SUPPORTING INFORMATION

Biologically Inspired Design of Nanoparticle Artificial Antigen-Presenting Cells for Immunomodulation

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Supplemental Tables 1-2

Table S1. Tabular summary of aAPC particle properties and conditions used within experiments.

aAPC name	Average size (nm)	Ligand density signals/μm^2	# effective ligand/aAPC	# aAPCs/T cell @ 2 pM	Chemistry and Particle Source	Figures particles were used in
50 nm	55	44	0.05	34,582	EDC/NHS, Ocean Nanotech	1, 2a, 3, 4, 5, S1, S6-12
HD 50 nm	44	3,440	4.2	411	Miltenyi	5, S9-12
300 nm	299	309	5.1	138	EDC/NHS, Ocean Nanotech	1, 2a, 3, 4, 5, S1, S6-12
600 nm	621	124	3.4	175	EDC/NHS, Ocean Nanotech	1, 2a, 3, 4, 5, S1, S6-12
4.5 μm	4640	393	375.2	0.1	Epoxy, ThermoFisher	1, 2a, 3, 4, 5, S1, S6-12
M 50 nm	31	127	0.1	12,044	Sulfo-SMCC, Micromod	2b-e, S2-5
M 300 nm	402	184	3.0	232	Sulfo-SMCC, Micromod	2b-e, S2-5
M 600 nm	578	164	7.6	65	Sulfo-SMCC, Micromod	2b-e, S2-5

Table S2. Tabular summary of 50- and 600-nm aAPC particle properties and conditions used to examine the impacts of ligand density and spacing.

Nanoparticle size	Ligand density signals/μm^2	# effective ligand/aAPC	Linear Ligand Spacing (nm)	# aAPCs/T cell @ 2 pM
50	44	0.05	150	34582
50	3438	4.2	16	411
600	942	38.74	33	15
600	237	11.0	65	54
600	164	7.6	78	79
600	112	5.2	95	115
600	13	0.6	279	1003
600	2	0.07	795	8129

Supplemental Figures 1-12

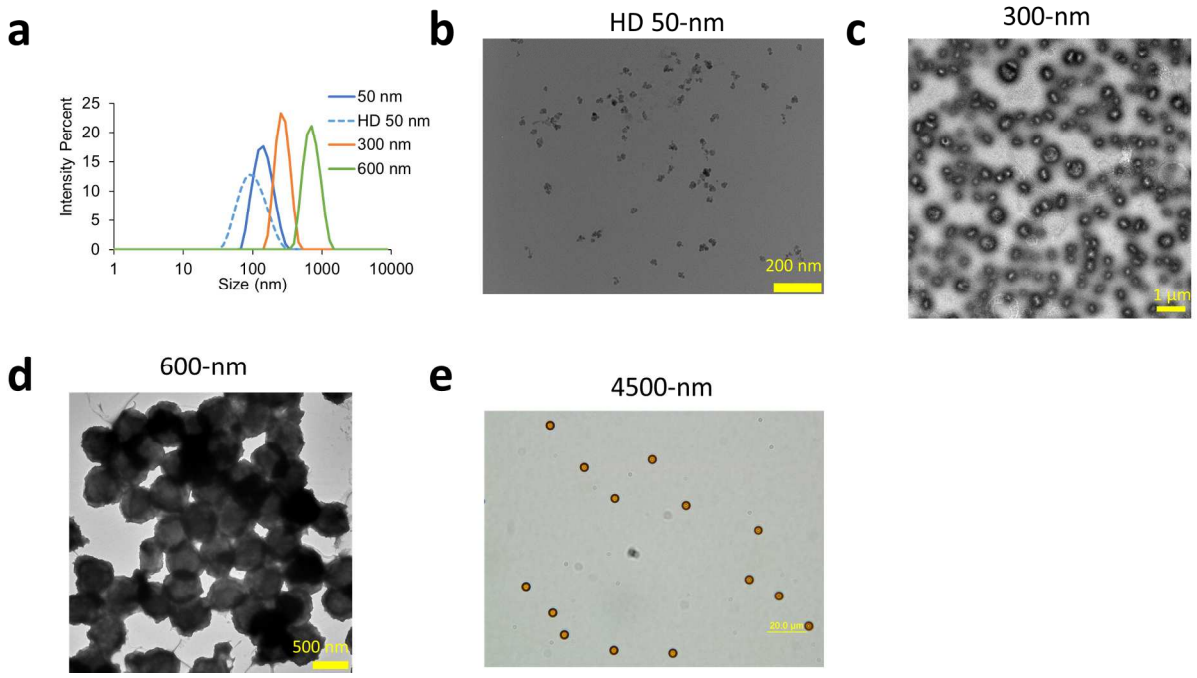


Figure S1. Further characterization of formed particle aAPCs. (**a**) Dynamic light scattering (DLS) measurements showing size distributions of nanoparticle aAPCs by Intensity Percent. (**b-e**) Representative images of functionalized aAPCs. (**b-d**) TEM images of (**b**) HD 50-nm (scale bar = 200 nm), (**c**) 300-nm (scale bar = 1 µm), (**d**) 600-nm (scale bar = 500 nm), and (**e**) of 4500-nm aAPCs with light microscopy (scale bar = 20 µm).

