ADVANCED MATERIALS

Supporting Information

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Cytokine Secreting Microparticles Engineer the Fate and the Effector Functions of T-Cells

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Experimental Procedure

Materials and Methods:

Unless noted otherwise, all chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). All glassware was cleaned overnight using concentrated sulfuric acid and then thoroughly rinsed with Milli-Q water. All the other cell culture reagents, solutions, and dishes were obtained from Thermo Fisher Scientific (Waltham, MA) except indicated otherwise. Alginate (Mw ≈250 kDa, high G blocks; Novamatrix UP MVG, FMC Biopolymer, Rockland, Maine) was first oxidized using sodium periodate (2%), overnight at room temperature, the reaction was quenched by drop-wise addition of ethylene glycol (Sigma) for 45 min. The sample was then dialyzed (MWCO 3.5 kDa) against deionized water for 3 d followed by lyophilization. Alginate-heparin (Alg-Hep) conjugation was done according to a previously published protocol [S1]. Briefly, alginate and heparin were dissolved separately in MES buffer (0.05 M, pH 5.4) in a concentration of 20 mg/ml overnight at room temperature. Carboxylic acid groups of alginates were activated using EDC and NHS at a fixed molar ratio of 0.6 to 1 for 10 minutes before addition of ethylenediamine (EDA, 31 mM) and running the reaction for 4 h under vigorous stirring at room temperature. This solution was then mixed with EDC/NHS activated heparin and allowed to react for 24 h with gentle stirring at room temperature. The resulting polymer conjugate was dialyzed (SpectraPor dialysis membrane MWCO: 8-14kDa; Spectrum Laboratories, Inc., Rancho Dominguez, CA) against 1M NaCl for a day followed by ultrapure water for an additional two days. The solution was then concentrated, lyophilized and kept at -20°C before further use. The amount of conjugated Heparin was determined using a toluidine blue assay reported by Hinrichs *et al*. [S2].

A glass hydrophobic microfluidic droplet junction chip (channel depth of 100 µm; Dolomite Microfluidics, Charlestown, MA) was utilized to make monodispersed Alginate-Heparin (Alg-Hep) microparticles. Alg-Hep solution (1% w/v) was used as the inner aqueous phase. Mineral oil containing 10 wt% surfactant Span 80 was used as the continuous phase. Flow rates of 5 and 12 µL.min^{-1} were applied using two syringe pumps (Fusion 200, Chemyx,

Stafford, TX) for the Alginate and oil flows, respectively. Images were taken at various time points using a Leica DMIL inverted microscope. The formed particles were collected in a bath of calcium ions (100 mM $CaCl_2$) and left at room temperature for 30 min for ionic crosslinking. The microgels were extensively washed with 10 mM NaCl solution and centrifuged (15,000 rpm for 10 min) twice before further use.

We also designed a second microfluidic platform using 3D printing and structure-removal [13] to control the coating of alginate-heparin microparticles. The acrylonitrile butadiene styrene (ABS) filament was extruded through a 400 μm nozzle using Flashforge Creator Pro Dual Extrusion 3D Printer (Rowland Heights, CA) to form the desired 3D shape. The ABS-based 3D structure was then immersed in a degassed solution of polydimethylsiloxane (PDMS, resin:curing agent ratio of 10:1; Sylgard 184; Dow Corning Corp., Auburn MI). The PDMS was then cured for 4 h at 65°C; the structure was immersed in acetone for several days to dissolve the ABS filaments. After dissolving the template, the channels were cleaned with acetone injection and air-dried. The resulting serpentine micromixer consisted of one inlet for pre-made Alg-Hep microparticles and one for the aqueous solution of chitosan polymer (0.7 wt%, pH 6.1) plus an outlet. The rhodamine-conjugated chitosan (RITC-Chitosan) coating was also performed to evaluate the coating efficiency and the thickness of the formed layer using fluorescent microscopy.

Binding kinetic of the studied cytokines, Interlukine-2 (IL-2; BRB Preclinical Repository, NCI, NIH, Frederick, MD, USA) and stromal cell-derived factor 1α (SDF-1α; Abcam, Cambridge, MA), with alginate, heparin and alginate-heparin surfaces were estimated using biolayer interferometry with a BLItz (Pall ForteBio LLC; Fremont, CA) device. The alginate, heparin and alginate-heparin polymers were immobilized onto Amine-functionalized biosensor tips using EDC/NHS chemistry. The concentration of reactants has been adjusted to have an approximate number of 2×10^6 molecules per chip. Different dilutions of IL-2 and SDF-1α samples, 20, 40, 60, 100, 140, and 200 nM, in HEPES buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4) were used for kinetic studies. Data analysis was performed with Fortebio Data Analysis software using a 1:1 binding model and global fitting analysis to report k_{on}, k_{off} and K_D values for the different surfaces.

To prepare IL-2 loaded Alg-Hep microparticles, our microparticles were incubated with cytokine in PBS buffer containing bovine serum albumin (BSA; 0.1 %w/v) and were gently shaken overnight at 4°C. The microparticles were then centrifuged and washed several times to remove unabsorbed cytokines. The concentration of IL-2 in the removed supernatant was measured using enzyme-linked immunosorbant assay (ELISA) to estimate the binding

capacity of microparticles. Differences in uptake values after the coating process were also evaluated and were on the order of 12-23% depending on the mixing time. Initial loadings were optimized to provide the same loading content for the non-coated particles.

To study the *in vitro* release profile, cytokine-loaded Alg-Hep microparticles were dispersed in PBS (pH 7.4) and 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 were replaced with an equivalent volume of fresh PBS solution. The concentration of released cytokine from microparticles was determined using a human IL-2 ELISA Kit.

For scaffolds preparation, the oxidized alginate (as described before) solution in MES (150 mM MES, 250 mM NaCl, pH 6.5) was covalently conjugated to the RGD peptide (GGGGRGDGS; GenScript USA Inc., Piscataway, NJ) using carbodiimide chemistry (NHS/EDC). After 24 h of reaction the solution was again dialyzed (MWCO 20 kDa) and lyophilized. The solution of 2% w/v alginate-RGD in PBS was mixed with 2×10^6 microparticles (IL-2 loaded) and at 60°C. The cross-linking was initiated by the addition of calcium chloride solution (20 mM) to the alginate-RGD/microparticle mixture while mixing. The mixture was poured in a pre-made PDMS mold, frozen at -80 °C, lyophilized for 3 d, and stored at 4 °C before cellular studies.

Scanning electron microscopy (SEM) was used to observe the cross-sectional microstructure of the alginate-RGD scaffolds. Lyophilized scaffolds were freeze-fractured (using liquid nitrogen) to expose a cross-section. The scaffold specimens were imaged without further coating using a ZEISS Supra 40VP scanning electron microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The pore sizes were calculated from SEM images by evaluating at least 40 pores using the ImageJ program (National Institutes of Health, Bethesda, MD).

Mice, cell culture and reagents: Five- to eight-week-old wild type mice were purchased from 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 policy on humane and ethical treatment of animals. Cells were cultured in IMDM supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 uM 2-ME. Total T-cells or CD8+ T-cells were purified using a negative enrichment kits (Affymetrix eBioscience).

