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Separating T Cell Targeting Components onto Magnetically Clustered Nanoparticles Boosts Activation

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Abstract

T cell activation requires the coordination of a variety of signaling molecules including T cell receptor-specific signals and costimulatory signals. Altering the composition and distribution of costimulatory molecules during stimulation greatly affects T cell functionality for applications such as adoptive cell therapy (ACT), but the large diversity in these molecules complicates these studies. Here, we develop and validate a reductionist T cell activation platform that enables streamlined customization of stimulatory conditions. This platform is useful for the optimization of ACT protocols as well as the more general study of immune T cell activation. Rather than decorating particles with both signal 1 antigen and signal 2 costimulus, we use distinct, monospecific, paramagnetic nanoparticles, which are then clustered on the cell surface by a magnetic field. This allows for rapid synthesis and characterization of a small number of single-signal nanoparticles which can be systematically combined to explore and optimize T cell activation. By increasing cognate T cell enrichment and incorporating additional costimulatory molecules using this platform, we find significantly higher frequencies and numbers of cognate T cells stimulated from an endogenous population. The magnetic field-induced association of separate particles thus provides a tool for optimizing T cell activation for adoptive immunotherapy and other immunological studies.

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Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://doi.org/10.1021/acs.nanolett.7b05284) at DOI: 10.1021/acs.nanolett.7b05284. T cell isolation and stimulation studies, nanoparticle cluster analysis, nanoparticle characterization, cytokine secretion analysis, costimulatory molecule analysis, and Materials and Methods (PDF)

The authors declare no competing financial interest.

Graphical Abstract

Keywords

Immunotherapy; nanoparticle; CD8+ T cell; artificial antigen presenting cell; magnetic clustering; immune synapse

Decades of research have built a framework for understanding T cell signaling and have led to protocols for the *in vivo* and *in vitro* activation of T cells for basic science studies as well as immunotherapeutic applications. However, it has become evident that current platforms are not optimized for effective T cell stimulation. T cell activation is sensitive to the composition and organization of a large family of signaling molecules, many of which are often absent or difficult to optimize using the current methods of synthetic or modified antigen presenting cells.

Minimally, T cells require two signals for activation; signal 1, specific peptide-MHC (pMHC), and signal 2, a costimulatory signal such as the B7 interaction with CD28. During endogenous T cell activation, signals 1 and 2 cocluster in the immune synapse, a dynamic microcluster of signaling molecules at the interface of the T cell and antigen presenting cell.¹ This coclustering is necessary, as micropatterned surfaces that separate signal 1 and signal 2 by several microns eliminate activation.²

Although the B7/CD28 interaction is thought of as the “prototypical” signal 2, dozens of different molecules can serve as costimulation for CD8+ T cell activation, among them 4-1BB, CD27, and OX40, and each plays a different role in the activation process.^{3–7} The specificity and phenotype of activated T cells is sensitive to costimulatory molecule composition^{5,6,8–12} and affects the ability to stimulate a long-lived memory population able to kill virally infected or tumor cells. Even simply changing the ratio of costimulatory molecules or their timing during stimulation alters T cell functionality.^{8,9}

One reductionist approach for CD8+ T cell stimulation utilizes artificial antigen presenting cells (aAPCs), three-dimensional platforms that minimally present the two signals necessary for T cell activation; signal 1, specific peptide-MHC (pMHC), and signal 2, such as B7/anti-CD28 monoclonal antibody (mAb).^{13,14} In contrast to cell-based methods such as K562-based antigen presentation,⁵ cell-free aAPCs provide an engineered, more cost-effective off-the-shelf therapeutic. These particles can be used to activate and expand a patient’s CD8+ and CD4+ T cells against tumor antigens for adoptive cell therapy (ACT).^{15–20} The signaling molecules are often randomly conjugated to the surface of the aAPC,^{13,14,21} which

leaves little control over the nanoscale organization of the molecules, despite this showing importance empirically.^{1,22} When length scales have been more systematically investigated, it has been performed on flat substrates that lack the properties of three-dimensional activation structures.^{22,23} Progress in understanding the dynamics of T cell activation requires methods of such analyses on three-dimensional platforms that can be systematically manipulated.

