Supporting Information for

Fine tuning and efficient T cell activation with stimulatory aCD3 nano-arrays

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Materials and Methods

Nano-array fabrication

Nano-arrays were fabricated by Block Copolymer Micellar NanoLithography (BCML) similar to previously described.[1] Briefly, piranha cleaned glass slides were dip-coted or spin-coated at defined velocities with toluene solution of $HAuCl_4$-loaded micelles of various diblock copolymers polystyrene-block-poly(2-vinylpyridine) (PS-b-PVP) (Polymer Source Inc.,

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Montreal, Canada) to achieve interparticle spacing from 35 nm to 150 nm. Upon drying, substrates were treated with forming gas (10% H_2 + 90% Ar) plasma (45 min, 0.4 mbar forming gas, 350 W; TePla 100-E, Wettenberg, Germany) and stored sealed at room temperature until further use. Few surfaces from each batch of nano-arrays were coated with ~5 mm carbon layer (Leica EM MED020 Modular high vacuum coating system, Leica Microsystems, Wetzlar, Germany) to reach conductivity and analyzed by Scanning Electron Microscopy (SEM) using Zeiss Ultra 55 electron microscope (Zeiss SMT, Oberkochen, Germany). Pattern quality (order parameter) and interparticle spacing were determined using the ImageJ (NIH, Bethesda, USA) plug-in written and kindly provided by Dr. Philippe Girard (European molecular biology laboratory (EMBL), Heidelberg, Germany). Global density of AuNPs was subsequently calculated as: $\rho = \frac{2*N\ particles}{\sqrt{3}*(spacing)^2}$.

Protein and antibody immobilization

Nano-arrays were activated with oxygen plasma (10 min, 0.4 mbar O_2, 150 W; TePla 100-E, Wettenberg, Germany) and passivated with in house synthesized mPEG2000-urea (CH_3-O-(CH_2-CH_2-O)$_{43}$-NH-CdO-NH-CH_2-CH_2-CH_2-Si(OEt)$_3$) as previously described.[2] Briefly, activated substrates were inserted into a flask containing 0.25 mM mPEG2000-urea solution in dry toluene (Merck KGaA, Darmstadt, Germany, dried over 3 Å molecular sieve). After adding 2.5 µM triethylamine (Fluka Analytical, Sigma-Aldrich Chemie GmbH, Munich, Germany) and 1 µL of MilliQ water per mL of final reaction solution, the flask was heated at 80˚C for 16-20h. All chemicals were stored and processed under inert nitrogen atmosphere and all solvents used were of analytical grade. To remove the physisorbed PEG residues, the samples were rinsed with ethyl acetate (Acros Organics, Fischer Scientific GmbH, Nidderau, Germany) and methanol (BASF SE, Ludwigshafen, Germany). The samples were finally dried under nitrogen stream and used

for coupling of biomolecules in a manner similar to previously described.[3] A 1mM ethanolic solution of thiol-NTA linker (HS-$(CH_2)_{11}$-EG_3-NTA) (Prochimia, Sopot, Poland) was incubated for 90 min at room temperature. When nano-arrays were used for T cell activation experiments, the surfaces were sterilized with 70% ethanol for 30 min and a custom-made 4-well Teflon chamber (well dimensions corresponding to 96 well plate format) was assembled on the surface with two-component dentist glue. Thiol-NTA linker was then loaded with 10 mM $NiCl_2$ solution in HBS pH 7.5 and 10 μg/mL of His_6-Protein G (BioVision, Milpitas, USA) in HBS for 2h at room temperature. After rinsing with HBS and PBS, human aCD3-AlexaFluor488 or purified human aCD3e OKT3 (both purchased from eBioscience, Frankfurt, Germany) was coupled to nano-arrays by incubating 10 μg/mL antibody solution in PBS pH 7.4 over night at 4ºC. Functionalized nano-arrays were extensively rinsed with PBS and immediately used for imaging or cell activation experiments.