For *in vitro* activation of CD8+ T-cells, cells were cultured at the concentration of 1x10e6/ml in 96-well plates coated with 5 μg/ml of anti-CD3 (2C11; Bio X Cell) in

combination with 2 μg/ml of soluble anti-CD28 (37.51; Bio X Cell) or 100 IU/ml of human IL-2 [S3]. After three days, cells were re-plated in 24 well plates at the concentration of 0.25 x10e6/ml with IL-2 or IL-2 containing particles. Particles were counted after about 20 times dilution and *via* hemocytometer before co-culture with T-cells. We prepared plain Alg-Hep, chitosan-coated Alg-Hep, and a mixture of both of these IL-2 containing particles. Each type of particle was added to the cells at the particle to cell ratios of 2:1, 1:1, or 1:2.

To seed mouse CD8+ T-cells on alginate-based scaffolds, 250 μl of cell suspension (8×10^6 cells/ml) was seeded on top of each lyophilized scaffold in a 48-well tissue culture plate. Then, the well plate was shaken at 150 rpm using an orbital microplate shaker (MS3, IKA) for 20 min in a 37°C incubator to help cells infiltrate the biomaterials. At the end of the culture period, T-cells were recovered from alginate-based scaffolds using dissolving buffer (55 mM sodium citrate, 30 mM EDTA, 0.15 M NaCl). The cells were then centrifuged and the number of viable cells in each culture were determined using dual-fluorescence viability assay by AO and PI.

For flow cytometry analysis, antibodies to mouse CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7), Thy1.2 (53-2.1), IL-7R (CD127), CCR7, CD62L, perforin Monoclonal Antibody (eBioOMAK-D), PE, Alexa Fluor® 647 anti-human/mouse granzyme B (GB11), Alexa Fluor® 647 anti-mouse/rat/human FOXP3 and CD16/CD32 (FC block) were purchased from eBioscience (San Diego, CA), BioLegend (San Diego, CA), or BD Biosciences (San Diego, CA). Propidium iodide (PI) and acridine orange (AO) were purchased from Calbiochem. Cells were analyzed on FACSVerse using FlowJo software (Treestar, Ashland, OR).

To analyze perforin and granzyme B expressions after treatment in 2D or 3D cultures using Western blot, T-cells were lysed using protein extraction buffer (Bio-Rad, Irvine, CA). The extracted proteins were then fractionated in 10% sodium dodecyl sulfate-polyacrylamide gels (PAGE) and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membranes were then incubated with antibodies against perforin and granzyme B as well as β-actin which was used as the housekeeping protein.

To perform stem cell cytotoxicity test, 5×10^5 human bone marrow mesenchymal stem cells (hBMMSCs) were cultured in a six-well plate in a regular stem cell medium. Recombinant mouse Interferon gamma protein (IFN-γ; Abcam) was loaded within the coated and non-coated microparticles and co-cultured with the stem cells at the ratio of 1:1 particle: cell. After seven days of coculturing relative number of apoptotic cells was evaluated using Annexin V-PE Apoptosis Detection Kit (BD Bioscience).

For iT-reg formation experiments CD4+ T-cells were purified from mouse spleen by EasySep immunomagnetic negative selection (Stem Cell Technologies). Cells were then activated on anti-CD3e antibody (8 mg/ml) coated plates with the anti-CD28 antibody (2 mg/ml) supplemented medium. At the same time TGF-β at different concentrations, either in a soluble format or via TGF-β loaded particles were added to the media. All the conditions expect for two of the controls were supplied with IL-2 loaded (20 U/ml) particles. After four days regulatory T-cells were removed from wells coated with anti-CD3e and stained with antibodies for flow cytometry analysis.

Five- to 10-wk-old TCR transgenic 318 mice were used for *in vitro* cytotoxicity assay and were kept under specific pathogen-free conditions, according to institutional guidelines. EL-4 thymoma cells (H-2b, Thy-1.2; ATCC® TIB-39) were cultured in Dulbecco's medium that contained 5% FCS and glutamine and penicillin/streptomycin was added to it.

A total of 4×10^6 cells/ml spleen cells from 318 TCR transgenic mice and p33 peptide was added to 1 mM final concentration. After 48 h, CD8+ T-cells were purified by negative selection, as mentioned before. Then 250 µl of cell suspension (8×10^6 cells/ml) was seeded on top of each lyophilized scaffold in a 48-well tissue culture plate. Then, the well plate was shaken at 150 rpm using an orbital microplate shaker for 20 min in a 37°C incubator to help cells infiltrate the biomaterials. At the end of the culture period, T-cells were recovered from alginate-based scaffolds using dissolving buffer (55 mM sodium citrate, 30 mM EDTA, 0.15 M NaCl). Finally, a total of 5×10^6 transgenic T-cells/well in a volume of 1ml were counted and serially diluted up to six times (T-Cell: tumor cell= 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.[S4] EL4 cells were coated with peptide p33 at a concentration of 10^{-6} M and were labeled in a total volume of 300 µl with 250 µCi ^{51}Cr for 2 h at 37°C on a rocking platform. The resulting labeled cells were washed three times, and 10^4 cells were added to the effector cells in a final volume of 200 µl. After a 0.5, 2 h, 4 h, 6 h, or 12-h incubation at 37°C, 60 µl of the supernatants was harvested and counted with a gamma counter (PerkinElmer, Richmond, CA).

S1 Zuo, Q. *et al.* Heparin-conjugated alginate multilayered microspheres for controlled release of bFGF. *Biomedical Materials* **10**, 035008 (2015).

S2 Hinrichs, W., Ten Hoopen, H., Wissink, M., Engbers, G. & Feijen, J. Design of a new type of coating for the controlled release of heparin. *Journal of controlled release* **45**, 163-176 (1997).

S3 Kidani, Y. *et al.* Sterol regulatory element-binding proteins are essential for the metabolic programming of effector T cells and adaptive immunity. *Nature immunology* **14**, 489-499 (2013).

S4 Hany M, *et al.* Anti-viral protection and prevention of lymphocytic choriomeningitis or of the local footpad swelling reaction in mice by immunization with vaccinia-recombinant virus expressing LCMV-WE nucleoprotein or glycoprotein. *Eur J Immunol* **19,** 417-24 (1989).

Supporting Information Figures

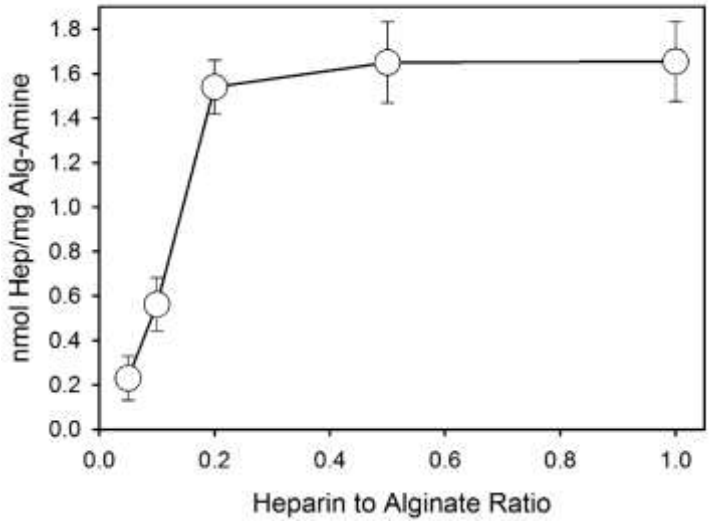

Figure SI. 1. Degree of heparinization of alginate with varying amount of initial added heparin content to the reaction.