Initially, traditional aAPCs were micron sized and thus have radii larger than the necessary degree of coclustering. This necessitates that all signaling molecules be conjugated to the surface of the same aAPC. Thus, for each type of costimulation condition studied, new aAPC must be synthesized which limits the ability to study large numbers of costimulatory conditions and increases time spent on quality controlling and potential for error.²⁴ Nano-aAPC with length scales closer to that required for signal clustering during T cell activation could allow for more control over signal distribution. Our previous work has demonstrated the ability to increase the effectiveness of nano-aAPCs which normally are not capable of activating a robust T cell response.²⁵ By constructing aAPC on paramagnetic iron-dextran nanoparticles, the aAPC can be clustered on the T cell surface using an external magnetic field to more closely mimic the clustering required for endogenous T cell activation. Because of the small length scales of nano-aAPC, we hypothesized that we could use this approach to separate the signaling components onto distinct particles.

Here, we develop and validate a reductionist T cell stimulation platform with each type of signaling molecule on separate paramagnetic nanoparticles. Previously, T cell signaling components could not be isolated on distinct particles unless embedded into a solid substrate or nanomatrix.²⁶ However, with the paramagnetic nanoparticle platform, the separately conjugated particles can be coclustered on the cell surface in the presence of a magnetic field²⁵ to mimic the signal microcluster and activate T cells. This new system provides a streamlined approach to T cell activation where a small number of homogeneous particles are easily synthesized and added into culture at different times, ratios, and amounts to customize stimulatory conditions. This platform can also be utilized to study biophysical aspects of T cell activation. We demonstrate that by altering nanoparticle size and composition, the separate particle platform can be used to assess the optimal degree of clustering between signaling molecules. This platform outperforms traditional methods to expand endogenous CD8⁺ T cell populations by enabling greater initial enrichment of the cells of interest and can be used to systematically study the biophysical effects of signal clustering on the cell surface.

To study the impact of altering composition and ratio of costimulatory signals, we developed a separate paramagnetic nanoparticle platform. The separate particle platform enables T cell activation by using an external magnetic field to cluster paramagnetic nanoparticles separately conjugated with signal 1 or signal 2. The separate particles are then added to culture at defined combinations and ratios to optimize T cell stimulation (Figure 1a).

One hundred nanometer paramagnetic iron-dextran particles were chosen as the base of the separate particle platform which we previously showed can be clustered on the T cell surface by an external magnetic field.²⁵ Thus, nanoparticles separately conjugated with T cell

stimulatory signals can be brought into the close proximity required for T cell activation. Although signal 1 and signal 2 separated onto distinct stimulatory complexes cannot activate T cells,^{27,28} clustering of paramagnetic particles mimics stimulation by a single, larger signal 1 and signal 2 complex.

We first demonstrated proof-of-principle of the system using separately conjugated signal 1 and signal 2 nanoparticles in the PMEL TCR transgenic murine model. While transgenic T cells may be less sensitive to changes in costimulation, the large numbers of identical T cell receptors provide an ideal system for validation. Anti-CD28 was chosen as the costimulatory molecule and Db-gp100 pMHC as the initial signal 1 to stimulate TCR transgenic PMEL CD8+ T cells, all of which are specific for the gp100 peptide presented by Db MHC I.

Separately conjugated Db-gp100 dimer only and anti-CD28 mAb only particles were incubated with naïve PMEL CD8+ T cells at a 1:1 molar ratio in the presence or absence of a magnetic field. There was significant expansion of the cells by the separate particle platform in the presence of a magnetic field ($p < 0.01$), which increased in a dose-dependent fashion with increasing signal 2/signal 1 ratio (slope = 0.2 ± 0.09 , $p = 0.02$). In contrast, in the absence of a magnetic field, there was little to no CD8+ T cell proliferation in the presence of low or high doses of signal 1, regardless of whether signal 2 was present. This was seen by both CFSE dilution (Figure 1b) and day 7 cell counts (Figure 1c). As expected, signal 2 alone did not cause T cell expansion, indicating that the magnetic field-induced clustering alone does not nonspecifically activate the cells.