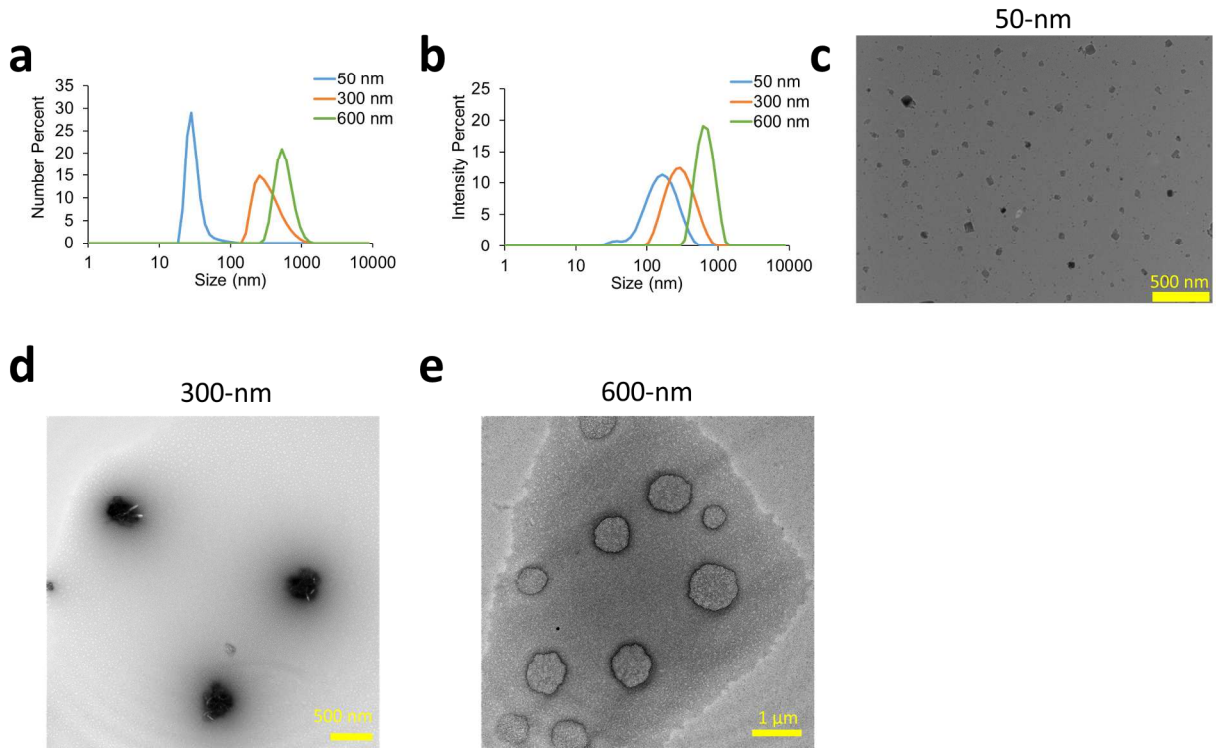


Figure S2. Particle characterization of formed aAPCs derived from another company (Micromod). (**a-b**) Dynamic light scattering (DLS) measurements showing size distributions of nanoparticle aAPCs by (**a**) Number Percent and (**b**) Intensity Percent. (**c-e**) Representative TEM images of functionalized aAPCs of 50-nm (scale bar = 500 nm), (**d**) 300-nm (scale bar = 500 nm), and (**e**) 600-nm (scale bar = 1 µm).

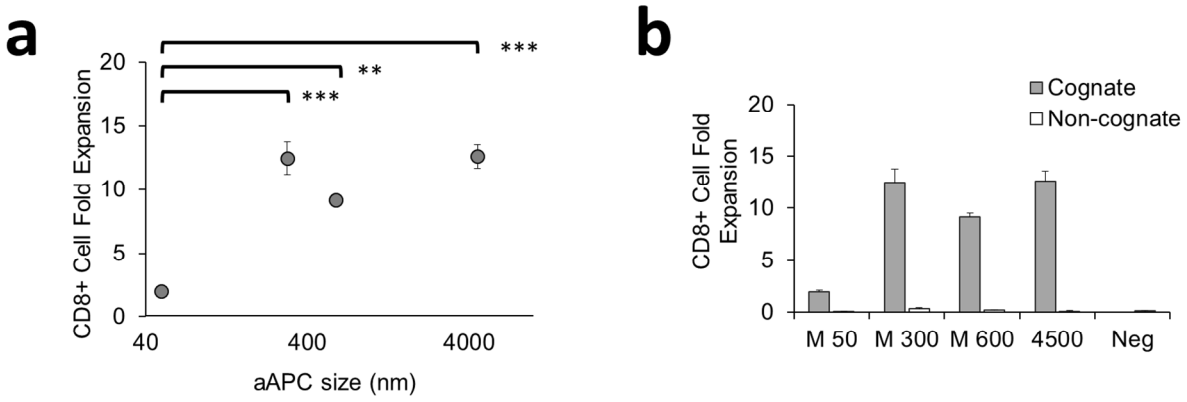


Figure S3. Size dependent effect is still observed when using another source of iron oxide particles, but not with non-cognate aAPCs. (**a-b**) Antigen-specific T cells are cultured with 2 pM of aAPC-bound pMHC and fold proliferation is measured 7 days later for (**a**) cognate (error bars show s.e.m.; ***$p < 0.0005$, **$p < 0.005$, *$p < 0.05$, $n = 3$, one-way ANOVA with Tukey's post test) and (**b**) non-cognate aAPCs (cognate values are replotted from Fig S3a for comparison purposes).

