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1 **Supplementary Information**

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3 In-situ formation of peptidic nanofibers can fundamentally 4 optimize the quality of immune responses against HIV vaccine

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9 **Materials and Methods**

10 **Assembly of nanofibers in cells**

11 Hela cells (1×10^5 cells/well) were seeded in 96-wells plate in 100
12 μL DMEM medium (10% fetal calf serum) overnight. Hela cells
13 (attached) were co-incubated with NMe precursors (0.1% WT) for
14 24 h. Hela cells cultured with medium alone were used as control.
15 Cell medium was removed, and cells were washed five times with
16 PBS. Cells were split by softly mechanical crush, and observed by
17 TEM (Tecnai G2 F20 U-TWIN TEM system, American FEI
18 company). We randomly chose 20 fibers in each sample. By
19 measuring the length (the distance between two nodes) and
20 diameter of each fiber via TEM, we estimated the rough range of
21 length and diameter of these fibers.
22

Isothermal titration calorimeter (ITC) test

Titration experiments were carried out in 10 mM sodium phosphate buffer (pH 9.0) at 25 °C on a Microcal Auto-ITC200 microcalorimeter. In a typical experiment, the host (HIV DNA vaccine) was in the sample cell at a concentration of 153 nM, and the guest (NMe precursor) was in the pipette at a concentration of 250 μM. The titration consisted of 20 consecutive injections of 2 μL with 120 s intervals between injections. The first data point was removed from the data set prior to curve fitting. Heats of dilution were checked by titration well beyond saturation or by titration of the guest into a buffer solution and subtracted from the normalized enthalpies, but relatively small in all cases. The data was analyzed using the one set of sites model in Origin software.

Preparation of HIV DNA vaccine and NMe

The antigen of vaccine is HIV envelope (gp145) which is derived from a major epidemic strain in China named CN54 (derived from Chinese isolate 97CN001, B/C recombinant strain). The protocol of synthesis and characterization of NMe follows previous work [20]. NMe precursors were dissolved in PBS solution (pH 9.0). NMe can assemble into nanofibers via directly adding alkaline phosphatase (Takara Co. Ltd) at room temperature.

1

2 **Mice vaccination**

3 Animal experiments were approved by the Animal Ethics
4 Committee of National Center for Nanoscience and Technology,
5 and were carried out in accordance with the Guidelines from the
6 Committee of Welfare and Ethics of Laboratory Animals in Beijing.
7 Female BalB/C mice of eight weeks old were purchased from Vital
8 River Co. Ltd in Beijing, China. Mice were randomly divided into
9 seven groups, with six mice per group. The groups contain: (1) HIV
10 DNA vaccine alone; (2) HIV DNA vaccine aided by NMe (Mode I);
11 (3) HIV DNA vaccine aided by NMe (Mode II); (4) HIV DNA
12 vaccine aided by NMe (Mode III); (5) HIV DNA vaccine aided by
13 NMe (injecting DNA vaccine alone first, followed by NMe); (6) HIV
14 DNA vaccine aided by NMe (injecting NMe first, followed by DNA
15 vaccine alone); (7) empty vector aided by NMe. All mice were
16 vaccinated by intradermal injection. The dose of DNA vaccine was
17 50 μg per mice each vaccination. The spacing interval between
18 two vaccinations was three weeks. Two weeks after the final
19 vaccination, mice were sacrificed, and the spleens and blood were
20 harvested. Fresh splenocytes were prepared for flow cytometric
21 assay, and serum samples were prepared for ELISA assay.

22

Detection of polyfunctional T cell response via flow cytometry

Mice splenocytes were isolated and washed twice with PBS containing 2% bull serum albumin (BSA). Cells were adjusted to the concentration of 1×10^6 cells per ml, and were incubated to stay overnight (18 h) at 37 °C and 5% CO_2 with 100 μl Env peptides (2μg/ml), dimethylsulfoxide/ DMSO (2μl per well, Sigma, negative control), or staphylococcal enterotoxin B/SEB (1μg/ml, Sigma, positive control). Brefeldin A/BFA (1μg/ml, Sigma) and monensin (1μg/ml, Sigma) were used for stopping the transportation of cytokines. Cells were stained with four anti-mouse surface marker antibodies (Anti-Mouse CD3e FITC, Anti-Mouse CD8a Alexa Fluor® 700, Anti-Mouse CD4 APC-eFluor® 780, Anti-Mouse CD107a (LAMP-1) PerCP-eFluor® 710) for 30 min at 4 °C. After fixing with 2% paraformaldehyde (Sigma) for 15 min at 4 °C, cells were washed twice with PBS (3% fetal calf serum, Gibco). Then they were stained with four monoclonal antibodies against intracellular targets (Anti-Mouse IFN gamma APC, Anti-Mouse IL-2 eFluor® 450, Anti-Mouse IL-4 PE, Anti-Mouse TNF alpha PE-Cyanine7) for 30 min at 4 °C (0.2% saponin for permeabilization). After washing twice with PBS (3% fetal calf serum, Gibco), samples were re-suspended with PBS and immediately analyzed on a FACS Calibur flow cytometer (Becton Dickinson). All