CD8+ T cells stimulated with the separate particle platform had equivalent cytotoxicity to those stimulated with traditional aAPC, maximally ~40% specific lysis (Figure 1d). Signal 1 only activated cells had significantly less cytotoxic activity as expected, as signal 1 alone has been demonstrated previously to produce anergic cells.²⁹ Thus, the separate particle platform stimulates CD8+ T cells equivalently to traditional aAPC and maintains the requirement for signal 2, recapitulating the natural biology of T cell stimulation.

Based on data that T cell signaling starts on a nanoscale level,²¹ we hypothesized that increasing the particle size would limit the activity of the separate particle platform because this would intrinsically limit the coclustering of the individual molecules. Thus, we next sought to evaluate the working parameters of particle diameter using the separate particle platform.

We synthesized signal 1 and signal 2 only particles ranging in diameter from 30 to 4500 nm. Naïve PMEL CD8+ T cells were stimulated with each pair of matching diameter signal 1 and signal 2 only particles within a magnetic field, normalized to total signal 1 amount, and fold expansion was measured after 7 days. Standard aAPC based activation (i.e., signals 1 and 2 on the same particle, in the absence of a magnetic field) results in increasing CD8+ T cell activation with increasing particle diameter (Figure S1). This is a well-documented phenomena due to the higher radius of curvature of larger particles and thus higher surface area of contact between the T cell and aAPC.^{30,31} In contrast, the opposite effect is seen with the separate particle platform; particles with a diameter of 30 nm resulted in the greatest expansion over the course of 1 week, almost three times more than expansion by

4500 nm particles (Figure 2a). Magnetic clustering had no effect on activation by particles with diameters of 500 nm and above (Figure 2b), whereas smaller diameter particles only stimulated a CD8+ T cell response when clustered within a magnetic field. T cell activation did not correlate with cluster size or the number of clusters on the cell surface (Figure S2a,c). One hundred and three hundred nanometer particles resulted in significantly fewer numbers of clusters per cell than 30 or 500 nm particles despite producing intermediate levels of T cell activation. Similarly, cluster size did not vary significantly based on particle size, although 300 nm trended toward the largest diameter clusters (Figure S2b,c). This data indicates that the activation molecules must be presented on particles less than 500 nm in diameter to enable signal 1 and 2 coclustering at sufficiently small length scales. Particles with diameters smaller than 500 nm enable the signaling molecules to cluster within the nanoscale proximity required for activation.

To study the importance of clustering both signal 1 and signal 2, we synthesized nanoparticles from paramagnetic or nonparamagnetic materials to control which particles, and thus which signals, cocluster when a magnetic field is applied. While it is known that during endogenous T cell activation the signal 1 and CD28 molecules colocalize within the immune synapse,^{32,33} since our platform utilizes external forces to cocluster the molecules, it may intrinsically change the properties of activation and alter the requirement for coclustering of signal 1 and signal 2 molecules.

Db-gp100 dimer and anti-CD28 mAb were separately conjugated to the surface of 100 nm paramagnetic iron-dextran particles or 100 nm nonparamagnetic polystyrene particles (Figure S3). Each type of signal 1 only and signal 2 only particle was combined with PMEL CD8+ T cells within a magnetic field to produce coclustering of the signaling molecules, signal 1 or 2 only clustering, or no clustering of either signaling molecule (Figure 3a).

Optimal expansion occurred when both signals were coclustered on the surface of iron-dextran particles and decreased when either signal was presented on nonparamagnetic polystyrene particles (Figure 3b). Clustering of signal 1 alone decreased cell expansion by approximately 17% and clustering of signal 2 alone decreased cell expansion by approximately 33%. All conditions resulted in little to no expansion in the absence of magnet-induced clustering.

While transgenic T cells provide an important tool for proof-of-principle validation studies, they do not fully recapitulate endogenous naïve T cell activation. Thus, we sought to assess the clustering of signal 1 and signal 2 on the separate particle platform to expand endogenous T cell populations from naïve wild-type mice. To study this, we conjugated the Kb-trp2 pMHC dimer or anti-CD28 mAb to the surface of iron-dextran or polystyrene 100 nm particles, as above. CD8+ T cells were expanded over the course of 1 week with each combination of paramagnetic and nonparamagnetic signal 1 and signal 2 only particles in a magnetic field.

