Supplementary data for "Engineering Dendritic Cells-based Vaccines and PD-1 Blockade in Self-assembled Peptide Nanofibrous Hydrogel to Amplify Antitumor T-cell Immunity"

Pengxiang Yang,[1, 2] Huijuan Song,[1] Yibo Qin,[1] Pingsheng Huang,[1] Chuangnian Zhang,[1] Deling Kong,*[1, 3, 4] Weiwei Wang*[1]

[1] Tianjin Key Laboratory of Biomaterial Research, Institute of Biomedical Engineering, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300192, China

[2] Institute of Cancer Prevention and Treatment, Heilongjiang Academy of Medical Science, Harbin Medical University, Harbin 150081, China

[3] State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin 300071, China

[4] Jiangsu Center for the Collaboration and Innovation of Cancer Biotherapy, Cancer Institute, Xuzhou Medical University, Xuzhou 221004, Jiangsu, China

1. Materials and methods

1.1 Reagent and antibodies

The peptide RADA16, Ac-RADARADARADARADARADA-$CONH_2$ (molecular weight, 1712 Da), was commercially synthesized by Bankpeptide biological technology co., Ltd. (Hefei, China) via solid-phase synthesis. The HPLC and MS spectra were shown in Figure S1, which proved the successful synthesis of peptide sequence. The working solution of RADA16 was prepared at various concentrations in sterile water (18 MΩ, Millipore Milli-Q system) and stored at 4 °C. The endotoxin level in the peptide was checked by using the Limulus Amebocyte Lysate

assay kit (GenScript Biotech Corp., Nanjing, China) and the result indicated that the endotoxin level in the hydrogel (10 mg/mL) was less than 0.1 EU/ml, which could exclude the influence of contamination. Trypsin-EDTA, 4', 6-diamidino-2-phenylindole (DAPI), calcein-AM, and phalloidin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-mouse IFN-γ ELISA kits were purchased from eBioscience. Recombinant mouse IL-4 and GM-CSF were purchased from PeproTech (Rocky Hill, NJ, USA). Fluorochrome-labeled anti-mouse monoclonal antibodies (CD4, CD8a, CD3, Foxp3, MHCII, CD11c and CD86) were received from eBioscience (San Diego, CA, USA). Immunomagnetic beads for isolating DCs were provided from Miltenyi Biotec (Bergisch Gladbach, Germany). The anti-PD-1 monoclonal antibody was offered by Bio X Cell (West Lebanon, NH, USA).

1.2. Cell lines and animals

C57BL/6 (6-8 weeks) and C57BL/6 mice expressing green fluorescence protein (GFP) (6-8 weeks) were purchased from Vital River Laboratory (Beijing, China). All animal procedures were reviewed and ethically approved by Center of Tianjin Animal Experiment Ethics Committee and Authority for Animal Protection (Approval No: SYXK (Jin) 2011-0008). Mouse EG7-OVA tumor cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, and cultured according to the manufacture's guidelines.

1.3 Characterization of peptide hydrogel

The secondary structure of peptide was investigated by circular dichroism (CD) spectrometer (Jasco J-810). RADA16 solution (5 mg/ml) was prepared and equilibrated for 30 min, the ellipticity change in the UV region of 190-260 nm was scanned with an average of three scans.

The modulus of hydrogel with or without the encapsulation of DCs (1×10^6 /mL) was examined by rheology analysis using the AR 2000ex rheometer (TA) at a concentration of 5 mg/ml according to our previously reported method.[1] Elasticity modulus (G′) and viscosity modulus (G″) were determined within a frequency sweep range of 0.1-10 rad/s. The strain was set at 1.0% and the temperature was 25 °C.

The structural morphology of self-assembled RADA16 hydrogel (5 mg/mL) was examined by transmission electron microscopy (TEM, Hitachi H-600). Several drops of the hydrogel were added on the surface of a TEM copper grid, which was observed by TEM after air-drying.

