Supporting Information

Magnetic Field-Induced T Cell Receptor Clustering By Nanoparticles Enhances T Cell Activation and Stimulates Anti-Tumor Activity In Vivo

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Supplementary Figure 1. Characterization of Protein Bound to Nano- and Micro-aAPC By Fluorescence.

(A) Mean fluorescence intensity (MFI) of antibody bound to nanoparticles and controls. Nano-aAPC and Micro-aAPC (cell-sized) particles were incubated with excess of monoclonal anti-mouse IgG1 (for MHC-Ig) and anti-antibody conjugated with PE for 30 minutes, and washed on a magnetic column. Fluorescent antibody bound to particles was detectable above background samples, including micro- and nanoparticles not stained with anti-IgG1 (No Ab) and particles which were not coupled to protein and stained with anti-IgG1 (Blank). Protein concentration in solution was determined by comparison to an IgG1-PE standard curve. Fluorescence is shown for anti-IgG1 and is representative of three experiments. HD – High Density. LD – Low Density. (B) Particles in solution do not interfere with antibody fluorescence. Soluble anti-IgG1 PE antibody was titrated and measured for fluorescence. Similar fluorescence emission was observed when soluble antibody was measured in the presence of blank micro- and nano-particles. (C) Washing in magnetic column was sufficient to remove free antibody. After three washes (Fraction 3), fluorescence is not detectable above background. Fluorescence of 0.63 ug/ml free antibody is provided for comparison. (D) Nanoparticle concentration was characterized by iron absorbance at 405 nm. Particle
concentrations were determined by Nanoparticle Tracking Analysis. Titrations of nanoparticles were measured for absorbance and a standard curve was calculated to determine particle concentration.
Supplementary Table 1

| Particle         | Particle Mean Diameter (µm) | MHC-Ig Dimers per Particle | MHC-Ig Density (protein/ µm²) | Anti-CD28 per Particle | Anti-CD28 Density (protein/ µm²) |
|------------------|-----------------------------|----------------------------|--------------------------------|------------------------|---------------------------------|
| Nano-aApc        | 0.1                         | 13 ± 3                     | 96 ± 10                        | 12 ± 5                 | 92 ± 12                          |
| Kb-SIY Alone     | 0.1                         | 29 ± 6                     | 214 ± 12                       | --                    | --                              |
| Nanoparticle     |                             |                            |                                |                        |                                 |
| Micro HD         | 4.5                         | 49,900 ± 2800              | 196 ± 11                       | 27,200 ± 4600         | 107 ± 18                        |
| Micro LD         | 4.5                         | 15,300 ± 1000              | 60 ± 11                        | 14,400 ± 4500         | 56 ± 17                          |

The amount and density of MHC-Ig and anti-CD28 on the surface of micro- (cell-sized) and nano-aApc. Protein was quantified as described in Supplementary Figure 1, and particle concentration determined by counts (micro-aApc) or Nanoparticle Tracking Analysis (nano-aApc). High (HD) and low (LD) density particles were synthesized by varying amount of protein per particle during synthesis. Signal 1 nanoparticles were synthesized without anti-CD28.
Supplementary Figure 2. pMEL T cell Proliferation Induced by Micro-aAPC

(A) CD8+ pMEL splenocytes include a population of memory-phenotype, CD44 positive cells (representative percentage shown as percentage of CD8, left). CD44lo naive cells were isolated by a no-touch negative selection enrichement with anti-CD44 antibody in a magnetic enrichment column. (B) Proliferation of Naive CD44lo (left) and activated (right) cells by CFSE dilution stimulated three days with micro-aAPC (dark red and blue lines) and nano-aAPC (light red and blue lines) or unstimulated (grey lines). Micro- and nano-aAPC were used at doses presenting equivalent total amount of MHC-Ig (8 ng). Nano-aAPC data are reproduced from Figure 1. (C) Proliferation of naive (red) and active (blue) cells seven days after stimulation with indicated doses of micro-aAPC. (D) Effect of MHC-Ig density on micro-aAPC induced stimulation. High density (HD, blue) and low density (LD, red) micro-aAPC were normalized for total MHC-Ig (4-16 ng). See Supplementary Table 1 for density. Proliferation assessed by CFSE dilution three days after activation. (E) Fold expansion of samples shown in D seven days after activation, representative of three experiments.
Supplementary Figure 3.

