Electronic Supplementary Material (ESI) for Materials Horizons.

Supporting Information

Augmenting T cell responses to tumors by in situ nanomanufacturing

Mohammad Mahdi Hasani-Sadrabadi, Fatemeh S. Majedi, Matthew L. Miller,

Timothy J. Thauland, Louis-S. Bouchard, Song Li, and Manish J. Butte**

Experimental Procedures

Chemicals and Biologicals

Unless noted otherwise, all chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All cell culture reagents, solutions, and dishes were obtained from Thermo Fisher Scientific (Waltham, MA), except as indicated.

Plasmids

Plasmid DNA Preparation and Caging

We used IL2-GFP plasmid DNA (Addgene #67053, 5280 bp). To add spatiotemporal control on encoding process, the plasmid DNA was caged using an ultraviolet-sensitive cleavable "caged ATP" (1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester (DMNPE). Activation of DMNPE and caging procedure followed as reported before. DMNPE (10 mg) and manganese (IV) oxide (MnO_2; 50 mg) were gently mixed in dimethyl formamide (1.5 mL) for 30 min at room temperature. MnO_2 was removed from the DMNPE through centrifugation followed by filtering. Purified and activated DMNPE mixed with plasmid DNA (0.5 mg DNA per mL of DMNPE) in Tris HCl buffer (10 mM, pH 5.5) at 4 °C for 24 h. Excess DMNPE was removed using Amicon Ultra-0.5 (Ultracel-3 membrane, MWCO 3 kDa). Caged plasmids were ultra-centrifugated to adjust concentration and stored in dark at 4 °C before use. Two other plasmid DNAs, *V72 ELP* (Addgene#68938, 6565 bp) and *Renilla luciferase* (Promega, 3320 bp), were also used to test encapsulation efficiency.

Super2 construct design

To construct our IL-2 superkine ("Super2"), we modified the pCellFree_G03_IL2 expression plasmid (AddGene #67053) using NEBuilder HiFi DNA Assembly. H9 IL-2 superkine cDNA (AddGene #41808) was amplified using primers: 5'-*TTATTTTATTTTATTTAACC*GGAGCCATGGGAGAATTCGC-3' and 5'-*GGAGGAGGGCGGCCGCTTAC*CTAAGTTAGTGTTGAGATGATGC -3'. Primers included 20 base pair overlaps for insertion into the pCellFree backbone (Promega). The pCellFree_G03_IL2 backbone was amplified in two-parts, with overlaps included for insertion of the IL-2 amplification and overlaps generated in the AmpR gene to serve as a positive marker for correct HiFi DNA assembly. The first section of the backbone was amplified using primers: 5'-

GCGAATTCTCCCATGGCTCCGGTTAAATAAAATAAAATAA-3' and 5'-TTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTAT-3'. The second section of the backbone was amplified using primers: 5'-ATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAA-3' and 5'-GCATCATCTCAACACTAACTTAGGTAAGCGGCCGCCCTCCTCC-3'. The amplification and assembly strategy excluded the GFP gene present on the original pCellFree_G03_IL2 plasmid. Once all three constructs were successfully PCR amplified they were assembled using NEBuilder HiFi DNA Assembly Master Mix (cat. # E2621).

Super2 construct sequence

GGAGCCATGGGAGAATTCGCACCTACTTCAAGTTCTACAAAGAAAACACAG
CTACAACTGGAGCATTTACTTCTGGATTTACAGATGATTTTGAATGGAATTAATAATT
ACAAGAATCCCAAACTCACCAGGATGCTCACATTTAAGTTTTACATGCCCAAGAAG
GCCACAGAACTGAAACATCTTCAGTGTCTAGAAGAAGAACTCAAACCTCTGGAGGA
AGTGCTAAATTTAGCTCAGAGCAAAAACTTTCACTTCGATCCCAGGGACGTCGTCA
GCAATATCAACGTATTCGTCCTGGAACTAAAGGGATCTGAAACAACATTCATGTGT
GAATATGCTGATGAGACAGCAACCATTGTAGAATTTCTGAACAGATGGATTACCTTT
TGTCAAAGCATCATCTCAACACTAACTCAT

Nanoliposome Formation

Bulk fabrication

Liposomes were formed according to the procedures reported previously[1,2]. with some modification. Briefly, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), and cholesterol (all from Avanti Polar Lipids) in a molar ratio of 6:2:4 were dissolved in dry chloroform and the solvent was evaporated at room temperature under a nitrogen flow to form a dry lipid cake. Afterwards the cake was further dried overnight in a vacuum oven (mixture#1).

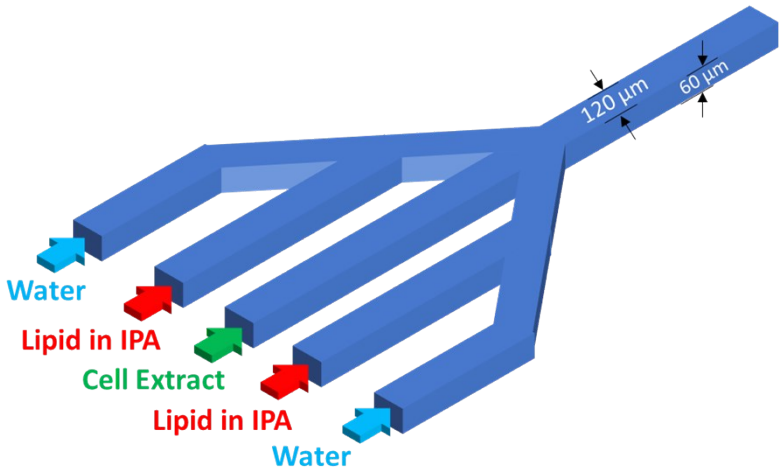
To form nanoliposomes, caged-ATP and DNA were mixed with S30 T7 High-Yield Protein Expression System (Promega). DNA containing solution was added to warmed mixture#1 and vortexed briefly. The non-encapsulated extracts and DNA separated from formed liposomes by centrifugation at 10,000 rpm for 20 min and washed at 4 °C with 5% dextrose twice. After 1 h of incubation under gentle shaking at 37 °C, the nanoliposome formed *via* extrusion technique of solution by 800, 400, 200, 100 nm pores track-etched polycarbonate membranes (Whatman Nuclepore Hydrophilic Membranes) at 37°C.