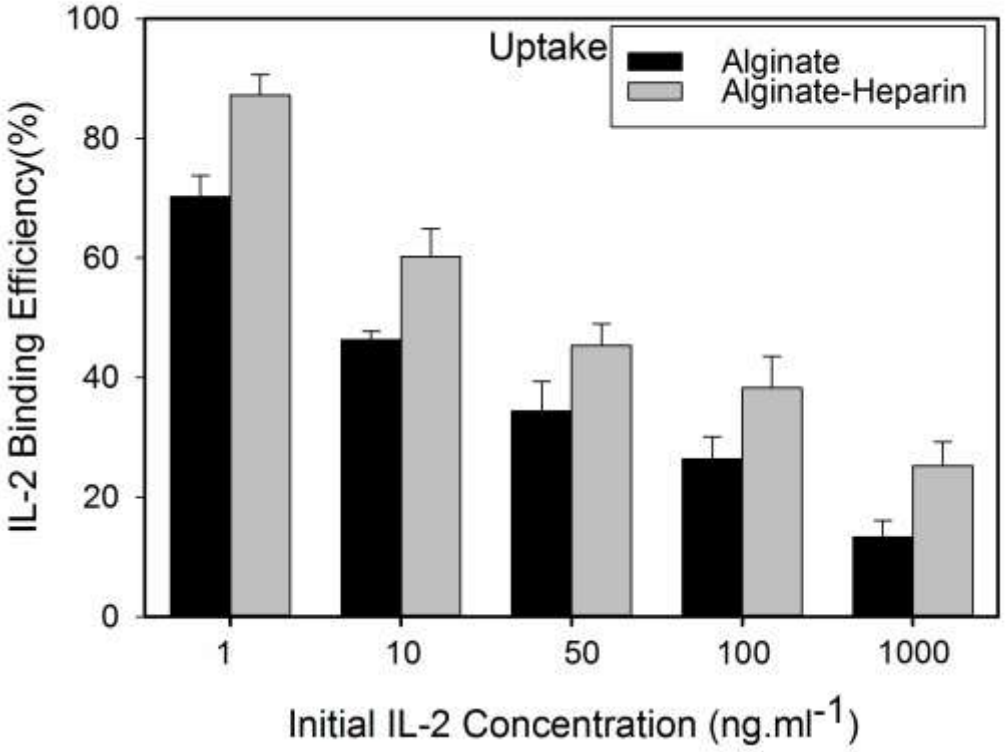

Figure SI. 2. IL-2 binding efficiency of alginate and Alg-Hep microparticles at various initial concentration of IL-2 which loaded to particles *via* uptake method. The presented data are expressed as average ± SD.

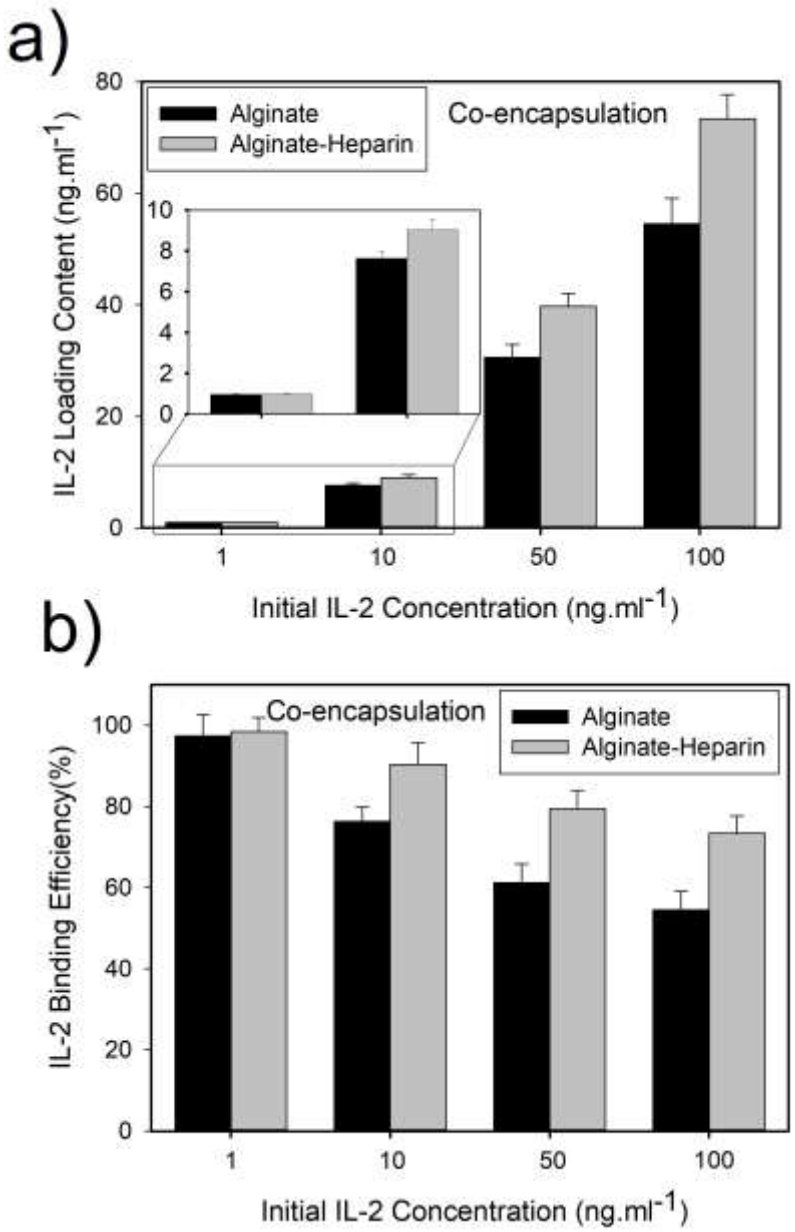

Figure SI. 3. IL-2 loading content (a) and binding efficiency (b) of alginate and Alg-Hep microparticles at various initial concentration of IL-2 which co-encapsulated during the particle formation. The presented data are expressed as average ± SD.

