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Potential of a novel peptide P16-D from membrane-proximal external region of human immunodeficiency virus type 1 to enhance retrovirus infection

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Abstract

The peptide P13 (Ac-^{671}NWFDITNWLWYIK^{683}-NH_2), derived from the membrane-proximal external region (MPER) of the human immunodeficiency virus type 1 (HIV-1) transmembrane protein and its derivative P16, have been shown to significantly boost HIV-1 infectivity by forming amyloid fibrils. Here, a new modified nanofibril peptide P16-D derived from P16 was demonstrated to have an enhanced ability to promote retroviral gene transfer. Moreover, the "networks" formed by P16-D nanofibrils could effectively capture and concentrate enveloped virus by low-speed centrifugation. In addition, the captured influenza virus H1N1 could elicit a stronger immune response in mice at a lower dose than that in the absence of the nanofibrils. The results implied a potential for P16-D to improve gene transfer rates and vaccine applications.

Keywords: HIV-1 MPER, P16, P16-D, amyloid fibrils, infection enhancement, virus enrichment

Introduction

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein mediates the fusion of viral and cellular membranes at the beginning of the infectious cycle. The demonstration that gp41 transmembrane subunits could form trimeric helical structures put forward the possibility of their function in bringing cell and viral membranes in close apposition [1, 2]. Multiple studies have revealed the function of a hydrophobic and conserved region known as the membrane-proximal external region (MPER) in the gp41 ectodomain, which is thought to perturb the bilayer organization of the cell membrane and allow it to be more prone for merging with the viral membrane [3, 4]. It contains a high proportion of aromatic amino acids that can favorably interact with the external monolayer of the viral membrane [5]. Several broadly neutralizing monoclonal antibodies (mAbs) against the MPER of HIV-1 have been successively identified. In particular, 4E10 is the most broadly neutralizing mAb yet discovered, and its epitope encompasses residues 671 to 683 of gp41 (NWFDITNWLWYIK) [6, 7].

P16, a synthetic peptide composed of the 4E10 epitope with a solubilizing lysine tail (Ac-^{671}NWFDITNWLWYIK^{683}KKK-NH_2), has been demonstrated to enhance HIV-1 infectivity [8]. Studies on the importance of individual amino acids of P16 have been conducted by substituting each residue with alanine, and the aromatic Trp residues and cationic Lys residues were shown to contribute to the enhancement of HIV-1 infection.

Previously, the amyloidogenic peptide SEVI derived from human semen was found to also significantly boost HIV-1 gene transfer [9, 10], mainly due to its ability to serve as a polycationic bridge to neutralize the negative charge repulsion between HIV-1 virions and target cells [11, 12]. The positive charge of the peptide facilitates its ability to enhance retroviral gene transfer. As was described with SEVI, a synthetic peptide termed enhancing factor C (EF-C) was also shown to form nanofibrils and enhance the efficiency of retroviral infection [13]. Recently, these functional amyloid fibrils were used to promote transduction of retroviral genes in clinical research of genetic disorders and infectious diseases [14]. These polymer-based transduction enhancers identified above provide broadened applications compared to common enhancers, including DEAE and polybrene [15-19]. Such efforts to successfully boost retroviral infection led to new perspectives and approaches for gene transfer.

The first pandemic of influenza in this century was caused by the H1N1 strains of swine origin first detected in humans in April 2009 [20]. The wide spread of the novel H1N1 influenza subtype has made the development of vaccines a global health priority, and related clinical and laboratory studies were subsequently conducted. Prevention of influenza by vaccination is crucial, especially in patients with a variety of pre-existing health

complications [21, 22]. Unfortunately, a high viral titer limits the laboratory diagnosis. Innovative approaches to reduce innoculation doses by the use of the intradermal route or new adjuvants have shown efficacy equivalent to that of a normal vaccine dose [23]. Therefore, we hypothesize that peptides which boost retroviral infection can provide a means to elicit effective immune responses to H1N1 vaccines at a lower dose.