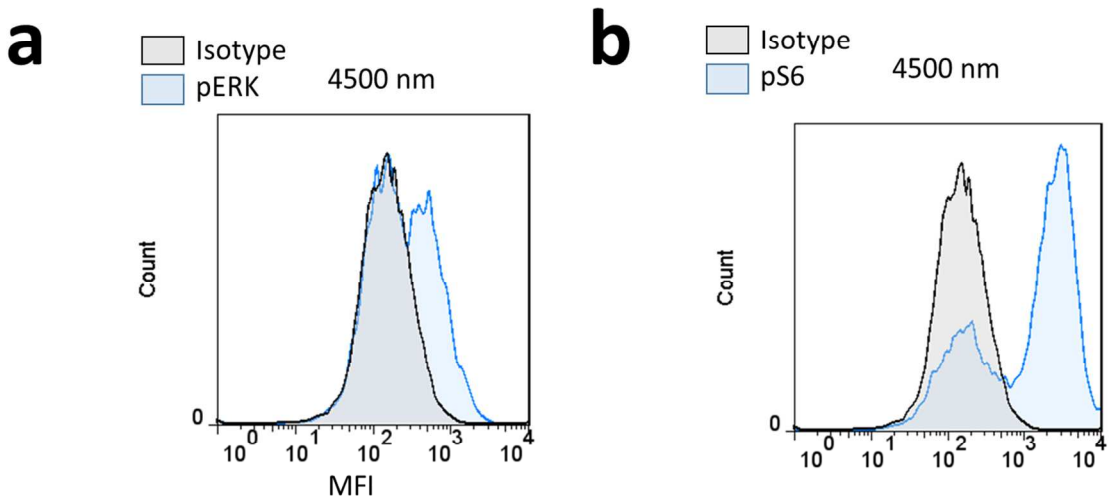


Figure S4. Isotype control staining for phosphorylation studies. (**a-b**) Example of isotype (grey) and phosphorylation (blue) staining for CD8+ T cells stimulated with 4500 nm aAPC at a 2 pM dose for (**a**) pERK and (**b**) pS6.

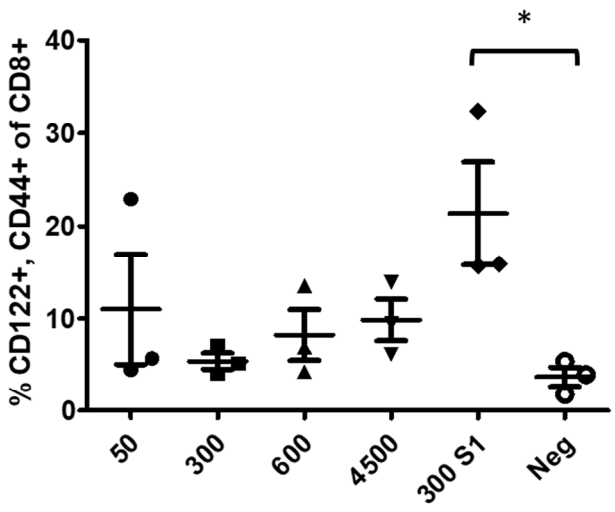


Figure S5. Only aAPCs with only pMHC KbSIY attached (no anti-CD28), 300 S1, showed a significant increase in the percent of CD122+, CD44+, CD8+ T cells after 7 days of culture with aAPCs at a 2 pM dose of aAPCs (error bars show s.e.m.; *$p < 0.05$, $n = 3$, One way ANOVA with Dunnett's post test).

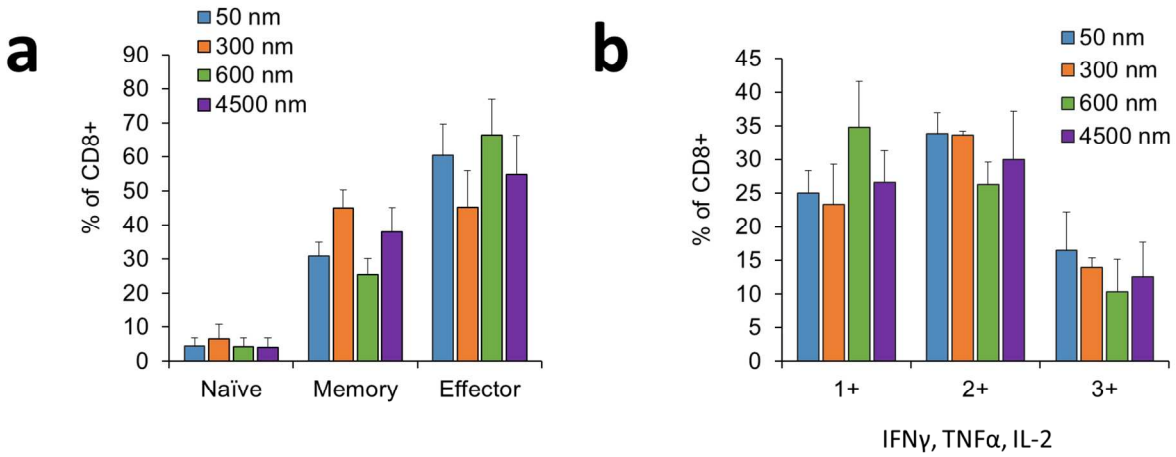


Figure S6. CD8+ T cells stimulated with particle aAPCs of different sizes result in similar CD8+ T cell phenotype and functionality. (**a, b**) CD8+ T cells were stimulated by particles of different sizes for 7 days at a concentration of 18 pM of particle-bound Kb-SIY and (**a**) phenotypic cell surface markers (CD62L, CD44) and (**b**) intracellular cytokine production as measured by triple, double, and single positive cells (IFNγ, TNFα, IL-2) were measured as a percent of CD8+ (n=3).