monoclonal antibodies were ordered from eBioscience Co. Ltd. The data was analyzed with flowjo software (Tree Star). At least 100,000 live cell events gated by scatter plots were analyzed for each sample.

Detection of B cell maturation via flow cytometry

Mice were injected three times with DNA vaccine alone, DNA vaccine aided by NMe (three models) and empty vector (as the control) at day 1, day 2 and day 3 via intradermal vaccination. Mice were sacrificed and their splenocytes were harvested at day 4. These cells were stained with Anti-Human/ Mouse B220 PerCP-eFluor® 710, Anti-Mouse MHC Class I-Ab FITC, Anti-Mouse CD86 (B7-2) PE, Anti-Mouse CD40 APC, Anti-Mouse CD69 APC-eFluor® 780 (eBioscience). The data were analyzed with flowjo software (Tree Star). At least 100,000 live cell events gated by scatter plots were analyzed for each sample.

Enzyme-linked immunosorbent (ELISA) assay

ELISA assay was used for measuring antibody titers in mouse serum samples. 96-wells flat bottom plates (Costar, Corning, NY) were coated with purified recombinant HIV Env proteins (the whole gp145 or V1/V2 loop of gp70) at a concentration of 0.01 μg/ml in

coating buffer (0.012 mol/L Na_2CO_3, 0.038 mol/L $NaHCO_3$, pH 9.6) at 4 °C overnight. The target protein was home-made in 293T expression system. The purity of protein was >95%. The plates were washed five times with PBST, and blocked with 3% BSA in PBST at 37 °C for two hours. Mouse serum samples were diluted with blocking solution, and added into each well (100 μl each well). After incubation at 37 °C for one hour, the plates were washed five times with PBST and then incubated with 1:5000 diluted HRP-labeled antibodies against mouse IgM, IgG, IgG1, IgG2a IgG2b or IgG3 (Santa Cruz Biotechnology) at 37 °C for one hour. The plates were washed five times with PBST. Fresh-prepared TMB substrate solution (100 μl, Sigma, St. Louis, MO) was added into each well, and the mixtures were incubated for 5 minutes. The reaction was stopped by additng H_2SO_4 (25 μl, 2 M). The optical density (OD) was measured at 450 nm or 630 nm by a Multiscan enzyme-linked immunosorbent assay plate reader (Thermo Life Sciences, Hampshire, United Kingdom). The cut-off value was determined: (1) OD value (D-value between 450 nm and 630 nm) >0.1 at the dilution of 1:100 (if not, the sample was considered as negative), (2) at least 2.1 folds that of the negative control is considered as positive. Endpoint titers were expressed as Log10 concentrations.

1 The serum samples from empty vector vaccination mice were

2 considered as negative control.