In the present work, we synthesized a series of peptides derived from P16 and determined their potential to enhance retroviral infection. In addition, we investigated the formation of amyloid fibril structures by one of those derivatives, P16-D, as a factor in its ability to enhance retroviral infectivity. To evaluate their application, we also explored the influence of P16-D nanofilbrils on immune responses against attenuated H1N1 in mice.

Materials and Methods

Design and synthesis of peptides

All synthetic peptides involved in the study are presented in Table 1. Mutated residues are underlined in the newly synthesized peptides. Peptides (>98% purity) were produced by GL Biochem Ltd. (Shanghai, China) using solid-phase chemical synthesis and purified by preparative reversed-phase high-performance liquid chromatography and mass spectrometry. All lyophilized peptides were resuspended in phosphate-buffered saline (PBS; 0.1 M, pH 7.0) or serum-free Dulbecco's modified Eagle's medium (DMEM) at a concentration of 1 mg/ml. Fibril formation was induced effectively with agitation at 1000 rpm overnight at 37°C. In the assay to determine the active component, agitated peptides subsequently were centrifuged at 3000 rpm to separate the precipitate. After removal of the supernatant, the pelleted precipitate was resuspended in the same volume as that of the initial solution of peptides before agitation [8].

Preparation of retroviral particles

Infectious HIV-1 stocks, Yu-2 (CCR5 tropic) and NL.4.3 (CXCR4 tropic) were generated by transient transfection of 293T cells with DNA proviral expression plasmids. Lipofectamine 2000 transfection regent (Invitrogen) was used according to the manufacturer's protocol. Virus stocks were harvested after 48 h and titrated by using an anti-p24 Gag enzyme-linked immunosorbent assay (ELISA) [24, 25] with anti-p24-coated plates (Perkin-Elmer).

HIV-1 infectivity assays

TZM-bl cells were mainly used for HIV-1 infection. TZM-bl cell contains the luciferase reporter gene which is expressed under the control of the HIV-1 promoter. As described for P16[8], the infection was performed in the

absence of FBS during the first 4 h. HIV-1 infectivity was monitored by measuring luciferase activities in the cell lysates (Promega) with a luminescence spectrometer (FLUOROSKAN ASCENT FL, Thermo Scientific).

Cytotoxicity

The cytotoxicity of peptides in TZM-bl cells was measured by the MTT assay. Briefly, cells were seeded at 10^4 cells/well in 96-well plates at 37°C overnight. The following day, serial dilutions of peptides were added to the wells, and untreated cells served as the negative control. After 48 h of incubation, 10% MTT solution (5 mg/ml) in phosphate-buffered saline was added, and the plate was incubated for 2 h with 5% CO_2 at 37°C. The supernatant was then removed, and 150 µl dimethyl sulfoxide was added. The absorbance of the supernatant was measured at a wavelength of 490 nm.

Inhibition of P16-D-mediated enhancement of HIV-1 infection by epigallocatechin-3-gallate (EGCG)

0.2 mM EGCG was incubated with P16-D nanofibrils for 1 h. The mixtures were centrifuged at 3000 rpm for 5 min. After removal of the supernatant, the precipitate was resuspended in fresh medium of an equal volume. TZM-bl cells were then added for 4 h in serum-free medium. Thereafter, 10% FBS (heat-inactivated) was added, and HIV-1 infectivity was determined by measuring luciferase activity.

Transmission electron microscopy (TEM)

The resuspended P16-D nanofibril solution was pelleted onto 200-mesh carbon-coated copper grids and subsequently stained with 2% potassium phosphotungstate for 1 min. The fibril morphology was characterized by TEM (JEOL-2000EX, JPN) with an accelerating voltage of 80 kV.

Thioflavin T binding assay

Peptide solutions (dissolved in PBS) were added to 5 µM thioflavin T (ThT) (Sigma-Aldrich) in PBS diluted at a concentration starting from 1000 µg/ml, and fluorescence was assayed at 482 nm in triplicate wells with a Perkin-Elmer Ls-5B luminescence spectrometer.