Microfluidic fabrication

Microfluidic devices were fabricated with poly(dimethylsiloxane) (PDMS) using a standard micro-molding process.[3] To make the master molds, silicon wafers were coated with 60 μm thick SU-8 photocurable epoxy. Baking, lithography, and development procedures were performed according to previously published reports[4] to obtain negative microchannels on the wafer. The wafers were then annealed and coated with trimethylethoxysilane to prevent PDMS from sticking to the mold. PDMS (Sylgard 184) oligomer and curing agent were mixed (10:1 ratio), casted on the mold, degassed in desiccators and cured in an oven at 60 ºC for 2 h. PDMS was removed from the mold and

in-/outlet holes were punched. The mixing channel was 120 µm wide, 60 µm high and 15 mm long. To bond PDMS to a glass slide, ambient-oxygen plasma was used at 100 mW for 1 min.

To form nanoliposomes using microfluidic technique, the mixture#1 was dissolved in dry isopropyl alcohol (IPA) at a 5 mg/mL. IPA injected into two middle channels (**Supplementary Scheme 1**). DMNPE-caged DNA was mixed with the transcription/translation extract and was used as source was injected into the center channel of the microfluidic platform. PBS was injected into two lateral channels. The flow rate ratio (FR), defined as volumetric flow rate of lipid-IPA solution to the overall (lipid-IPA + cell extract + PBS) volumetric flow rate.



Supplementary Scheme 1. Schematic representation of microfluidic inlets.

Nanoliposome formation at different flow conditions was studied by tuning the flow rates of the PBS streams. The flow rates are summarized in the **Supplementary Table 1**. Dynamic light scattering (DLS) measurements were performed using a Zetasizer (Zetasizer 3000HS, Malvern Instruments Ltd., Worcestershire, UK) in backscattering mode at 173° for water-diluted systems.

Microparticle (cell mimicking particles) Formation.

Alginate-Heparin conjugate was synthesized using EDC/NHS chemistry and via ethylenediamine[5]. The amount of conjugated Heparin was optimized to provide enhanced affinity toward cationic proteins like IL-2 cytokine. Microfluidic droplet junction chip (glass hydrophilic; channel depth of 100 µm; Dolomite Microfluidics, Charlestown, MA) was utilized to make monodispersed Alginate and Alginate-Heparin microparticles. Alginate-Heparin solution (10 mg/mL) was mixed with nanoliposomes (2.5 mg/mL) and used as the inner aqueous phase. Mineral oil containing surfactant (10 wt% Span-80) was used as the continuous phase. Flow rates of 6.5 and 14 µL/min were applied using two syringe pumps (Harvard Apparatus PHD 2000) for the polymer and oil flows, respectively. Images were taken at various time points using a Nikon inverted microscope to check and tune the flow properties. The formed particles were collected in a bath of calcium ions (100 mM $CaCl_2$) and left at room temperature for 30 min in dark for ionic crosslinking. Inserting of magnetic nanoparticles (SPION; 50 nm, carboxylated, Chemicell GmbH) also utilized to facilitate separation and washing of particles after formation. The microgels

were extensively washed with 20 mM NaCl solution and centrifuged (15,000 rpm for 15 min) twice before further use.

Microparticle coating

For the preparation of lipid-coated alginate-heparin microparticles, POPC phospholipid was dissolved in chloroform. Rotary evaporation of the phospholipid was used to form a homogeneous lipid film. The film was further dried under nitrogen and followed by high vacuum overnight. The pre-warmed dried lipid was then wetted using microparticles containing PBS at 60 °C and agitated at 37 °C for 15 min. Samples were cooled to room temperature and then centrifuged to remove extra lipids and re-dispersed in PBS for further use.

Protein Production and Detection

The solutions of free nanoliposomes or encapsulated inside Alginate(-Heparin) microparticles were kept at 4 °C before the UV triggering. To activate the protein production machinery, UV light (360–480 nm) was used to illuminate the particles for 10 s at 80 mW/cm^2 (pH 7.4 and 37 °C). Confocal imaging was used to visualize synthesis of green fluorescence protein (GFP) inside the nanoliposomes. A 100× Plan Apo 1.4 NA objective (Nikon) was used for the fluorescent imaging.

In vitro Functional Assays

T cell isolation and activation

Five- to eight-week-old wild type mice were purchased from the University of California, Los Angeles (UCLA) and maintained in pathogen-free facilities at UCLA. All experiments on mice and cells collected from mice were performed in strict accordance with UCLA's institutional policy on humane and ethical treatment of animals. Cell culture media was RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 µg/mL streptomycin, 100 U/mL penicillin, 50 µM 2-mecaptoethanol. T cells were purified from spleens using the appropriate negative enrichment kits (Affymetrix eBioscience).