After 7 days of stimulation, CD8+ T cells were harvested and analyzed (Figure 3c). When both signals 1 and 2 were clustered on iron-dextran particles, almost 30% of cells were specific for Kb-trp2. This is an increase in specificity of up to 300,000-fold, as the estimated

precursor frequency for Kb-trp2 is approximately 10–100 cells per 10 million, or 0.001–0.0001%.³⁴ This decreased to less than 20%, a reduction of almost one-half, when signal 1 and/or 2 was not clustered on polystyrene particles, indicating the necessity for coclustering of the signals in endogenous CD8+ T cell expansion.

Next, we sought to visualize and quantify the coclustering of signal 1 and signal 2 particles in the presence and absence of a magnetic field. Fluorescently labeled Db-gp100 (red) and anti-CD28 (green) particles were coincubated with naïve PMEL CD8+ T cells for 1 h in the presence or absence of a magnetic field. Cells were then fixed and analyzed by confocal microscopy. Visually, it was evident that the two types of particles were coclustered when incubated within a magnetic field (Figure 3d). Coclustering was quantified in Matlab by assigning each cell from each condition a colocalization score that indicates the level of clustering between the two particle types at the cell level, with a higher colocalization score indicating increased clustering (see Supporting Information). The median colocalization score for cells within a magnetic field was more than double that for cells in the absence of a magnetic field, 0.560 and 0.271, respectively, and the two groups differed significantly by a Mann–Whitney test (Figure 3e).

We have shown previously that paramagnetic nano-aAPC can be used to enrich for clonal populations of CD8+ T cells from an endogenous repertoire on a magnetic column prior to *in vitro* expansion.³⁵ Enrichment prior to expansion reduces the competition for cytokines and MHC binding and has been shown to increase the expansion of cognate CD8+ T cells greater than 10-fold over nonenriched cells.³⁵ However, traditional aAPC are conjugated with anti-CD28 mAb in addition to the cognate signal 1 molecule and thus may lead to nonspecific binding and decreased enrichment potential. We hypothesized that since we can separate signal 1 and signal 2 on the nanoparticle platform, the separate particle platform could be utilized to obtain more highly enriched cells with the use of signal 1 only particles, followed by the addition of signal 2 particles to stimulate expansion.

We first modeled the difference in enrichment potential of traditional aAPC versus signal 1 only particles in PMEL CD8+ T cells. To study this, fluorescently labeled naïve PMEL CD8+ T cells were doped into CD8+ T cells from a naïve C57BL/6 mouse at a 1:1000 ratio. aAPC expressing both Db-gp100 and anti-CD28 or Db-gp100 alone were then used to enrich for the cognate PMEL CD8+ T cells, normalized by the total amount of Db-gp100. Signal 1 only particles resulted in a final purity of 1% PMEL CD8+ T cells, a 10-fold enrichment over the original cell mixture (Figure 4a). Traditional aAPC enriched PMEL CD8+ T cells to a final purity of only 0.5%, 50% less than signal 1 alone. Noncognate particles did not enrich for PMEL CD8+ T cells. Both signal 1 alone and traditional aAPC resulted in approximately 80% or more recovered PMEL CD8+ T cells (Figure S4).

Next, we sought to determine if the increased enrichment by signal 1 only particles corresponds with a greater expansion of endogenous CD8+ T cell populations from naïve C57BL/6 mice by the separate particle platform compared to traditional aAPC. CD8+ T cells isolated from naïve C57BL/6 mice were enriched with either traditional aAPC or signal 1 only Kb-trp2 particles. Anti-CD28 mAb signal 2 only particles were added to cells enriched with signal 1 only Kb-trp2 particles, and both traditional aAPC and separate signal

1 and 2 particle cultures were expanded in a magnetic field for 1 week. All cultures were normalized by the total amount of signals 1 and 2, which were present at an approximate 1:1 molar ratio.

