Supporting Information

Controlling T cell activation with synthetic Dendritic Cells using the multivalency effect

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Table S1. Characteristics of the polymers.

Polymer	Catalyst ratio	N_3 ratio	M_v (kg/mol)[a]
P1'	1:200	1:100	200
P2'	1:10,000	1:100	628
P3'	1:10,000	1:70	577

[a]Determined from viscosity measurements.

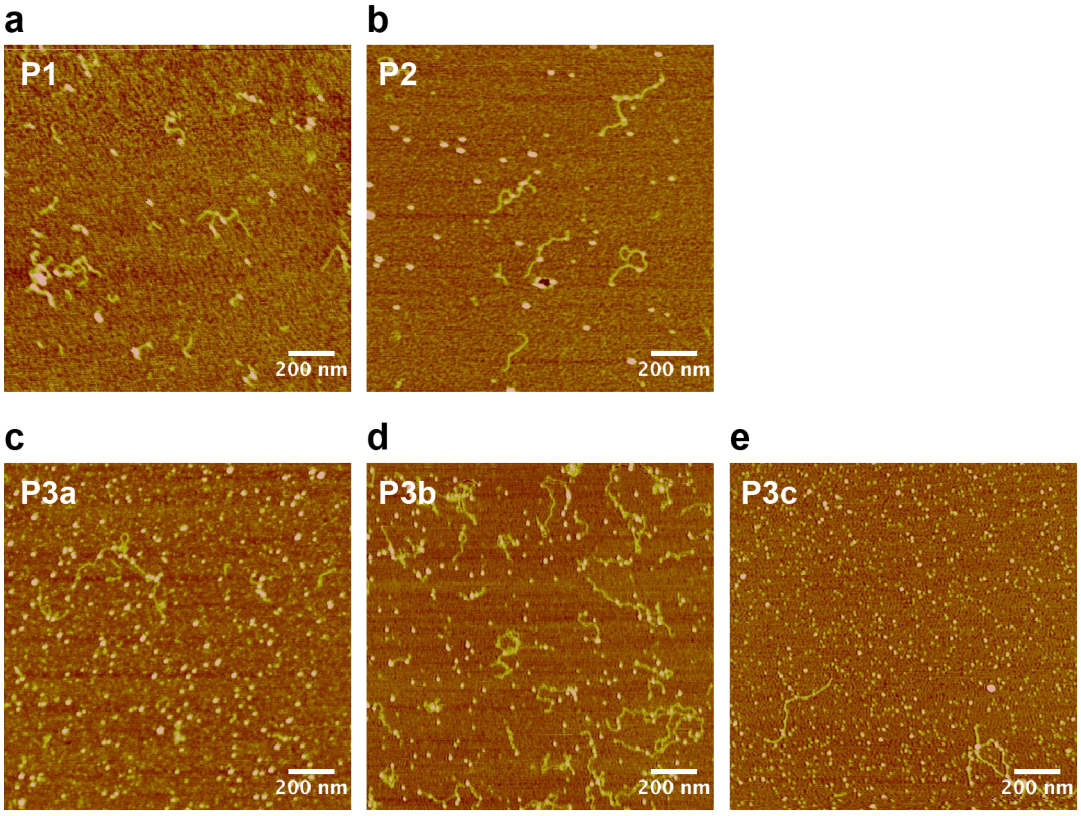

Figure S1. Representative AFM images of the polymer-SAv conjugates (**P1-P3c**) drop-casted on polylysine treated mica. Scale bar: 200 nm for (a)-(e).