For *in vitro* activation of purified T cells (CD8+ or CD4+), 96-well plates were prepared with anti-CD3 (2C11; Bio XCell) coating plates at a concentration of 5 µg/mL overnight, followed by washing with PBS. T cells at a concentration of 1x10^6/mL were mixed with 2 µg/mL soluble anti-CD28 (37.51; Bio XCell) in the presence of nanoliposomes (free or encapsulated in microparticles) or 100 IU/mL of soluble human IL-2. Microparticles were added a 1:1 ratio of particles to cells or else the equivalent number of nanoliposomes (using a ratio of ~400 nanoliposomes / microparticle) was added to the media. For flow cytometry analysis, antibodies to mouse CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7), CD62L, and CD16/CD32 (FC block) were purchased from eBioscience, BioLegend, or BD Biosciences. Propidium iodide and acridine orange were purchased from Calbiochem. Cells were measured on Cytek DxP flow cytometer and analyzed using FlowJo software (Treestar).

To study the *in vitro* release profile of produced cytokine, nanoparticle-loaded microparticles were dispersed in PBS (pH 7.4) and exposed to UV for 10 s. 500 µL of microsphere dispersion were placed in Eppendorf tubes, gently shaken, and incubated at 37 °C. At predetermined time points, samples were collected using centrifugation and the supernatant was replaced with an equivalent volume of PBS. To detect the production of

protein, we included FluoroTect GreenLys tRNA (Promega) in the nanoliposomes, which replaces a portion of the available lysines with a fluorescently-tagged amino acid during synthesis (excitation maximum of 502 nm, emission maximum of 510 nm). To eliminate the signal arising from unincorporated green lysine, we pelleted the nanoparticles and took only the supernatant – the fraction that should contain diffused cytokine but could also contain fluoro-Lys. We then filtered the supernatant samples using Amicon Ultra centrifugal filter units (Ultra-4, MWCO 5 kDa). Free fluoro-lysine is much smaller than 5 kDa. The concentration of fluorescent cytokine was determined using a plate reader.

Chromium functional assay

Transgenic OT-I mice (5-10 weeks old) were used for *in vitro* cytotoxicity assay and were kept under specific pathogen-free conditions, according to institutional guidelines. B16F10-ova and B16F10 were cultured in Dulbecco's medium that contained 5% FCS and glutamine, with penicillin/streptomycin added to it. OT-I CD8+ T cells were purified by negative selection as mentioned before, and co-cultured with various formulations of designed particles microparticles at a one-to-one ratio of microparticles. At the end of the culture period (day 4 or day 10), T cells were recovered. Finally, a total of 5×10^6 transgenic T cells/well in a volume of 1 mL were counted and serially diluted up to seven times (T Cell: tumor cell of 100:1, 30:1, 15:1, 7:1, 3:1, 1.5:1, 0.75:1) in MEM supplemented with 2% FCS. Specific cytotoxicity of the dilutions were then determined in a standard ^{51}Cr release assay, as described elsewhere[6]. 10^4 cells were added to the effector cells in a final volume of 200 µL. After a 12 h incubation at 37 °C, 60 µL of the supernatant was harvested and counted with a gamma counter (PerkinElmer, Richmond, CA).

In vivo Functional Assays

In vivo Protein Production

5×10^6 protein producing microparticles (150 µL) subcutaneously injected into right flank of C57BL/6J WT mice (6-8 weeks old) mice. 24 h after the injection the injection site was divided in half. One half was covered with UV-absorbing film and the other half subjected to UV irradiation at 300 mW/cm^2 power using a 365 nm UV source (OmniCure S2000) for 300 s. Protein (GFP) production was visualized using whole animal fluorescence scanning 3 h post irradiation.

In vivo Tumor Suppression Assay

2×10^5 B16F10-OVA tumor cells subcutaneously injected into both right and left flanks of C57BL/6J WT mice (6-8 weeks old) mice. These melanoma-derived cells are transfected to express chicken ovalbumin peptide (OVA)[7]. Three days after tumor cell injection, 5×10^6 Super2 microfactories (Alginate-Heparin microparticles encapsulating protein producing nanoparticles) were adoptively transferred subcutaneously into the same approximate region of the tumors in both flanks. 500×10^3 OT-I T cells were activated *ex vivo* with anti-CD3/anti-CD28 3 days and then transferred intravenously on day 6 by retro-orbital injections (100 µL per animal). That same day, right flanks were exposed to UV for 300 s using OmniCure S2000 (365 nm; 300 mW/cm^2). On the same day (day 6), control animals (sides) were injected with soluble IL-2 (5,000 units/kg) at sites immediately proximate to the tumor.

Tumor size was assessed over time using a digital caliber until day 22 at which animals were sacrificed and the tumor was extracted. Tumor mass was measured using a digital balance before digesting the tumor tissue for flow cytometry. Tumors were digested by incubating in collagenase and DNase I (50 µg/mL) at 37 °C for 15 min. These enzymes were inactivated with EDTA (20 µL/mL of solution). Tissues then were mechanically disaggregated and passed by a 0.7 µm cell strainer to obtain a single-cell suspension. Cells were then stained with the fluorochrome-conjugated antibodies on ice. For intracellular staining (e.g., Granzyme B), cells were permeabilized with Fix/Perm buffer according to manufacturer instructions (BioLegend) before staining.