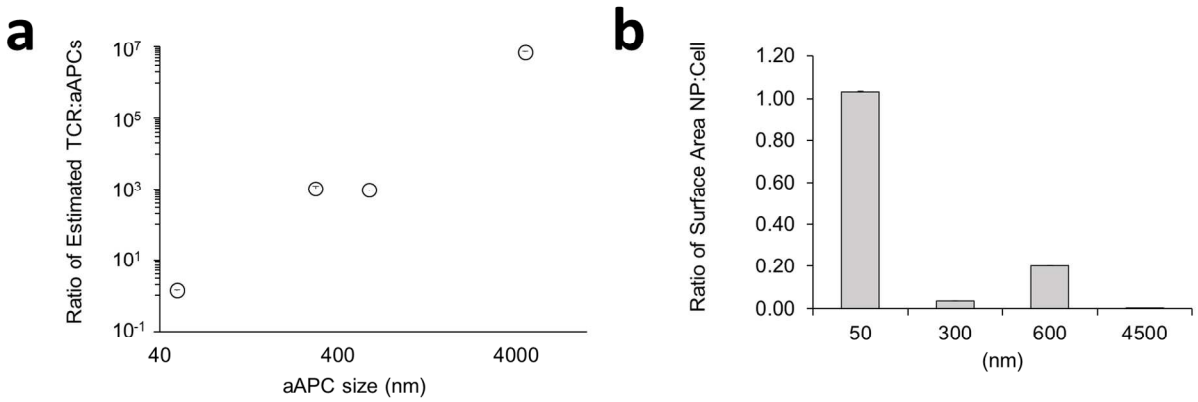
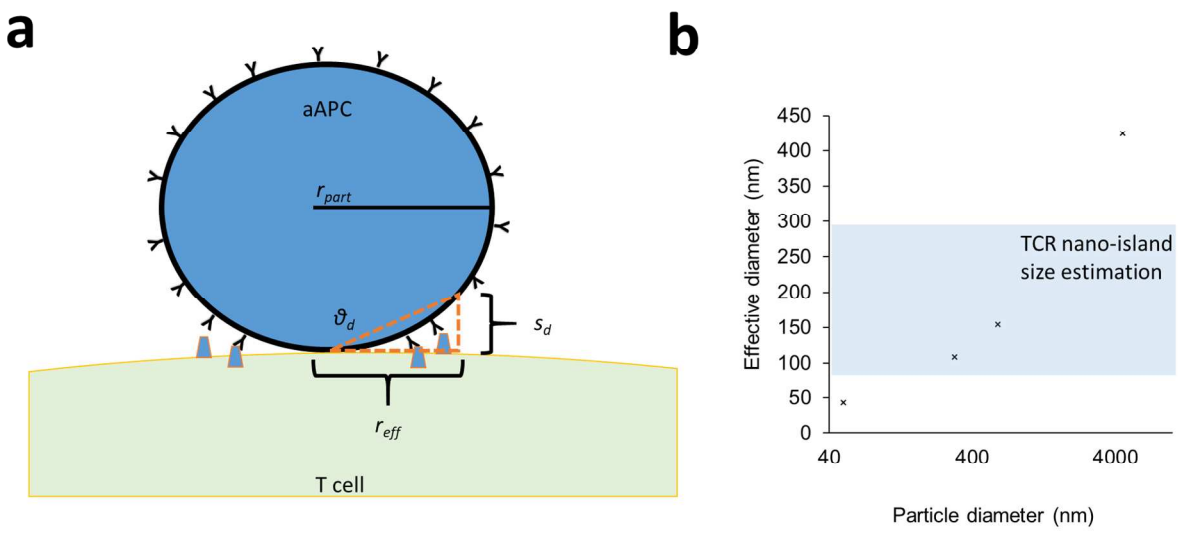


Figure S7. 50-nm aAPC require saturating dose of particles to provide T cell activation. (**a-b**) Dose-titrating amounts of particle aAPCs to activate T cells for 7 days determined lowest dose (number of aAPCs) and therefore (**a**) number of estimated TCRs per particle aAPC needed. The number of TCRs per T cell was estimated to be 50,000 based on literature values, and (**b**) surface area ratio of particle aAPC to T cell needed. The size of the T cell was simulated to be 10 μm (error bars show s.e.m., n=4).



(1) $$r_{eff} = \sqrt{{r_{part}}^2 - (r_{part} - s_d)^2}$$

(2) $$\theta_d = \tan^{-1}\frac{s_d}{r_{eff}}$$

(3) $$SA_{eff} = \frac{\theta}{\pi/2} \cdot \pi \cdot r_{part}^2$$

(4) $$\#\ ligand_{eff} = lig.dens./NP \cdot SA_{eff}$$

Figure S8. Spherical geometry of particle aAPC limits the actual size and number of ligands interacting with the T cell. (**a**) Schematic showing the defined effective radius (r_{eff}) of the particle aAPC. This is governed by the separation distance (s_d) between the pMHC on the surface of the aAPC. This is the distance that prevents attached pMHC from engaging TCR on the surface of the T cell due to distance. We find the r_{eff} using Equation 1, which is defined from the geometry of a circle. In order to define effective surface area as a percent of surface area we used Equations 2 and 3. Equation 2 finds the angle of the effective surface by using the sides of the defined triangle r_{eff} and s_d. We then use the effective surface area and nanoparticle ligand density to find the effective number of stimulatory ligands/particle from Equation 4. (**b**) Calculated effective diameters are plotted versus actual diameters to show estimated size of interaction for

aAPCs. Lightly shaded blue region indicates estimated T cell receptor nano-island estimations previously described.

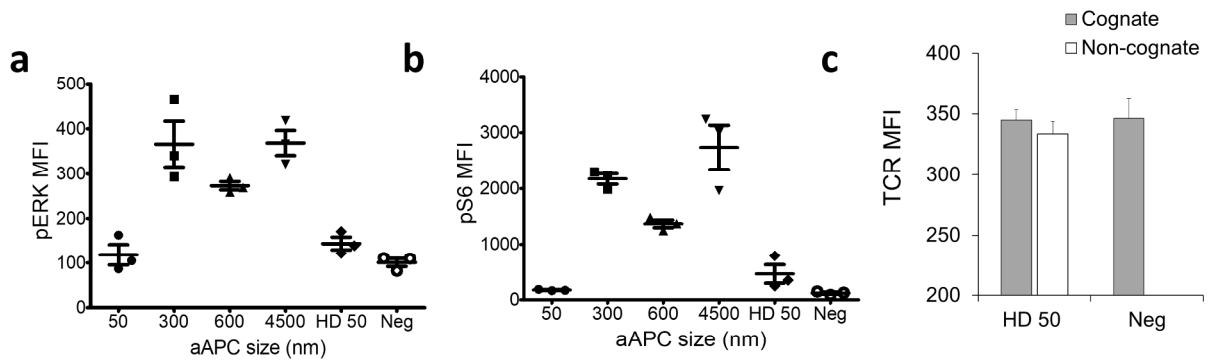


Figure S9. Ineffective activation of CD8+ T cells also observed for HD 50 nm aAPCs at a 2 pM dose with early activation events. (**a-b**) MFI of phosphorylation of (**a**) ERK and (**b**) ribosomal protein S6 for HD 50 nm aAPCs. Other groups were replotted for comparison purposes. (**c**) MFI for TCRβ of CD8+ T cells cultured with aAPCs at 37 °C for 5 hours for cognate and non-cognate aAPCs. Negative control was replotted for comparison purposes.