(A) Kb-SIY nanoparticle binding to cognate 2C T cells. Binding to activated cells, seven days after peptide activation (activated, blue, MFI 89) as compared to naive, CD44lo isolated 2C T cells (naive, red, MFI 179) and control non-cognate CD44lo pmel T cells (non-specific binding, grey, MFI 21). Binding is characterized as mean fluorescence intensity of Alexa 647 labeled particles bound to cells. (B) Surface TCR expression of naive (MFI 137) and activated (MFI 128) cells measured with fluorescent anti-TCRβ. (C) Disassociation of Kb-SIY MHC-Ig dimers from activated (dark blue) and naive (dark red) cells. Disassociation of nano-aAPC from activated (light blue) and naive (light red) cells are reproduced from Figure 1 for comparison. (D) Disassociation curves of nano-aAPC bound to naive CD44low cells before (red) and after (black) one hour of incubation in a magnetic field. Figure is representative of 2 experiments.
## Supplementary Table 2

| Ligand             | T Cells       | Off-Rate ($s^{-1}$)$^A$ | Half-Life (s)$^B$ | TCR-MHC Contacts$^C$ |
|--------------------|---------------|--------------------------|-------------------|----------------------|
| MHC-Ig Dimer       | Naive         | $8.9 \times 10^{-3}$     | 78                | 1                    |
|                    | Activated     | $5.2 \times 10^{-3}$     | 112               | 1.7                  |
| Nanoparticle       | Naive         | $(2.0 \pm 0.5) \times 10^{-3}$ | $531 \pm 149**$ | 6.8                  |
|                    | Activated     | $(0.9 \pm 0.2) \times 10^{-3}$ | $984 \pm 221**$ | 12.6                 |

$^A$Off-rates experiments were performed by incubating naive or activated 2C TCR transgenic T cells with APC-labeled MHC-Ig or APC-labeled nanoparticles bearing K$^b$-SIY alone. After incubation for one hour at 4°C, cells were washed, a Time 0 fluorescence measurement was taken, and 1B2, an anti-clonotypic antibody, was added to prevent re-binding. Fluorescence measurements were then repeated at 2-10 minute intervals. Off-rates were calculated from a one-dimensional exponential fit in GraphPad Prism.

$^B$Half-lives were derived from off-rates in column A. Particles bound to activated cells had a significantly longer half-life (**p<0.02 by paired t-test, where measurements were paired by experiment) than particles bound to naive cells. Three experiments were performed for each condition.

$^C$Unbinding of individual MHC-Ig on either dimer or particle can be stochastically modeled as a Poisson (aka memoryless or exponential) Process. For a Poisson Process with rate constant $r$, the departure time of the $n$th event is characterized by a gamma distribution with shape parameter $n$ and single-event rate parameter $r$:

$$f_n(t) = r^n \frac{t^{n-1}}{(n-1)!} e^{-rt}, \quad 0 \leq t < \infty$$

The mean of this distribution is $E[t] = \frac{n}{r}$. If MHC-Ig dimer is assumed to make one contact with a naive T cell$^1$, then $r$ can be estimated from the off-rate of MHC-Ig on naive cells $(8.9 \times 10^{-3})$. Thus, for any given condition, $E[t]$ is derived from the half-life of MHC-Ig dimer or particle on naive or active cells ($t_{1/2}$), and $r$ is assumed constant. The number of TCR-MHC contacts is estimated as $n$:

$$n = \frac{t_{1/2} \times r}{\ln(2)}$$

The true number of contacts is likely to be higher than this estimate, as MHC-Ig are likely to make more than one contact with naive cells.
Supplementary Figure 4: TCR Clustering and Expansion by Micro-aAPC in a Magnetic Field