After 1 week, cells were harvested, counted, and stained with Kb-trp2. Cells enriched with signal 1 only particles then expanded with signal 1 and signal 2 only particles contained 35.8% Kb-trp2+ cells, more than double that of traditional aAPC, 15.6% (Figure 4b). This effect was consistent in multiple independently repeated experiments (Figure 4c) as well as with different signal 1 peptides (Figure S5). The total number of Kb-trp2+ cells also increased nearly 2-fold when enriched and expanded with the separate particle platform (Figure 4d).

The functionality of the cells expanded by the separate particle platform was assessed by measuring cytokine secretion and degranulation markers. Seven days after stimulation, the cells were harvested and restimulated with trp2-pulsed RMA-S cells and cytokine secretion and CD107 expression, a degranulation marker, was measured. Cells expanded with the separate particle platform secreted the same or higher level of all cytokines measured, IFN- γ , IL-2, and TNF- α , and had equivalent expression of CD107 (Figure S6). Thus, the separate particle platform results in greater expansion of highly functional antigen-specific CD8+ T cells compared to traditional aAPC.

A valuable goal of the development of this platform is the ability to easily customize the type, combination, and specific ratios of costimulatory signal 2 molecules during CD8+ T cell expansion. We thus sought to use the separate particle platform to investigate how altering costimulatory molecule type and ratio affects endogenous CD8+ T cell expansion and phenotype. Four stock particle types were synthesized expressing anti-CD3, anti-CD28, anti-4-1BB, or anti-CD27 mAb. Anti-CD3 bypasses the pMHC-TCR recognition and was used as the signal 1 to stimulate endogenous polyclonal CD8+ T cells.

CD8+ T cells were isolated from C57BL/6 mice on day 0 and incubated with signal 1 particles and a constant total amount, but varied ratio, of anti-CD28 and anti-4-1BB mAb signal 2 particles within a magnetic field. After 7 days, total cell counts were recorded. Despite the total amount of signal 2 in all stimulatory conditions being equivalent, changing the ratio of signal 2 molecules significantly impacted CD8+ T cell proliferation (Figure 5a). All combinations of anti-CD28 and anti-4-1BB resulted in equivalent or greater CD8+ T cell expansion than either signal 2 molecule alone. At a 75/25% ratio of anti-CD28/anti-4-1BB, CD8+ T cells reached nearly 13-fold expansion, approximately 50% more proliferation than anti-CD28 alone and 95% more than anti-4-1BB alone. Also of note, signal 1 alone induced little to no cell proliferation of endogenous antigen-specific CD8+ T cells. Similarly, we showed how the separate particle platform allows for streamlined signal 2 customization by also testing various combinations of anti-CD28 and anti-CD27 mAb during a seven-day activation. Interestingly, at a 50/50% ratio of anti-CD28/anti-CD27 mAb, there was a significant increase in the number of CD8+ T cells with a memory phenotype despite no significant difference in total CD8+ T cell expansion (Figure S7). This data indicates that altering signal 2 composition can impact skewing toward specific T cell subsets in addition to overall expansion.

Finally, we assessed the ability to use the separate particle platform to study how different signal 2 ratios change T cell phenotype when expanding endogenous antigen-specific CD8⁺ T cells. C57BL/6 mice were enriched with signal 1 only Kb-trp2 particles, and different ratios of anti-CD28 and anti-4-1BB mAb signal 2 only particles were added during expansion, as above.

Both a 25/75% and 75/25% ratio of anti-CD28 and anti-4-1BB mAb increased the expression of CD127, the IL-7R α chain, on Kb-trp2⁺ CD8⁺ T cells compared to anti-CD28 alone (Figure 5b). Signaling CD8⁺ T cells through 4-1BB has previously been shown to increase CD127 expression in CD8⁺ T cells and may be indicative of cells with a memory rather than exhausted phenotype.³⁶ Thus, the separate particle platform presents a streamlined method to customize stimulatory conditions to optimize the phenotype of T cells for immunotherapeutic applications.