P16-D-mediated concentration of virions

HIV-1 Yu-2 stock was incubated with P16-D nanofibrils at 37°C for 30 min and subjected to low-speed centrifugation for 5 min at 4000 rpm. The p24 levels of the collected supernatants as well as that of the original virus stock were determined by an anti-p24 Gag ELISA as described above.

Hemagglutination (HA) assay

A round-bottomed 96-well plate was employed for the HA assay. To each well, 50 µl PBS/P16-D nanofibril solution (1000 µg/ml) was added. In the first column, 50 µl of H1N1 virus sample was added and mixed well before transferring 50 µl to the next well on the right. In this manner, the virus was repeatedly mixed and serially diluted by transferring 50 µl to the adjacent well down the length of the plate. From the last well, 50 µl was discarded into a bleach solution. Fifty microliters of 1% guinea pig red blood cell suspension was then added to each well, mixed gently and left at room temperature for 30-60 minutes to develop. Negative results appeared as dots in the center of the round-bottomed plates, while positive results formed a uniform reddish color across the well. The virus HA titer is the highest dilution factor that produced a positive reading.

Immunization

BALB/c mice (4–6 weeks) were immunized with P16-D treated/untreated H1N1 influenza virus (pandemic A/California/2009/38, 5×10^5 $TCID_{50}$/ml) by nasal cavity injection on day 0, and a booster vaccination was given on day 14. Serum samples were collected from the caudal vein 2 days before the next immunization. Serum samples were heat-inactivated for 30 min at 56°C and stored at -80°C until use. All animals were housed under approved Institutional Animal Care and Use Committee protocols. The animal trials in this study were carried out in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China (11-14-1988). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University (permit number: SCXK 2013-0001).

ELISA of mouse antisera

96-well Maxisorp plates (Jet Biofil, Guangzhou, China) were coated with 100 ml/well of H1N1 in physiological saline solution and then incubated overnight at 4°C. After washing with 150 µl of wash buffer (25 mM Tris–HCl, 150 mM NaCl, pH 7.2, 0.05% Tween-20) per well three times, serial dilutions of mouse antisera were added and incubated for 1 h at 37°C. Plates were washed five times before incubating with 100 ml/well of horseradish peroxidase (HRP)-conjugated anti-mouse IgG for 1 h at 37°C. Lastly, peroxidase substrate [0.3 mg/ml tetramethylbenzidine (TMB) in 0.18 M sodium citrate, pH 3.95] was added after five rinses with wash buffer and one with PBS. The reaction was quenched using an equal volume of 2 M H_2SO_4, and the optical density was read at 450 nm.

Results and Discussion

P16-D is a better enhancer of retroviral infection than the parent peptide P16

We have previously confirmed that P16 could enhance HIV infectivity by forming amyloid fibril structures [8]. The stabilization of the amyloid fibril structure is based on hydrogen bonding in the monomer peptide. The amount of basic groups plays a key role in the conformation of hydrogen bonds, number of electric charges and isoelectric points [26, 27]. The acidic Asp located in the P-16 sequence was substituted by a hydrophobic amino acid, basic amino acid or nonpolar amino acid to evaluate their abilities to enhance HIV-1 infection in this study. In addition several mutated peptides with different pI based on the peptide P16 were synthesized and tested as shown in Table 1.

HIV-1 Yu-2 infection could be significantly enhanced by P16-D, P16-K and P16-N (Figure 1A). The results indicated that basic groups may play a role in enhancing infection. As shown in Figure 1B, P16-D exhibited lower cytotoxicity than the other peptides. Thus, the P16-D peptide was chosen over P16-K and P16-N for further investigation. Common enhancers, including DEAE and polybrene were also chosen for comparison. Indeed, P16-D boosted retroviral infection more efficiently than conventional additives, such as DEAE and polybrene (Figure 1C).