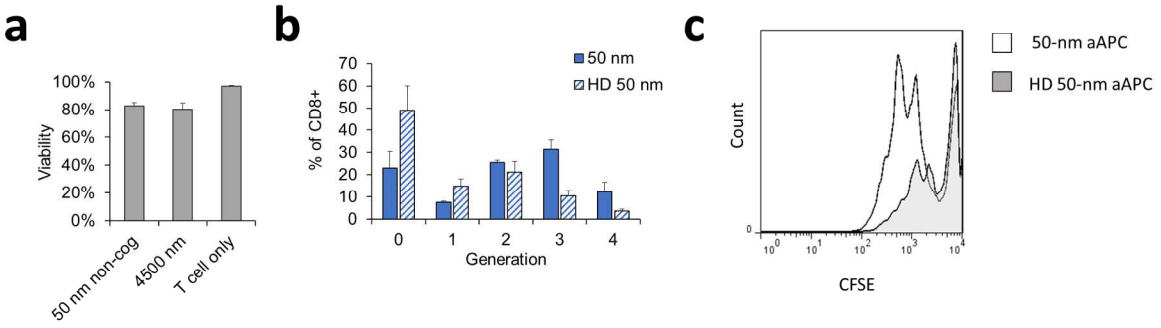


Figure S10. High concentrations of 50-nm aAPCs are not toxic due to particle doses, but to overactivation-induced cell death. (**a**) CD8+ T cells were incubated with 90 pM controlled non-cognate pMHC aAPCs for 7 days and viability was measured. T cells without aAPCs and T cells stimulated with traditional 4500 nm aAPCs are shown for comparison purposes for a normal antigen-specific stimulation (n=3). (**b**) CD8+ T cells were stimulated for three days and proliferation is characterized by CFSE dilution and characterization of percent of cells in each generation (n=3). (**c**) Representative flow cytometry plot of CFSE dilution experiment.

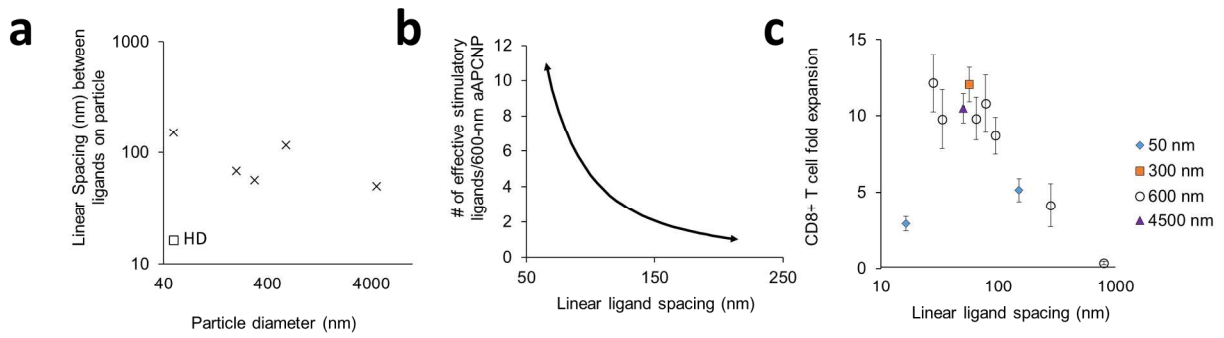


Figure S11. Linear ligand density and size of nanoparticle aAPCs are important for T cell stimulation. (**a**) Linear spacing between stimulatory molecules on the surface of the aAPC for different sized aAPCs. (**b**) Graph representing the relationship between the linear ligand density and the number of effective ligands presented from a 600-nm aAPC. (**c**) 600-nm aAPCs with different ligand densities were incubated with T cells at 2 pM concentration and fold expansion of CD8+ T cells was measured on day 7 and 50 nm, 300 nm, and 4500 nm data replotted for comparison (error bars show s.e.m.; n = 5).