3

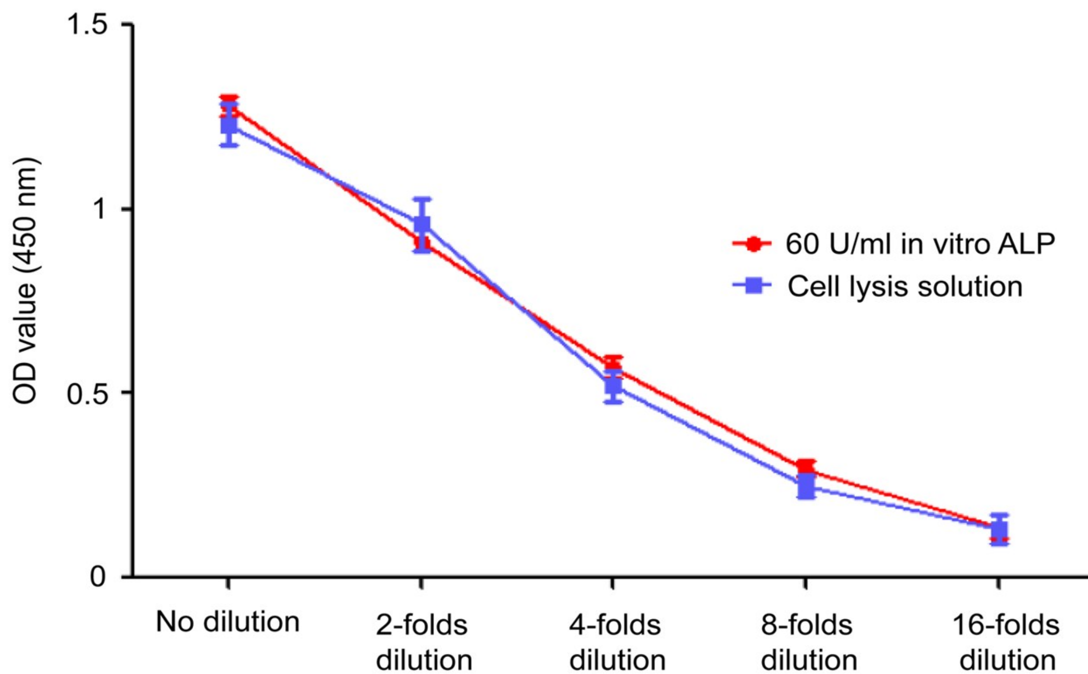
4 **Statistical analysis**

5 Values were expressed as means ± standard deviations (SD).

6 Analysis of differences in means between groups was conducted

7 by two-way ANOVA and post-hoc test; $P<0.05$ was significant.

8



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10 **Supplementary Figure 1. The catalytic capability between in**

11 **vitro ALP and Hela cell lysis solution**

12 In vitro ALP (60 U/ml) and Hela cell lysis solution (1×10^5 cells)

13 were double diluted with purified water. P-nitrophenyl phosphate

14 (PnPP, the substrate of phosphatases) was added. Five minutes

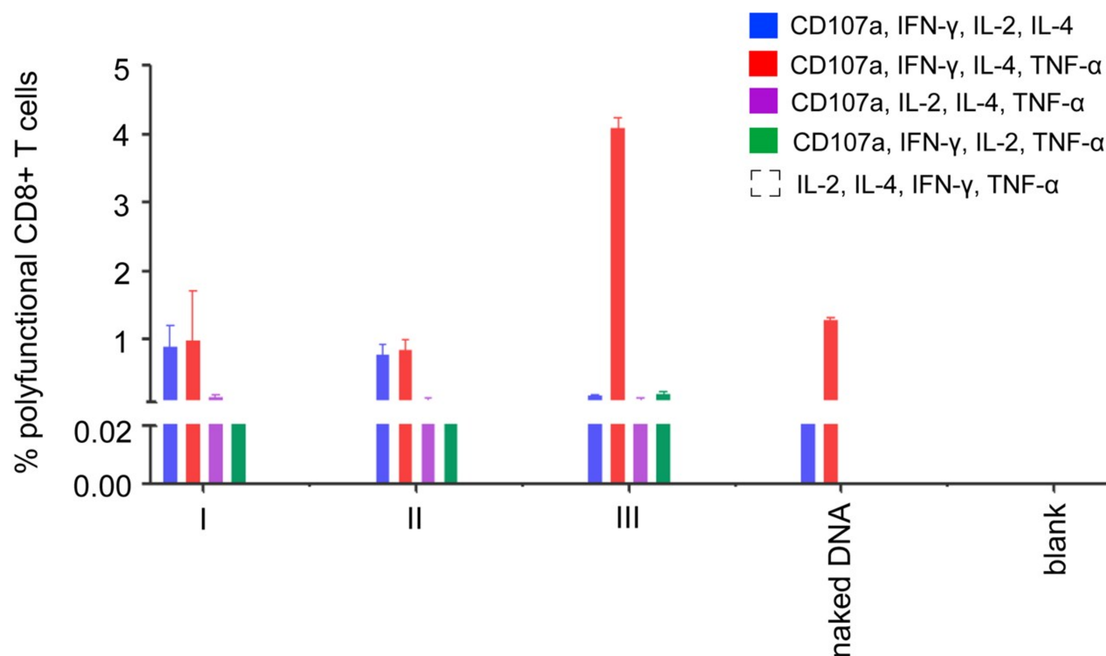
15 later, the reaction was stop, and OD (450 nm) was detected by a

1 Multiscan enzyme-linked immunosorbent assay plate reader

2 (Thermo Life Sciences, Hampshire, United Kingdom). The

3 experiment was repeated once.