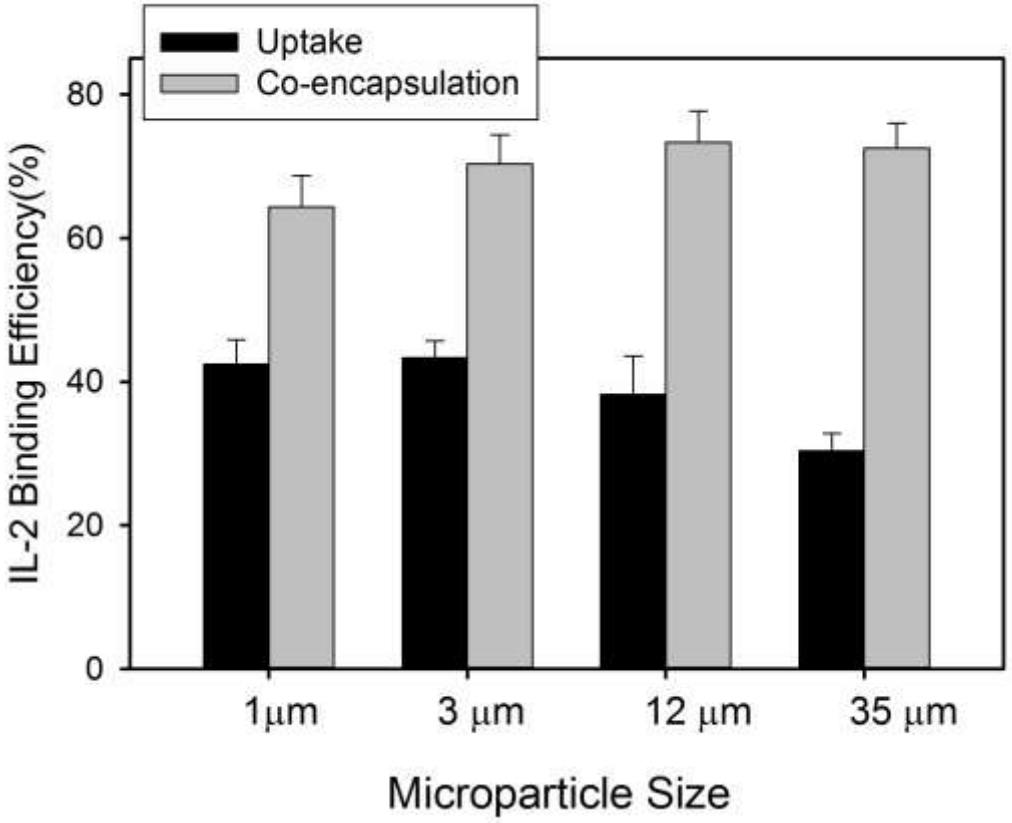

Figure SI. 4. Effect of microparticle size on IL-2 binding efficiency of Alg-Hep microparticles at initial IL-2 concentration of 100 ng/ml which was studied through particles that uptake IL-2 after their formation or IL-2 was co-encapsulated within them during their formation procedure. The presented data are expressed as average ± SD.

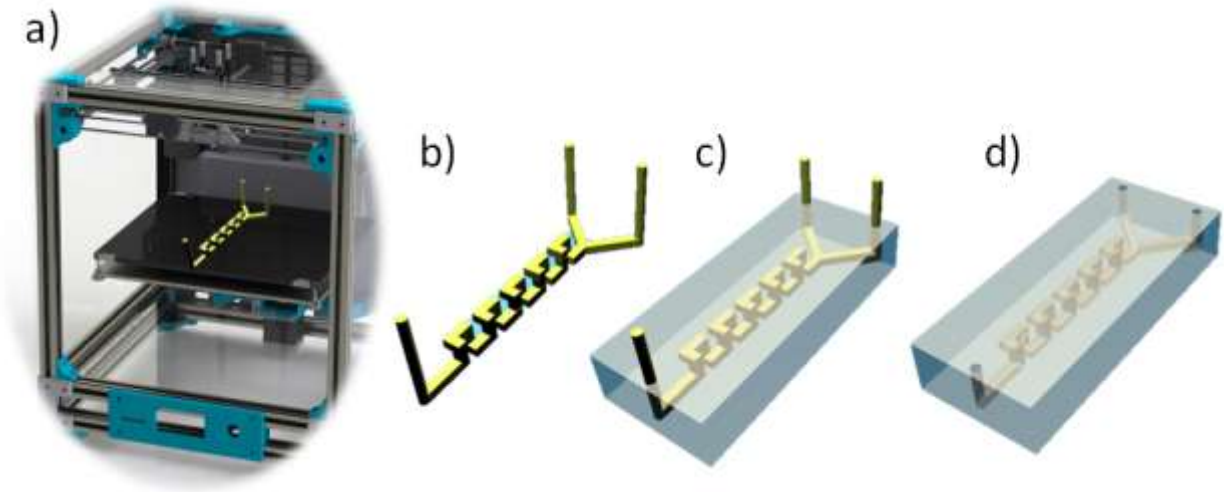

Figure SI. 5. Schematic representation of 3D printed and structure removal method that is used to prepare a micromixer platform for coating of Alg-Hep particles includes: (a) 3D printing of mold, (b) PDMS casting and (c) dissolution of ABS filaments to provide microchannels inside PDMS (d).

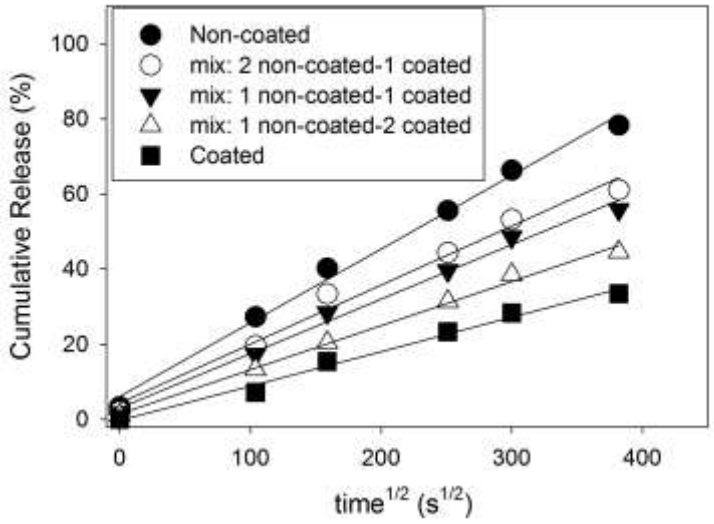

Figure SI. 6. *In vitro* release profile of IL-2 from microfluidic Alg-Hep –based microparticles in PBS at 37°C.

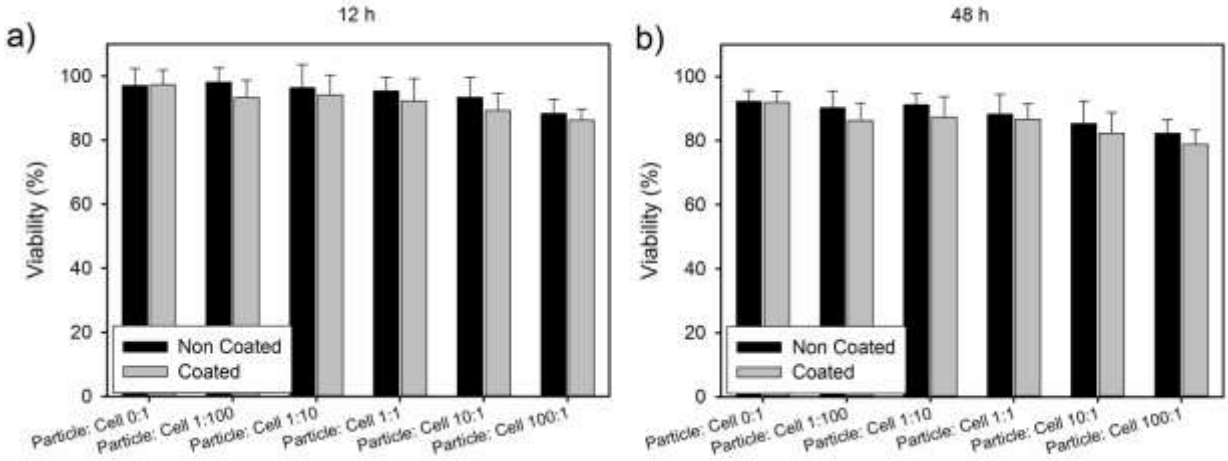

Figure SI. 7. Cell viability evaluation of non-coated and coated particles at different ratio to cells after 12 (a) and 48 h (b) of co-culturing with CD8+ T-cells and in absence of IL-2. The presented data are expressed as average ± SD.