1.4 Generation of BMDCs

Bone marrow-derived DCs (BMDCs) were generated according to the previously reported procedure.[2]

1.5 Phalloidin/DAPI staining

DCs were encapsulated in RADA16 hydrogel by adding peptides to the cell suspension and cultured at 37 °C for 24 h and rinsed with PBS buffer for triple times with each for 5 min. Afterwards, cells were fixed with 4% paraformaldehyde for 15 min, and washed with PBS. Then 10 μl phalloidin (8.3 μg/ml) was added and incubated for 20 min, and washed with PBS. Finally, recovered cells were stained with 10 μl DAPI (2.5 μg/ml) and fluorescence images were recorded by a confocal laser scanning microscopy (CLSM, TCS SP5II, Leica).

1.6 In vitro DC activation, antigen uptake and release

To evaluate the viability by live/dead assay and the activation of BMDCs, cells were incubated in blank hydrogels, or OVA-loaded hydrogels or in complete culture medium containing OVA for 24 h. The dose of OVA and positive control LPS was 2.5 μg/ml. Briefly, isolated BMDCs in triplicate were incubated with the dye solution (Molecular Probes) containing 0.5 μl calcein AM and 2 μl ethidium homodimer in 1 ml PBS. After incubation for 30 min, cells were rinsed with PBS, and the cell viability was quantified by flow cytometer and 3D construction of cells was captured by CLSM. The maturation of BMDCs was evaluated by flow cytometer after surface staining by fluorescence markers conjugated CD86 antibody (2.5 μg/ml). For antigen uptake study, immature BMDCs (1×10^6 cells/ml) were cultured with OVA-FITC in culture medium or in hydrogels containing OVA-FITC at 37 °C for 6 h. The uptake efficiency was determined by measuring the mean fluorescence intensity (MFI) using flow cytometer. Total fluorescence intensity (FI) = the percentage of positive cells × MFI. The release profile of OVA from RADA16 hydrogel was also examined. Briefly, 200 μL hydrogel containing 200 μg OVA were placed in a tube and then 200 μL PBS solution was added on top of the gel. At desired time points, 100 μL PBS were removed and equal volume of fresh PBS solutions was supplemented. The concentration of OVA in the extracted sample was measured by BCA assay and cumulative OVA release was calculated.

1.7 DCs delivery in hydrogel in vivo

GFP^+ DCs (2×10^6) and 20 μg OVA were mixed in 200 μl RADA16 hydrogel for each mouse and the mixture was kept on ice before injection. C57BL/6 mice were immunized with blank hydrogel or GFP^+ DCs-loaded hydrogel by subcutaneous injection at the back flank. Animals were euthanized and DCs-loaded hydrogels, and lymph nodes were explanted at days 0 (2 h), 2, 4 and 6 after injection. The recovered gels were diluted in PBS and passed through 40 μm nylon

mesh cell strainer (BD Falcon, San Jose, CA). After centrifugation, recovered cells were stained by MHCII and CD11c antibodies and re-suspended in flow cytometer buffer at 4°C and determined by flow cytometer. For histological examination, the lymph nodes were isolated and embedded by optimal cutting temperature compound (OCT) at -20°C, then cut into 6 μm sections, stained by DAPI and observed by fluorescence microscope.

1.8 Vaccination and tumor challenge in vivo

C57BL/6 mice (6-8 weeks) were randomly distributed into one of the following treatment groups: PBS, blank hydrogel (Gel), hydrogel vaccine encapsulated with OVA alone (Gel-OVA), OVA-pulsed DCs (DC-OVA), or hydrogel vaccine encapsulated with OVA and DCs (Gel-DC-OVA), and Gel-DC-OVA in addition with anti-PD-1 antibody (Gel-DC-OVA+anti-PD-1). DC-OVA formulations were prepared by sorting $CD11c^{+}MHCII^{+}$ DCs with immunomagnetic beads after culturing OVA with DCs for 24 h and the obtained $CD11c^{+}MHCII^{+}$ cells were centrifuged and re-suspended in medium. DC-OVA formulations were prepared by mixing DCs and OVA in the medium, which was cultured for 24 h, and then adding certain amount of peptide to the mixture to form hydrogels, which was treated by gentle vortex before in vivo injection. Gel-DC-OVA+anti-PD-1 formulation was prepared by simultaneously adding anti-PD-1 antibody and peptide to the mixture of DCs and OVA. The injection volume of hydrogel vaccine was 200 μl. The number of DCs in each vaccine was $\sim 1\times10^{6}$. The dose of antigen and anti-PD-1 antibody was 20 μg and 200 μg, respectively. Mice were received three times of subcutaneous vaccination at day 0, 14 and 21 in the left flank. At day 25, three mice of each group were euthanized and lymph nodes and spleens were retrieved. To assess T cell infiltration and activation single cell suspensions were prepared. Tissues were

treated accroding to our previous approach to obtain single-cell suspensions for antobody staining and flow cytometer analysis.[2]