(A) Micro-aAPC aggregation in a magnetic field. Representative confocal images of micro-aAPC (red) shown before (left) and after (right) application of a magnetic field. (B) Micro-aAPC magnetic aggregation does not induce CD3 aggregation. Cells were labeled with antibodies against LFA-1 (green), MHC-Ig on micro-aAPC (red), and CD3ε (magenta). Micro-aAPC displayed auto-fluorescence in all three channels, particularly in the red and magenta channels. Representative images are shown for cells incubated with cognate micro-aAPC (No Magnet), both not in contact (top) and in contact (bottom) with micro-aAPC, and cells incubated with cognate nano-aAPC in a magnetic field (Magnet). (C) Average cluster area and clusters per cell identified with cluster detection algorithm (20 cells/group, divided evenly between cells in contact and not in contact with particles). Control samples include cells prior to incubation (Time 0) and cells incubated with non-cognate microparticles (Non-Cognate) (p>0.05 by ANOVA). (D) Pmel T cells incubated with 5 ng (left) and 10 ng (right) MHC-Ig dose of micro-aAPC with (red) and without (black) a 0.2 T magnetic field for 3 days. Proliferation assessed by CFSE dilution at day 4. (E) Fold expansion of pmel T cells incubated with increasing doses of micro-aAPC with and without a 0.2 T magnetic field seven days after stimulation (p>0.05 by two-way ANOVA).
Supplementary Figure 5: Magnetic Field Strength Generated in Culture by Neodymium Disk Magnets

Density plots of field strength in culture as estimated by finite element analysis with FEMM (Finite Element Method Magnetics) software. Disk magnets (magenta) ¾”, ½”, and ¼” in thickness were used to generate fields of up to 0.225 T, 0.200 T, and 0.150 T, respectively.
Supplemental Methods

Preparation of MHC-Ig Dimers
Soluble MHC-Ig dimers, K\textsuperscript{b}-Ig and D\textsuperscript{b}-Ig, were prepared and loaded with peptide as described \textsuperscript{2}. Briefly, K\textsuperscript{b}-Ig molecules were loaded with peptide by stripping at alkaline condition (pH 11.5), and then refolded in the presence of 50 fold molar excess peptide. D\textsuperscript{b}-Ig molecules were stripped under mildly acidic conditions (pH 6.5) and refolded in the presence of 50 fold molar excess peptide and 2-fold molar excess of human β\textsubscript{2}-microglobulin\textsuperscript{3}. Peptides SIY (SIYRYYGL, synthetic), SIIN (SIINFEKL, derived from ovalbumin protein), GP100 (KVPRNQDWL, from melanocyte GP100 protein) and ASN (ASNENMETH, from influenza A nucleoprotein) were purchased from Genscript (Piscataway, NJ). Protein concentration was determined after labeling by size exclusion high performance liquid chromatography (HPLC).

Micro-aAPC Synthesis
Micro-aAPCs were fabricated as described previously\textsuperscript{4} by direct chemical coupling of protein to 4.5 μm Dynal Magnetic Microbeads (Life Technologies, Carlsbad, CA). For the initial coupling step, 25 μg antibiotin antibody (Sigma, St. Louis, MO) was added to 100 million microbeads in 0.1 M sodium borate buffer. After washing in a magnetic column, biotin labeled MHC-Ig and CD28 were added in equimolar amounts to form aAPC.

Nanoparticle Tracking Analysis
A Nanosight LM10 equipped with a sensitive CCD camera was used for characterizing the size distribution of nano-aAPC by NTA. 50 μL of diluted nanoparticle solution was loaded into the sample chamber, which was connected to a 405 nm laser source. A 60 s movie containing the Brownian motion tracking of the scattering centroids (particles) was recorded using NTA software (Version 2.0). The movie was processed using the manufacturer recommended auto settings with manual adjustment of the gain, blur and brightness as recommended. The nanoparticle solution was diluted in phosphate buffered saline to adjust the sample concentration to 5×10\textsuperscript{12} particles mL\textsuperscript{-1}.

Micro-aAPC Microscopy
T cells were incubated with micro-aAPC, spun at 1000 RPM for 1 minute to pack cells and particles, and incubated for 60 minutes at 4\textdegree C. Cells were subsequently transferred to a 96-well plate at 37\textdegree C in the presence or absence of a magnetic field generated by Neodymium N52 disk magnets. After 30 minutes, cells were washed and stained at 4\textdegree C with Alexa488 anti-LFA1, monoclonal PE anti-mouse IgG1, and Alexa 647 anti-CD3ε. Samples were washed and fixed immediately with 2% paraformaldehyde. Images were acquired on a Zeiss LSM 510 META (Zeiss, Oberkochen, Germany) laser scanning confocal at 100x magnification at the Johns Hopkins School of Medicine Microscopy Facility. CD3ε cluster size was determined using a particle-detection algorithm written in ImageJ (National Institutes of Health) using the built-in Particle Analyzer. Particle auto-fluorescence for cells bound to particles was removed manually.

References

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