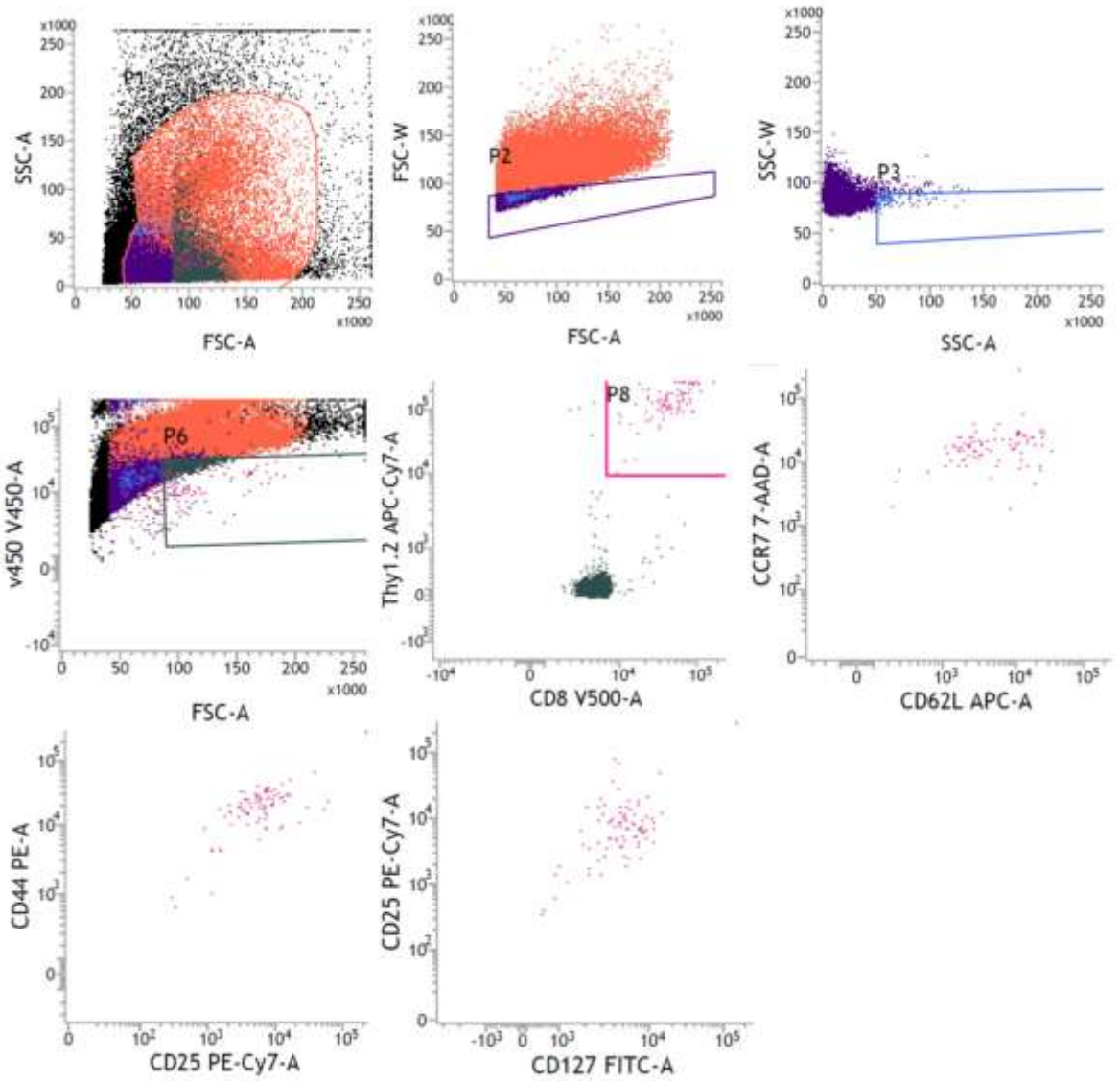

Figure SI. 8. Flow cytometry results of microparticles in the absence of T-cells. The results confirm there is a negligible level of particles staining.

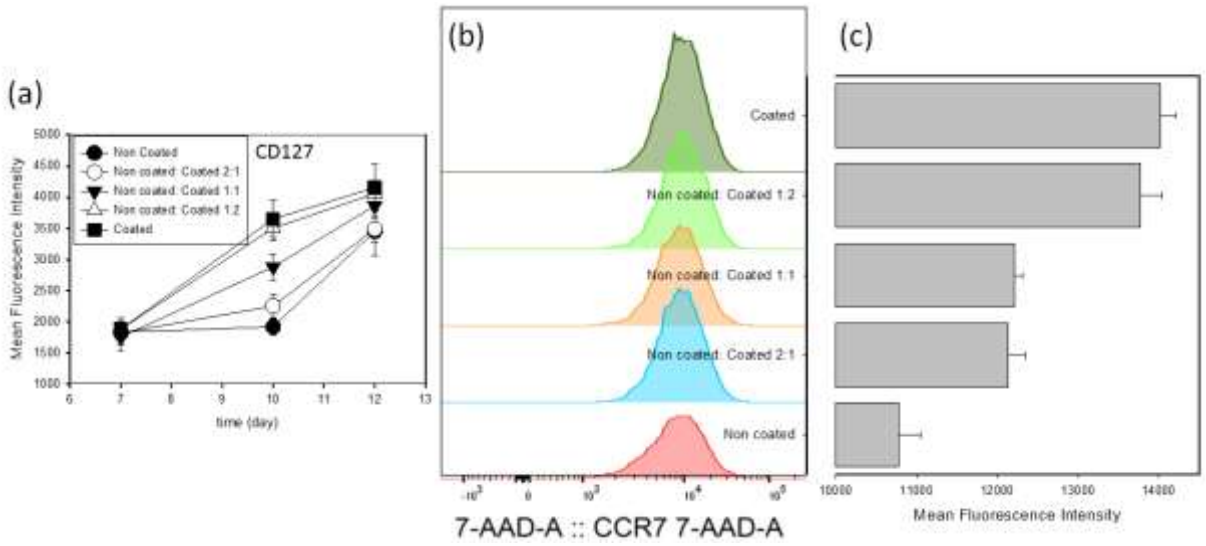

Figure SI. 9. (a) Regulation of IL-7Ra (CD127) re-expression after priming in three different cell-to-particle ratios at day 7, 10, and 12. (b) Histograms of CCR7 expression of memory cells subset when cells are treated with designated mixtures of coated vs non-coated particles. The corresponding MFIs are shown in (c). The presented data are expressed as average ± SD.

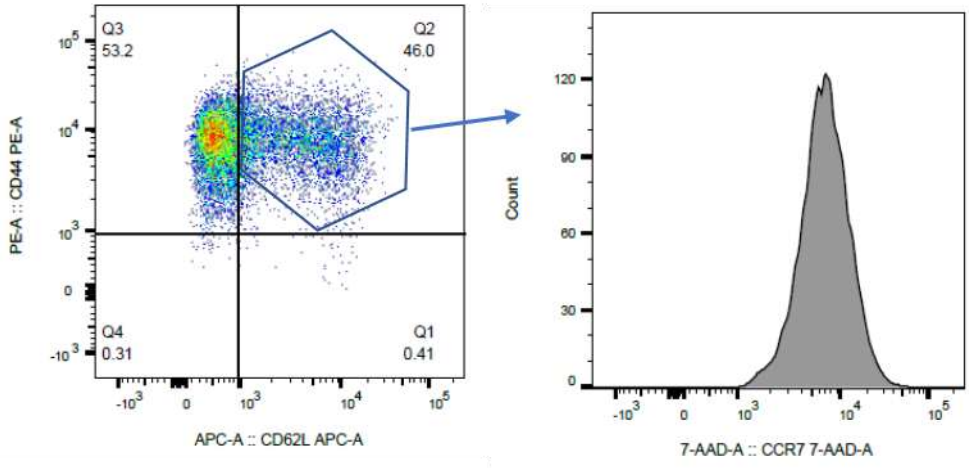

Figure SI. 10. Gating procedure for CCR7 surface marker.