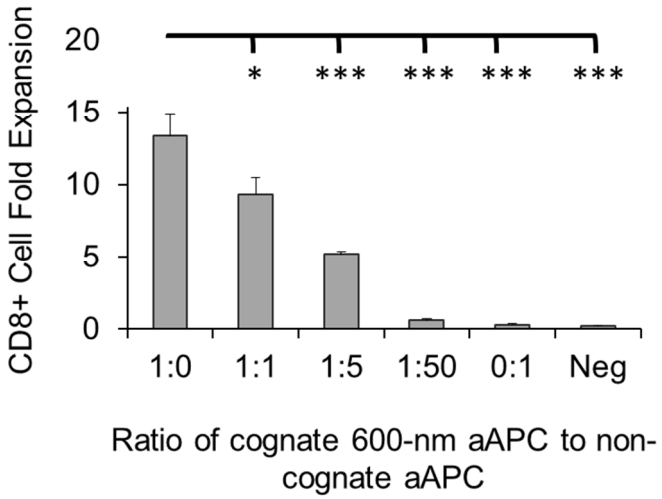


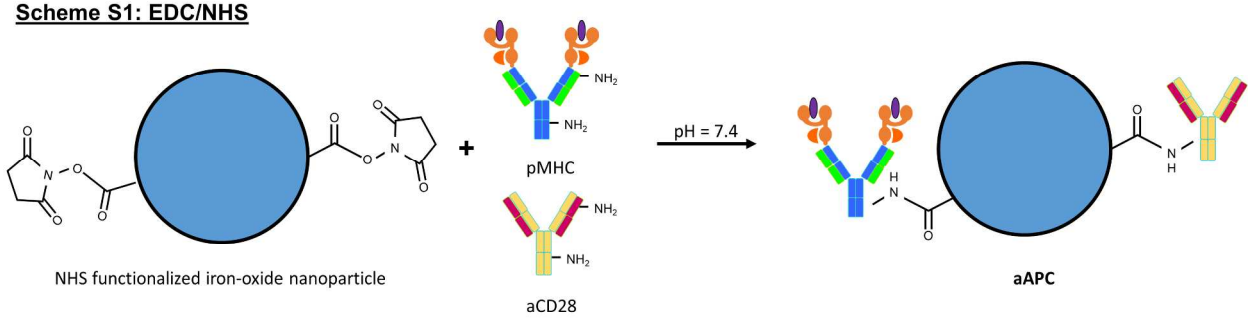
Figure S12. Addition of non-cognate aAPCs decreases the activation of antigen-specific T cells in a dose dependent manner. 600-nm cognate (Kb-SIY/anti-CD28) and 600-nm non-cognate (Kb-TRP2/anti-CD28) were mixed at the indicated ratios respectively and cultured for 7 days (error bars show s.e.m.; ***$p < 0.0005$, **$p < 0.005$, *$p < 0.05$, $n = 3$, one-way ANOVA with Dunnett's post test).

MATERIALS AND METHODS

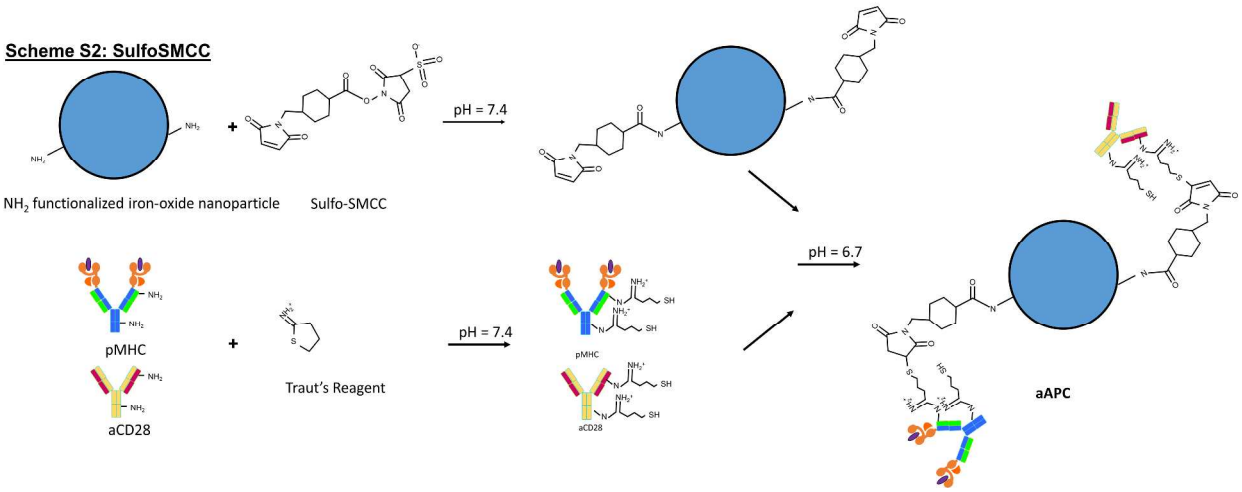
Mice and Reagents. 2C TCR transgenic mice were kept as heterozygotes by breeding on a C57/BL6 background. C57BL/6j mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were maintained per guidelines approved by the Johns Hopkins University's Institutional Review Board.

Particle Fabrication. Soluble MHC-Ig dimers, Kb-Ig, was produced in-house and loaded with peptides as described[1] and anti-CD28 antibody was purchased from Biolegend (37.51; BioLegend, San Diego, CA, USA). NHS (NHS) labeled super paramagnetic iron-oxide particles (SPIONs) were purchased from OceanNanotech (Springdale, AR, USA). Conjugation of MHC-Ig dimer and anti-CD28 proteins to the particles was carried out per manufacturer's recommendations (Scheme S1). Briefly, SPIONs were washed while on a STEM-cell magnetic column (Vancouver, Canada) with PBS three times and then incubated with equimolar amounts of MHC-Ig and anti-CD28 at ratios of 0.1 mg of total protein to 1 mg of SPIONs. SPIONs and protein were allowed to react for 24 hours at 4°C. Tris-HCl buffer was added to the SPIONs to quench any unreacted functional groups and SPIONs were subsequently magnetically washed three times with PBS with 2% bovine serum albumin (BSA). 4500 nm particles—Dynal Particles—were purchased from ThermoFisher (Halethorpe, MD). MHC-Ig and anti-CD28 were conjugated to the surface as established previously[2].

