 complexes were phosphorylated after stimulation with a low dose of antigen [125,135]. Enhanced clustering after activation may also partially explain the increased sensitivity of previously activated T cells to antigen [136,137], acting in concert with changes in downstream signaling [138]. Similarly, clustering MHC on an APC surface augments T cell recognition [139]. Thus, receptor organization likely plays a significant role in the exquisite sensitivity and specificity of the TCR-MHC interaction, motivating its careful consideration in aAPC design.

4.1. MHC Valency and Density

With the importance of MHC clustering and spatial organization in endogenous T cell activation, it is no surprise that this parameter has been carefully explored for the design of artificial T cell activation platforms. Soluble MHC monomers, separated from their natural context in the APC membrane, have low, micromolar affinity for cognate TCR. This prompted the development of multimeric MHC constructs such as dimers and tetramers, which enhance overall binding avidity [140,141]. T cell triggering by these soluble multimeric MHC is highly dependent on intramolecular distances between MHC within the protein construct, with shorter intramolecular cross-linkers being significantly more

effective than longer cross-linkers, and a significant decrease in stimulatory activity occurring at a distance of approximately 8 nm [142].

By analogy, while monomeric MHC coupled to aAPC microspheres can trigger T cell activation [48,88], studies with multivalent MHC suggest that multivalency induces stronger responses [49,143]. However, no comprehensive examination between such constructs has been performed. Furthermore, multivalent MHC constructs can have other biophysical disadvantages: commonly used MHC tetramers have been coupled aAPC [144–146], but their rigid tetrahedral geometry orients a fraction of MHC molecules toward the particle surface and consequently away from the interacting T cell.

A similar effect to MHC multivalency may be mediated by controlling density of monomeric MHC proteins on an APC surface. Antigen density on APC membranes is known to affect subsequent T cell response [147–150]. This principle may also apply to aAPC design, although antigen presented on aAPC is often presented at supraphysiologically high doses compared to the small numbers of cognate MHC-peptides that trigger T cell activation.

On planar lipid arrays functionalized with anti-CD3 antibody in a controlled and homogenous fashion, anti-CD3 spacing of less than 70 nm is required to observe T cell activation [151]. Using peptide-MHC “corrals” patterned on lipid arrays, the Groves group demonstrated that thresholds for T cell triggering are determined by the number of activating ligands available to individual TCR clusters, not the total MHC available to the entire cell [152]. A similar density threshold is observed for monomeric MHC presented on spherical microparticles, and the addition of more beads at a sub-threshold density is not sufficient to overcome this effect [10,49]. Additionally, the density of MHC may control the avidity of the resulting cultured T cells, with an inverse relationship between T cell avidity and MHC density. Higher avidity T cells result from aAPC with a lower MHC density, likely via clonal competition, and high density aAPC increase activation of low-avidity clones [153]. Thus, if high avidity T cell responses are desired, investigators must titrate density to achieve a balance between T cell quantity and quality.

Furthermore, there may be advantages to manufacturing aAPC with heterogenous or clustered protein signals which mimic the distribution of MHC on endogenous APC membranes. One study used anti-CD3, -CD28, and -LFA-1 monoclonal antibodies pre-clustered on liposomes using neutravidin rafts to efficiently activate MART-1 specific CD8 T cells [50], although the precise role of clustering in enhancing T cell responses could not be clearly defined. In a separate liposome-based study, CD4 stimulation was significantly stronger from aAPC presenting clustered compared to unclustered MHC [154]. A high density (5–8 nm separating distances) of clustered *non-cognate* MHC presented on the surface of nano-sized quantum dots is required to enhance binding and activation by cognate pMHC [155], a finding that may explain conflicting results obtained with the “pseudo-dimer” theory, which states that numerous weak binding events with non-cognate MHC augment less frequent strong binding events mediated by cognate MHC.

Further application of heterogeneous MHC clustering on aAPC will require the development of biocompatible, readily synthesized platforms with spatially controlled receptor patterning [109]. For example, the Little group recently reported a technique based on interfacial condensation of a liquid mask to create microspheres with “patchy” protein islets [156].

4.2. Nanoparticle aAPC Can Activate T cells and Sense Membrane Organization

Studies on synthetic bead-based aAPC have largely focused on the development of cell-sized, micro-scale aAPC in order to better mimic T cells interaction with antigen presenting cells. This choice is theoretically reinforced by microscale T cell-APC interactions described above. In fact, early studies by the Mescher group suggested only bead-based aAPC larger than 2 microns in diameter are able to induce T cell proliferation [88,157]. Steenblock et al. [38] demonstrated that polymer-based nanoparticles were much less efficient than microbeads in inducing short-term functional responses, with no reported proliferation. However, recent discoveries of TCR organization at the nanoscale reignited interest in developing nano-sized aAPC particles, which might theoretically be able to interact with individual TCR nanoclusters to stimulate T cells and “sense” TCR distribution.