Plastic well controls were prepared by incubating high-binding polystyrene wells (96 wp, FLUOTRAC 600 with μClear bottom; Greiner Bio One, Frickenhausen, Germany) with 10 μg/mL purified human aCD3e OKT3 (im.aCD3), 10 μg/mL purified human aCD3e OKT3 and 50 μg/mL human aCD28 functional grade purified (clone 9.2, eBioscience, Frankfurt, Germany) (im.aCD3+im.aCD28) or 10 μg/mL aCD3 isotype control (eBioscience) (aCD3 isotype) in PBS pH 7.4 for 2h at 37°C, followed by extensive rinsing with PBS. Soluble aCD28 (2 μg/mL, im.aCD3+s.aCD28) and phorbol 12-myristate 13-acetate (10 ng/mL) + ionomycin (0.5 μM) (both purchased from Sigma-Aldrich) (PMA/iono) were added where indicated in complete RPMI medium together with the suspended cells. Non-treated cell were cultured in complete medium alone.

Quartz Crystal Microbalance with Dissipation (QCM-D)

The measurements were performed with a Q-sense E4 device (Q-Sense, Stockholm, Sweden). AT-cut quartz crystals with Au electrodes (Q-sense) were cleaned for 45 min in oxygen plasma (0.4 mbar O_2, 150 W; TePla 100-E, Wettenberg, Germany) and mounted into the flow system. A flow rate of 50 µL/min and a temperature of 22°C were kept constant throughout all measurements. After the system was equilibrated in ethanol (p.a., Merck KGaA, Darmstadt, Germany), the sensors were incubated under constant flow with following solutions: 1 mM ethanolic solution of thiol-NTA , 10 mM $NiCl_2$ solution in HBS pH 7.5 and 10 µg/mL His_6-Protein in PBS pH 7.4, respectively. Finally, the sensors were incubated with the 500 mM imidazole solution in HBS. Prior to each incubation step the system was equilibrated in the appropriate solvent to avoid any frequency shift due to solvent exchange and each incubation was followed by the rinsing step with the same solvent. The incubation time intervals were arbitrary – the sensors were incubated with each solution until the frequency shift became steady (reached a plateau). All values are taken from higher overtones (f=7).

Fluorescence microscopy of fabricated substrates

Laser Scanning Confocal Microscopy (LSCM) was performed on Leica TCS SP5 inverted microscope (Leica Microsystems, Wetzlar, Germany) equipped with HCX PL APO CS 20x/0.7 objective. For imaging, the samples were mounted upside-down on a standard microscopy glass slide with ProlongGold mounting media (Invitrogen™, Life Technologies GmbH, Darmstadt, Germany). A white laser was used to excite the labeled aCD3 at 488 nm. Acquired images were further processed with ImageJ.

T cell isolation

Human CD4+ T cells were isolated via a two-step procedure from the peripheral blood of healthy volunteers after informed consent. Peripheral Blood Mononuclear Cells (PBMCs) were first acquired by density gradient centrifugation on Lymphocyte Separation Media (PAA, Piscataway, USA) according to manufacturer's instructions and were further enriched for CD4+ T cell by negative selection with CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched population was stained with aCD3-FITC, aCD4-PE and the appropriate isotype controls (all antibodies for flow cytometry were purchased from Immunotools, Friesoythe, Germany) and analyzed by FACS for purity. The purity of isolated CD4+ T cells was consistently >97% and the viability >90% (based on Trypan Blue exclusion assay). CD4+ T cells were resuspended in complete RPMI medium (RPMI 1640 supplemented with 10% HI FBS, 2 mM L-glutamine and 1% Penicillin-Streptomycin, all purchased from Gibco®, Life Technologies GmbH, Darmstadt, Germany) and immediately used in activation experiments.

Bright-field microscopy of T cells on substrates

Bright-field images were acquired after 17h and 4 days of T cell culture on aCD3 nano-arrays and PEG controls, as well as 96 well controls on Axiovert 40 CFL (Zeiss) equipped with LD A-Plan 20x/0.3 Ph1 objective. Collected images were further processed with ImageJ.

CD69 expression

Freshly isolated CD4+ T cells (10^5/well) were cultured for 17 h on aCD3 nano-arrays of different spacing or PEG control in 200 µL complete RPMI medium with or without 2 µg/mL aCD28 (clone 9.2, eBioscience, Frankfurt, Germany) as designated, as well as plastic well controls. Harvested cells were washed with PBS, stained with aCD69-PE in FACS buffer (PBS/0.1% HI FBS/0.1% NaN3) for 30 min on ice and fixed with 4% formaldehyde in FACS

buffer. FACS analysis was performed on FC 500 Cytometer (Beckman Coulter GmbH, Krefeld, Germany) with CXP software using blue/green laser to excite PE at 488 nm. Dead cells and cell debris were excluded from analysis by forward- and side-scatter gating. Collected data were further analyzed by FlowJo (Tree Star Inc., Ashland, USA).