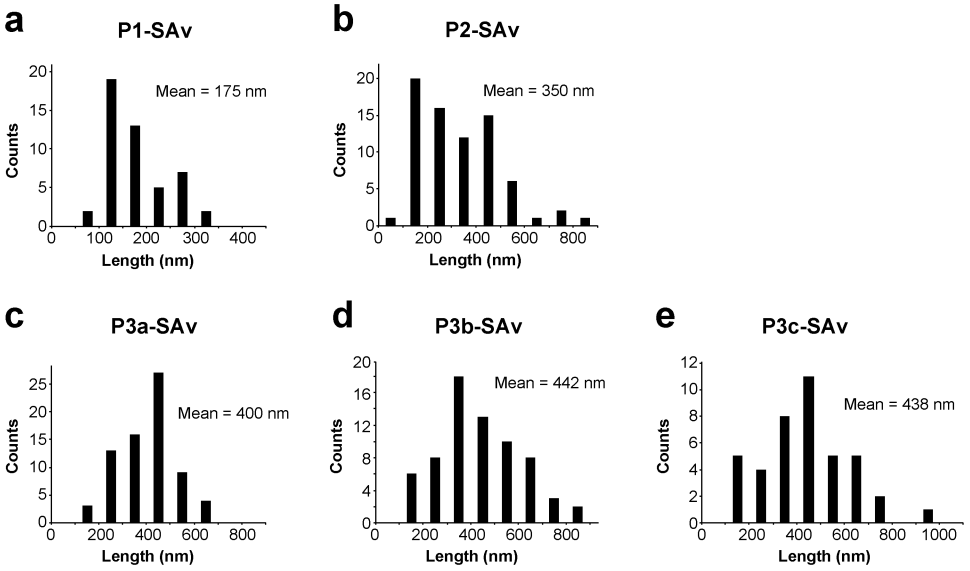

Figure S2. Length distribution of the different polymer-SAv conjugates as determined from AFM images. Each histogram contains at least 41 polymers: (a) **P1-SAv**, n = 48; (b) **P2-SAv**, n = 74; (c) **P3a-SAv**, n = 72; (d) **P3b-SAv**, n = 68; (e) **P3c-SAv**, n = 41.

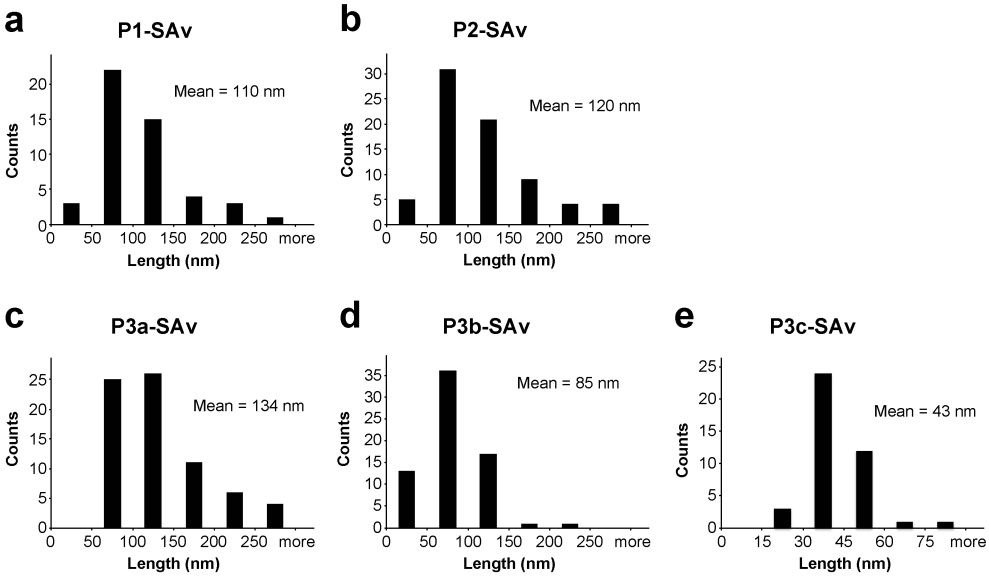

Figure S3. Average distance between SAv molecules determined from the AFM images. Each histogram contains at least 41 polymers: (a) **P1-SAv**, n = 48; (b) **P2-SAv**, n = 74; (c) **P3a-SAv**, n = 72; (d) **P3b-SAv**, n = 68; (e) **P3c-SAv**, n = 41.

Table S2. Characteristics of the polymer-SAv conjugates.