Several publications have now demonstrated that nanoparticles less than 100 nm in diameter can trigger robust T cell activation. For example, our group developed “nano-aAPC” by coupling dimeric MHC-immunoglobulins and anti-CD28 to the surface of iron-dextran nanoparticles. These nano-aAPC are able to induce robust T cell expansion *in vitro* and have significant advantages over micron-sized particles. Unlike microparticles, nanoparticles of approximately 50–100 nm diameter can be transported by lymphatics to the lymph nodes [158,159], thus gaining access to a larger pool of T cells. Enhanced drainage of 50–100 nm aAPC compared to micron sized aAPC was observed in our hands [160], although even smaller nanoparticles may be required for optimal lymphatic transport [161]. In addition, nanoscale delivery vehicles may preferentially accumulate in tumors through the enhanced permeability and retention effect which is attributed to the tumor’s tendency toward leaky vasculature and poorly organized lymphatic drainage [162,163]. By delivering an immunostimulatory signal *in situ*, aAPC in the tumor microenvironment may address one of the most prominent hurdles in cancer immunotherapy, the immunosuppressive tumor microenvironment [164].

In addition to inducing antigen-specific T cell activation, nano-aAPC binding to TCR nanoclusters can be used to gain qualitative information on TCR clustering of T cells in various physiological states, such as naive and activated cells. In particular, TCR undergo a state-dependent, persistent increase in the extent of TCR clustering days to weeks after activation. Binding assays using dimeric MHC-Ig fusion proteins demonstrated that T cells activated four days previously showed enhanced binding of low concentrations of MHC, with a high degree of cooperativity [136,140]. Since this effect was not observed with monovalent MHC, the authors determined that a higher degree of clustering led to the enhanced binding of MHC dimers. Persistent TCR clusters on activated cells was directly visualized using electron [137] and k-Space Image Correlation microscopy [165], with a distinct increase in clustering noted as compared to naive cells.

Differences in TCR clustering modify the T cell's ability to bind to and be activated by cognate pMHC. CD3 functionalized nanoparticles preferentially augment the expansion of antigen-experienced but not naive T cells that are concurrently stimulated with endogenous APC [166]. Recent work by our group demonstrates that nano-aAPC favor binding to clusters which are characteristic of activated rather than naive CD8 T cells [167]; furthermore, by exploiting the paramagnetic properties of iron-dextran nanoparticles, we demonstrated that magnet induced clustering of the cell-bound nanoparticles, and consequently their associated TCR clusters, was sufficient to induce robust activation of otherwise poorly sensitive naive T cells. This technique can be used to boost nano-aAPC mediated activation of naive T cells, and suggests an additional mechanism by which T cells more efficiently detect cognate antigen subsequent to activation.

5. Applications of aAPC: Past and Future

Despite these insights into the complex interactions underscoring T cell activation, most aAPC designs remain straightforward applications of the Signal 1 + 2 paradigm, with T cell activating proteins fixed in an uncontrolled manner to spherical microscale particles. This choice reflects the ease and simplicity of developing such systems, but does not reflect the rapid pace at which our understanding of T cell-APC interactions is advancing.

aAPC have been utilized as therapeutic platforms primarily in one of two ways. The first, direct vaccination, involves the direct administration of aAPC into a host, where aAPC must traffic to and co-localize with host T cells. The earliest reports of this approach involved 5 μm diameter silica microbeads coated with MHC-antigen but no Signal 2, injected intraperitoneally into tumor-bearing mice [48]. However, no antigen-specific responses or tumor activity could be detected unless a cell-based tumor vaccine was co-administered, suggesting that aAPC could only boost an existing response. This platform was subsequently assessed in a Phase I trial of patients with disseminated melanoma [168], with 8/15 patients developing antigen-specific cytolytic T cells, but only one partial tumor response. In mice, iron-dextran micro-aAPC bearing dimeric MHC-peptide and anti-CD28 injected intravenously [15] can mediate regression of both subcutaneous melanoma and intravenous lung metastases. Similarly, latex particles administered both intravenously and subcutaneously generated robust antigen-specific T cell responses in mice, and subcutaneously administered microparticles mediated B16 melanoma rejection [146].

This approach is particularly relevant to disease states like cancer, where endogenous antigen-presenting mechanisms are defective [169–171] and therefore motivate a desire to replace or supplement the endogenous APC compartment. Within the tumor microenvironment, APC can go beyond dysfunction to active immune inhibition [172–174]. Thus, even if antigen is delivered in a vaccine platform to the host APC, it may not lead to T cell activation, motivating the use of aAPC *in vivo*.

