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# How to Enrich and Expand Rare Antigen-specific T cells with Magnetic Nanoparticles

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June 18, 2018

Dear Editors:

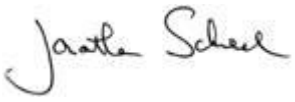
We are pleased to submit our manuscript titled "**Making Magnetic Nanoparticle Artificial Antigen-presenting Cells to Enrich and Expand Rare Antigen-specific T cells.**" for publication in *JoVE* as invited by Dr. Jialan Zhang, a science editor at *JoVE*.

We have centered our manuscript around our recent method of isolating and stimulating rare antigen-specific CD8+ T cells to high numbers and percentages after only one week. First, we describe in detail the loading of different antigens into the MHC. Second, we provide several different conjugation methods to attach peptide-MHC and co-stimulatory molecules to the surface of paramagnetic nanoparticles to form artificial antigen presenting cells. Third, we specify how to characterize these particle artificial antigen-presenting cells for quality control. Fourth, we outline the process for using a magnetic field to isolate these nanoparticles to enrich antigen-specific cells. Fifth, we describe how to expand the enriched fraction and detect antigen-specific CD8+ T cells after only seven days of culture.

*JoVE's* broad audience and video methods platform makes it an ideal journal for this work. Furthermore, we believe that this specific technical description of particle fabrication and assay with immunotherapeutic applications will draw interest from both clinical and biological fields to *JoVE*.

Thank you for your time and consideration.

Sincerely,



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**TITLE:**

# Enrich and Expand Rare Antigen-specific T cells with Magnetic Nanoparticles

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**KEYWORDS:**

Nanoparticles, magnetic enrichment, artificial antigen-presenting cells, T cells, immunotherapy, cancer, rare cell enrichment, T cell expansion, adoptive T cell therapy, Neoantigen, immunoengineering, bioengineering

**SUMMARY:**

Antigen-specific T cells are difficult to characterize or utilize in therapies due to their extremely low frequency. Herein, we provide a protocol to develop a magnetic particle which can bind to antigen-specific T cells to enrich these cells and then to expand them several hundred-fold for both characterization and therapy.

**ABSTRACT:**

We have developed a tool to both enrich and expand antigen-specific T cells. This can be helpful in cases such as to A) detect the existence of antigen-specific T cells, B) probe the dynamics of antigen-specific responses, C) understand how antigen-specific responses affect disease state such as autoimmunity, D) demystify heterogeneous responses for antigen-specific T cells, or E) utilize antigen-specific cells for therapy. The tool is based on a magnetic particle that we conjugate antigen-specific and T cell co-stimulatory signals, and that we term as artificial antigen presenting cells (aAPCs). Consequently, since the technology is simple to produce, it can easily be adopted by other laboratories; thus, our purpose here is to describe in detail the fabrication and subsequent use of the aAPCs. We explain how to attach antigen-specific and co-stimulatory signals to the aAPCs, how to utilize them to enrich for antigen-specific T cells, and how to expand antigen-specific T cells. Furthermore, we will highlight engineering design considerations based on experimental and biological information of our experience with characterizing antigen-specific

T cells.

## INTRODUCTION:

With the rise of many immunotherapies, there is a need to be able to characterize and control immune responses. In particular, the adaptive immune response is of interest because of the specificity and durability of the cells. Recently, chimeric-antigen-receptor T cell therapies have been approved for cancer therapy; however, the antigen-receptors are based off the common cell surface antigen CD19, instead of the antigens specific to the cancer[1]. Beyond the specificity, immunotherapies can also suffer from the lack of control, and limited understanding the dynamic immune response within cancer or autoimmunity.

One of the challenges of studying antigen-specific responses is their extremely low frequency, *e.g.*, antigen-specific T cells are 1 of every $10^4$ to $10^6$ T cells[2,3]. Thus, to investigate which T cells are present or responding, the cells need to either be enriched and expanded, or their signal need to be amplified. It is expensive and difficult to maintain the feeder cells using current techniques that focus on the expansion of antigen-specific cells. Current techniques that focus on amplifying the signal of antigen-specific T cells, like the enzyme-linked immunospot (ELISPOT) assay, limit the re-use of those T cells[4]. Finally, because of low sensitivity, often these two techniques need to be combined for antigen-specific enumeration.

To address these issues, we have developed the magnetic nanoparticle-based artificial antigen presenting cell (aAPC)[5–7]. The aAPC can be functionalized with an antigen-specific signal—peptide loaded major histocompatibility complex (pMHC)—and co-stimulatory molecules—*e.g.*, an anti-CD28 antibody—to both enrich antigen-specific T cells and then subsequently stimulate their expansion (**Figure 1**). The particles can thus be a cost-effective off-the-shelf product that can be both customized to meet antigen-specific stimulations yet standardized across experiments and patients. Performing the enrichment and expansion process results in hundreds to thousands-fold expansion of antigen-specific CD8+ T cells and can result in frequencies up to 60 percent after just one week, enabling the characterization or therapeutic use of the large number of cells. Herein, we describe how to make nanoparticle aAPCs, some critical design considerations in choosing the nanoparticle properties, and demonstrate some typical results from utilizing these particles in isolating and expanding rare antigen-specific CD8+ T cells.

## PROTOCOL:

All mice were maintained per guidelines approved by the Johns Hopkins University's Institutional Review Board.

### 1. Load Dimeric Major Histocompatibility Complex Immunoglobulin Fusion Protein (MHC-Ig) with Desired Antigen Peptide Sequence.

Note: If using H-2Kb:Ig, then follow the protocol detailed in Step 1.1; if using H-2Db:Ig, then follow the protocol detailed in Step 1.2.

1.1. Active loading of peptide sequence into H-2Kb:Ig.

1.1.1. Prepare necessary buffers. Prepare the denaturation buffer by making a solution of 150 mM NaCl and 15mM $Na_2CO_3$ in deionized water and then adjusting the pH to 11.5. Prepare the renaturation buffer by making a solution of 250 mM Tris HCl in deionized water and adjusting the pH to 6.8.

Note: Typically, it will require about 5 mL of both the denaturation and renaturation buffers for 1 mg of H-2Kb:Ig.

1.1.2. Denature H-2Kb:Ig to allow enhanced peptide binding. Bring the H-2Kb:Ig concentration to between 0.5-2 mg/mL with phosphate-buffered saline (PBS). Then dilute the H-2Kb:Ig to a final concentration of 100-200 µg/mL with 5-10 volume equivalents of denaturation buffer and allow to incubate at room temperature for 15 min.

1.1.3. Add 50 molar excess of peptide sequence (usually stock peptide is kept at 1 mM at -80 °C) to the H-2Kb:Ig solution.

Note: Usually peptide antigens will need to be dissolved within at least 10% dimethyl sulfoxide (DMSO) and then added slowly to PBS to remain soluble. Depending on the amino acid sequence, the amount of DMSO may need to be increased.

1.1.4. Renature H-2Kb:Ig with peptide. Immediately following the addition of the peptide, bring the solution to a pH of 7.4 by adding renaturation buffer. Allow the neutralized solution to incubate for 48 h at 4 °C.

1.1.5. Concentrate and wash peptide-loaded H-2Kb:Ig. Utilizing a centrifugal concentrator with a 50 kDa molecular weight cut-off (MWCO), follow the manufacturer's directions to wash the peptide-loaded H-2Kb:Ig solution 3 times with PBS, concentrate to at least 1 mg/mL and quantify the concentration on a spectrophotometer.

1.2. Active loading of peptide sequence into H-2Db:Ig.

1.2.1. Prepare necessary buffers. Prepare the denaturation buffer by making a solution of 131 mM citric acid, 150 mM NaCl, and 124 mM $Na_2HPO_4$ in deionized water and then adjusting the pH to 6.5. Prepare the renaturation buffer by making a solution of 120 mM Tris HCl in deionized water and adjusting the pH to 8.8.

Note: Typically, it will require about 5 mL of the denaturation buffer and 1 mL of the renaturation buffer for 1 mg of H-2Db:Ig.

1.2.2. Denature H-2Db:Ig to allow enhanced peptide binding. Bring the H-2Db:Ig concentration to a final concentration of 0.5-2 mg/mL with PBS. Then, dilute the H-2Db:Ig to a final concentration of 100-200 µg/mL with 5-10 volume equivalents of denaturation buffer.

1.2.3. Add 50 molar excess of peptide sequence (usually stock peptide is kept at 1 mM at -80 °C) to the H-2Db:Ig solution and allow to incubate for 1 h at 37 °C.

1.2.4. Add β2 microglobulin and renature H-2Db:Ig with peptide. Add 2-fold molar excess of β2 microglobulin. Then, bring the solution to a pH of 7.4 by adding renaturation buffer. Allow the neutralized solution to incubate for 24 h at 4 °C.

1.2.5. Concentrate and wash peptide-loaded H-2Db:Ig. Utilizing a centrifugal concentrator with a 50 kDa MWCO, follow the manufacturer's directions to wash the peptide-loaded H-2Kb:Ig solution 3 times with PBS, concentrate to at least 1 mg/mL and quantify the concentration on a spectrophotometer.