Statistics

Permutation testing was used for all statistical comparisons of flow cytometry data, survival curves, fluorescence colocalization, and stiffness. This method reduces the potential influence of outliers and relaxed the requirement of knowing the distribution of observations by comparing the value of the test statistic to a reference distribution generated from the data themselves, rather than to a standard distribution [8]. To ensure that permutation testing was a suitable way to compare means, we showed first that the variances of the two groups were similar by the non-parametric Ansari-Bradley test. We used the permutationTest2 function of the "resample" package of R to calculate two-sided p-values and determine the 95% confidence intervals, performing typically 50,000 permutations. All average values in this paper are bootstrapped means, calculated using the "bootstrap" function of the resample package in R. All boxes in figures show the bootstrapped mean and the calculated 95% confidence interval. Confidence intervals are calculated using the "CI.t" function of the resample package in R.

Where multiple groups were compared (e.g., Fig. 4, Supplemental Fig. 9), the comparison was found significant first by ANOVA where treatment was compared to the outcome measure (e.g., tumor mass). Then, post-hoc pair-wise comparisons are shown with p-values calculated by permutation testing as above. Adjustment of multiple comparison testing p-values was done by the Benjamini-Hochberg method using the p.adjust function in R.

Supporting Information References

1 A. Jahn, W. N. Vreeland, D. L. DeVoe, L. E. Locascio and M. Gaitan, *Langmuir*, 2007, **23**, 6289–93.

2 M. M. Hasani-Sadrabadi, S. Taranejoo, E. Dashtimoghadam, G. Bahlakeh, F. S. Majedi, J. J. Vandersarl, M. Janmaleki, F. Sharifi, A. Bertsch, K. Hourigan, L. Tayebi, P. Renaud and K. I. Jacob, *Adv. Mater.*, 2016, **28**, 4134–4141.

3 D. Qin, Y. Xia and G. M. Whitesides, *Nat. Protoc.*, 2010, **5**, 491–502.

4 F. S. Majedi, M. M. Hasani-Sadrabadi, J. J. Vandersarl, N. Mokarram, S. Hojjati-Emami, E. Dashtimoghadam, S. Bonakdar, M. A. Shokrgozar, A. Bertsch and P. Renaud, *Adv. Funct. Mater.*, 2014, **24**, 432–441.

5 F. S. Majedi, M. M. Hasani-Sadrabadi, Y. Kidani, T. J. Thauland, A. Moshaverinia, M. J. Butte, S. J. Bensinger and L.-S. Bouchard, *Adv. Mater.*, 2018, **30**, 1703178.

6 M. Hany, S. Oehen, M. Schulz, H. Hengartner, M. Mackett, D. H. L. Bishop, H.

Overton and R. M. Zinkernagel, *Eur. J. Immunol.*, 1989, **19**, 417–24.

7 M. Bellone, D. Cantarella, P. Castiglioni, M. C. Crosti, A. Ronchetti, M. Moro, M. P. Garancini, G. Casorati and P. Dellabona, *J. Immunol.*, 2000, **165**, 2651–6.

8 L. M. Chihara and T. C. Hesterberg, *Mathematical statistics with resampling and R*, John Wiley & Sons, Hoboken, NJ, 2018.

Supplementary Table 1. Flow Conditions used in this study

	Volumetric Flow Rate (µL/min)				**Flow Rate Ratio (FR)**
	Extract	*Lipid-IPA*	*PBS*	*Total*	
µF-1	2	5	55	62	0.08
µF-2	2	5	45	52	0.10
µF-3	2	5	30	37	0.14
µF-4	2	5	15	22	0.23

Supplemental Table 2. Details of used particle formulations in this study.

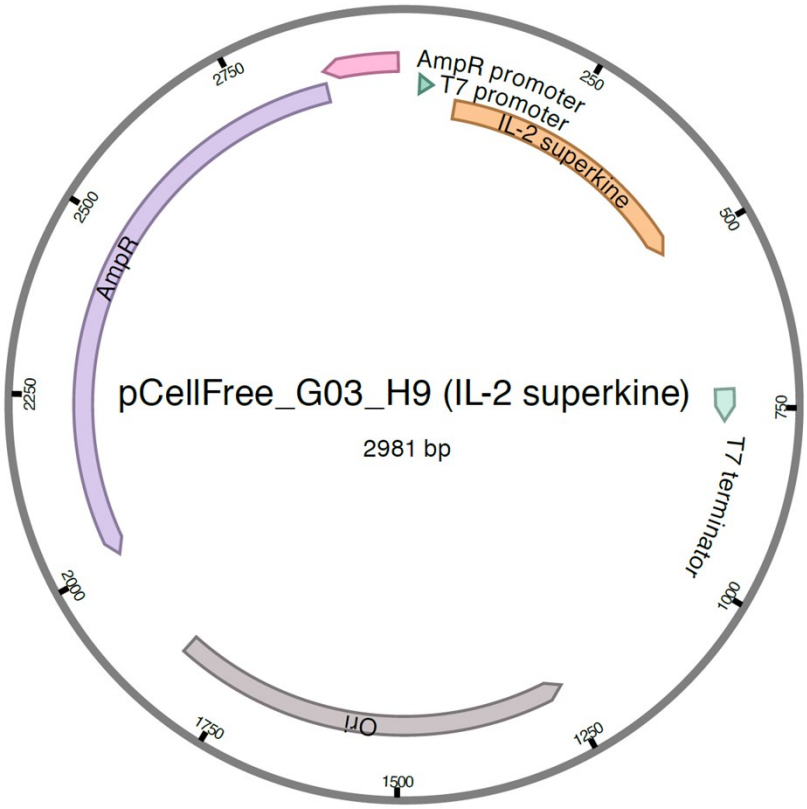
Sample	Size*	Morphology	Method	Stability (days)		Cited in paper
				**	***	
μP-1	45±12 nm	Spherical	DLS	9-14	7-12	Fig. 1 b, c, d
μP-2	94±28 nm	Spherical	DLS	9-14	7-12	Fig. 1 b, c, d
μP-3	166±43 nm	Spherical	DLS	9-14	7-12	Fig. 1 b, c, d, f
μP-4	380±90 nm	Spherical	DLS	9-14	7-12	Fig. 1 b, c, d, e, f. Fig. 3
Bulk 1	191±122 nm	Spherical	DLS	6-10	6-12	Fig. 1 b, c, d, f
Bulk 2	371±184 nm	Spherical	DLS	4-7	4-6	Fig. 1 b, c, d, f
Liposome (μP-4) encapsulated (Alginate)+lipid membrane	5.8±1.0 μm	Spherical	Microscopy	>30	>21	Fig. 3
Liposome (μP-4) encapsulated (Alginate-Heparin)	5.1±0.6 μm	Spherical	Microscopy	>30	>21	Fig. 3
Liposome (μP-4) encapsulated (Alginate-Heparin)+lipid coated	6.4±0.9 μm	Spherical	Microscopy	>30	>21	Fig. 2 b, c, d. Fig. 3. Fig. 4