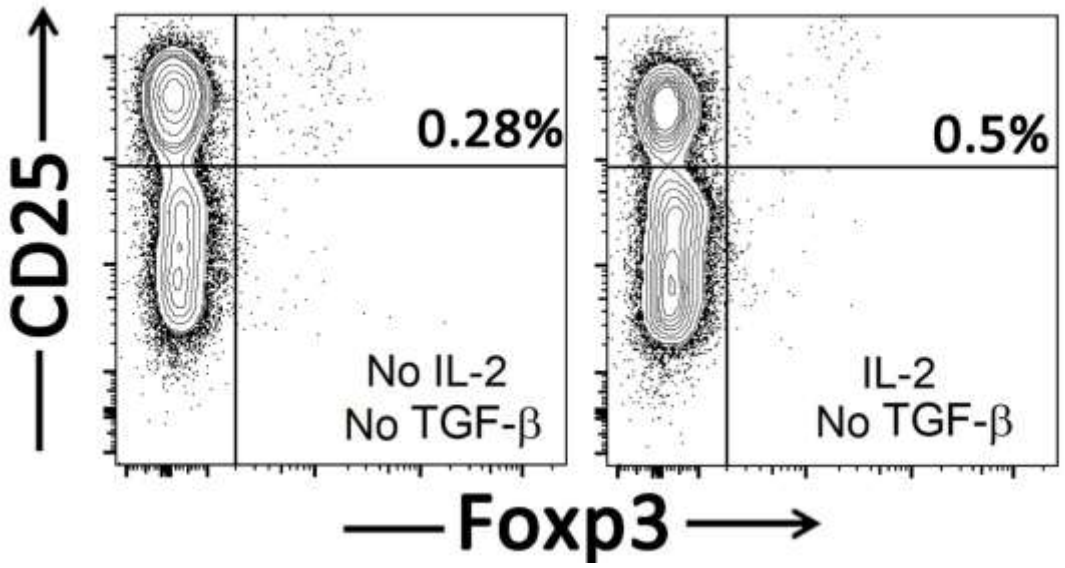

Figure SI. 11. Flow cytometric analysis of Foxp3 and CD25 co-expression were performed to show the effect of IL-2 presence in absence of TGF-β which did not cause any difference in the amount of iT-reg formation.

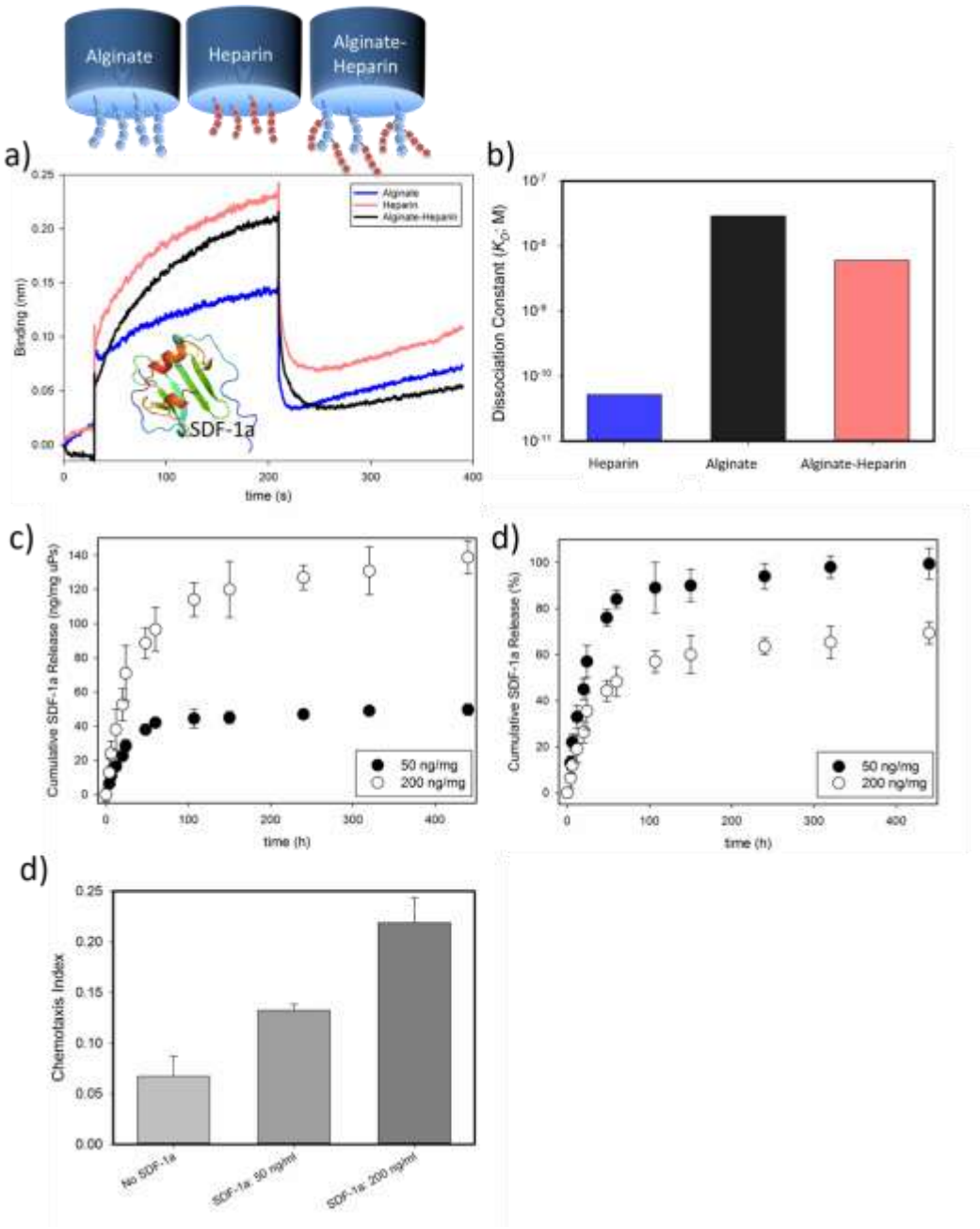

Figure SI 12. (a) Binding kinetic of SDF-1a cytokine (100 nM) to alginate, heparin, and Alg-Hep functionalized surfaces. (b) Calculated dissociation constants (K_D). Release profile of SDF-1a cytokine from non-coated Alg-Hep microparticles into a PBS solution at 37°C at different initial loading of 50 and 200 ng/ml as presented by cumulative released amount (c) and percentage (d). (e) Quantification of relative T-cells migration (Chemotactic index) for naïve and active T-cells after 2 h of culturing with non-coated Alg-Hep microparticles at different initial loading. The presented data are expressed as average ± SD.