**2.** Conjugate MHC-peptide Complexes and Co-Stimulatory Molecules to the Surface of Magnetic Nanoparticles to Form Nanoparticle Artificial Antigen Presenting Cells. Use One of Three Different Methods Depending on Particle Size and Application.

Note: A number of different techniques can be used to conjugate the proteins to the surface of the particles. Herein, 3 separate approaches are described: amine-coated particles (Step 2.1), N-hydroxysuccinimide (NHS)-coated particles (Step 2.2), and anti-biotin-coated particles (Step 2.3). These processes have also been described in detail within the methods section of two papers published[6,7]. Perform all steps in a biosafety fume hood with sterile solutions to maintain the sterility of stock aAPC particles.

2.1. Coat antigen-specific and stimulatory signals to amine-coated magnetic particles (**Figure 2**). This process is described for 100 nm, amine-coated superparamagnetic nanoparticles.

Note: Detailed protocols for attaching the antibody to the surface of an amine coated particle can be found at https://www.micromod.de/en/technotes-2.html, where Technote 201 describes how to thiolate antibodies and conjugate to maleimide functionalize particles and 202 describe the process needed to functionalize amine-coated particles with maleimide functional groups. Here, only slight modifications are highlighted for the MHC-Ig and co-stimulatory signals attached to these particles.

2.1.1. Thiolate the antibodies with Traut's reagent (Technote 201).

2.1.1.1. Prepare a 10x PBS-ethylenediaminetetraacetic acid (EDTA) buffer (0.1 M PBS and 100 mM EDTA). Add the 10x PBS-EDTA buffer to the antibodies at a 1:10 ratio to prevent the oxidation of free thiols added to the antibodies.

2.1.1.2. Add 20 molar excess of Traut's reagent (2-iminothiolane) to the antibody and incubate for 2 h at room temperature with mixing. Measure out the Traut's reagent (dry powder) within a chemical fume hood to avoid respiration as it converts amine groups to thiol groups.

2.1.1.3. Wash thoroughly with 1x PBS-EDTA buffer 3 times using a centrifugal concentrator with

a 50 kDa MWCO, and follow the manufacturer's directions, concentrating until a final volume of 500 µL. Measure the concentration of the antibody solution using a spectrophotometer.

2.1.2. Convert the amine functional groups on the magnetic nanoparticle to maleimide groups using sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Technote 202).

2.1.2.1. Add the 10x PBS-EDTA buffer to the antibodies at a 1:10 ratio to the particles.

2.1.2.2. Dissolve Sulfo-SMCC in deionized water and vortex to resuspend at a concentration of 1 mg/mL. Measure out the Sulfo-SMCC (dry powder) within a chemical fume hood to avoid respiration as it converts amine groups to maleimide groups.

2.1.2.3. Add 0.016 nmol of the Sulfo-SMCC solution for every square millimeter surface area of the particles. For 1 mg of the 100 nm particles, use 0.3 mg of Sulfo-SMCC. Allow to react for 1.5 h at room temperature.

2.1.2.4. Wash the particles with 1x PBS-EDTA buffer 3 times using a magnetic field with a magnetic column and resuspend in 500 µL of 1x PBS-EDTA buffer.

Note: If using particles smaller than 200 nm, such as the 100 nm particles described herein, most likely permanent magnets will not be powerful enough to pull the particles for washing or concentrating purposes. Thus, to wash the smaller particles, use a magnetic column composed of ferromagnetic spheres to amplify the magnetic field.

2.1.3. React maleimide-functionalized particles with thiolated antibodies (Technote 201).

2.1.3.1. Add the particles to a glass scintillation vial, add a mini-magnetic stir bar, place just an inch above a magnetic stir plate, and induce magnetic mixing of the particle solution.

2.1.3.2. To the mixing solution, add the thiolated antibodies (0.5 mg of antibody for every 1 mg of particles) dropwise. Allow to react overnight at room temperature.

2.1.3.3. Wash with 1x PBS buffer 3 times using a magnetic field and resuspend in 500 µL of 1x PBS. Label and store at 4 °C for up to 6 months.

Note: Maximum conjugation efficiency occurs when maleimide-functionalized particles are immediately mixed with thiolated protein.

2.2. Coat antigen-specific and stimulatory signals to NHS-coated magnetic particles (**Figure 3**). This process is described for 200 nm, NHS-coated superparamagnetic nanoparticles.

2.2.1. Prepare the resuspension buffer, quenching buffer, and storage buffer. The resuspension buffer is 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) with 0.01% Tween 20 adjusted to

pH 6.0. The quenching buffer is a 100 mM solution of Tris-HCl at pH 7.4. The storage buffer is a solution of 10 mM PBS and 0.01% Tween at pH 7.4.

2.2.2. Resuspend the lyophilized particles in 1 mL of the resuspension buffer. Vortex vigorously for at least 15 min until no aggregates are visible.

2.2.3. Place the magnetic particles on a magnetic stand to remove the supernatant, resuspend with 0.5 mL of resuspension buffer and transfer to a glass scintillation vial. Vortex until no aggregates are visible.

2.2.4. Add 0.1 mg of total protein per 1 mg of resuspended particles. Vortex to mix and react at room temperature for 2.5 h while mixing.

2.2.5. Add 0.1 mL of quenching buffer and react at room temperature for 30 min while mixing.

2.2.6. Place the scintillation vial on a magnetic stand and wash the particles. Wait until the supernatant is clear to remove. Remove the particles from the magnetic stand, add 1 mL of resuspension buffer and vortex until no aggregates are visible. Repeat this process three times and resuspend the particles in 1 mL of resuspension buffer. Store the particles at 4 °C for up to 6 months.

2.3. Coat antigen-specific and stimulatory signals to anti-biotin-coated magnetic particles (**Figure 4**). This process is described for 50-100 nm, anti-biotin superparamagnetic nanoparticles.

2.3.1. Biotinylate MHC-Ig or co-stimulatory molecules.

2.3.1.1. Adjust the protein concentration to 0.5-2 mg/mL in PBS buffer. Resuspend sulfo-NHS-biotin at a concentration of 10 mg/mL in deionized water and add 20-fold molar excess to stimulatory antibody. Incubate at room temperature for 45 min.

2.3.1.2. Wash thoroughly with PBS 3 times using a centrifugal concentrator with a 50 kDa MWCO, follow the manufacturer's directions, concentrating until a final volume of 500 µL. Measure the concentration of the antibody solution using a spectrophotometer.

2.3.2. Conjugate biotinylated MHC-Ig and/or co-stimulatory signals to anti-biotin nanoparticles. For 500 µL of stock anti-biotin particles, add 0.5 nmol of stimulatory antibody and incubate at 4 °C overnight.

2.3.3. Wash conjugated aAPC nanoparticles. Because these particles are smaller than 200 nm, wet a magnetic column and put on a magnetic stand.

2.3.4. Add the particle/protein suspension to the column. Allow all the protein/particles to completely enter the column.

2.3.5. Wash by adding 0.5 mL of PBS three times to the column.

2.3.6. Elute by removing the column from the magnetic stand, adding 0.5 mL of PBS to the column, and using the plunger, expulse the particle aAPCs into a glass scintillation vial. Store particles at 4 °C for up to 6 months.

Note: Particles are stable at 4 °C (should not be frozen) for up to 6 months. Higher temperatures decrease the functionality of the particles and some particle aggregation have been observed (data not shown). Do not keep at room temperature for extended periods of time as this significantly decreases the shelf-life of the particles.

**3. Characterize the Protein Content on Artificial Antigen Presenting Cell Nanoparticles Using Fluorescent Antibody Detection.**

Note: This is a useful quality control of the produced artificial antigen presenting cells. Also, the amount of stimulatory signal is used to produce equivalent aAPC doses across batches and various aAPC types (*e.g.*, different sizes).

3.1. Measure the particle concentration of coated aAPCs.

3.1.1. Use unconjugated particles from the stock solution and make a 1:2 dose titration in a solution of PBS across a 96-well flat-bottomed tissue culture plate with 100 µL per well.

3.1.2. Read the particles on a plate-reading spectrophotometer at 405 nm to create a standard curve from a known particle concentration.

3.1.3. Take a sample of the conjugated aAPCs, dilute in PBS to a total volume of 100 µL and read on the spectrophotometer.

3.2. Remove a sample of fabricated aAPCs and stain with fluorescent antibodies.

3.2.1. To calculate how much sample to remove, estimate the number of antibodies on the surface of the particle.

Note: For these techniques, assume a density of around 1000 antibodies/$\mu m^2$ of particle surface area. To be able to detect the fluorescence, it requires about $10^{11}$ MHC-Ig or CD28 molecules total per fluorescent test.

3.2.2. Bring the total volume of aAPCs up to 100 µL in PBS, add the staining antibodies at a 1:100 dilution and incubate for 1 h at 4 °C.

Note: Example antibodies successfully used are FITC conjugated rat-anti mouse Ig λ1, λ2, λ3 light chain, clone R26 46 to detect MHC-Ig, and FITC conjugated mouse anti armenian/Syrian hamster IgG, clone G192-1, to detect anti-mouse CD28.

3.3. Wash the particles and read the fluorescence on a fluorescent plate reader.

3.3.1. Magnetically wash (as described in Step 2) the stained aAPC fractions three times with 0.5 mL of PBS.

3.3.2. Elute the washed aAPCs with 0.5 mL of PBS.

3.3.3. Add 100 µL of the eluted aAPCs to a 96-well flat-bottomed plate to read the concentration using the absorbance as in Step 3.1.