Scheme S1: EDC/NHS



Using NHS labeled magnetic nanoparticles worked effectively for a facile conjugation with our pMHC-Ig and anti-CD28; however, we were not able to substantially increase the density of the ligand by adding additional ligand to the reaction. To control the density and increase the ligand density on the particles we aimed to functionalize amine-coated iron oxide particles with a higher density of reactive groups. SPIONS were purchased from Micromod (Rostock, Germany) and functionalized according to manufacturer's recommendations (see https://www.micromod.de/daten/File/Technotes/Technote_202_1.pdf and https://www.micromod.de/daten/File/Technotes/Technote_201_2.pdf) (Scheme S2). Briefly, amines were then reacted with Sulfo-SMCC (Proteochem, Hurricane, UT) and then magnetically washed. MHC-Ig and anti-CD28 were modified with 2-iminothiolane (Traut's reagent) purchased from Sigma Aldrich (St. Louis, MO). Excess 2-iminothiolane was washed away from protein solution by using a Vivaspin 20 50kDa MWCO concentrator (GE Healthcare, Little Chalfont, United Kingdom). Traut's reagent targets the same free amines that would be targeted in the conjugation of the antibodies to NHS-labeled particles. Therefore, the orientation of the antibodies or Signals does not change between chemistries, only the linkers. Washed and activated particles and MHC-Ig and anti-CD28 were then mixed and reacted. The resultant product was washed and stored at 4°C. HD 50 nm aAPCs were conjugated by Miltenyi Biotec (Cologne, Germany).



Particle Characterization. NP size and zeta potential was measured using a Zetasizer DLS. Protein surface density was measured through a fluorescent detection assay. Briefly, particles were either stained with FITC-conjugated rat anti-mouse Ig λ1, λ2, λ3 light chain, clone R26-46, or FITC-conjugated mouse anti-armenian/syrian hamster IgG, clone G192-1 (BD Pharmingen, Franklin Lakes, New Jersey) for 1 hour at 4°C. Particles were washed three times with 1x PBS. Particles were then collected in 1x PBS and subsequently the fluorescence was quantified using a Synergy HTX Multi-mode florescent plate reader (BioTek, Winooski, VT, USA). Particle concentration was measured using a spectrophotometer at a 405 nm reference. Imaging of particle aAPCs by Transmission Electron Microscopy (TEM), was done on a FEI Tecnai 12 and Philips EM 420 electron microscopes. Samples were allowed to adhere to discharged nickel grid covered with carbon film for 30 minutes. Solution was removed with a Kim wipe and then the samples were stained with 2% uranyl acetate for 45 seconds. The grids were then washed three times and allowed to dry at room temperature.

***In Vitro* T Cell Activation.** Lymphocyte isolations were completed as previously described[3]. Briefly, CD8+ T cells were isolated from the spleen and lymph node from a 2C transgenic mouse by using a mouse CD8+ T cell negative isolation kit from Miltenyi Biotec (Cologne, Germany)

and following manufacturer's instructions. 2C transgenic mice have CD8+ T cells with identical TCRs, which recognize the SIY peptide loaded into the mouse pMHC Kb. All aAPCs had pMHC Kb loaded with SIY and co-stimulatory molecule anti-CD28, except for non-cognate aAPCs which had an irrelevant peptide, TRP2, loaded into pMHC Kb and co-stimulatory molecule anti-CD28, and 300 s1 particles which only contained pMHC (no costimulatory molecules). CD8+ T cells were activated by NPs at indicated doses as previously described[4]. Briefly CD8+ T cells were plated with nano-aAPCs at 100,000 cells/mL in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and T cell growth factor, a cytokine cocktail derived from condition media produced from stimulated human PBMC as previously described[5]. T cells were fed with media with double concentration of T cell growth factor on day 3. Fold expansion and viability was determined by harvesting and counting cells by Trypan Blue staining and hemocytometry. For magnetic stimulations, T cells were isolated and plated as described above, but tissue culture plates were set between two neodymium N52 disk magnets (K&J Magnetics, Jamison, PA, USA) as described previously[4].

For experiments determining the minimum surface area ratio of NP aAPC to CD8+ T cells, T cells were incubated with decreasing ratios of NP aAPCs. The activation cut-off was defined as CD8+ proliferation above 1-fold the initial number of CD8+ T cells as measured on day 7. The graphs were generated by a custom-MATLAB script, with the grid size showing the representative surface area of a NP aAPC and the shading demonstrating NP aAPC needed for activation.

Cell proliferation was also characterized by dilution of CellTrace™ CFSE Cell Dye per manufacturer's recommendations (ThermoFisher). Briefly, isolated CD8+ T cells were suspended in 1 mL of PBS, and 1 μL of CellTrace™ CFSE Cell Dye suspended at 5 mM was

added to the solution and placed in a cell incubator for 20 minutes at 37°C. Then 5 mL of complete RPMI-1640 medium supplemented with 10% fetal bovine serum was added and allowed to incubate in a cell incubator for 5 minutes at 37°C. Cells were then washed twice in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and counted. Cells were then stimulated for three days. On day 3, cells were counted and 200,000 cells were taken and stained with a solution of 1:100 APC-conjugated rat anti-mouse CD8a, clone 53-6.7 (BD Pharmingen) for 15 minutes at 4°C. Cells were then washed with FACS was buffer and read on a BD FACSCalibur.

T Cell Phosphorylation Analysis. 2C CD8+ T cells were isolated as described above and particle aAPCs were incubated with T cells for 30 minutes at 4°C and then activated for 30 minutes at 37°C. Samples were then washed in a 96 well plate with PBS and fixed with pre-warmed (37°C) BD Phosflow 1x Lyse/Fix Buffer (BD Biosciences, San Jose, CA) for 10 minutes at 37°C. Fixative was washed twice from the samples with PBS and pre-chilled (-20 °C) BD Phosflow Perm Buffer III was added dropwise to each sample and gently mixed and incubated at -20 °C for 30 minutes. This mixture was spun down and the supernatant was removed and then washed three times with FACS wash buffer. Samples were then stained with a solution of FACS wash buffer with 1:50 PE-conjugated rat anti-mouse CD8a, clone 53-6.7 (BD Pharmingen) and either a 1:100 Rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236), clone D57.2.2E, or Rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), clone 9101, or Rabbit IgG Isotype Control, clone DA1E (Cell Signaling Technology, Danvers, Massachusetts) for 45 minutes at room temperature. Samples were then washed with FACS wash buffer and then stained with a solution of FACS wash buffer with 1:250 of Alexa Fluor 647-conjugated Goat

anti-Rabbit IgG, polyclonal (ThermoFisher) for 45 minutes at room temperature. Samples were washed and resuspended with FACS wash buffer and read on a BD FACSCalibur.