Polymer	SAv equivalents	Mean length (nm)[a]	Mean SAv distance (nm)[a]
P1	1	175	110
P2	1	350	120
P3a	0.5	400	134
P3b	1	442	85
P3c	5	438	43

[a]Determined with AFM imaging.

Table S3. Treatment conditions for the single-cell Ca^{2+}-signaling experiments.

Experiment	Stimulant	Imaging time	αCD3 concentration[a]
1. sDC length	i. **P1** ii. **P2** iii. free αCD3 iv. untreated	5 min baseline Ca^{2+} 1 h treatment 5 min ionomycin	0.005, 0.5, 5, 25 ng/ml
2. αCD3 density	i. **P3a** ii. **P3b** iii. **P3c** iv. free αCD3 v. untreated	5 min baseline Ca^{2+} 1 h treatment 5 min ionomycin	0.005, 0.5, 5, 25 ng/ml
3. dose-response curves	i. **P3c** ii. free αCD3 iii. untreated	5 min baseline Ca^{2+} 1 h treatment 5 min ionomycin	0.001, 0.01, 0.1, 5, 10, 25, 50, 100 ng/ml

[a]The concentration always refers to the amount of αCD3 bound to the αCD3-sDCs or present in freely soluble form (free αCD3).

Table S4. Number of cells analyzed for the single-cell Ca^{2+}-signaling experiments.

Experiment	Stimulant	c(αCD3)	Number of cells analyzed		
			Donor 1	Donor 2	Donor 3
1. sDC length	i. **P1**	0.005 ng/ml	12	7	5
		0.5 ng/ml	12	5	6
		5 ng/ml	11	5	6
		25 ng/ml	6	7	6
	ii. **P2**	0.005 ng/ml	12	6	6
		0.5 ng/ml	12	5	6
		5 ng/ml	12	7	6
		25 ng/ml	7	8	5
	iii. αCD3	0.005 ng/ml	7	5	4
		0.5 ng/ml	6	3	6
		5 ng/ml	5	8	5
		25 ng/ml	7	7	6
2. αCD3 density	i. **P3a**	0.005 ng/ml	4	4	4
		0.5 ng/ml	2	4	6
		5 ng/ml	4	4	4
		25 ng/ml	12	6	5
	ii. **P3b**	0.005 ng/ml	6	6	5
		0.5 ng/ml	5	5	8
		5 ng/ml	8	5	7
		25 ng/ml	6	5	6
	iii. **P3c**	0.005 ng/ml	6	6	6
		0.5 ng/ml	10	10	6
		5 ng/ml	8	8	5
		25 ng/ml	14	10	5
	iv. αCD3	0.005 ng/ml	6	5	4
		0.5 ng/ml	7	4	6
		5 ng/ml	5	8	5
		25 ng/ml	7	7	6
3. dose-response curves	i. **P3c**	0.001 ng/ml	44	31	33
		0.01 ng/ml	17	39	19
		0.1 ng/ml	22	21	22
		5 ng/ml	22	17	18
		10 ng/ml	23	14	22
		25 ng/ml	13	12	25
		50 ng/ml	14	24	24
		100 ng/ml	21	26	31
	ii. αCD3	0.001 ng/ml	10	30	23
		0.01 ng/ml	24	26	26
		0.1 ng/ml	14	33	33
		5 ng/ml	17	16	15
		10 ng/ml	13	42	26
		25 ng/ml	8	21	15
		50 ng/ml	12	20	31
		100 ng/ml	11	25	21