3.3.4. Take the remaining 400 µL, split up into two 200 µL aliquots and add to two wells in a black, polystyrene 96-well, flat-bottomed plate. Titrate at a 1:2 ratio down the plate by taking 100 µL of the solution and mixing with the next well that has 100 µL of PBS in each well at least four times.

Note: Average multiple replicates of the measurement to reduce the noise in the measurement.

3.3.5. On the same black 96-well plate, make a standard curve of the fluorescent antibody used to stain, by adding it at 1:200 in a well with 200 µL of PBS and titrating down at 1:2 ratio for at least 12 wells.

3.3.6. After both aAPC and fluorescent antibodies are on the plate, read the plate with a fluorescent plate reader.

3.4. Calculate the amount of protein per particle. Determine the concentration of antibody by comparing the values to the standard curve, where the antibody concentration is known, assuming a 1:1 ratio of staining antibody to detected antibody. Then dividing this concentration of detected antibody with the concentration of particles determined by the absorbance assay will give the number of antibodies per particle.

## 4. Enrich Antigen-specific CD8+ T Cells with Prepared Nanoparticle Artificial Antigen Presenting Cells.

4.1. Isolate CD8+ T cells.

4.1.1. Euthanize the animals by exposure to isoflurane followed by cervical dislocation.

4.1.2. Remove the spleen and lymph nodes from wildtype C57BL/6j mice and place in a solution of PBS. Macerate the organs and elute the cells through a sterile 70 µm cell strainer with frequent washes of PBS.

4.1.3. To eliminate non-CD8+ T cells, use a no-touch CD8+ T cell isolation kit and follow the manufacturer's instructions.

Note: Each antigen condition requires at least 3 x $10^6$ CD8+ T cells.

4.2. Add the nanoparticle aAPCs to bind to the CD8+ T cells.

4.2.1. Following the isolation, concentrate to a volume of 100 µL in PBS with 0.5% bovine serum albumin (BSA) and 2 mM EDTA.

4.2.2. Determine the number of aAPCs to add by calculating based on the ratio of $10^{11}$ aAPC-bound, peptide-loaded MHC-Ig for every $10^6$ CD8+ T cells.

4.2.3. Incubate aAPC particles and CD8+ T cells for 1 h at 4 °C with continual mixing in a sterile 5 mL polystyrene round bottom tube.

4.3. Prepare supplemented media and T cell growth factor (TCGF) to elute and culture CD8+ T cells.

4.3.1. For supplemented media, supplement complete RPMI 1640 media (with glutamine) with 1x non-essential amino acids, 1 mM sodium pyruvate, 0.4x vitamin solution, 92 µM 2-mercaptoethanol, 10 µM ciprofloxacin and 10% fetal bovine serum (FBS).

4.3.2. To make TCGF, follow the protocols already established and referenced here[8].

Note: TCGF is an in-house cocktail of human immune cytokines which is essential to provide T cells with additional stimulation signals needed to grow. TCGF could be exchanged for known T cell stimulatory cytokines such as IL-2, IL-7, or IL-15; however, each may polarize the T cell response accordingly. The protocol described herein has not been optimized with these cocktails; thus other techniques should be consulted for concentrations and combinations if TCGF is not used with examples listed[9, 10].

4.4. Wash and enrich aAPC and CD8+ T cell mixture.

4.4.1. Wash the magnetic particle aAPCs as described in Step 2. However, wash first using the PBS buffer with 0.5% BSA and 2 mM EDTA, second using supplemented media, and third using supplemented media with 1% TCGF.

4.4.2. Elute aAPCs and enriched CD8+ T cells in 500 µL of supplemented media with 1% TCGF.

4.4.3. Count the cells using a hemocytometer and plate in a 96 U-bottomed plate in 160 µL per well of supplemented media with 1% TCGF at a concentration of 2.5 x $10^5$ CD8+ T cells/mL.

4.4.4. If isolating using aAPCs with only peptide-loaded MHC-Ig on the surface (no co-stimulatory signals), then complete Step 4.5. If isolating using aAPCs with both peptide-loaded MHC-Ig on the surface and co-stimulatory signals, then proceed to Step 5.

4.5. Add the magnetic particles coated with co-stimulatory signals on the surface to the enriched fraction and add a magnetic field to co-cluster the stimulatory signals on the surface of the T cells.

4.5.1. To the enriched fractions, add an equimolar (or greater depending on the application—see section about aAPC properties to control) of stimulatory antibody to the number of peptide-loaded MHC-Ig on the particle.

4.5.2. Allow co-stimulatory magnetic particles to bind to enriched CD8+ T cells for 1 h at 4 °C.

4.5.3. Add magnetic field by placing the culture plate between two neodymium N52 disk magnets of 1.9 cm (0.75 inches) in length.

Note: N52 disk magnets have an extremely strong field. Care should be taken both to store them with spacers between magnets, as it is hard to remove from one another, and when putting them on the culture plates. To minimize the magnets from sticking to the metal components of the incubator, place them in 50 mL conical tube Styrofoam containers on both the bottom and the top.

**5. Expand and Detect Antigen-specific CD8+ T Cells with Prepared Nanoparticle Artificial Antigen Presenting Cells.**

5.1. Add the 96 U-bottomed well plate with aAPCs and CD8+ T cells in a humidified 5% $CO_2$, 37 °C incubator for 3 days. On day 3, feed the cells with 80 µL per well of supplemented media with 2% TCGF and place back into the incubator until day 7.

5.2. On day 7, harvest the stimulated cells into a 5 mL round bottom tube for counting.

5.3. Once all the of solution is harvested, spin down the harvested cells to resuspend in 0.5 mL of PBS with 0.05% sodium azide and 2% FBS. Count viable cells by staining with trypan blue and counting on a hemocytometer.

5.4. Remove 50,000-500,000 counted cells into two new 5 mL round bottom tubes for antigen-specific staining. One tube will be used for the cognate peptide-MHC stain, and the other tube will be used for the non-cognate stain to determine background staining.

5.5. Add 1 µg of biotinylated MHC-Ig (using the technique described in Step 2) to the respective cognate and non-cognate tubes in 100 µL of PBS with 0.05% sodium azide and 2% FBS with allophycocyanin (APC)-conjugated rat anti-mouse CD8a, clone 53-6.7 (dilution ratio of 1:100) for 1 h at 4 °C.

5.6. Add secondary streptavidin and live dead stain. Wash out excess biotinylated MHC-Ig with PBS through centrifugation. Then stain all samples with a 1:350 ratio of phycoerythrin (PE)-

labeled streptavidin and 1:1000 ratio of a live/dead fixable green dead cell stain for 15 min at 4 °C.

5.7. Read all samples on a flow cytometer to determine the specificity and the number of antigen-specific cells.

5.7.1. Wash out excess secondary and live/dead stain by centrifugation and resuspend with 150 µL of PBS buffer with 0.05% sodium azide and 2% FBS to read on a flow cytometer.

5.8. Determine the number and percent of antigen-specific cells with data analysis software.

5.8.1. To determine the percent of antigen-specific cells, use the following gates in the respective order live+, lymphocyte+ (forward scatter by side scatter), CD8+, and Dimer+. Determine the Dimer+ gate by comparing the non-cognate to the cognate stain.

5.8.2. Determine the percentage of antigen-specific cells in a sample by subtracting the percentage of Dimer+ of the cognate MHC-Ig stain from the non-cognate MHC-Ig stain.

5.8.3. Using this percentage of antigen-specific cells, multiply it by the number of cells counted, yielding the number of antigen-specific cells resultant from the enrichment and expansion.

Note: Compensation will have to be set up on the flow cytometer since there is spectral overlap with the fluorophores used in this panel.

**REPRESENTATIVE RESULTS:**

To complete a successful enrichment and expansion of antigen-specific T cells, the peptide-loaded MHC-Ig and co-stimulatory molecules should be successfully attached to the aAPC particle. Based on the 3 methods of particle attachment, we provide some representative data for a successful conjugation procedure outcome (**Figure 5a**). Indeed, if the ligand density is too low, then there will not be effective stimulation of antigen-specific CD8+ T cells where this occurs around linear spacing between the ligands above 100 nm in our experience (**Figure 5b**)[7].

Besides both quantitative fluorescent antibody readouts and transgenic CD8+ T cell expansions, nanoparticle aAPCs can be checked for quality control by doping in cognate transgenic antigen-specific CD8+ T cells. This can be done by isolating CD8+ T cells from a transgenic mouse such as a Pmel mouse which has gp100-specific antigen-specific CD8+ T cells and doping into a B6 background at a 1:1000 ratio. Counting and staining before and after enrichment allows the enumeration of both the fold enrichment (**Figure 6a**) and percent recovery (**Figure 6b**)[6]. In these representative results, we demonstrate that signal-1 only aAPCs provide the most efficient enrichment (nearly 10-fold) and around 80% cell recovery, which is enhanced over traditional signal 1 and 2 aAPCs which have non-specific anti-CD28 on the particle as well.