*: Average hydrodynamic size

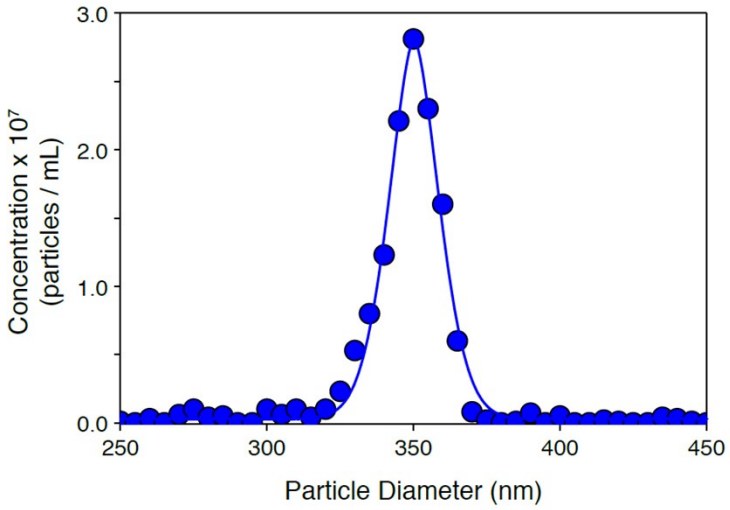
**: in PBS supplemented with physiological calcium