Thus, induction of tumor-specific cytolytic T cells does not necessarily correlate with tumor regression in human studies, a finding at least partially attributed to the suppressive mechanisms of the tumor microenvironment. Direct vaccination with aAPC therefore has significant unexplored potential, particularly as immunomodulatory strategies such as

"checkpoint blockade" are developed to temper these suppressive mechanisms that doomed the first generation of cancer vaccines [175]. Considerations of aAPC size and shape are likely to feature prominently in this approach, as they influence both the *in vivo* trafficking and subsequent T cell interactions of administered aAPC.

The second approach, adoptive immunotherapy, involves the generation of antigen-specific T cells *in vitro* and adoptive transfer into a patient or animal model. For example, adoptive transfer of large numbers of tumor specific T cells generated from melanoma cultures can mediate complete and durable regression of even large and metastatic tumors [7], and adoptive transfer of CMV-specific lymphocytes has been studied as a means of limiting immunosuppression post-transplant [14,176]. This approach has several advantages, including complete control of T cell culture environment during growth, and avoids the problem of attaining optimal aAPC biodistribution *in vivo*. On the other hand, *in vitro* T cell culture is costly and labor-intensive compared to direct aAPC administration, and can be described as designing a "new drug" for each patient.

Moving forward, aAPC have tremendous potential as a platform for inducing T cell expansion and for studying fundamental aspects of TCR organization and signaling. New breakthroughs in controlled micropatterning and shape manipulation on the nanoscale will allow for optimization of T cell activation mechanisms. aAPC can be produced for many patients in bulk and designed from easily manufactured, biocompatible platforms, creating off-the-shelf reagents that has significantly reduced cost compared to cellular APC. Adoptive T cell immunotherapy is limited in part by the need to generate large numbers (up to 10^{10}) of tumor-specific T cells quickly and reliably [177–179], which motivates the application of more advanced, biophysical understanding of T cell activation to optimize T cell expansion. The most rapid and robust T cell proliferation may only be possible when micro- and nanoscale considerations are taken into account. Thus, regardless of the therapeutic strategy chosen, size, shape, scale and surface distribution should be carefully considered as elements of effective aAPC design.

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Highlights

- Micro- and nanoscale interactions at the membrane shape T cell responses
- Fixing T cell stimulating molecules to solid substrates induces robust activation
- Valency and spatial arrangements of signaling ligands modify T cell activation
- Artificial antigen presenting cell shape affects cell interaction and biodistribution
- Future aAPC design will incorporate biophysical insights

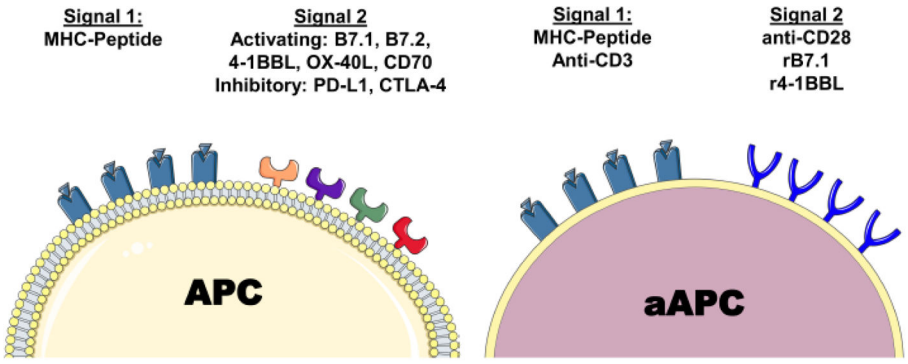

Figure 1. The Signal 1+2 Paradigm

Endogenous APC present two necessary and sufficient signals for T cell activation. Signal 1 is cognate peptide presented in the context of MHC, whereas Signal 2 comprises numerous activating and inhibitory co-stimulatory ligands that bind receptors on T cells. aAPC are synthesized by coupling either specific MHC-peptide complexes or polyclonally activating anti-CD3 antibody as Signal 1, and either activating antibodies against co-stimulatory molecules such as CD28 or recombinant co-stimulatory molecules such as B7.1 (rB7.1).