Once particle aAPCs have been sufficiently characterized and quality controlled, then they can be used in the enrichment and expansion of rare antigen-specific CD8+ T cells from wildtype mice.

For accurate results, it is critical to have functional detection reagents, such as the biotinylated dimer. The quality control of the biotinylated dimer can also be done on transgenic CD8+ T cells to verify staining. Here, representative results show the positive staining with gp100-specific CD8+ T cells with B6 CD8+ T cells as a background control (**Figure 7**). **Figure 7** also demonstrates that if there are too high of levels of the biotinylated dimer, then it will decrease its avidity as it will compete with itself and exhibit mono-valent binding.

After the enrichment and expansion of mouse CD8+ T cells for seven days, one might expect between 5 and 50 percent antigen-specific CD8+ T cells, with nearly 20,000 to 200,000 antigen-specific CD8+ T cells after starting with 5 x $10^6$ CD8+ T cells per condition (**Figure 8**)[6]. Specifically, when staining for antigen-specific CD8+ T cells, it is critical to know the background staining of the biotinylated dimer, where in this case it was 4.15%; any percentage lower than this from the cognate stain is considered a negative result (**Figure 8a**). Additionally, this will show where to draw the flow cytometry gates to determine the actual percentage of antigen-specific CD8+ T cells. This is important in cases where antigen-specific CD8+ T cells do not have distinct populations (as shown in **Figure 8a**) but may appear as a broad smear.

The same process can be used to isolate and stimulate human antigen-specific CD8+ T cells. Similar quality control and results should be seen where substantial increases in percentages and numbers of antigen-specific CD8+ T cells are observed after only one week of expansion following the enrichment (**Figure 9**)[5].

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic of the process of antigen-specific enrichment and expansion using nanoparticle artificial antigen-presenting cells.** First, complete a no-touch CD8+ T cell isolation. Then, add nanoparticle aAPCs to the CD8+ T cells. Enrich with a magnetic field, culture, and stimulate with aAPCs. Finally, detect enriched and expanded antigen-specific CD8+ T cells by flow cytometry.

**Figure 2: Schematic for conjugating peptide-loaded MHC-Ig and co-stimulatory molecules to the surface of amine-coated magnetic particles.** Briefly, Sulfo-SMCC crosslinker is used to functionalize the magnetic particle surface with maleimide functional groups. MHC-Ig and co-stimulatory molecules are simultaneously functionalized with Traut's reagents to produce thiol functional groups. The activated particles and protein signals are reacted together and then washed to produce antigen-specific artificial antigen-presenting cell magnetic nanoparticles. This figure has been modified from supplemental material of our laboratory's publication in *Nano Letters*[7].

**Figure 3: Schematic for conjugating peptide-loaded MHC-Ig and co-stimulatory molecules to the surface of NHS-coated magnetic particles.** Briefly, the NHS-coated particles are reacted together with peptide-loaded MHC-Ig and co-stimulatory molecules and then washed to produce antigen-specific artificial antigen-presenting cell magnetic nanoparticles. This figure has been modified from supplemental material of our laboratory's publication in *Nano Letters*[7].

**Figure 4: Schematic for conjugating peptide-loaded MHC-Ig and co-stimulatory molecules to the surface of anti-biotin-coated magnetic particles**. MHC-Ig and co-stimulatory molecules are functionalized with NHS-biotin to produce biotin functional groups. Then the anti-biotin-coated particles are reacted together with the functionalized peptide-loaded MHC-Ig and co-stimulatory molecules. Afterwards, these particles are washed to produce antigen-specific artificial antigen-presenting cell magnetic nanoparticles. This figure has been modified from supplemental material of our laboratory's publication in *Nano Letters*[7].

**Figure 5: Conjugation efficiency is critical for the enrichment and expansion of antigen-specific T cells.** (a) Representative data for conjugation efficiency with the three conjugation methods to three different base magnetic particles described in the paper: amine-coated particles, NHS-coated particles, and anti-biotin-coated particles. Each data point represents a different particle preparation technique and error bars represent S.E.M. (b) How ligand density affects transgenic CD8+ T cell stimulation, where the ligand density is represented as linear spacing between ligands in nanometers on 600 nm and 50 nm aAPCs (n = 5 and error bars represent S.E.M.). This figure has been modified from our laboratory's publication in *Nano Letters*[7].

**Figure 6: Quality control of aAPC enrichment.** Transgenic Pmel gp100-specific CD8+ T cells were doped in at a 1:1000 ratio into wildtype B6 CD8+ T cells. (a) Fold enrichment was measured using flow cytometry following the enrichment by staining the congenic marker Thy1.1 and CD8. Here was a comparison between signal 1 only particles or Db-Ig loaded with gp100, traditional signal 1 and 2 particles or Db-Ig loaded with gp100 and anti-CD28, and non-cognate signal 1 and 2 particles. (b) Cells were also counted before and after to measure the cell recovery by each of the methods. Data represents three independent experiments and error bars represent S.E.M. Data combined was measured by one-way ANOVA with Tukey's post-test (*$p$<0.05, **$p$<0.01). This figure has been modified from our laboratory's publication in *Nano Letters*[6].

**Figure 7: Quality control of biotinylated dimer.** Gp100-specific CD8+ T cells were isolated from a transgenic Pmel mouse and stained in 100 μL of PBS with three concentrations of biotinylated Db-Ig loaded with gp100 and APC anti-CD8a, using wildtype B6 CD8+ T cells as a negative control.

**Figure 8: Enrichment and expansion of antigen-specific CD8+ T cells.** B6 wildtype CD8+ T cells were enriched with either signal 1 only (Kb-Ig loaded with TRP2) or signal 1 and 2 (Kb-Ig loaded with TRP2 and anti-CD28 conjugated to the surface of the particle). Signal 2 was then added to the enriched fraction of signal 1 only aAPCs and all cells were cultured for 7 days. (a) CD8+ T cells are stained and gated on a live/dead fluorescent stain, then gated CD8+ and KbTRP2+, and compared to a non-cognate Kb-Ig to detect antigen-specific CD8+ T cells. (b) percentage and (c) number of TRP2-specific CD8+ T cells could thus be determined, where higher percentages and numbers of antigen-specific CD8+ T cells could be detected from the signal 1 only enrichment approach (n=7, error bars represent standard deviation, two-tailed paired t test *$p$ < 0.05, **$p$ < 0.01). This figure has been modified from our laboratory's publication in *Nano Letters*[6].

**Figure 9: Enrichment and expansion of human antigen-specific CD8+ T cells.** (a) Representative flow cytometry plots on day 0 before the enrichment and day 7 show the dramatic effects of

enriching and expanding antigen-specific CD8+ T cells from healthy donors with traditional nanoparticle aAPCs where A2-Ig loaded with NY-ESO1 and A2-Ig loaded with MART1 antigens are shown. (b) This generates high percentages (~10-20%) and numbers (0.5-1 x $10^6$) of antigen-specific CD8+ T cells by day 7 (n = 3 from independent donors, error bars represent S.E.M.). This figure has been modified from our laboratory's publication in *ACS Nano*[5].

**Supplementary File 1-Box 1.**

**Supplementary File 2-Box 2.**

**DISCUSSION:**

We have created a novel antigen-specific T cell isolation technology based on nanoparticle artificial antigen presenting cells (aAPCs). Nanoparticle aAPCs have peptide-loaded MHC on the surface that allows antigen-specific T cell binding and activation alongside co-stimulatory activation. aAPCs are also paramagnetic, and thus can be used to enrich rare antigen-specific T cells using a magnetic field. We have optimized and studied key nanoparticle properties of size, ligand density, and ligand choice and their influence on binding, enrichment, activation, and cell-enrichment (**Supplementary File 1-Box 1**).

Thus, the enrichment and expansion procedure results in antigen-specific CD8+ T cell expansion of several thousand-fold producing antigen-specific percentages as high as 60% and, can be used in both murine and human settings (**Supplementary File 2-Box 2**). Such high numbers and percentages of antigen-specific T cells enable the characterization of immune responses for diseases (*e.g.*, cancer, autoimmune, etc.), allow for the discovery of novel immune targets and mechanisms, and offer the opportunity to be used in adoptive immunotherapy. An example of a specific application is to sequence a patient's tumor, identify mutations, locate potential MHC-binders from the mutant sequences, produce aAPCs with those top candidate antigens, and then utilize the aAPCs to determine whether the patient has any tumor-specific neoantigens.

Indeed, methodological limitations have been a key barrier to studying and identifying antigen-specific responses. Current techniques (a) require substantial time- and work-intensive procedures, (b) present difficulty in maintaining cell lines such as the need to collect autologous dendritic cells, (c) require weeks of T cell expansion prior to obtaining results, (d) result in low specificities (1-2%) and low numbers of antigen-specific CD8+ T cells, (e) often with significant background signal, and (f) the CD8+ T cells that are produced often cannot be used or studied in further assays. One method requires immunization with antigen prior to ELISPOT to characterize the presence of antigen-specific response[13–16]. Another method utilizing tandem-mini-gene expression plasmids to transfect antigen presenting cells requires multiplexing tetramer stains with cytokine+ responses such as IFNγ to increase the sensitivity[17]. Even peptide pulsing endogenous antigen presenting cells in *in vitro* culture, only results in a 0.5% increase in antigen specificity[14].