***: in T cell media (RPMI/10%FBS)

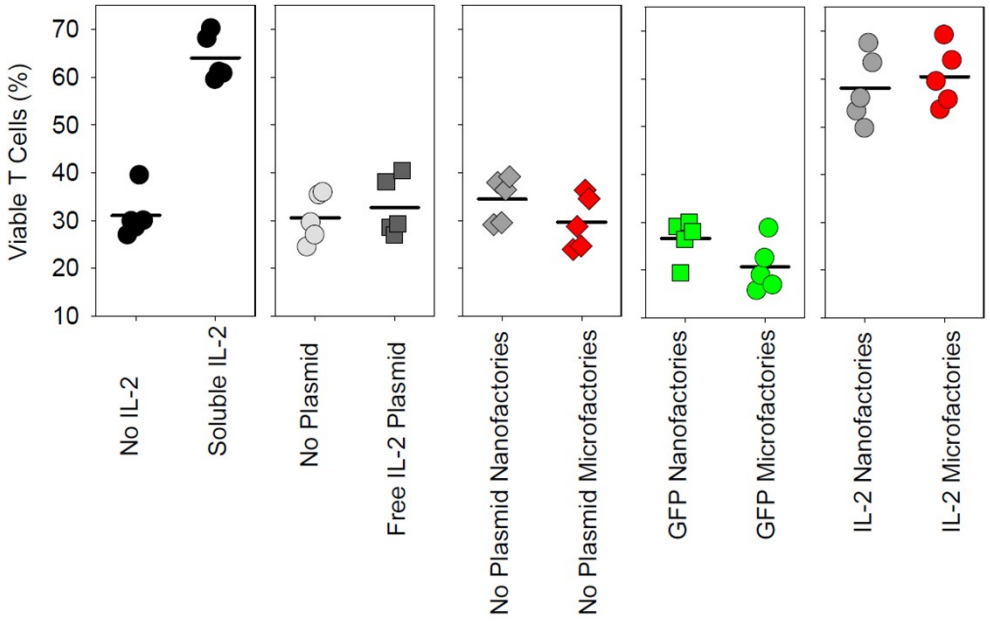
Supporting Information Figures



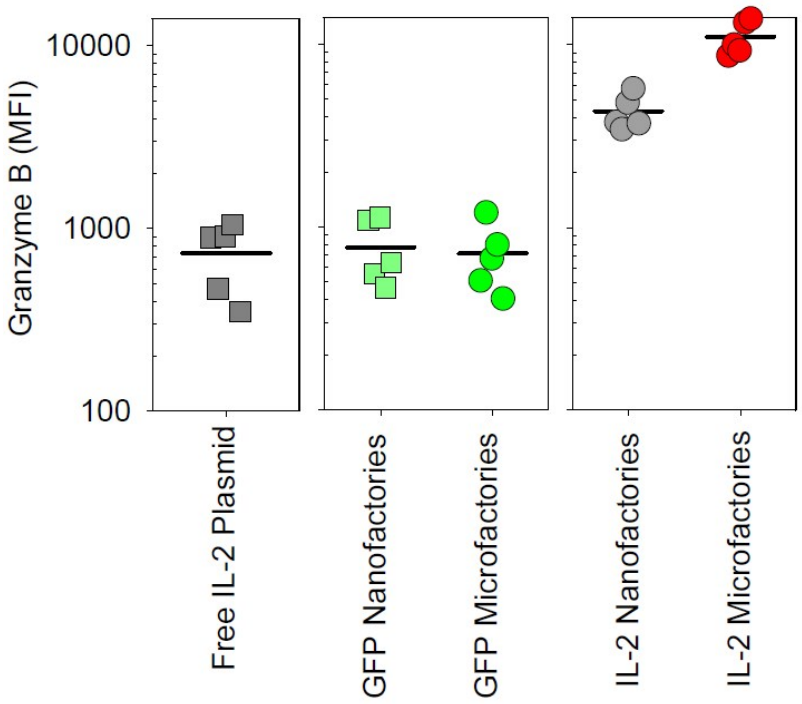
Supplemental Figure 1. Plasmid of the super2 cytokine gene, flanked by T7 sites for in vitro transcription.



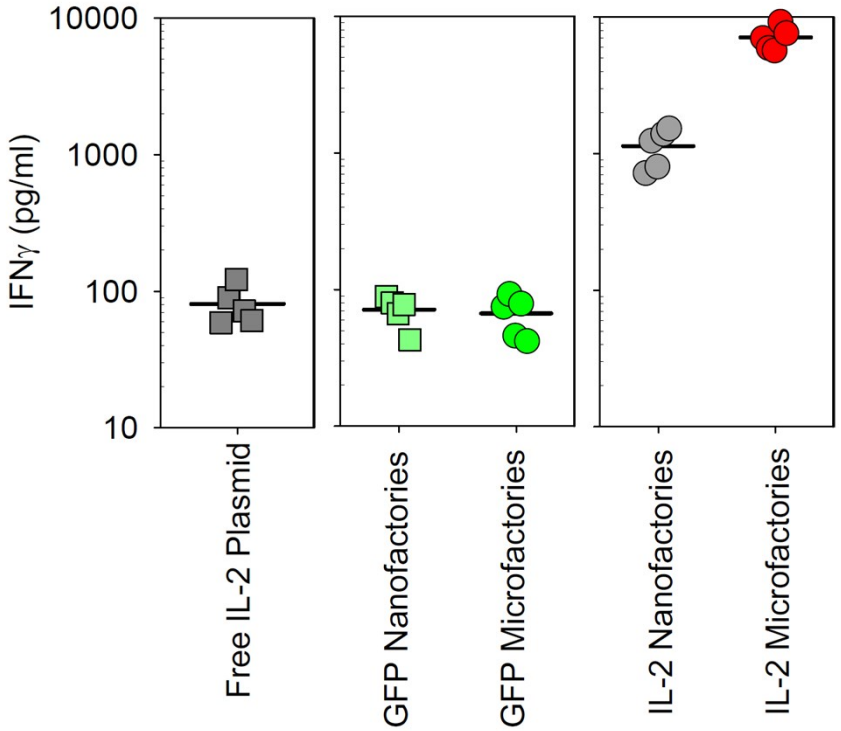
Supplemental Figure 2. Calculated concentration of NPs within microfactories is based on DLS measurement of 6.64×10^8 NP / mL, which were extracted from solution containing 1.5 x 10^6 microfactories / mL, thus ~443 NP per microfactory.



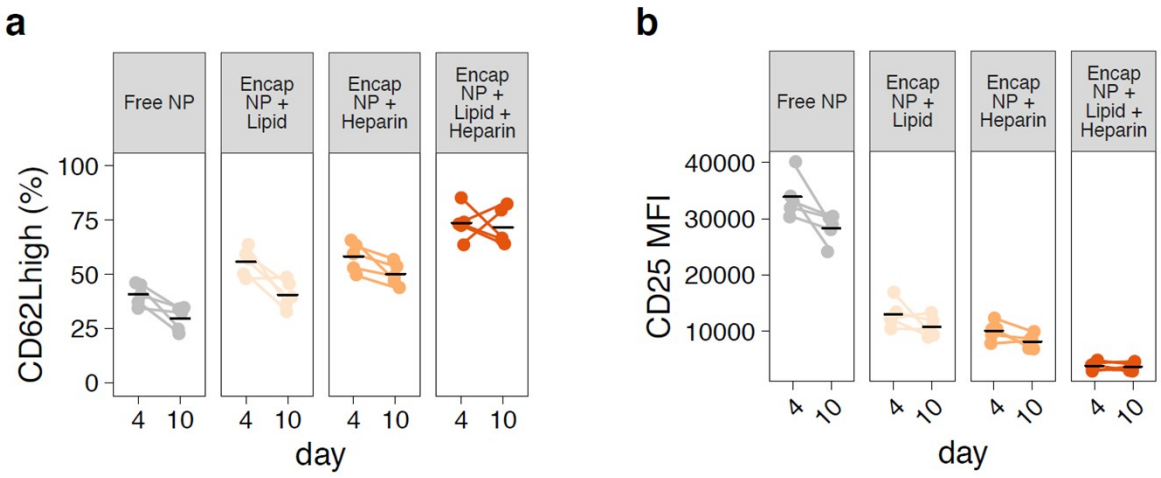
Supplemental Figure 3. T cell viability after co-culture with nanofactories or encapsulated nanofactories (microfactories) with IL-2 or GFP plasmids after 4 days of co-culture with CD8+ T cells.