IL-2 assay

Freshly isolated CD4+ T cells (10^5/well) were cultured for 17 h on aCD3 nano-arrays of different spacing or PEG control in 200 µL of complete RPMI medium with or without 2 µg/mL aCD28 as designated, as well as plastic well controls. IL-2 concentration was determined in culture supernatants by human IL-2 ELISA Ready-Set-Go! kit (eBioscience, Frankfurt, Germany) according to manufacturer's instructions.

Cell Proliferation

Fluorescent dye 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester CFSE (Invitrogen™, Life Technologies GmbH, Darmstadt, Germany) was stored as 5 mM stock solution in DMSO until used. Cell labeling was performed as previously described for low cell numbers (0.5-10x10^6cells/mL).[4] Freshly isolated CD4+ T cells in 1 mL complete RPMI medium were stained with 5 µM dye solution in PBS for 5 min at room temperature in the dark. The labeling was stopped by adding 10 vol of 20ºC PBS containing 5 % HI FCS, followed by centrifugation for 10 min at 300xg. The cells were washed once again with 20ºC PBS containing 5 % HI FCS and resuspended in complete medium to be seeded on the substrates. 5x10^4 labeled cells/well were cultured for 4 days on aCD3 nano-arrays or PEG controls in 200 µL complete RPMI medium with or without 2 µg/mL soluble aCD28, as well as plastic well controls. Harvested cells were washed with PBS, stained with a fixable viability dye eFluor780 (eBioscience, Frankfurt, Germany) according to manufacturer's instructions and fixed with 4%

formaldehyde in FACS buffer. FACS analysis was performed on the following day on FC 500 Cytometer with CXP software using blue/green laser to excite CFSE at 488 nm and red laser to excite the viability dye at 633 nm. Dead cells and cell debris were excluded from analysis by forward- and side-scatter gating and viable cells were further gated based on viability dye low signal. Collected data were further analyzed by FlowJo for different proliferation parameters.

Statistics

Data were analyzed in Origin Pro 8.6 by Kruskal-Wallis ANOVA for multiple comparison and Mann-Whitney U-test for specific sample pairs (* $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$).

1. (a) Spatz, J. P.; Mössmer, S.; Hartmann, C.; Möller, M.; Herzog, T.; Krieger, M.; Boyen, H.-G.; Ziemann, P.; Kabius, B., Ordered Deposition of Inorganic Clusters from Micellar Block Copolymer Films. *Langmuir* **1999,** *16* (2), 407-415; (b) Glass, R.; Moeller, M.; Spatz, J. P., Block copolymer micelle nanolithography. *Nanotechnology* **2003,** *14*, 1153-1160.

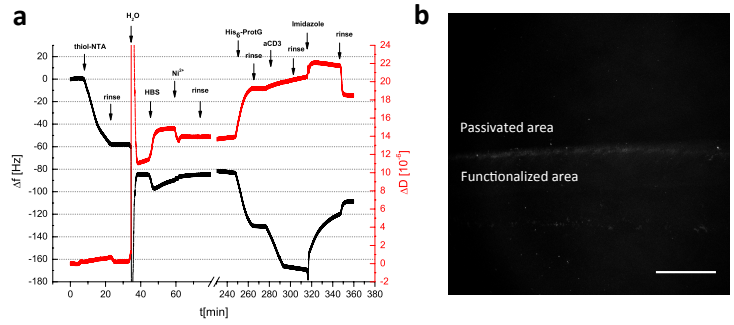
2. Blümmel, J.; Perschmann, N.; Aydin, D.; Drinjakovic, J.; Surrey, T.; Lopez-Garcia, M.; Kessler, H.; Spatz, J. P., Protein repellent properties of covalently attached PEG coatings on nanostructured SiO2-based interfaces. *Biomaterials* **2007,** *28* (32), 4739-4747.