Our approach solves these methodological limitations and can thus act as a diagnostic and therapeutic tool. Critical steps to ensuring antigen-specific CD8+ T cell enrichment and expansion

are to 1) effectively load MHC-Ig with peptide antigen, 2) conjugate stimulatory signals to the surface of nanoparticles, 3) bind the particles to T cells, 4) enrich the cells bound to the nanoparticles with a magnetic field, 5) expand eluted nanoparticle-bound T cells in culture, and 6) detect antigen-specific CD8+ T cells on day 7 with biotinylated, peptide-loaded MHC.

The main problems that emerge in the enrichment and expansion protocol arise from either improper production or expired detection reagents or nanoparticle aAPCs. Ensure that the biotinylated dimer can stain antigen-specific CD8+ T cells with testing on transgenic antigen-specific CD8+ T cells. If the peptide-MHC-Ig does not have a corresponding transgenic mouse model, it can be helpful to load a positive control peptide and test the positive control to verify loading. However, some peptides may not load into the MHC-Ig; this can be simulated with MHC-loading algorithms such as Net-MHC, or experimentally with RMAS-cell based assays[12]. aAPC particle stability may decrease after 6 months, so if there is some variability in enrichment and expansion results, then another fluorescent plate reader assay may be performed to verify the stability.

In future work, we aim to extend the capabilities, breadth, and depth of the assay. We are working on increasing both the throughput and the ability to multiplex with multiple antigens investigated at one time in a 96-well plate format. Currently, a main limitation is that only a few antigens can be investigated simultaneously. We are working this by investigating how the size of the particle aAPC and ligand density influences enrichment. Additionally, we are examining how different cell compositions effect CD8+ T cell expansion within culture. Finally, we aim to mimic this technology within MHC class II to be able to enrich and expand antigen-specific CD4+ T cells.

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**DISCLOSURES:**

The authors declare the following competing financial interest(s): under a licensing agreement between NexImmune and The Johns Hopkins University, Jonathan Schneck is entitled to a share of royalty received by the university on sales of products described in this article. He was also a founder of NexImmune and owns equity in the company. He serves as a member of NexImmune's Board of Directors and scientific advisory board. The terms of these arrangements have been reviewed and approved by The Johns Hopkins University in accordance with its conflict of interest policies.

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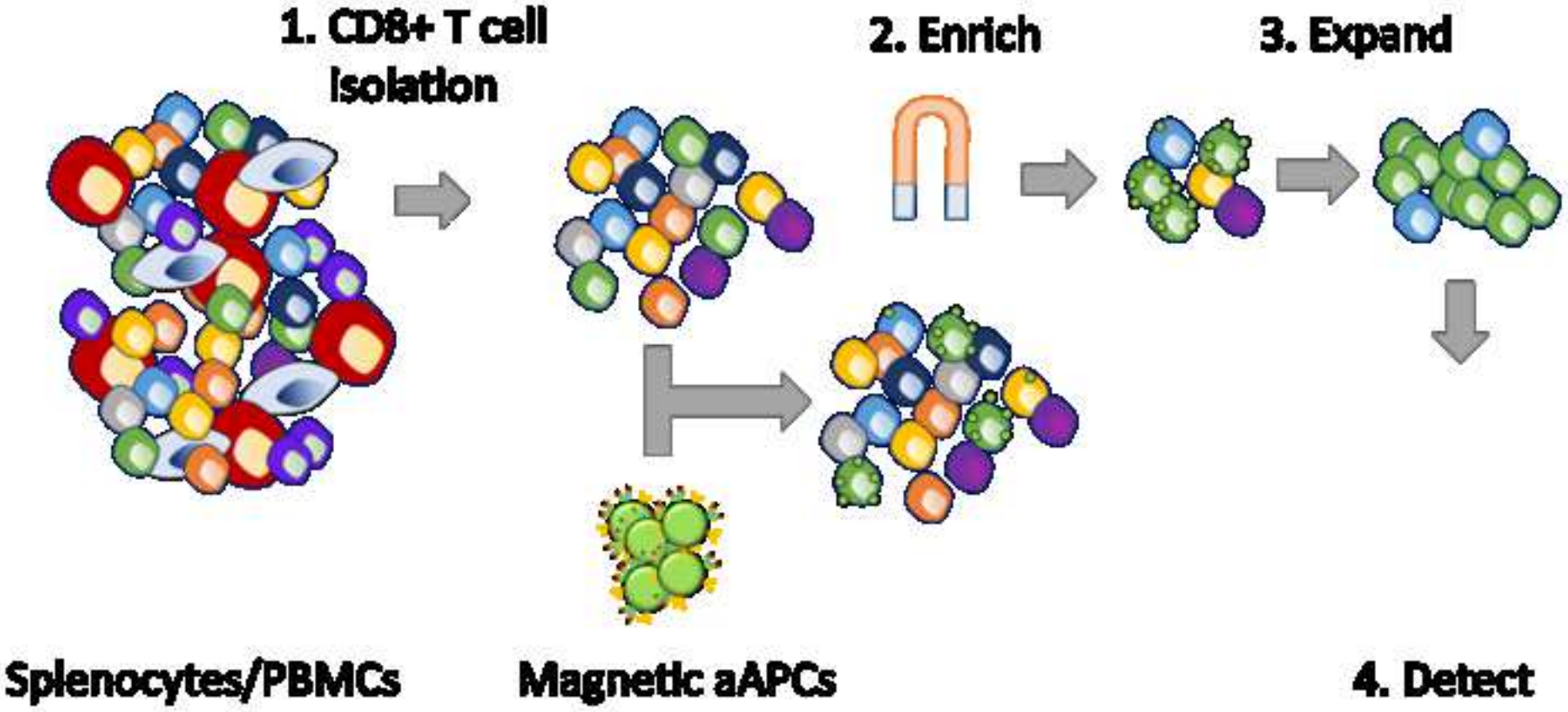

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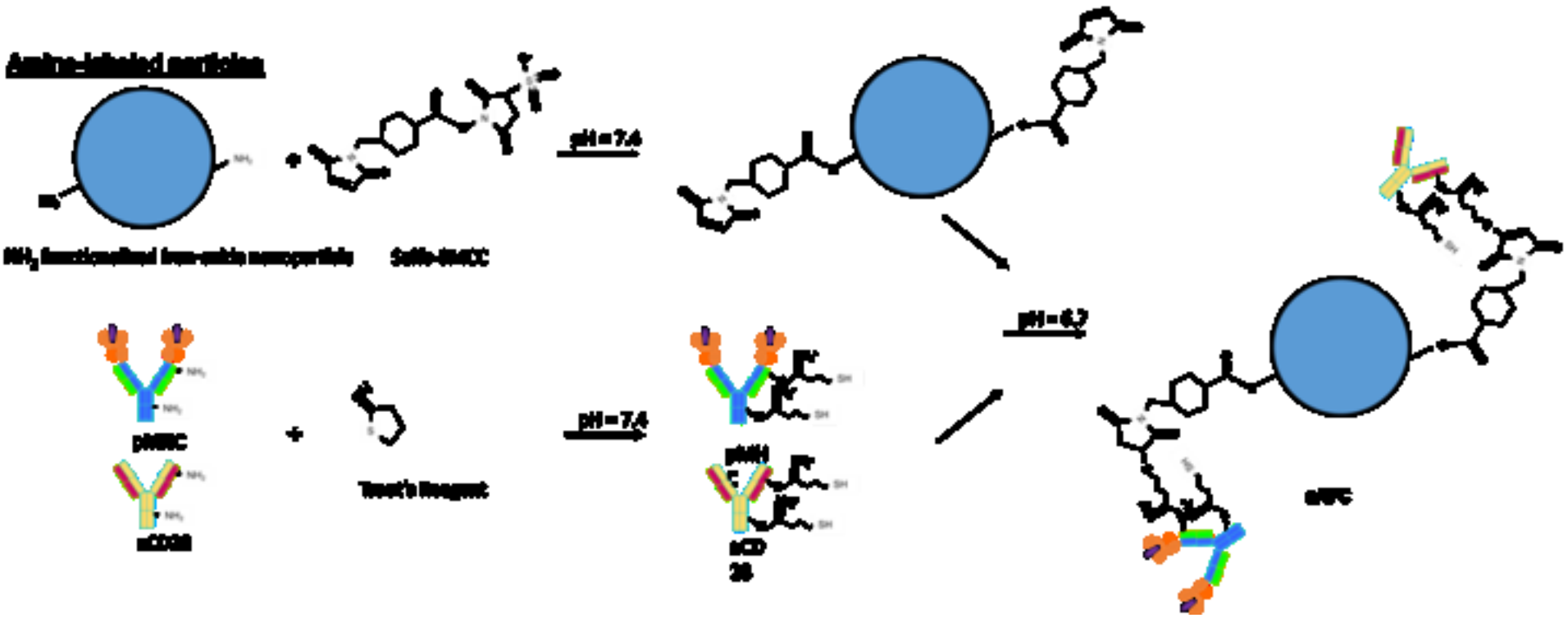