Here, we have developed and validated a system that can combine high throughput synthesis of single-molecule paramagnetic “aAPC” with magnetic fields to demonstrate a reductionist platform capable of studying and optimizing T cell activation. While upcoming cancer immunotherapies such as adoptive cell transfer are promising for generating patient-specific cytotoxic T cell responses, approaches that use homogeneous microscale aAPC cannot take full advantage of the nanoscale organization and costimulus diversity that have demonstrated importance in generating long-lived functional T cell populations. Additionally, few platforms exist that enable the more fundamental study of T cell costimulus clustering in a three-dimensional system. We show proof of concept for a new platform to easily customize costimulatory signals, study the clustering of signaling molecule clustering during activation, and isolate and activate higher purity endogenous T cell populations.

Various recent studies have shown the impact of the composition of costimulatory molecules on the expansion and phenotype of activated T cells. Simply changing the ratio of costimulatory molecules without altering the total amount of signal on aAPC can impact the expansion of T cells.⁹ The previous approach to study costimulus composition on three-dimensional platforms involves synthesizing new aAPC for each condition.^{5,9} This limits throughput and can present difficulty in accurately studying such effects as it has been documented that unexpected experimental artifacts in the preparation of aAPC with defined ratios of molecules are common.²⁴ The separate particle platform simplifies nanoparticle synthesis and quality control assessment while maintaining a similar, if not higher, level of T cell activation. This platform allowed us to easily investigate ten different costimulatory conditions, whereas previous reports have investigated five or fewer.^{5,9} We demonstrated proof of concept for the ability to alter the frequency and phenotype of CD8⁺ T cells by incorporating anti-4-1BB and anti-CD27 mAbs at defined ratios, although it could easily be extended to study additional conditions. This platform also provides a technique to customize costimulation on a case-by-case basis. For example, it can be hypothesized that in the future of adoptive cell therapy, one may wish to costimulate with molecules that synergize with checkpoint blockade⁴ in some patients, but molecules that improve expansion and function in the presence of signal 1 alone in others.^{5,6} Information gained about costimulatory requirements using this approach may also help to guide optimization of approved therapies such as CAR T cells in terms of which costimuli to incorporate.

In addition to providing a mechanism for high-throughput costimulus customization, by design, this platform also enables the three-dimensional study of signal coclustering. Prior studies that manipulate signal clustering at the nanoscale often utilize two-dimensional platforms that poorly simulate endogenous activation.^{2,23} By incorporating the separate signaling molecules on different sized paramagnetic and nonparamagnetic particles, we showed that pMHC and anti-CD28 require coclustering at length scales smaller than 500 nm. This particle size-dependent effect was not associated with a change in cluster size, as 30 nm particles did not result in any significant change in cluster size or number as compared to 500 nm particles. Thus, the ability of the separate particle platform to induce T cell activation is associated with the intermolecular distance of the signaling molecules dictated by particle size, but not with the size of the microscale cluster. Likewise, this approach can be used to investigate clustering requirements for less studied costimulatory or coinhibitory molecules. While some previous reports analyzed 4-1BB colocalization at the immune synapse during T cell activation,³⁷ little to no information is known about the clustering of other costimulatory molecules such as CD27, CD40L, and OX40. The clustering induced by this approach also may affect particle internalization. Particles with diameters of 40–60 nm have been shown to have the shortest internalization times compared to those with larger diameters.³⁸ In our studies, all particles resulted in cluster sizes that average 1 μm^2 or greater, correlating with a cluster radius greater than 500 nm. It is thus likely that one aspect of magnetic field-induced clustering is potentially a decrease of particle internalization by the T cells and prolonged extracellular activation time, especially of the small 30–100 nm particles.

Finally, the separate particle platform results in greater expansion of specific T cell clones from an endogenous repertoire. Previous work from our lab established an enrichment and expansion protocol to enrich for specific CD8+ T cell clones using paramagnetic aAPC prior to expansion.³⁵ The success of enrichment prior to expansion was demonstrated to be due to reduced competition for growth factors and pMHC binding and results in approximately a 10–40-fold increase in frequency after 1 week compared to expansion alone. Likewise, we showed that by further increasing enrichment using particles that only have the cognate signal 1 molecule followed by the addition of costimulatory particles for expansion, we doubled the frequency and total number of activated cognate CD8+ T cells. This is especially relevant as next generation expansion protocols could incorporate other costimulatory molecules that are not specific to the T cells of interest and would decrease enrichment. This platform enables a mechanism to eliminate that obstacle.