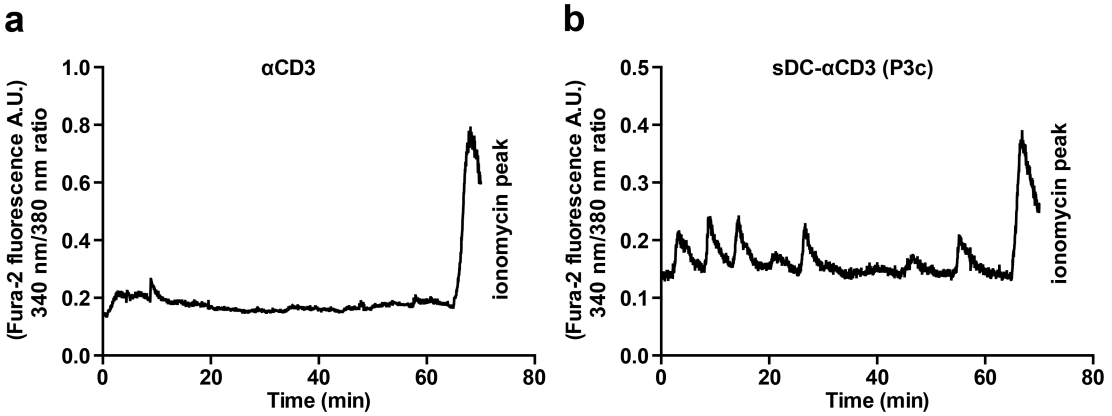

Figure S4. Single-cell Ca^{2+}-signaling experiments. Representative time traces (340 nm/380 nm ratio) for PBLs treated with free αCD3 (a) or **P3c** (b).

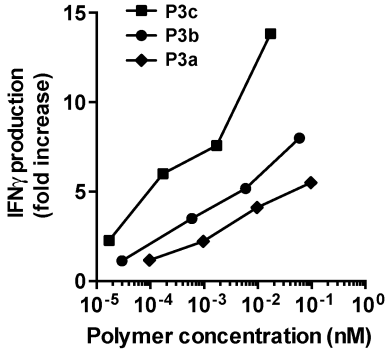

Figure S5. IFNγ secretion of PBLs treated with **P3a**, **P3b** or **P3c** normalized to polymer concentration.