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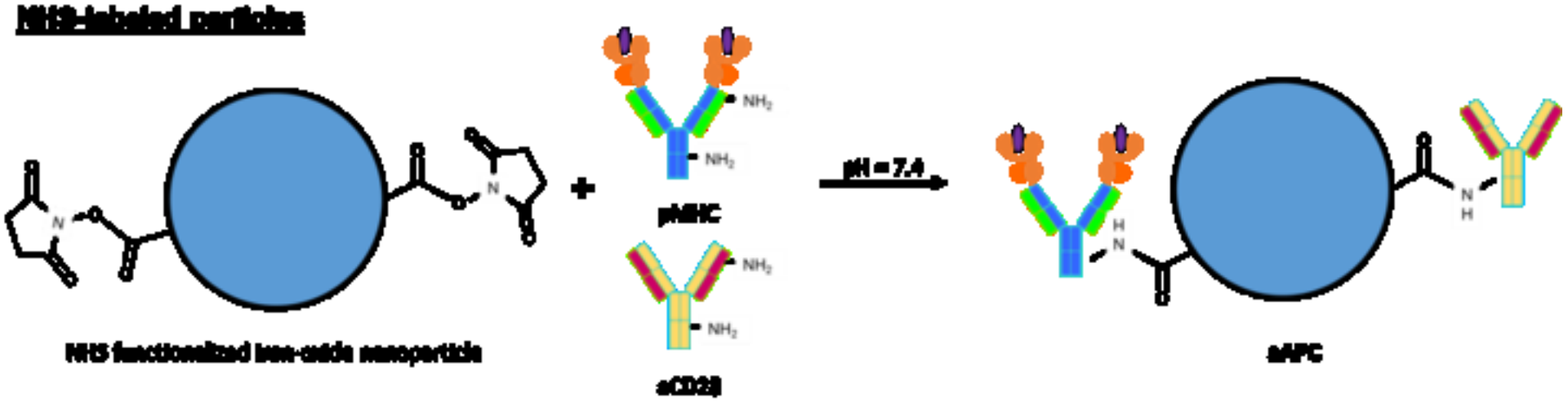

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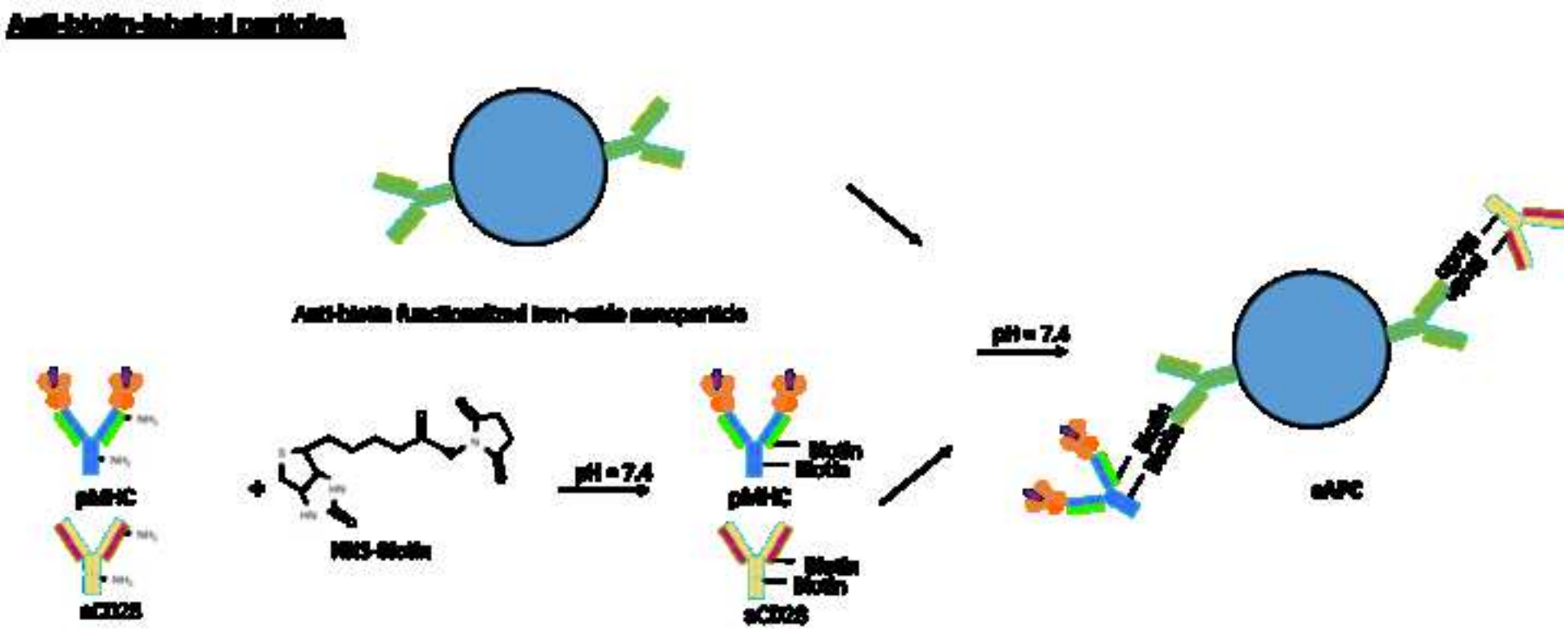

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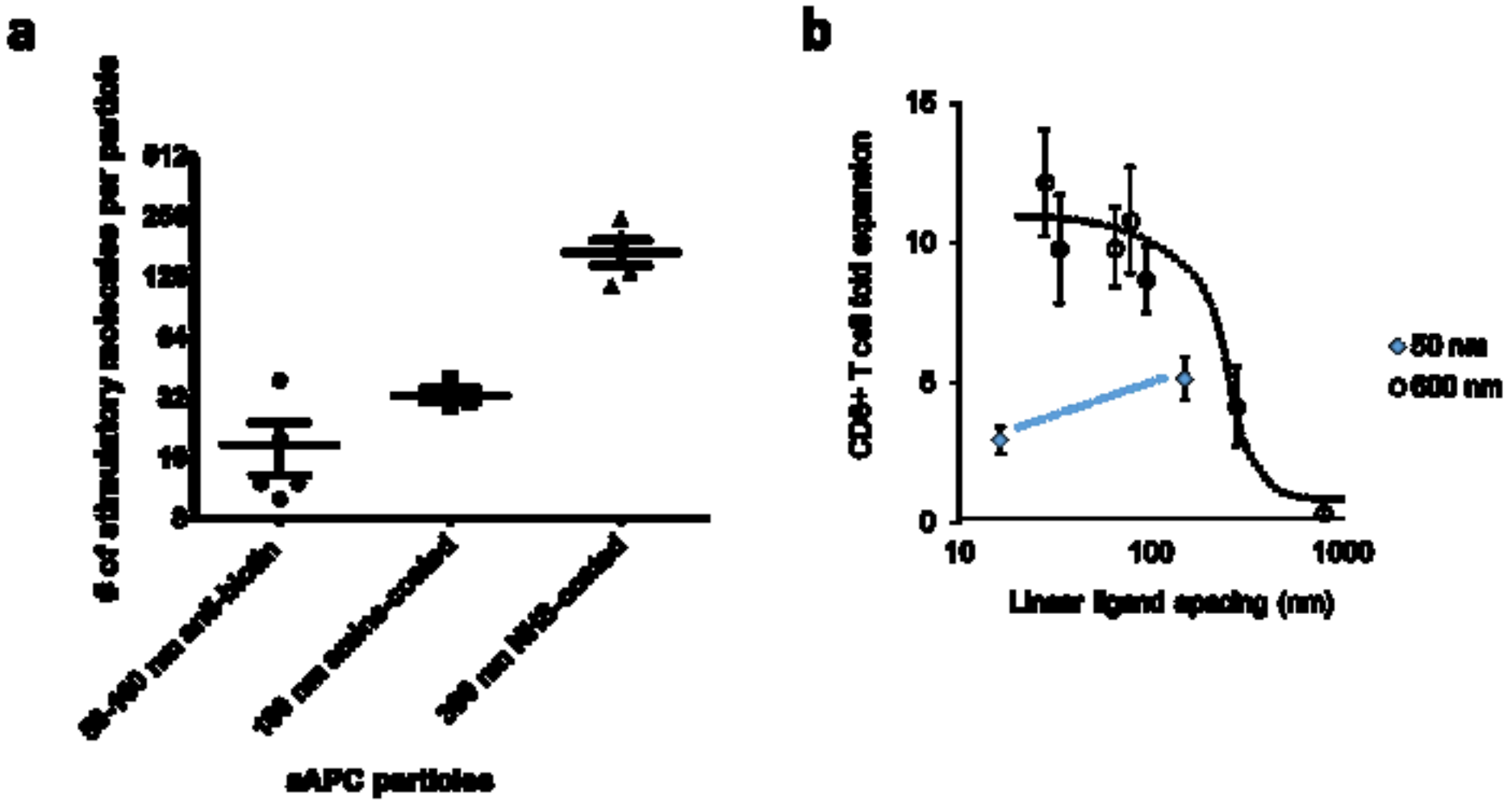