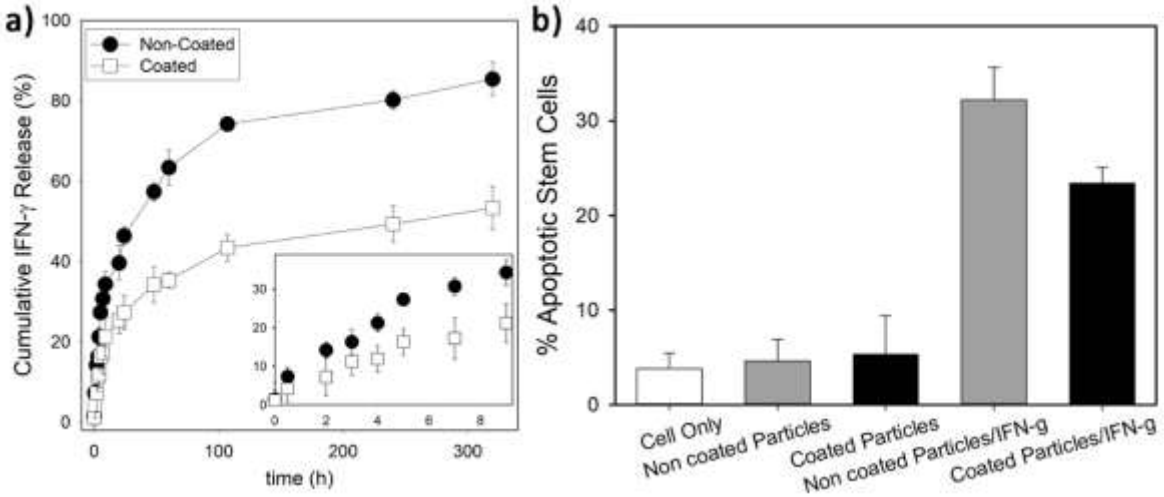

Figure SI. 13. (a) Cumulative release of IFN-γ Pro-inflammatory cytokines at 37 °C. (b) Apoptosis of hBMMSCs in the presence of IFN-γ-loaded microparticles after a week of co-culturing, as tested using Annexin V-FITC apoptosis detection kit.

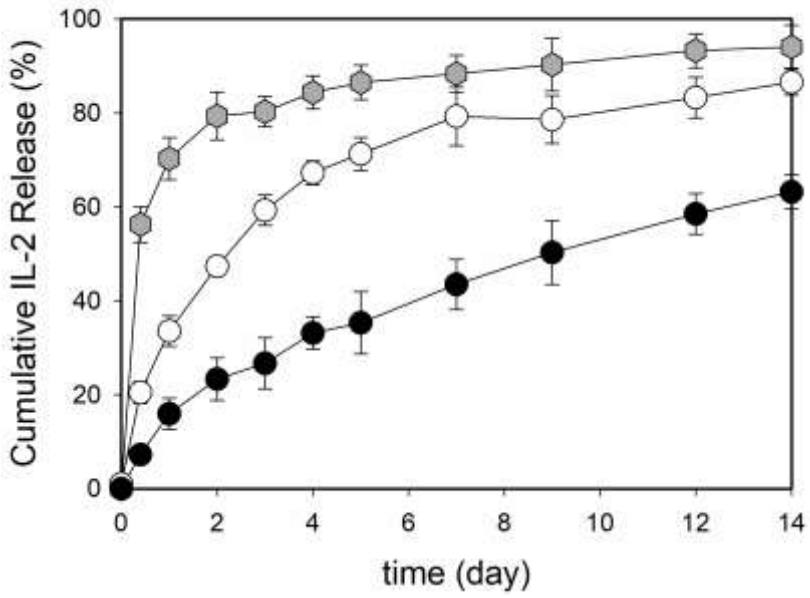

Figure SI. 14. IL-2 release kinetic from alginate-based scaffold after encapsulation of free IL-2 (gray hexagon), IL-2 loaded microparticles without (open circles) and with (filled circles) coating in PBS at 37°C. The presented data are expressed as average ± SD.

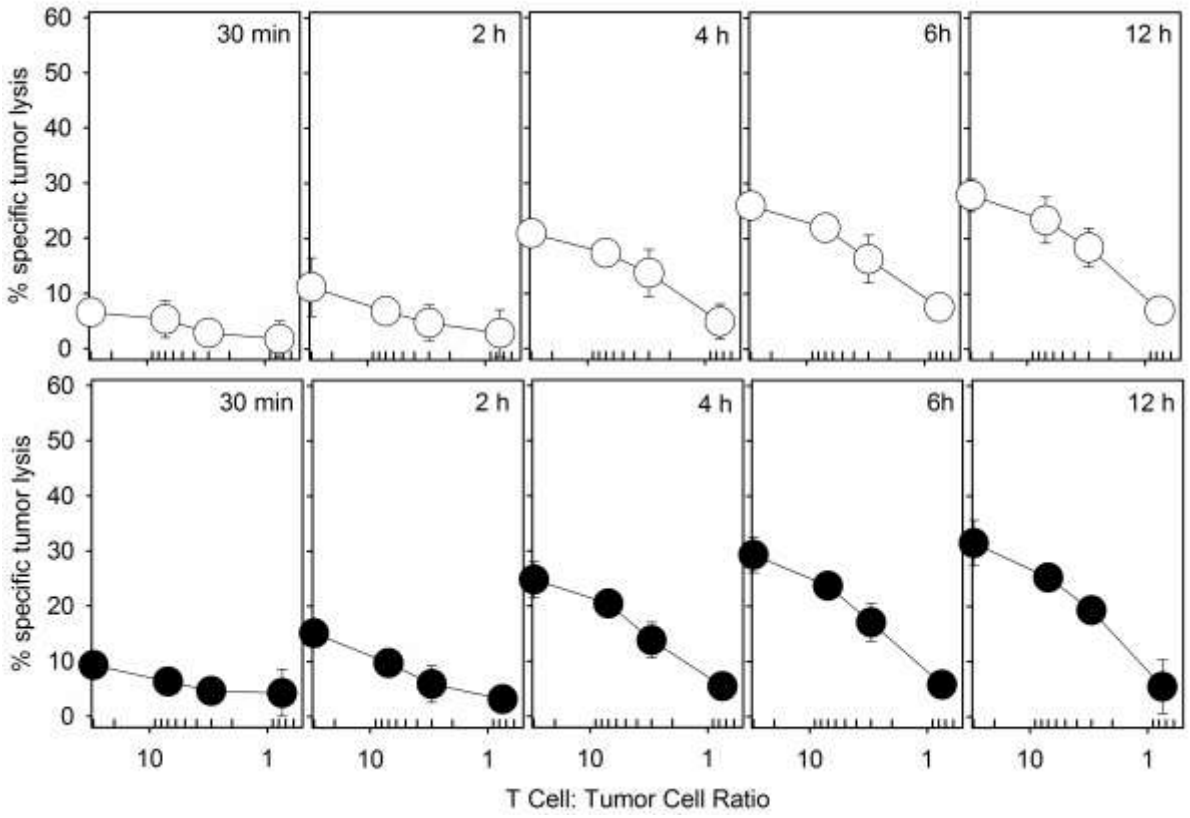

Figure SI. 15. Kinetics of cytotoxic activity of p33-specific T-cells after treatment with soluble IL-2 in 2D (upper panels; empty circles) and in the 3D alginate-RGD scaffold (lower panels; filled circles). Cytotoxic activity of spleen-derived, *in vitro* treated CD8+ T-cells was analyzed using chromium assay. Specific tumor lysis was determined at the indicated time points (30 min, 2 h, 4 h, 6 h, and 12 h) of incubation. Cytotoxic activity was examined at four different ratios (30:1, 7:1, 3:1, and 0.75:1) of treated T-cells to tumor cells. The data are presented as average ± SD of five independent samples. The presented data are expressed as average ± SD.