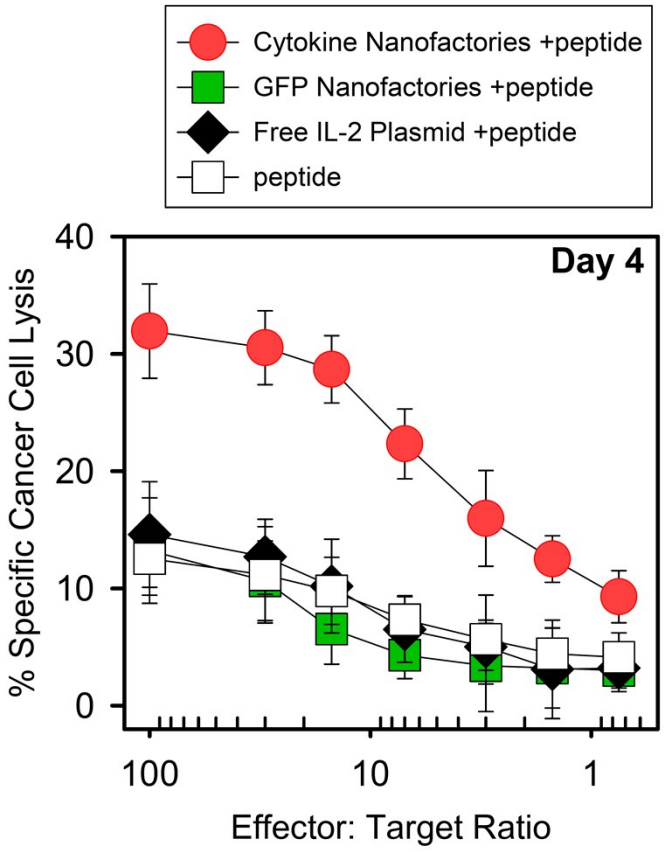
Supplemental Figure 4. Secretion of granzyme B as an indicator of activation level of CD8+ T cells after 4 days of co-culture with particles as listed. Intracellular cytokine staining was used to detect presence of granzyme B inside CD8+ T cells.



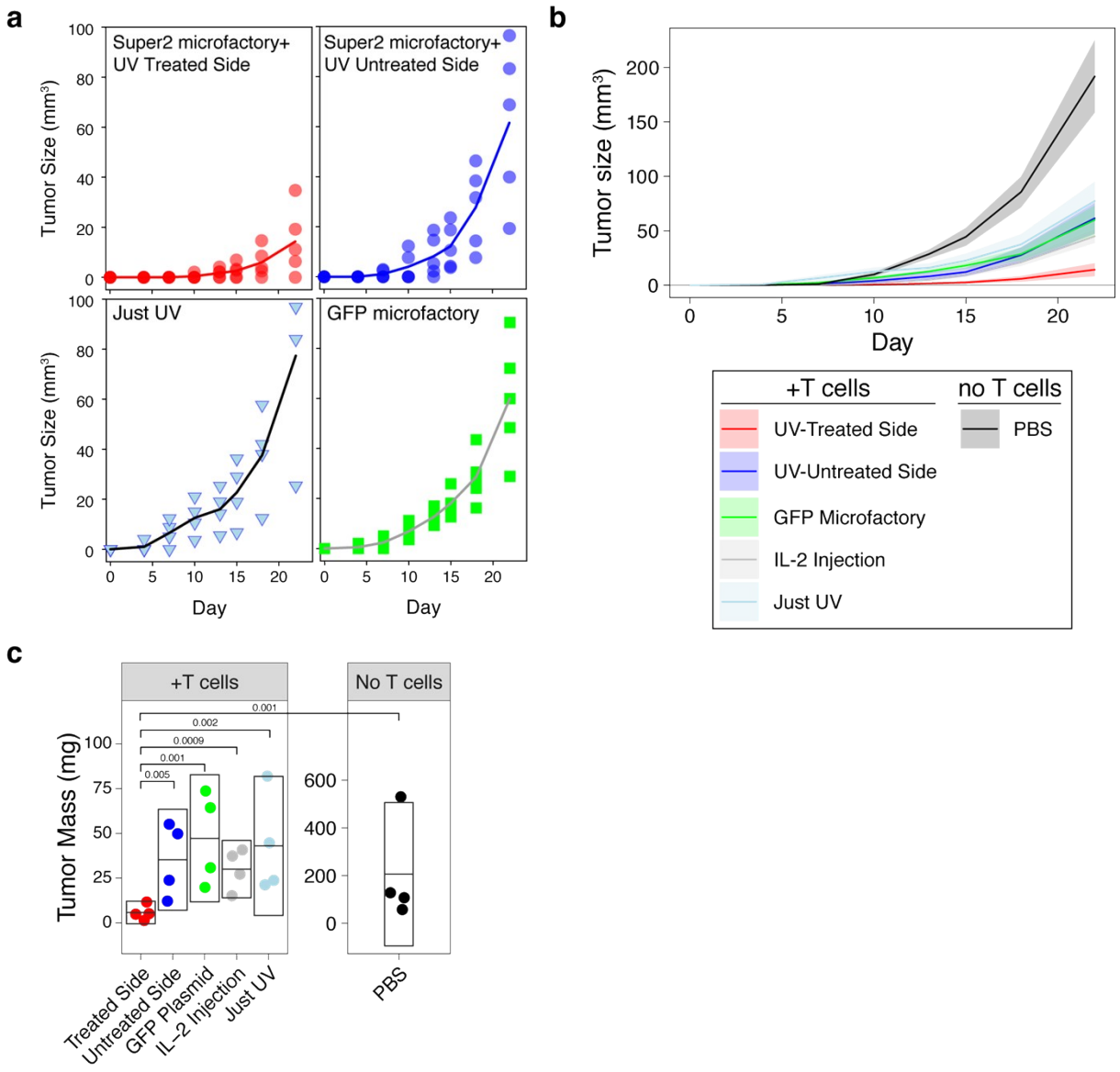
Supplemental Figure 5. Secretion of IFN-γ on day 4 of co-culture as an indicator of activation level as measured *via* cytokine ELISA assay.