4



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6 **Supplementary Figure 2. Combination ways of 4-cytokines in**

7 **mice groups**

8 Five (C_5^4=5) possible combination ways for 4-cytokines. Hidden-

9 line boxes mean the combinations of cytokines which fail to be

10 induced in our current study. DNA vaccine aided by peptidic

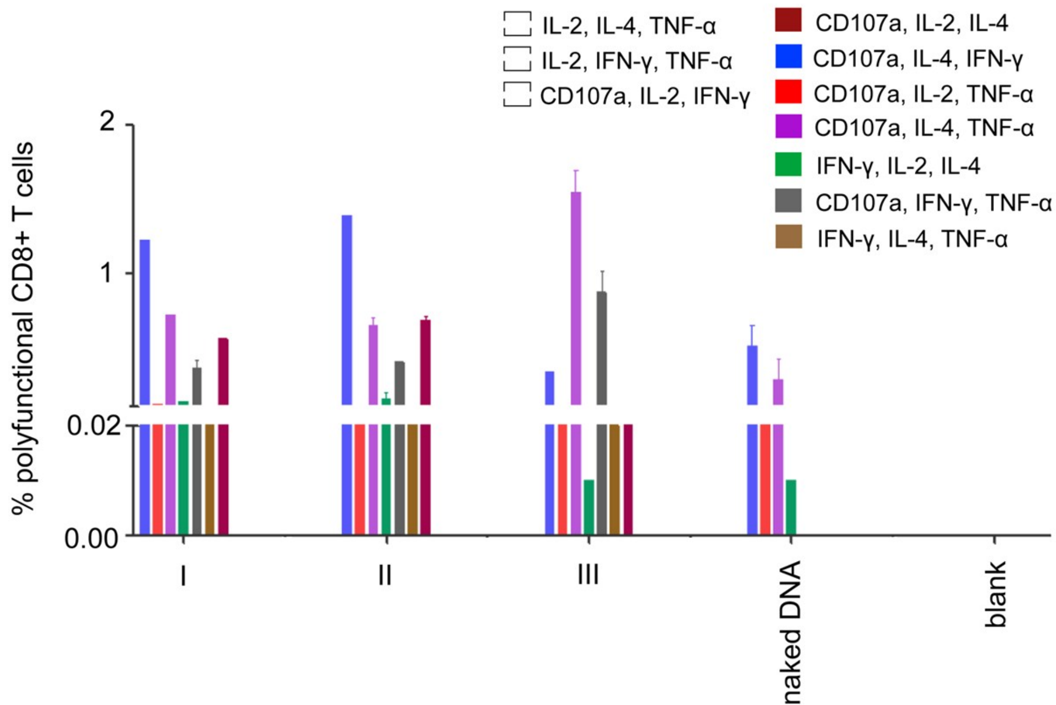
11 nanofibers via mode I, II and III induced four types of 4-cytokines

12 combinations which include 'CD107a, IFN-γ, IL-2, IL-4', 'CD107a,

13 IFN-γ, IL-4, TNF-α', 'CD107a, IL-2, IL-4, TNF-α' and 'CD107a, IFN-

14 γ, IL-2, TNF-α'. DNA vaccine alone only triggered two

combinations of 4-cytokines ('CD107a, IFN-γ, IL-2, IL-4' and 'CD107a, IFN-γ, IL-4, TNF-α') respectively.



Supplementary Figure 3. Combination ways of 3-cytokines in mice groups

Ten (C_5^3=10) possible combination ways for 3-cytokines. Hidden-line boxes mean the combinations of cytokines which fail to be induced in our current study. DNA vaccine aided by peptidic nanofibers via mode I, II and III induced seven types of 3-cytokines combinations which include 'CD107a, IL-4, IFN-γ', 'CD107a, IL-2, TNF-α', 'CD107a, IL-4, TNF-α', 'IFN-γ, IL-2, IL-4', 'CD107a, IFN-γ, TNF-α', 'IFN-γ, IL-4, TNF-α' and 'CD107a, IL-2, IL-4'. DNA vaccine

1 alone triggered four combinations (‘CD107a, IL-4, IFN-γ’, ‘CD107a,

2 IL-2, TNF-α’, ‘CD107a, IL-4, TNF-α’ and ‘IFN-γ, IL-2, IL-4’) of 3-

3 cytokines respectively.