At day 28, immunized mice were inoculated with 1×10^6 EG7-OVA cells suspended in 30 μl PBS in the right flanks. The tumor volume and body weight were closely monitored after cell inoculation. Tumor volumes (V, mm^3) were calculated using the following formula: $(W^2\times L)/2$, where W (width) is the short perpendicular dimension, and L (length) is the longest dimension. The survival of mice was observed over 56 days following the inoculation of tumor cells.

1.9 Splenocytes proliferation and cytotoxicity T lymphocyte (CTL) assay

Single cell suspensions of splenocytes were prepared from mice three days after the last immunization. Splenocytes (5×10^5 per well) were seeded in the 96-well plate and retreated with 50 μg/ml OVA for 72 h. Splenocytes proliferation was measured using CCK-8 kit analysis. OD values at 450 nm were recorded by a microplate reader. The production of IFN-γ in culture supernatants was measured using ELISA. For CTL assays, splenocytes and EG7-OVA cells denoted as effector (E) and target (T) cells, respectively, were co-cultured in U-bottomed 96-well plates with the cell number ratios of E/T varied from 5:1 to 50:1. After incubation for 4 h at 37°C, the lactate dehydrogenase (LDH) levels in the cell culture supernatants were determined by CTL assay kit (Promega) following the manufacture's instruction.

1.10 Generation and isolation of tumor cell lysates

EG7-OVA tumor cell lysate were prepared by using our previously reported method [3] and protein concentration in the obtained lysates was measured by BCA assay.

1.11 Therapeutic antitumor effect of Gel-DC-TCL vaccine in combination with anti-PD-1 immunotherapy

C57BL/6 mice (6-8 weeks) were subcutaneously injected with 1×10^6 EG7-OVA cells suspended in 30 μl PBS in the right flanks. On day 7, mice were randomly assigned into the following six groups (n=6). On day 7, 14, and 21 post the tumor inoculation, mice were subcutaneously vaccinated with PBS, Gel, Gel-TCL, DC-TCL, Gel-DC-TCL or Gel-DC-TCL+anti-PD-1. The antigen dose and the number of DCs were 20 μg and 1×10^6, respectively. The tumor volume and body weight were closely monitored. Tumor volumes were calculated according to the method described in Section 1.8. Mice were sacrificed at day 28 and tumors tissues were removed and weighted. For the analysis of T cells subpopulations, single-cell suspensions were prepared. The tumor tissues were separated, cut into small pieces, put into a dish containing Ficoll, and then they were centrifuged with discontinuous Ficoll gradient. TILs were collected from the interphase, washed and first stained with the specific antibodies including CD4 and CD8, and then fixed and permeabilized with the Perm/Fix solution (eBioscience), and stimulated with the mixture of phorbol-12-myristate-13-acetate, ionomycin, brefeledin and monensin for 4 h before intracellular IFN-γ and Foxp3 staining. These samples were re-suspended in flow cytometer buffer at 4°C and analyzed by flow cytometer.

1.12 Statistical analysis

Animal survival was estimated by the Kaplan-Meier method and evaluated with a log-rank test. Data are presented as mean ± standard deviations (SDs). The differences between two or more groups were assessed by student's t-test, or one-way ANOVA analysis in Prism (GraphPad Software, La Jolla, CA, USA), respectively. $p<0.05$ was considered to be statistical difference.