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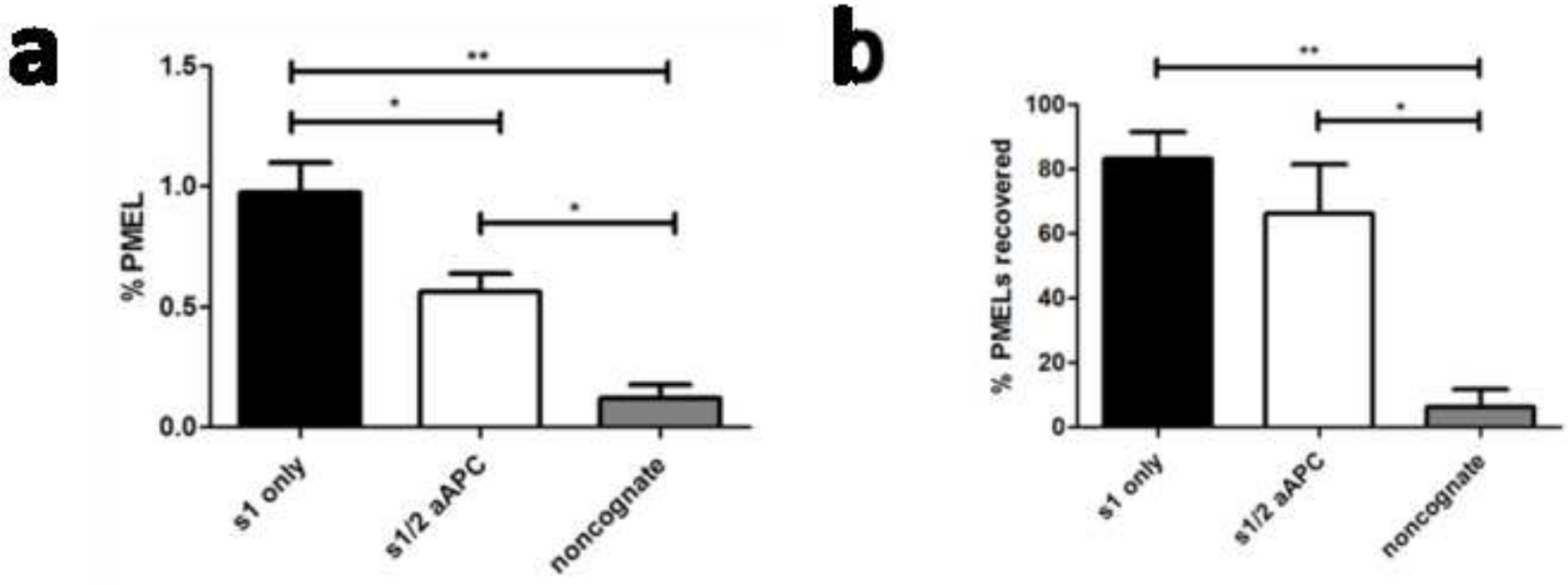

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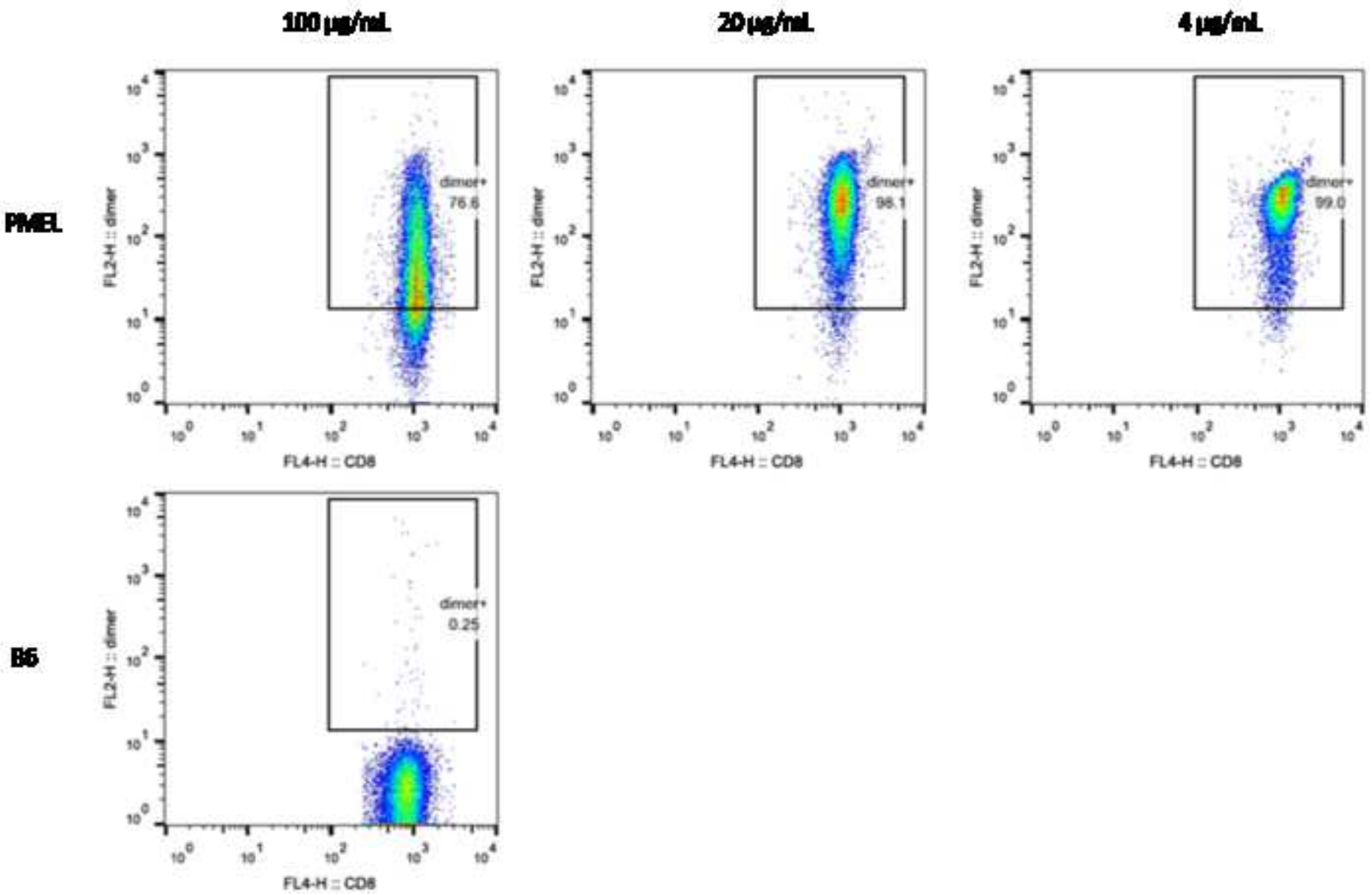

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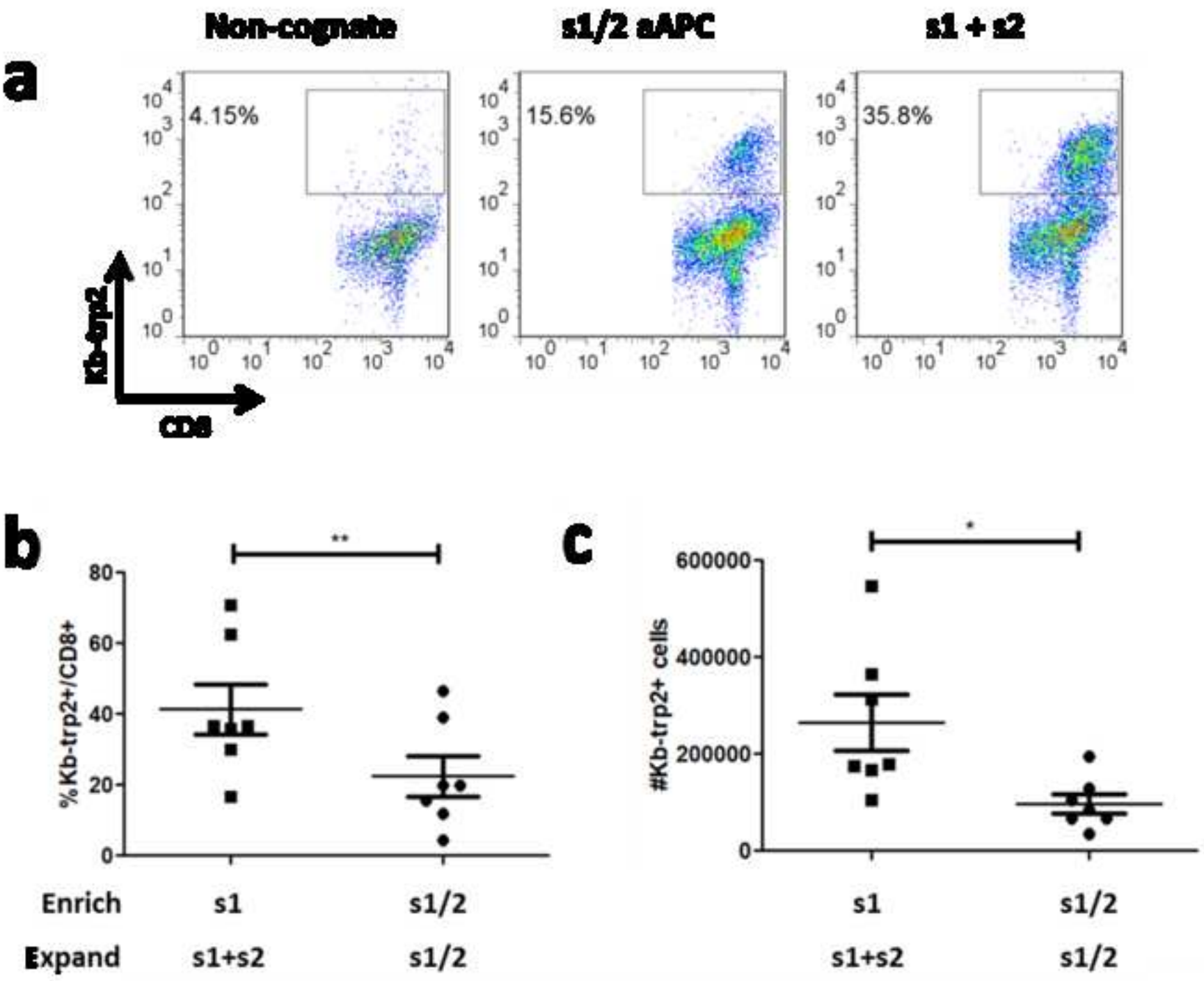