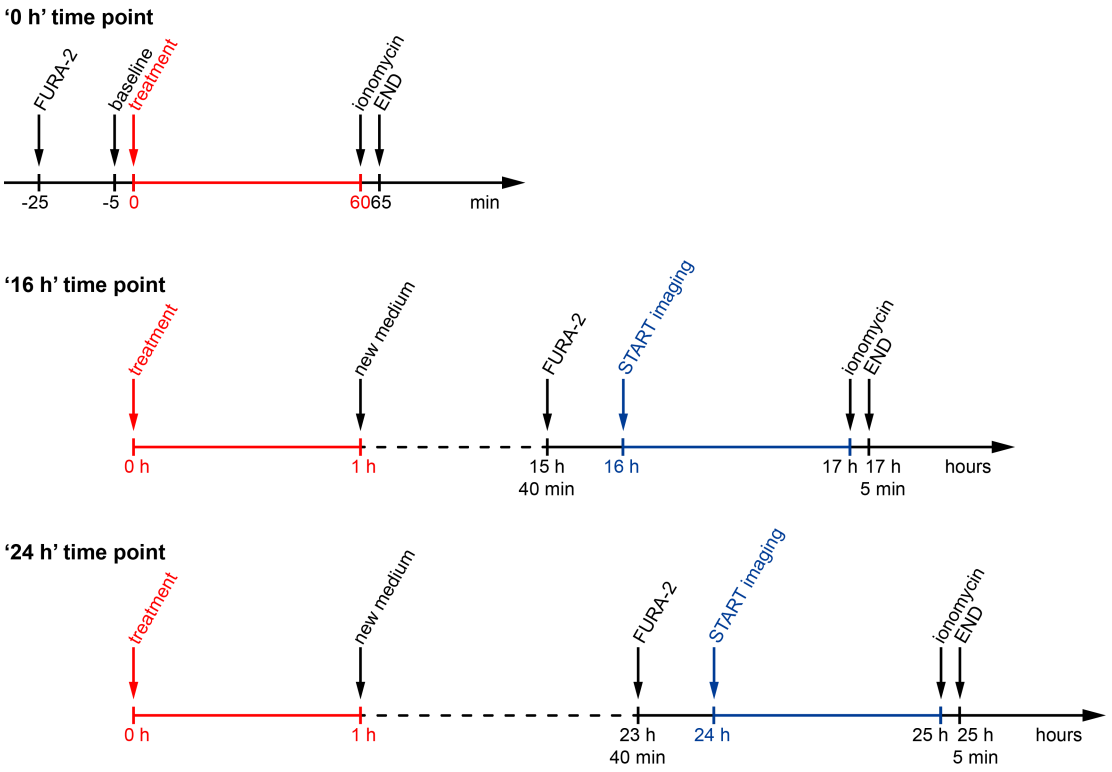

Figure S6. Timeline of the post-stimulation single-cell Ca^{2+}-signaling experiments. To obtain the '0 h' time point, the Fura-2 loaded cells were treated with the stimulant directly under the microscope and imaged for 1 h. For the '16 h' and '24 h' time points the cells were first treated with the respective stimulant for 1 h, washed to remove the stimulant and then incubated without stimulant for 15 h or 23 h, respectively. During the last 20 min of this incubation period, the cells were loaded with Fura-2. Imaging was started at the indicated time point. At the end of the 1 h imaging period, the cells were treated with ionomycin to determine cell viability. In this post-stimulation experiment, the '5 min' baseline was only determined for the '0 h' time point.

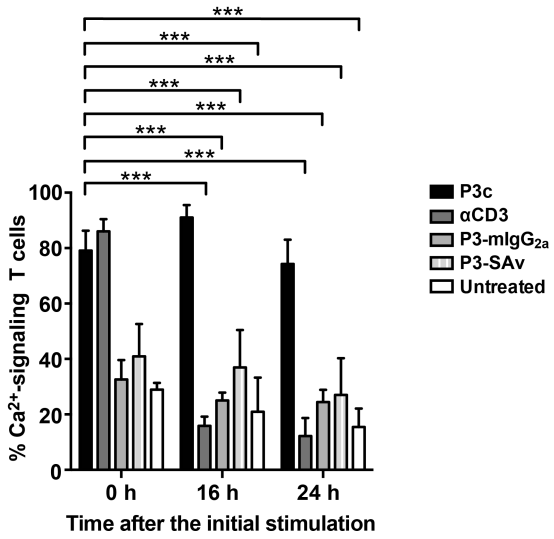

Figure S7. Results of the post-stimulation single-cell Ca^{2+}-signaling experiments. PBLs were treated with 12.5 ng/ml **P3c** or free αCD3 for 1 h. The stimulant was removed and the cells were incubated in fresh medium. Ca^{2+}-imaging was performed 16 h or 24 h after starting the treatment (15 h or 23 h after removal of the stimulant). As a reference, PBLs were also imaged under the microscope while the stimulant was added (0 h). The graph further shows data obtained for PBLs treated with the following controls: **P3**-SAv, **P3**-$mIgG_{2a}$ (isotype control) or medium (untreated). For all experiments the data represents the mean ± SEM of 3 independent experiments performed with PBLs from different donors. The number of cells analyzed is summarized in Table S5. The asterisks represent the statistical significance at 'p' values of 0.001 (***). A clear difference is observed between the post-stimulation signaling of αCD3-sDC treated cells and the cells treated with the free αCD3 antibody or any of the controls. Of note, the untreated control shows a small fraction of PBLs (~30 %) that display spontaneous increases in the cytosolic Ca^{2+}-concentration (white bars). A similar percentage of PBLs (~26%) showing spontaneous Ca^{2+} oscillations (Ca^{2+} oscillations that occur in the absence of added stimulant) has been reported before (Donnadieu, E; Bismuth, G; Trautmann, A. The intracellular Ca^{2+} concentration optimal for T cell activation is quite different after ionomycin or CD3 stimulation. *Pfluegers Arch. – Eur. J. Physiol.* **1995**, 429 (4), 546-554).

Table S5. Number of cells analyzed for the post-stimulation Ca^{2+}-signaling experiments.