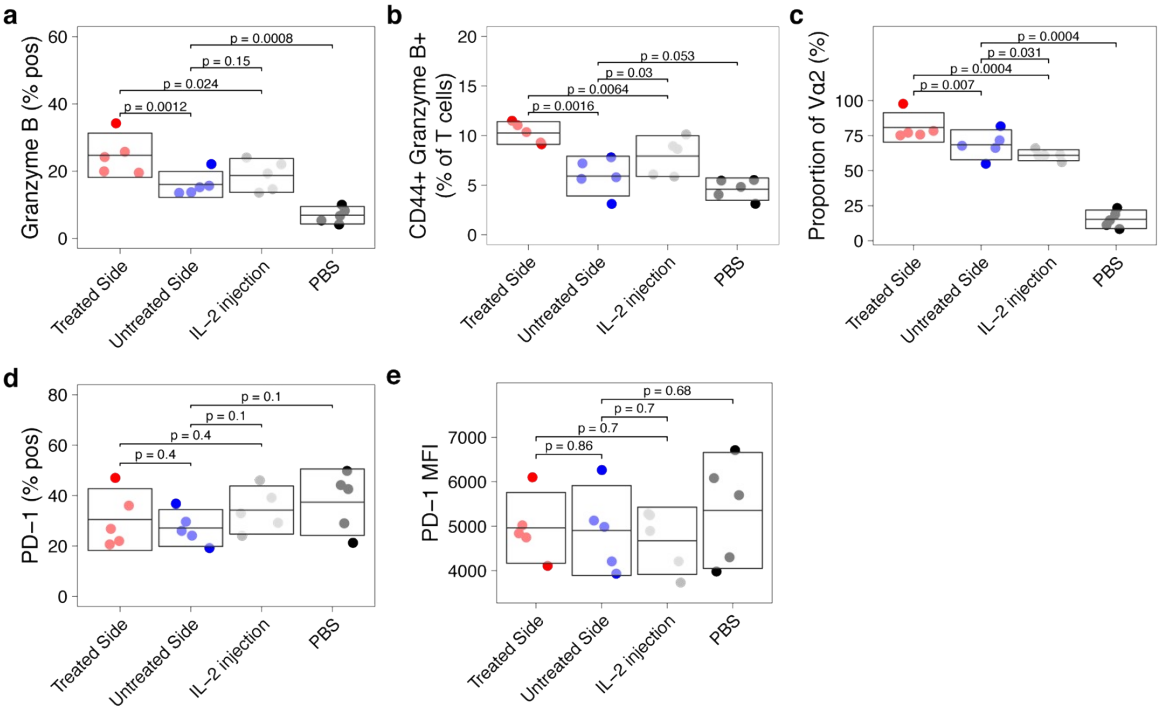
Supplemental Figure 6. Activation and differentiation markers on day 4 and 10 of co-culture including (a) CD62L, and (b) CD25.



Supplemental Figure 7. Chromium (^{51}Cr) release assay shows antigen peptide-specific and cytotoxicity of TCR transgenic CD8+ T cells after 4 days of co-culture with particles as listed. Cytotoxic activity was examined at different ratios of 100:1, 30:1, 15:1, 7:1, 3:1, 1.5:1, and 0.75:1 of treated T cells to tumor cells. The data are presented as Mean ± SD of 3 independent experiments.



Supplemental Figure 8 (Accompanies Figure 4). Mice were injected as per the diagram in Figure 4 with microfactories carrying Super-2, microfactories containing an irrelevant plasmid (GFP), with or without UV activation. Mice with "Just UV" are shown as a control to demonstrate the effect of UV alone on tumor growth. All mice received intravenous transfer of T cells, except the "PBS" mice. These data include the data from Figure 4 plus additional control groups. **a)** Tumor growth was monitored over time. **b)** Averages (lines) and 95% confidence intervals (ribbons) of tumor sizes for each treatment group over time. **c)** Tumor masses measured upon sacrifice on day 22. Each dot is a mouse. Boxes are permuted mean and 95% CI. P-values were calculated by permutation testing to reduce the impact of outliers, and p-values shown were subsequently adjusted for multiple testing. There are no statistically significant differences between the tumor masses on day 22 for the UV-Untreated, "Just UV" treated, GFP (irrelevant) plasmid, or IL-2 only treated mice.



Supplemental Figure 9 (Accompanies Figure 4). In vivo activity of microfactories in tumor model. All measured by flow cytometry on tumor-infiltrating CD8+ T cells. **a,** Granzyme B percent positivity. **b,** Co-expression of CD44 and Granzyme B measured. **c,** Proportion of TILs that showed binding of Vα2 antibody (binds to the T cell receptor Vα region of OT-I T cells). **d-e,** Proportion of CD8+ T cells expressing PD-1 and MFI. Each dot is a mouse, boxes show permuted mean and 95% CI. All p values have been adjusted for multiple comparisons.