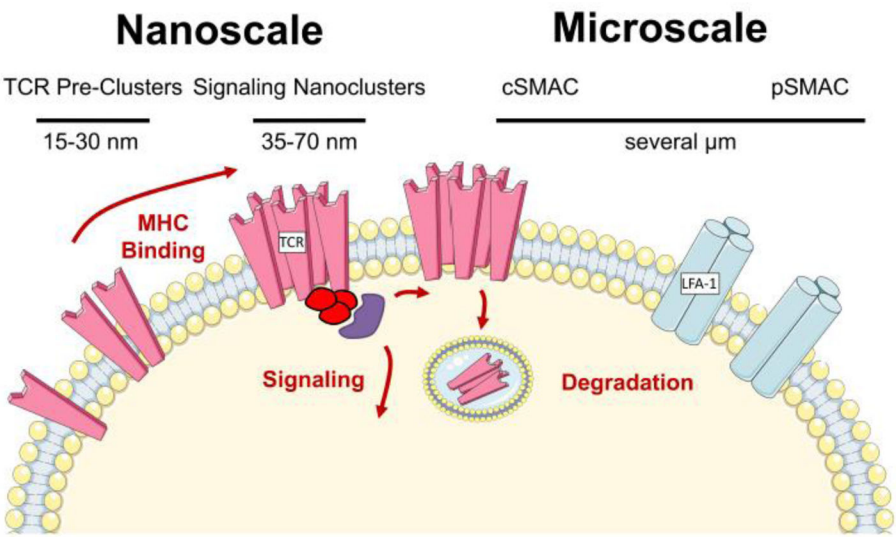

Figure 2. Scales of Organization

T cell membrane organization at several scales contributes to signaling and activation. TCR are pre-clustered in 15–30 nm nanoclusters prior to T cell activation, particularly on the surface of previously activated or memory cells. Upon MHC binding, 35–70 nm signaling nanoclusters form that drive downstream signaling. These clusters migrate to the center of the T cell-APC contact site, forming the cSMAC, and are subsequently internalized and degraded. Adhesion molecules such as LFA-1 are distributed in the pSMAC, which together with the cSMAC forms the micron-scale immune synapse.

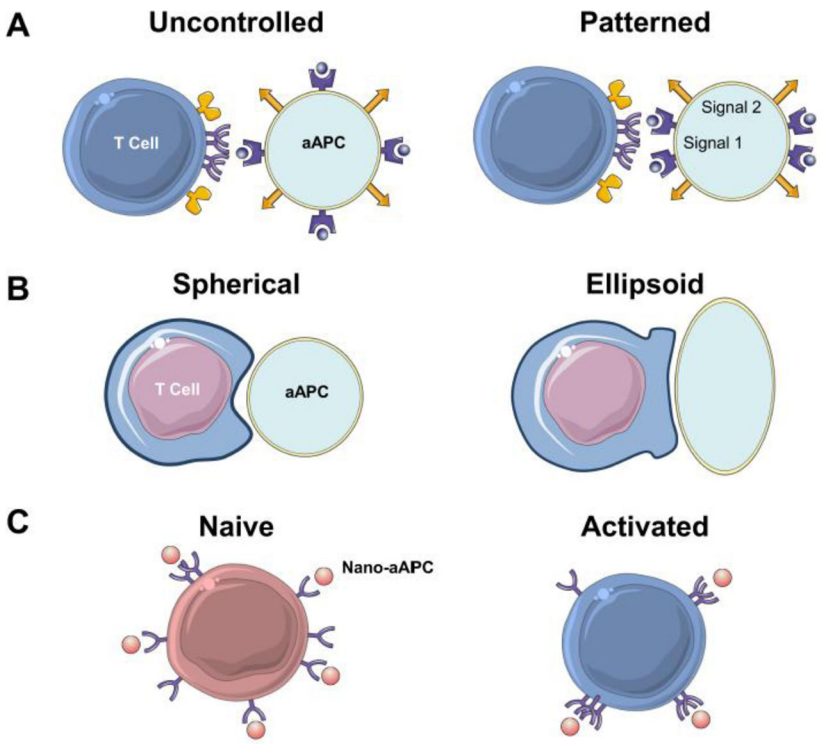

Figure 3. Scales of Organization

A. Studies from patterned lipid bilayers suggest that T cells preferentially activate against surfaces with MHC-peptide (purple) forming the center of the contact site, and anti-CD28 (orange) distributed in the periphery. In contrast, most aAPC are synthesized with uncontrolled protein distribution

B. Ellipsoid rather than spherical micro-aAPC provide a greater surface area and decreased surface curvature for optimal T cell engagement, leading to enhanced antigen-specific T cell activation and proliferation.

C. Nanoscale aAPC, less than 100 nm in diameter, have recently been shown to be capable of activating T cells. These nano-aAPC (orange) can “sense” nanoscale TCR distribution, as they preferentially bind to activated T cells which have more clustered TCR (purple) than naive cells. This leads to higher avidity for aAPC binding, but fewer aAPC bound to each T cell, since each aAPC binds multiple TCR. This effect enhanced activation of activated cells by nano-aAPC, whereas this preference was not observed with micro-aAPC.