We have demonstrated the ability of this platform to activate all T cells or specific T cell subsets depending on the type of signal 1 stimulation by utilizing anti-CD3 mAb or MHC-Ig, respectively. Dependent on the clinical indication, this separate particle approach could be used for adoptive cell therapy to activate antigen-specific T cell populations using an MHC-Ig dimer loaded with specific peptides, or could be used in combination with anti-CD3-coated particles to nonspecifically activate T cells in a more high-throughput manner. Due to the ability to customize the composition and clustering of signaling components, this platform may also have applications in basic science research to study T cell activation or clustering of signaling molecules on other cell types. While our current studies focused on the murine model, it is relevant to human T cell expansion with possible modifications in the

selection of costimulatory molecules. Finally, it is important to note that our separate particle approach creates homogeneous single signal “islands”, which may interact with the T cell differently than endogenous antigen presenting cells. Further studies could investigate how these two approaches compare in the context of T cell activation.

We have developed a reductionist T cell activation platform that simplifies and streamlines customized costimulatory conditions. This platform is a robust tool to study the composition and clustering of signaling molecules for T cell activation by altering particle size, material, and molecules conjugated to their surface. This allows for further study of T cell signal clustering and composition to better understand the signaling pathways as well as optimization of activation for cell-based immunotherapies such as adoptive cell transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

aAPC	artificial antigen presenting cell
mAb	monoclonal antibody
ACT	adoptive cell therapy
pMHC	peptide-major histocompatibility complex

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Figure 1.

CD8⁺ T cells are activated by nanoparticles separately expressing signal 1 and signal 2 when particles are clustered within a magnetic field. (a) Schematic comparing standard aAPC coexpressing stimulatory signals on the same nanoparticle, and separate signal 1 + signal 2 particle platform with each stimulatory signal conjugated to distinct nanoparticles. (b) TCR transgenic PMEL CD8⁺ T cells were stimulated with signal 1 only Db-gp100 dimer particles in the presence (black) or absence (gray) of signal 2 only anti-CD28 particles. CFSE dilution was measured after 3 days. Decreased fluorescence indicates increased proliferation. Cells proliferated when signal 1 and signal 2 were presented on separate particles, only when clustered within a magnetic field. One representative experiment is shown. (c) PMEL CD8⁺ T cells were stimulated with anti-CD28 signal 2 (s2) only particles, standard signal 1/2 aAPC (aAPC) expressing Db-gp100, or signal 1 (s1) only Db-gp100 particles with 0–8× molar ratio of signal 2 (s2) only anti-CD28 particles. Significance measured by two-way ANOVA with Bonferroni post-test. Signal 2 only particles and s1 + 8× s2 particles induce significantly different expansion compared to traditional aAPC within a magnetic field. Data combined from six independent experiments. (d) PMEL CD8⁺ T cells were incubated with signal 1 only particles, signals 1 and 2 expressed on separate particles (s1 + s2), or traditional signal 1/2 aAPC with 12 ng total signal 1. On day seven poststimulation, cells were isolated and coincubated with cognate B16–F10 tumor cells for 4 h. Cytotoxicity was measured. Significance measured by two-way ANOVA with Bonferroni post-test. Analysis performed on three independent samples (* $p < 0.05$, ** $p < 0.01$).

Figure 2.

Separate signal 1 and signal 2 particle platforms require particles on the nanometer size scale for efficient CD8⁺ T cell stimulation. (a) TCR transgenic PMEL CD8⁺ T cells were stimulated with different size Db-gp100 and anti-CD28 particles presented on separate particles within a magnetic field. Cell counts were taken on day seven and fold expansion calculated. Maximal expansion occurred when cells were stimulated with 30 nm separately conjugated particles. Significance measured by one-way ANOVA with Tukey's post-test. Data combined from three independent experiments. (b) CFSE labeled PMEL CD8⁺ T cells were stimulated with different size Db-gp100 and anti-CD28 particles presented on separate particles in the presence (black) or absence (gray) of a magnetic field. CFSE dilution was read by flow cytometry on day three post-stimulation. Data from one representative experiment of five is shown (* $p < 0.05$).