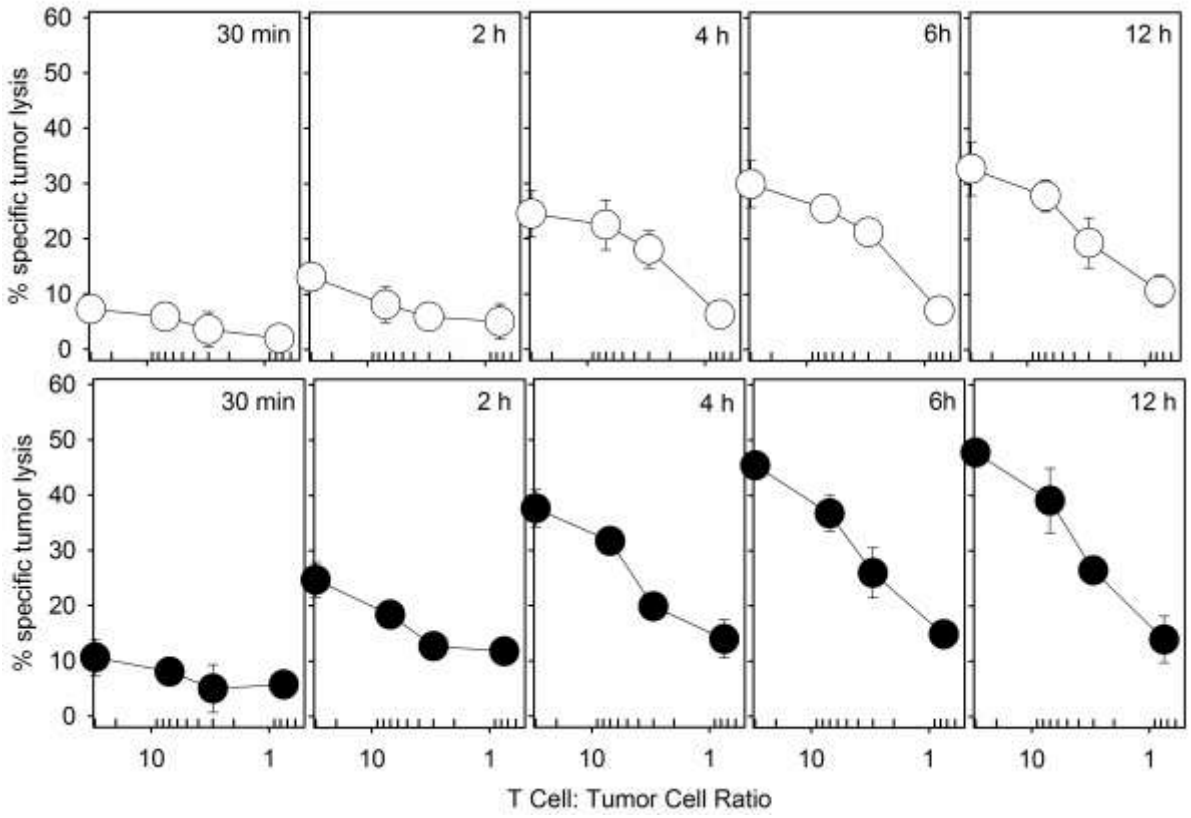

Figure SI. 16. Kinetics of cytotoxic activity of p33-specific T-cells after treatment with non-coated IL-2 loaded microparticles in 2D (upper panels; empty circles) and in the 3D alginate-RGD scaffold (lower panels; filled circles). Cytotoxic activity of spleen-derived, *in vitro* treated CD8+ T-cells was analyzed using chromium assay. Specific tumor lysis was determined at the indicated time points (30 min, 2 h, 4 h, 6 h, and 12 h) of incubation. Cytotoxic activity was examined at four different ratios (30:1, 7:1, 3:1, and 0.75:1) of treated T-cells to tumor cells. The data are presented as average ± SD of five independent samples. The presented data are expressed as average ± SD.

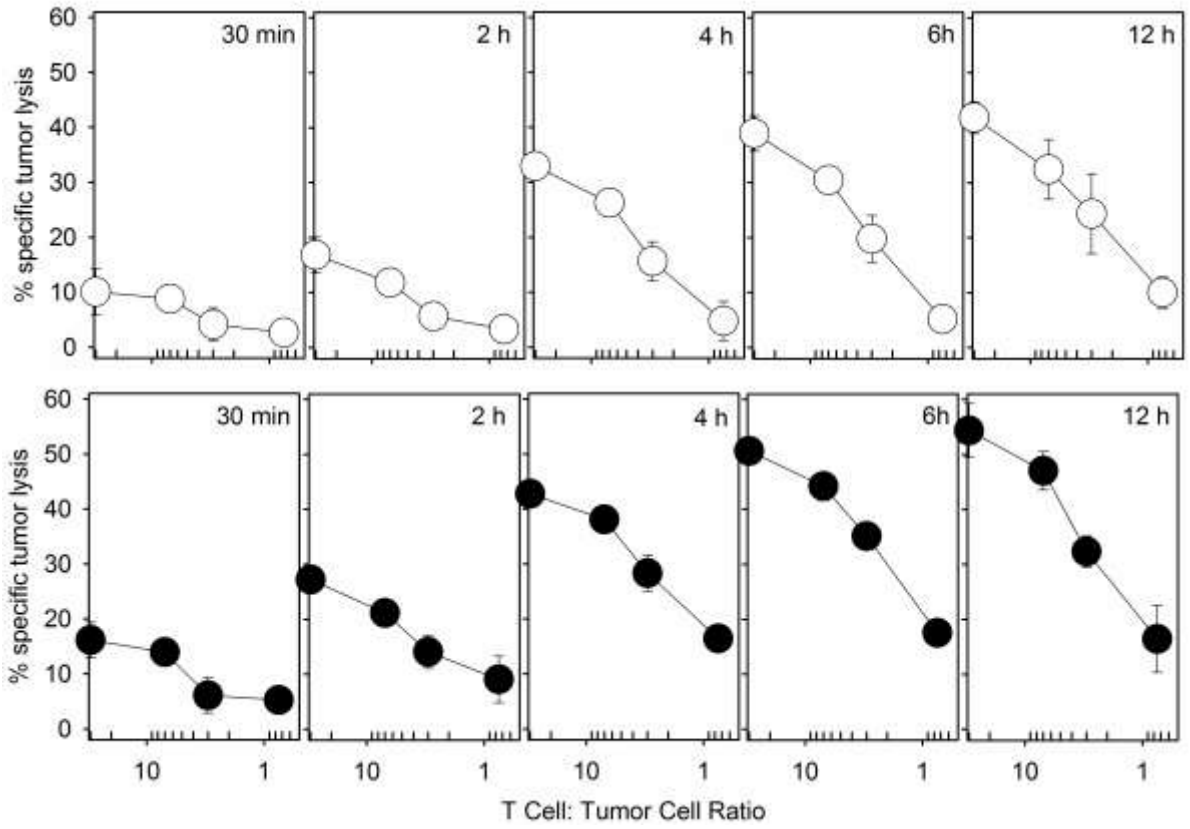

Figure SI. 17. Kinetics of cytotoxic activity of p33-specific T-cells after treatment with coated IL-2 loaded microparticles in 2D (upper panels; empty circles) and in 3D alginate-RGD scaffold (lower panels; filled circles). Cytotoxic activity of spleen-derived, in vitro treated CD8+ T-cells was analyzed using chromium assay. Specific tumor lysis was determined at the indicated time points (30 min, 2 h, 4 h, 6 h, and 12 h) of incubation. Cytotoxic activity was examined at four different ratios (30:1, 7:1, 3:1, and 0.75:1) of treated T-cells to tumor cells. The data are presented as average ± SD of five independent samples. The presented data are expressed as average ± SD.