3. (a) Wolfram, T.; Belz, F.; Schoen, T.; Spatz, J. P., Site-specific presentation of single recombinant proteins in defined nanoarrays. *Biointerphases* **2007,** *2* (1), 44-48; (b) Aydin, D.; Schwieder, M.; Louban, I.; Knoppe, S.; Ulmer, J.; Haas, T. L.; Walczak, H.; Spatz, J. P., Micro-nanostructured protein arrays: a tool for geometrically controlled ligand presentation. *Small* **2009,** *5* (9), 1014-8.

4. Quah, B. J.; Parish, C. R., The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. *J Vis Exp* **2010,** (44).

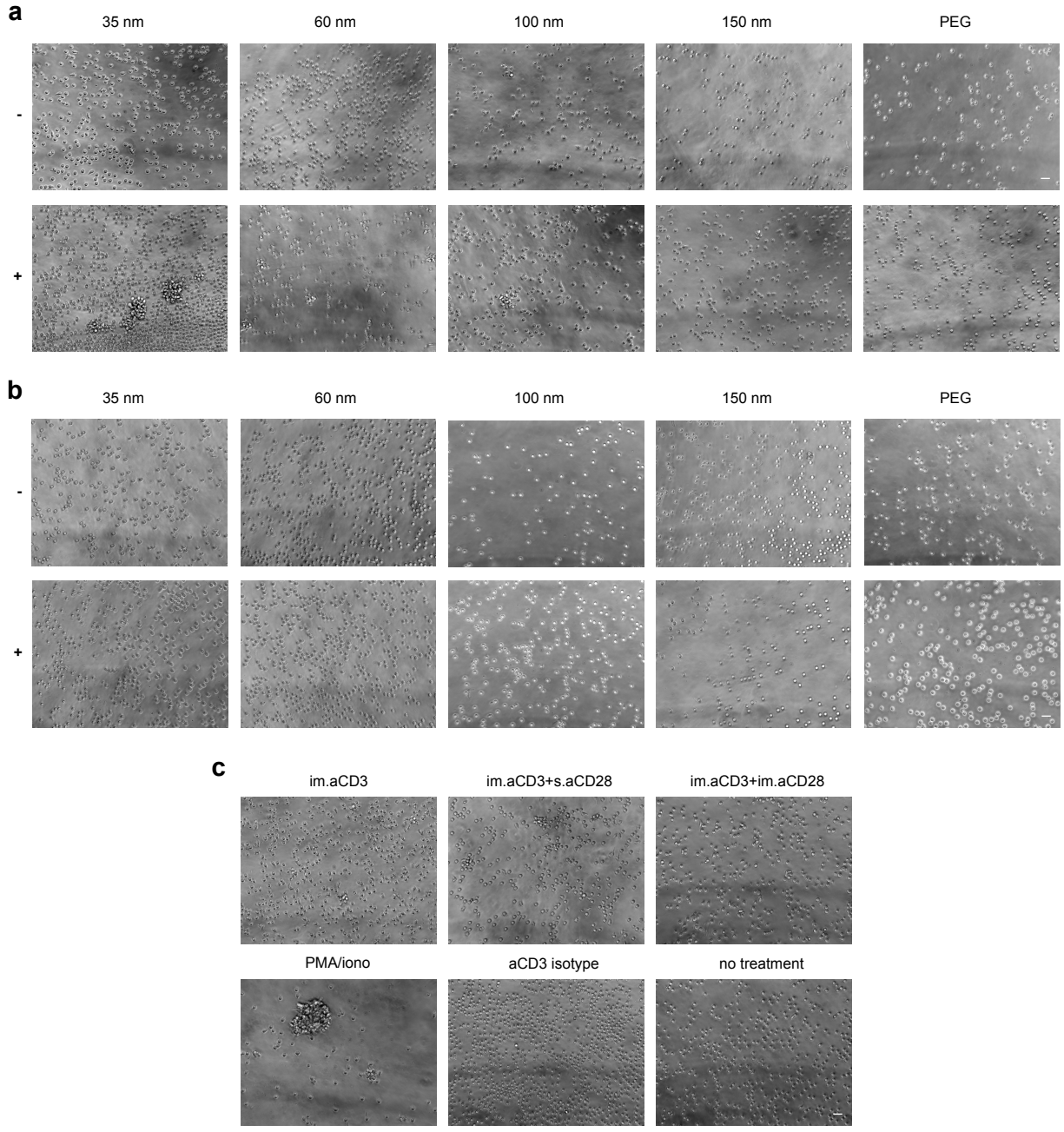
Supplementary table 1. Nano-arrays used for T cell activation