Figure 3.

Signal 1 and signal 2 only nanoparticles must be coclustered on the cell surface for efficient CD8+ T cell activation. (a) Schematic of stimulatory conditions and subsequent CD8+ T cell activation measured in (b). (b) PMEL CD8+ T cells were stimulated with signal 1 only Db-gp100 and signal 2 only anti-CD28 nanoparticles with either an iron or polystyrene (PS) core. Cells were counted after 7 days. The greatest proliferation was measured when both signal 1 and signal 2 were presented on paramagnetic iron-dextran particles. Significance measured by twoway ANOVA with Bonferroni post-test. Data combined from five independent experiments. (c) C57BL/6 CD8+ T cells were stimulated with separately conjugated Kb-trp2 signal 1 only and anti-CD28 signal 2 only particles with an iron or polystyrene core. Cells were stained for CD8 and Kb-trp2 cognate dimer after 7 days. When both signals were presented on iron-dextran particles, there was a maximum of 29.9% Kb-trp2+ CD8+ T cells. Data from one representative experiment is shown. (d) Naïve PMEL CD8+ T cells were incubated with fluorescently labeled Db-gp100 signal 1 only (red) and anti-CD28 signal 2 only (green) nanoparticles for 60 min ± a magnetic field. (e) Cells

incubated $\pm$ magnetic field were analyzed and each given a colocalization score quantifying the colocalization between signal 1 and signal 2 particles (see Materials and Methods). Cells incubated within a magnetic field had significantly higher colocalization between signal 1 and signal 2 measured by a Mann–Whitney test ($p = 0.0401$). More than 45 cells per condition were analyzed ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

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Figure 4.

The separate particle platform results in greater expansion of functional cognate CD8⁺ T cells. (a) Thy1.1⁺ TCR transgenic PMEL CD8⁺ T cells were doped into Thy1.2⁺ wild type C57BL/6 CD8⁺ T cells at a 1:1000 ratio. Cell mixture was enriched with signal 1 only Db-gp100 particles, signal 1/2 Db-gp100/anti-CD28 aAPC, or noncognate aAPC. Signal 1 only enrichment resulted in a final purity of approximately 1% cognate PMEL cells, while signal 1/2 aAPC had a final purity of approximately 0.5%. Noncognate cells did not enrich for PMEL cells. Significance measured by one-way ANOVA with Tukey's post-test. Data combined from three independent experiments. (b) C57BL/6 cells were enriched and expanded with Kb-trp2 signal 1 dimer presented on traditional aAPC or the separate particle platform within a magnetic field. Representative staining shows that separate particle platform increases the percentage of expanded cognate cells from an endogenous response after 7 days. (c) Percent of cognate dimer positive cells 7 days post-enrichment and -expansion. (d) Number of Kb-trp2⁺ CD8⁺ T cells 7 days post-enrichment per one million expanded CD8⁺ T cells. Significance measured by two-tailed paired *t* test of seven independent experiments (**p* < 0.05, ***p* < 0.01).

Figure 5.

The separate particle platform allows for manipulation of costimulatory signal 2 composition. (a) Naïve CD44^{lo} CD8⁺ T cells from C57BL/6 mice were stimulated for 7 days with the separate particle platform. Anti-CD3 mAb was used as signal 1, and anti-CD28 and/or anti-4-1BB mAb were used as signal 2. Total signal 2 amount remained constant, only the ratio varied. A 75/25% anti-CD28/anti-4-1BB ratio significantly increased expansion over either signal 2 alone. Data from three independent experiments shown. (b) C57BL/6 cells were enriched and expanded with Kb-trp2 signal 1 dimer presented on the separate particle platform within a magnetic field. Signal 2 ratio varied as in panel (a). On day seven, cells were harvested and stained for Kb-trp2 and CD127. The addition of anti-4-1BB significantly increased the percent of Kb-trp2⁺ cells expressing CD127. Significance measured by one-way ANOVA with Dunnett's post-test. Data from five independent experiments shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).