References

1. Wang, W.; Chang, L.; Li, X.; Wu, Y.; Xing, J.; Deng, L.; Dong, A.; Soft Matter **2012**, 8, (5), 1575-1583.

2. Song, H.; Huang, P.; Niu, J.; Shi, G.; Zhang, C.; Kong, D.; Wang, W.; Biomaterials **2018**, 159, 119-129.

3. Shi, Gao.; Zhang, C.; Xu, R.; Niu, J.; Song, H.; Zhang, X.; Wang, W.; Wang, Y.; Li, C.; Wei, X.; Kong, D.; Biomaterials **2017**, 113, 191-202.

2. Supplementary figures

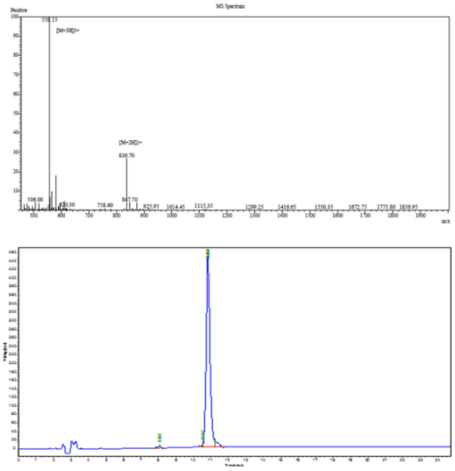
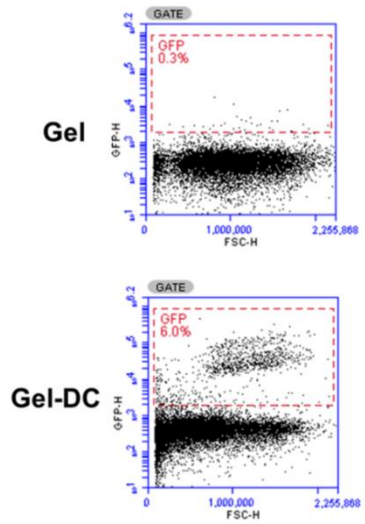


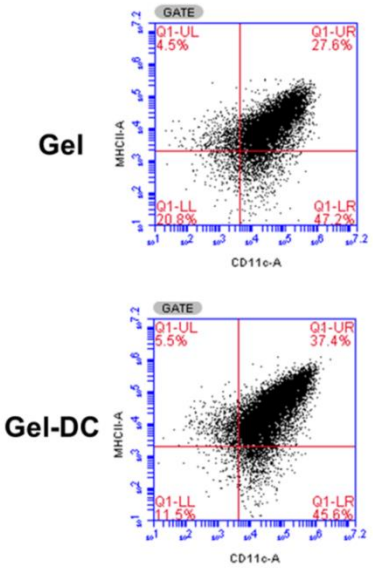
Figure. S1 Top) HR-MS spectra and down) HPLC spectrum of RADA16. The molecular weight was 1712 Da and the purity was more than 97%.



Groups	Days	Density (Num/μl)	Total Volume (ml)	Total GFP$^+$ Cell ($\times10^4$)	Normalized GFP$^+$ Cell ($\times10^4$)
Gel	0	2.73 ± 0.22	30	8.18 ± 0.65	0
	2	2.12 ± 0.08	22	4.67 ± 0.18	0
	4	5.26 ± 0.67	17	8.94 ± 1.14	0
	6	2.13 ± 0.34	2.5	0.53 ± 0.08	0

Groups	Days	Density (Num/μl)	Total Volume (ml)	Total GFP$^+$ Cell ($\times10^4$)	Normalized GFP$^+$ Cell ($\times10^4$)
Gel+DCs	0	14.18 ± 0.52	30	43 ± 1.55	34 ± 1.50
	2	12.64 ± 0.96	22	28 ± 2.10	23 ± 2.26
	4	11.67 ± 0.56	17	20 ± 0.95	11 ± 1.44
	6	4.03 ± 0.64	2.5	1 ± 0.16	0.5 ± 0.12

Figure. S2 Representative flow cytometry profiles and FACS analysis of the GFP$^+$DCs (in red rectangle) recovered from gels at day 0, 2, 4, 6 after injection. Quantification of numbers of GFP$^+$DCs retrieved from hydrogels was performed by flow cytometry based on at least three independent experiments. Data are presented as mean ± standard deviations (SDs), n=3.