Interparticle spacing [nm]	Particle density [AuNPs/μm^2]	Referred to in text as
35	1010	35 nm
41	680	40 nm
63	316	60 nm
98	115	100 nm
152	59	150 nm



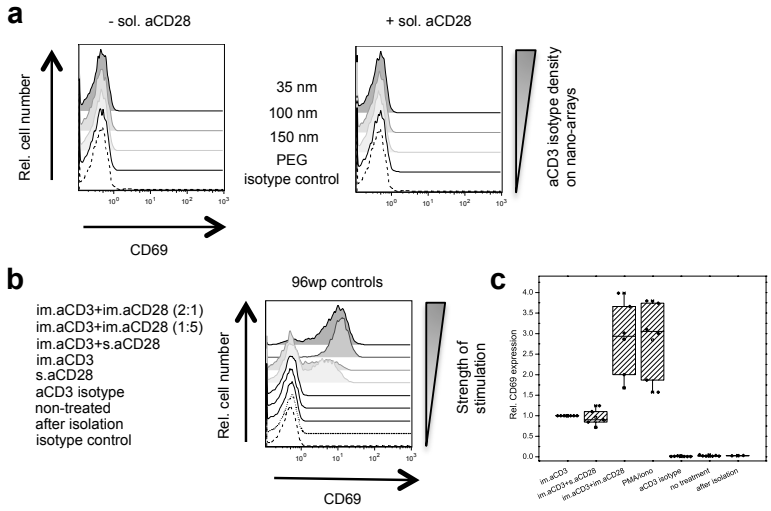
Supplementary figure 1. Probing the site-directed immobilization strategy on homogenous gold and gold nano-arrays. (**a**) QCM-D data showing frequency and dissipation shifts during the successive binding of linkers and proteins on the surface of gold-coated quartz sensor. (**b**) Fluorescence image of aCD3 AlexaFluor488 functionalized nano-arrays after rinsing with 250

mM aqueous solution of imidazole, which competes with His-tag for NTA-Ni^{2+} binding eluting the site-directed bound proteins from the nano-arrays (scale bar = 50 μm).



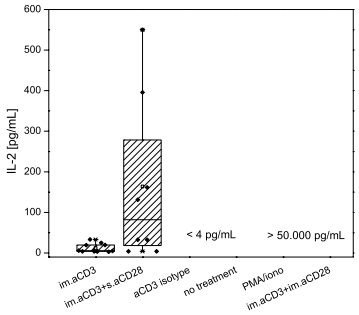
Supplementary figure 2. Cell morphology on various substrates after 17h of culturing. Bright-field images of CD4+ T cells on (**a**) aCD3 nano-arrays (**b**) matching isotype control nano-

arrays and (**c**) plastic control wells (im. = immobilized, s. = soluble) (scale bar = 20 μm). Enlarged, 'blast-like' cells can be observed on aCD3 nano-arrays, but not on PEG surfaces and negative controls. Also, the elongated morphology of activated cells in contact with the stimulating surface (uropod structure) can be noticed, as opposed to inactivated, round phenotype of stimulus-deprived cells. However, formation of clusters typical of activated T cells could not be observed until co-stimulaton was provided to the substrates in the form of soluble aCD28.