T Cell Receptor Downregulation. 2C CD8+ T cells were isolated as described above and particle aAPCs were incubated with T cells for 30 minutes at 4°C and then activated for 5 hours at 37°C. Cells were washed with FACS wash buffer in a 96 well plate and then stained with a solution of FACS wash buffer with 1:100 PE-conjugated rat anti-mouse CD8a, clone 53-6.7 (BD Pharmingen) and 1:100 Alexa Fluor 647-conjugated anti-mouse TCR β chain, clone H57-597 (Biolegend) for 30 minutes at 4°C. Samples were then washed and resuspended with FACS wash buffer and read on a BD FACSCalibur.

T Cell Phenotype Characterization. After 7 days of culture, T cells were counted using a hemocytometer. 200,000 T cells were taken to stain for phenotypic markers. Cells were stained with a 1:100 solution of PE-conjugated rat anti-mouse CD62L, clone MEL-14 (BD Pharmingen), APC-conjugated rat anti-mouse CD8a, clone 53-6.7 (BD Pharmingen), PerCP-conjugated rat anti-mouse CD44, clone IM7 (Biolegend), and 1:1000 of LIVE/DEAD® Fixable Green Dead Cell Stain (ThermoFisher) for 15 minutes at 4°C. Cells were then washed with FACS wash buffer and read on a BD FACSCalibur. For CD122+, CD44+, CD8+ T cell experiments, the same protocol was followed, but a 1:100 solution of PE-conjugated Rat Anti-Mouse CD122, clone TM-Beta 1 (BD Pharmingen) was used instead of the PE-conjugated anti-mouse CD62L.

T Cell Cytokine Functionality Characterization. After 7 days of culture, T cells were counted using a hemocytometer. 500,000 T cells were taken per condition and separated into restimulation or no-stimulation groups. A solution of 1:350 BD GolgiStop Protein Transport Inhibitor (BD Biosciences) and 1:350 BD GolgiPlug Protein Transport Inhibitor (BD Biosciences) was added to the cells in RPMI-1640 medium supplemented with 10% fetal bovine

serum. Microparticle Dyanl-based aAPCs were added to cells to be restimulated at a 1:1 ratio. Cells were then allowed to incubate in a cell incubator for 6 hours at 37°C. Following the incubation, cells were washed and then stained with 50 μL of a 1:100 solution of PerCP-conjugated rat anti-mouse CD8a, clone 53-6.7 (Biolegend) and 1:1000 LIVE/DEAD® Fixable Aqua Dead Cell Stain (ThermoFisher) for 30 minutes at 4°C. Cells were then washed with PBS and 100 μL of BD Cytofix/Cytoperm Fixation and Permeabilization Solution was added to the cells and allowed to sit overnight at 4°C. Following the fixation step, 100 μL of 1x BD Perm/Wash Buffer (10x solution diluted to 1x in a solution of 2% bovine serum albumin in PBS) was added to the cells and washed. Cells were again washed with 200 μL 1x BD Perm/Wash Buffer. Cells were then stained with a solution of 1:100 solution of PE-conjugated rat anti-mouse IFNγ, clone XMG1.2 (BD Pharmingen), APC-conjugated rat anti-mouse IL2, clone JES6-5H4 (BD Pharmingen), and PE-Cy7-conjugated rat anti-mouse TNFα, clone MP6-XT22 (Biolegend) for 1 hour at 4°C. Cells were washed with FACS wash buffer and then read on a BD LSR II flow cytometer. Background cytokine staining was accounted for by subtracting cytokine positive cells in non-stimulated conditions from the re-stimulated cells.

Transmission Electron Microscopy (TEM) of T cells and Particles. Thin sections, 60 to 90 nm, were cut with a diamond knife on the Reichert-Jung Ultracut E ultramicrotome and picked up with Formvar coated 2x1 mm copper slot grids. Grids were stained with 2% uranyl acetate (in 50% methanol) followed by lead citrate, and observed with a Philips CM120 at 80 kV. Images were captured with an AMT XR80 high-resolution (16-bit) 8 Mpixel camera.

Effective Particle Diameter and Ligands Available Calculations. To calculate the average number of available ligands it is assumed the particles and the CD8+ T cells are spherical. The separation distance from the particle and cell is assumed to be 5 nm to be the limit where a

productive interaction to occur. This distance is used to calculate the effective radius of a particle's interaction with the CD8+ T cell with Equation 1, where r_{eff} is the effective radius, r_{part} is the radius of the particle and s_d the separation distance.

1) $$r_{eff} = \sqrt{{r_{part}}^2 - (r_{part} - s_d)^2}$$

The effective radius is used to find the angle of interaction between the particle and the CD8+ T cells using Equation 2, where θ_d is the angle between the particle and the CD8+ T cell to where s_d, and r_{eff} is the effective radius.

2) $$\theta_d = tan^{-1}\frac{s_d}{r_{eff}}$$

The angle of interaction between the particle and CD8+ T cell can be used to calculate the effective surface area engaged between the particle and the CD8+ T cell using Equation 3, where SA_{eff} is the effective surface area engaged, θ is the angle of interaction, and r_{part} is the radius of the particle.

3) $$SA_{eff} = \frac{\theta}{\pi/2} \cdot \pi \cdot r_{part}^2$$

The effective number of ligands presented by each particle can then be calculated with the effective engaged surface area of each particle with Equation 4, where $lig.\ dens./NP$ is the ligand density of each NP aAPC, and SA_{eff} is the effective surface area of each particle.

4) $$\#\ ligand_{eff} = lig.\ dens./NP \cdot SA_{eff}$$

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