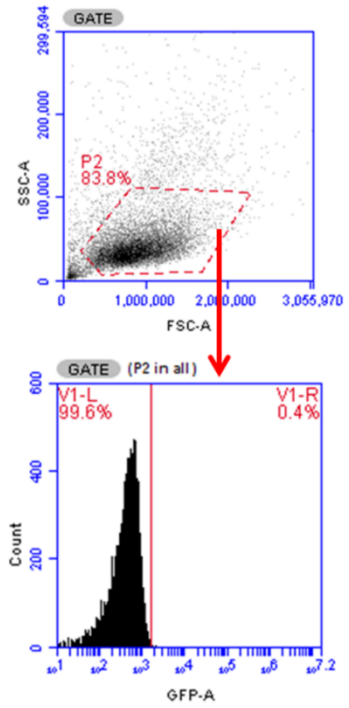


Groups	Days	Density (Num/μl)	Total Volume (ml)	Total MHCII$^+$CD11c$^+$ ($\times10^4$)
Gel	0	0.05 ± 0.02	30	0.16 ± 0.07
	2	3.56 ± 1.02	22	7.83 ± 2.25
	4	10.52 ± 1.84	17	17.88 ± 3.13
	6	84.81 ± 7.94	2.5	21.20 ± 1.99

Groups	Days	Density (Num/μl)	Total Volume (ml)	Total MHCII$^+$CD11c$^+$ ($\times10^4$)
Gel+DCs	0	13.06 ± 1.70	30	39.18 ± 5.11
	2	22.17 ± 4.18	22	48.78 ± 9.19
	4	22.05 ± 4.00	17	37.48 ± 6.80
	6	129.41 ± 21.71	2.5	32.35 ± 5.43

Figure. S3 FACS analysis of the numbers of MHCII$^+$CD11c$^+$ DCs recovered from gels at predetermined times after injection. Quantification of numbers of MHCII$^+$CD11c$^+$ DCs retrieved

from hydrogels was determined by flow cytometry based on at least three independent experiments. Data are presented as mean ± standard deviations (SDs), n=3.



Groups	Days	Density (Num/μl)	Total Volume (ml)	Total GFP$^+$ Cell
DCs	0	0.58 ± 0.06	2	1157 ± 119
	1	5.88 ± 0.70	2	11763 ± 1402
	2	2.37 ± 0.41	2	4738 ± 819
	4	1.46 ± 0.34	2	2925 ± 674
	6	0.89 ± 0.12	2	1779 ± 248

Groups	Days	Density (Num/μl)	Total Volume (ml)	Total GFP$^+$ Cell
Gel+DCs	0	0.41 ± 0.15	2	814 ± 304
	1	5.58 ± 0.64	2	11168 ± 1277
	2	3.63 ± 0.59	2	7265 ± 1186
	4	2.26 ± 0.38	2	4512 ± 768
	6	1.18 ± 0.28	2	2369 ± 564

Figure. S4 FACS analysis of the GFP$^+$DCs recovered from dLNs at predetermined times after injection. Quantification of numbers of GFP$^+$DCs retrieved from hydrogels was examined by flow cytometry on the basis of at least three independent experiments. Data are presented as mean ± standard deviations (SDs), n=3.

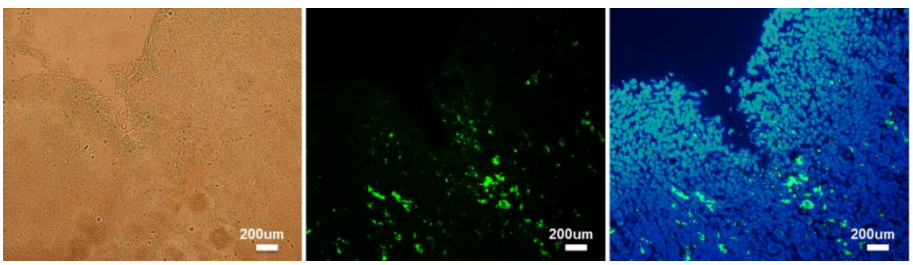


Figure. S5 Representative optical sectioning of dLNs explanted at day 1 after injection GFP^+ DCs. DCs in the dLNs were indicated by GFP (green) and the nuclei of T cells in the dLN were stained by DAPI (blue).