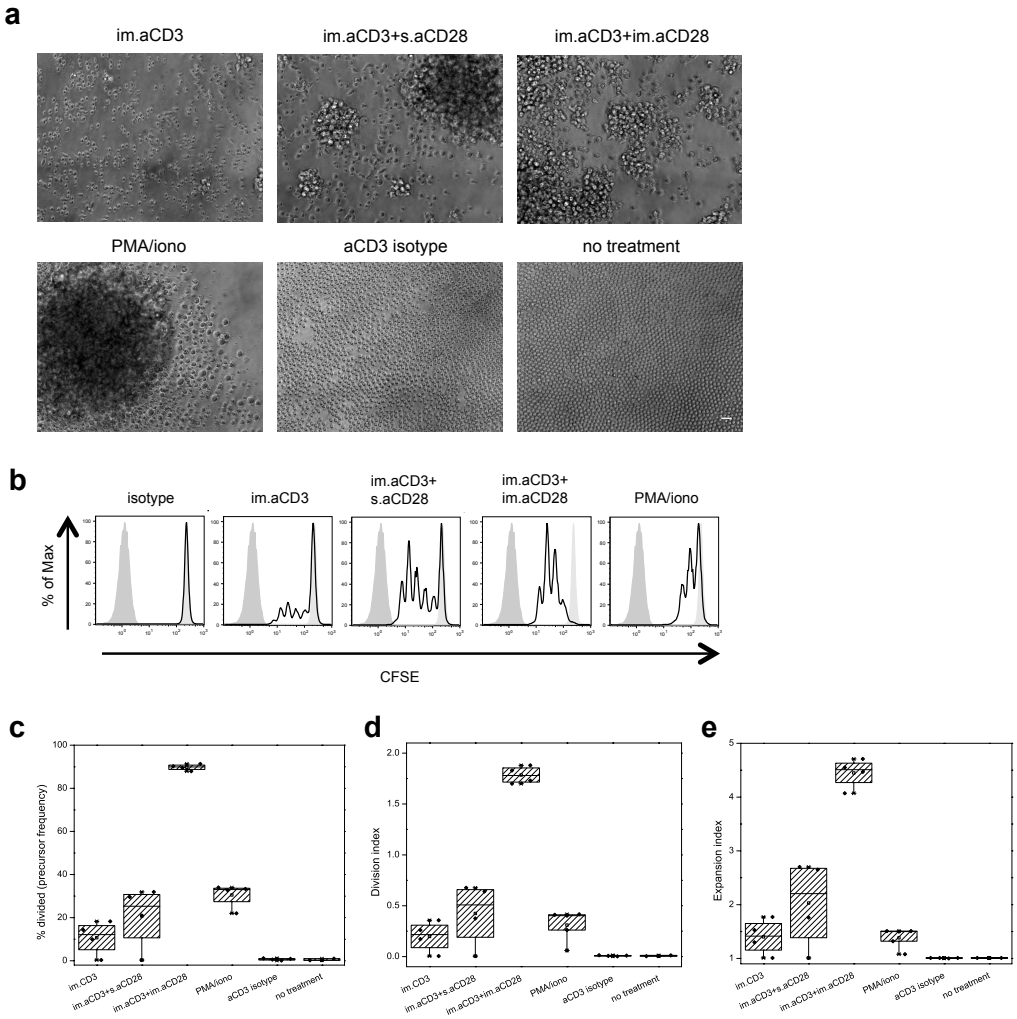


Supplementary figure 3. Cellular response on functionalized nano-arrays is specifically triggered by aCD3, and consequently CD69 expression on T cells is generally inducible only by specific stimuli. Freshly isolated CD4+ T cells (10^5/well) were seeded on aCD3 isotype control nano-arrays of different spacing or PEG control with or without 2 μg/mL aCD28 as designated, as well as plastic well controls (im. = immobilized, s. = soluble). After 17h of culture, the cells were analyzed for CD69 expression by anti-CD69PE staining and flow cytometry. (**a**) Representative histograms showing relative sizes of activated, CD69 expressing and non-activated, CD69 negative T cell populations on aCD3 isotype nano-arrays of different

spacing or PEG control and (**b**) different plastic well controls. (**c**) Box plots summarizing CD69 expression in plastic well controls from six independent experiments.



Supplementary figure 4. IL-2 secretion by human CD4+ T cells is co-stimulation dependent. Freshly isolated CD4+ T cells (10^5/well) were seeded on plastic well controls (im. = immobilized, s. = soluble) and IL-2 concentration was measured in the culture supernatant by ELISA after 17h of culture. Box plot summarizing IL-2 secretion on different plastic well controls from four independent experiments.



Supplementary figure 5. Long-term T cell survival and proliferation in control samples. CFSE-labeled CD4+ T cells ($5x10^4$/well) were cultured for 4 days in wells treated with different stimuli or negative controls. (**a**) Bright-field images showing T cell blasts and clusters where activation stimuli were provided (scale bar = 20 μm) (**b**) Representative histograms showing non-dividing cells (light grey) and auto-fluorescence of non-labeled cells (dark grey). Each fluorescent peak in between represents a distinct cell population that entered another division. (**c, d, e**) Box plots indicating different proliferation parameters from three independent experiments (% divided (precursor frequency) = the original fraction of T cells that divided at least once;

division index = average numbers of divisions cells have undergone; expansion index = average fold expansion of cell culture).