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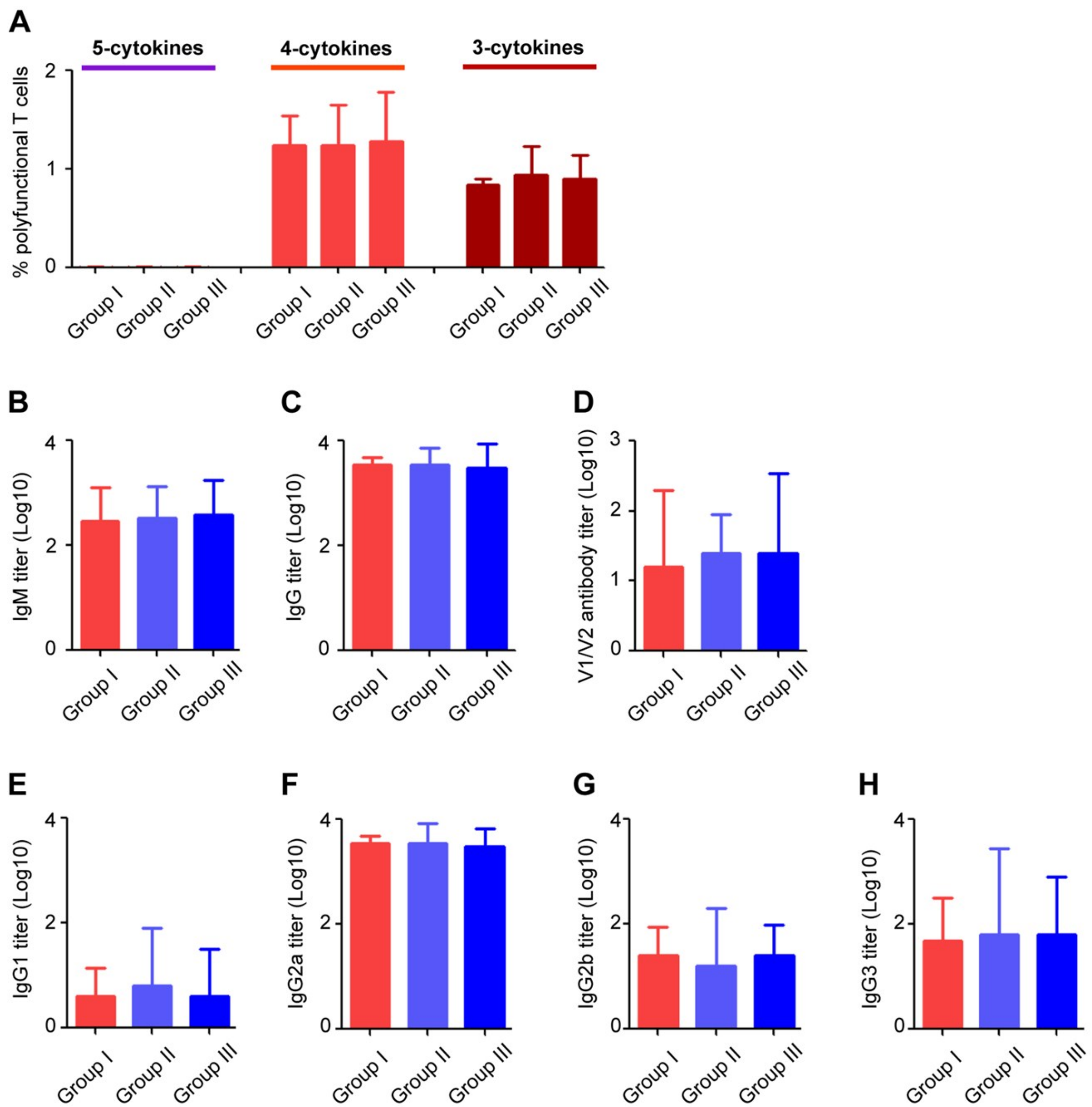

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6 **Supplementary Figure 4. Immunities triggered by separated**

7 **injections of NMe precursors and HIV DNA vaccine**

8 Three mouse groups (six mice per group) were used. Group I was

9 injected with HIV DNA vaccine alone (control group). Group II was

1 injected with NMe precursors first, followed by HIV DNA vaccine.
2 The time interval between two injections is one hour. Group III was
3 injected with HIV DNA vaccine first, followed by NMe precursors
4 one hour later. Comparing with control group, neither Groups I nor
5 Group II significantly improve HIV DNA vaccine-triggered
6 immunities, including polyfunctional T cell response **(A)**, IgM **(B)**,
7 IgG **(C)**, V1/V2 loop-specific antibody **(D)** and IgG subclasses
8 responses **(E-H)**.