Stimulant	Incubation time	Number of cells analyzed		
		Donor 1	Donor 2	Donor 3
i. **P3c**	0 h	15	14	11
	16 h	7	8	6
	24 h	10	7	7
ii. αCD3	0 h	14	9	8
	16 h	9	9	7
	24 h	10	9	7
iii. **P3**-mIgG$_{2a}$	0 h	10	9	6
	16 h	10	8	6
	24 h	10	9	5
iv. **P3**-SAv	0 h	10	14	7
	16 h	11	11	5
	24 h	9	9	6
v. untreated	0 h	9	7	8
	16 h	9	7	5
	24 h	9	9	7

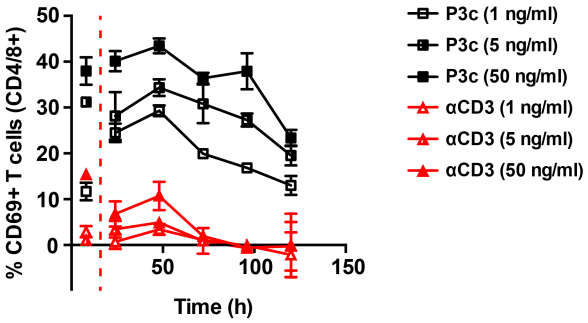

Figure S8. Post-stimulation analysis of CD69 expression. Percentage of CD69 expressing T cells after treatment with different concentrations of **P3c** or αCD3.

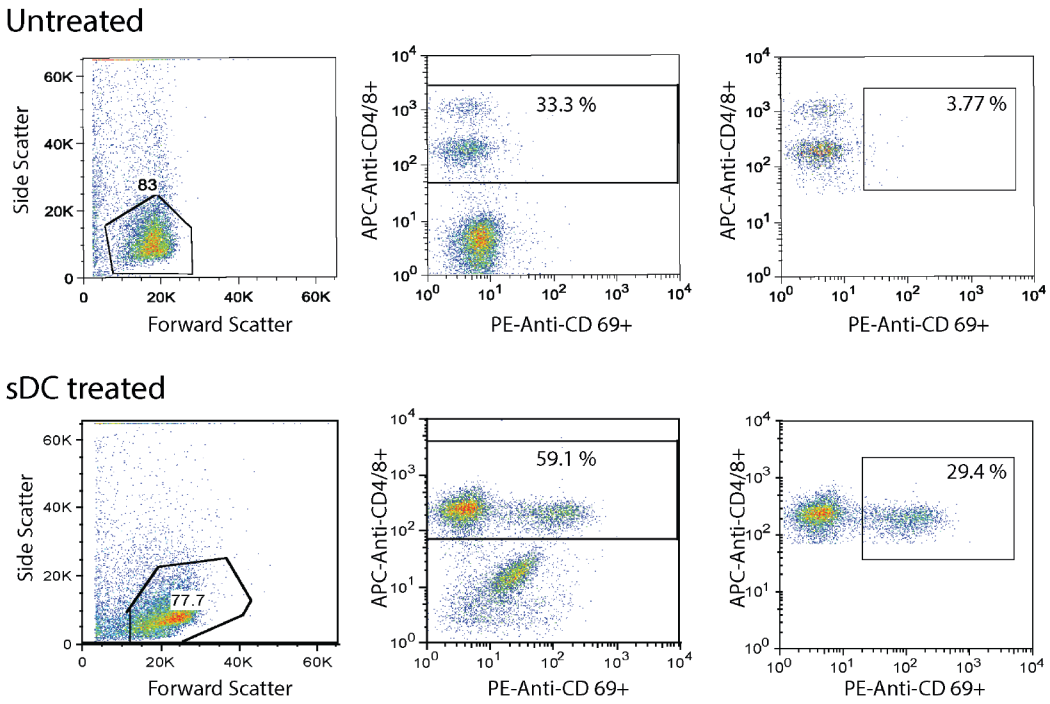

Figure S9. FACS analysis of sDC-treated (**P3c**) and untreated PBLs. After identification of CD4/8+ T cells, the percentage of CD69+ cells was determined.