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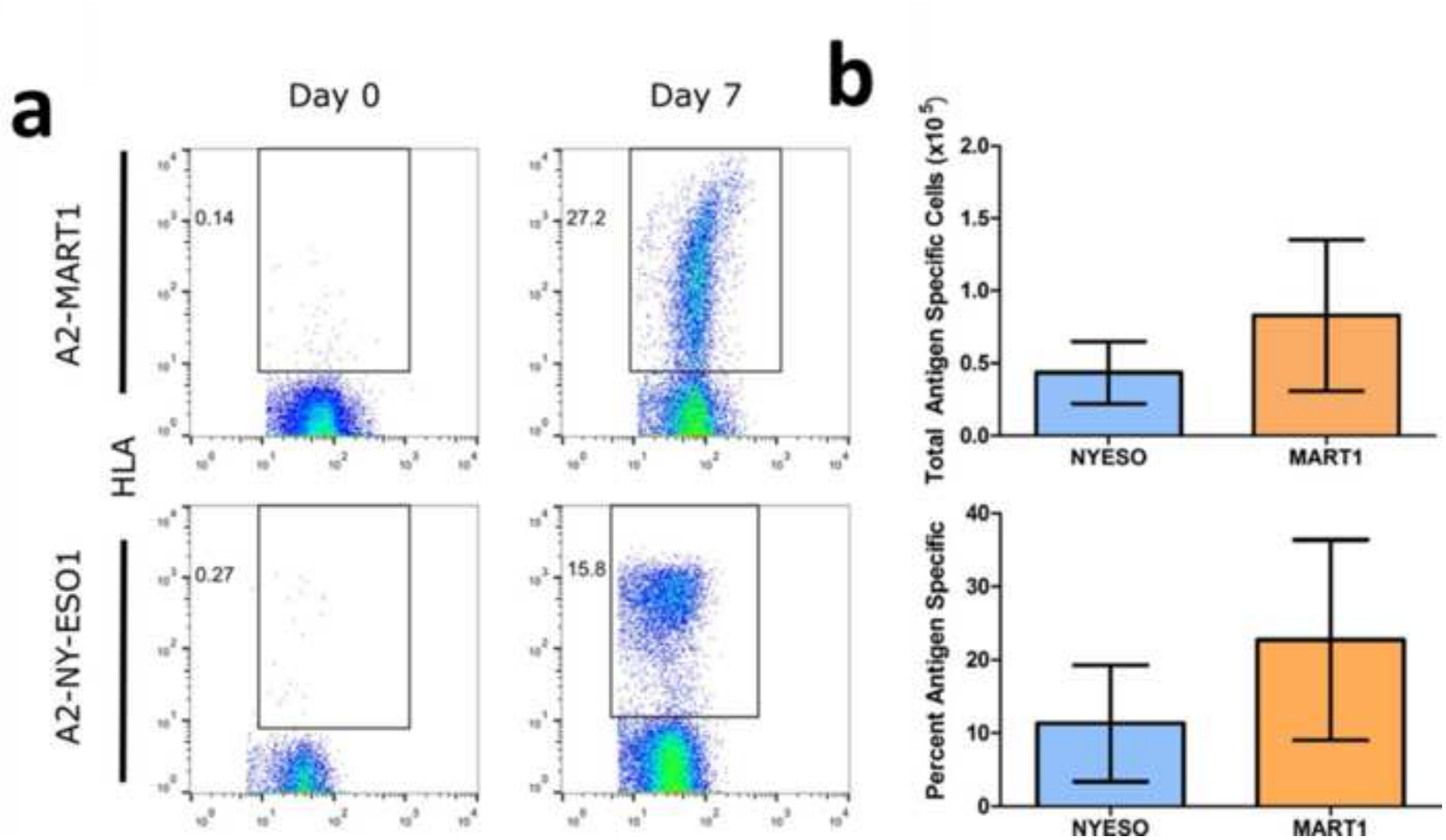

Table of Materials

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| Name of Material/ Equipment | Company |
|---|---|
| DimerX I: Recombinant Soluble Dimeric Human HLA-A2:Ig Fusion Protein | BD Biosciences |
| DimerX I: Recombinant Soluble Dimeric Mouse H-2D[b]:Ig | BD Biosciences |
| DimerX I: Recombinant Soluble Dimeric Mouse H-2K[b]:Ig Fusion Protein | BD Biosciences |
| Vivaspin 20 MWCO 50 000 | GE Life Sciences |
| Vivaspin 2 MWCO 50 000 | GE Life Sciences |
| Purified Human Beta 2 Microglobulin | Bio-Rad |
| nanomag-D-spio, NH2, 100 nm nanoparticles | Micromod |
| Super Mag NHS Activated Beads, 0.2 µm | Ocean Nanotech |
| Anti-Biotin MicroBeads UltraPure | Miltenyi |
| EZ-Link NHS-Biotin | ThermoFisher |
| Sulfo-SMCC Crosslinker | ProteoChem |
| 2-Iminothiolane hydrochloride | Sigma-Aldrich |
| 96 Well Half-Area Microplate, black polystyrene | Corning |
| FITC Rat Anti-Mouse Ig, λ1, λ2, & λ3 Light Chain Clone R26-46 | BD Biosciences |
| FITC Mouse Anti-Armenian and Syrian Hamster IgG Clone G192-1 | BD Biosciences |
| B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J (transgenic PMEL) mice | Jackson Laboratory |
| C57BL/6J (B6 wildtype) mice | Jackson Laboratory |
| CD8a+ T Cell Isolation Kit, Mouse | Miltenyi |
| MS Columns | Miltenyi |
| LS Columns | Miltenyi |
| Streptavidin-Phycoerythrin, SAv-PE | Biolegend |
| N52 disk magnets of 0.75 inches | K&J Magnetics |
| APC anti-mouse CD8a Antibody, clone 53-6.7 | Biolegend |
| LIVE/DEAD Fixable Green Dead Cell Stain Kit, for 488 nm excitation | ThermoFisher |

| Catalog Number | Comments/Description |
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| 551323 | |
| 550750 | |
| 28932362 | |
| 28932257 | |
| PHP135 | |
| 79-01-102 | |
| SN0200 | |
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| Signature: | Norma Stocker FOR DR. JONATHAN SCHNECK |
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We thank the editors for their comments to improve the manuscript and get it ready for filming. Based on these comments, we have made changes throughout the manuscript as suggested by the editors. These changes are highlighted by *tracked changes* in the manuscript. With these additional edits, we hope that our revised manuscript will be acceptable for publication in JoVe.

Our point by point response to the editor's comments is found below in blue.

**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have gone through the document and have edited for spelling and grammar issues.

2. The highlighted protocol steps are over 2.75 page limit (including headings and spacing). Please reduce the amount of highlighted protocol steps.

We have reduced the highlighted portions so that there is only 2.2 pages highlighted.

3. Please revise the text in Protocol to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have eliminated personal pronouns in the protocol sections of the manuscript.

4. Reference 7: Please include the source (journal name).

We provided the journal name of *Nano Letters* and change it in the text.

5. Step 4.5: Please ensure that all text is written in imperative tense.

We have altered Step 4.5 to a note, because it needs to be a conditional statement/inflection point.

6. 4.1.1: Please specify the euthanasia method.

We have added the following statement to 4.1.2: "Animals are euthanized by exposure to isoflurane followed by cervical dislocation."

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We have added the following statement before the numbered steps:

"All mice were maintained per guidelines approved by the Johns Hopkins University's Institutional Review Board."

**Title:** Enrichment and Expansion with Nanoscale Artificial Antigen Presenting Cells for Adoptive Immunotherapy

**Author:** Karlo Perica, Joan Glick Bieler, Christian Schütz, et al

**Publication:** ACS Nano

**Publisher:** American Chemical Society

**Date:** Jul 1, 2015



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**Author:** John W. Hickey, Fernando P. Vicente, Gregory P. Howard, et al

**Publication:** Nano Letters

**Publisher:** American Chemical Society

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**Author:** Alyssa K. Kosmides, Kevin Necochea, John W. Hickey, et al

**Publication:** Nano Letters

**Publisher:** American Chemical Society

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