P16-D forms nanofibrils to boost retroviral infection

The stock solution of P16-D became turbid after agitation for 16 h. Previous studies showed that both SEVI and P16 formed amyloid fibrils to promote retroviral infection. These fibrils can capture HIV virions and direct them to target cells, where they facilitate the fusion of virus and host cell. And the major green tea ingredient EGCG could redirect amyloidogenic polypeptides into unstructured oligomers and abrogate semen-mediated enhancement of HIV-1 infectivity. The exact mechanism by which EGCG disrupts amyloid formation is unclear. Some studies proposed that EGCG can bind to the backbone exposed in the disordered conformation of the monomeric species of many amyloid proteins, redirecting the aggregation pathway to amorphous aggregates that are nontoxic and do not share many of the common features of amyloid-based structures [28-31]. In this study, P16-D was pre-incubated with EGCG to inhibit its enhancing effect on HIV-1 infectivity. The EGCG concentration of 0.2 mM was used to rule out toxicity (data not shown), and 60 μg/ml was chosen as the optimal concentration of P16-D according to the results shown above to incubate with EGCG. The augmentation of HIV-1 infection was drastically inhibited by treatment with EGCG, which clearly showed that P16-D formed amyloid fibrils to boost HIV-1 Yu-2 infection (Figure 2A). TEM also confirmed effective fibril formation with diameters of 4–10 nm (Figure 2B).

P16-D nanofibrils can be used to concentrate virions rapidly by low-speed centrifugation

The HIV-1 Yu-2 stock was treated with P16-D nanofibrils and subjected to low-speed centrifugation. Analysis by anti-p24 Gag ELISA of the original virus stock (A) and the supernatants (B) derived after centrifugation of P16-D-treated virus indicated that P16-D nanofibrils effectively captured and concentrated the virions (Figure 3).

Potential enhancement of H1N1 immune response at a less virus dose

P16-D nanofibril has been shown to also enhance infection of other enveloped viruses, including influenza and FIV/VSV-G, and its influence on influenza was measured by a nonspecific HA test [30, 31]. The HA assay is a method for titering influenza viruses based on their ability to attach to molecules present on the surface of red blood cells [34, 35]. In this study, P16-D nanofibrils could increase the HA titer compared with that in the absence of the peptide fibrils (data not shown), demonstrating their potential ability to enhance the infection of influenza virus. Notably, TEM showed that P16-D nanofibrils could capture H1N1 virions like a net (Figure 4). In addition, the ThT binding experiment was performed to further confirm the formation of nanofibrils [36]. The fluorescence intensity could increase in the absence of EGCG, suggesting that stabilized amloyid fibrils could be formed by P16-D (Figure_S_1).

To further explore the potential application of P16-D fibrils in the immune response to influenza vaccines, BALB/c mice were challenged with attenuated influenza H1N1 by nasal cavity injection. Antibody responses in the mouse antisera were assessed after the second immunization by ELISA, and all groups showed elevated antibody titers in the presence of P16-D nanofibrils (Figure 5). The results indicated that using a lower dose of the H1N1 vaccine was feasible to stimulate immune responses in the presence of P16-D nanofibrils, which themselves were non-immunogenic in mice.

Conclusions

The aim of this study was to introduce a novel functional nanofibril and its potential use in stimulating immune responses to influenza. We synthesized several derivative peptides based on P16, and the analysis of their effects on infection showed that P16-D had a striking ability to promote retroviral gene transfer at a lower level of cytotoxicity by forming nanofibrils. In addition, these nanofibrils could effectively capture virion, which provides a means to concentrate virions by low-speed centrifugation. The captured H1N1 influenza virus could elicit immune responses in animals at a lower dose than those immunized without the nanofibrils. Although the possible role of P16-D nanofibrils will need to be further investigated, the results of this study provide us with a new perspective

on amyloid fibrils and optimization of viral vaccines.

Acknowledgements

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Table 1. Synthetic peptides used in this study

Peptides	Sequence	pI
P16	Ac-NWFDITNWLWYIKKKK-NH_2	9.52
P16-D	Ac-NWFAITNWLWYIKKKK-NH_2	9.87
P16-K	Ac-NWFKITNWLWYIKKKK-NH_2	10.03
P16-N	Ac-NWFNITNWLWYIKKKK-NH_2	9.87
P16-2W	Ac-NWFDITNKLWYIKKKK-NH_2	9.75
P16-W3K	Ac-NWFDITNWLWKYIKKK-NH_2	9.52

Mutated residues are underlined.

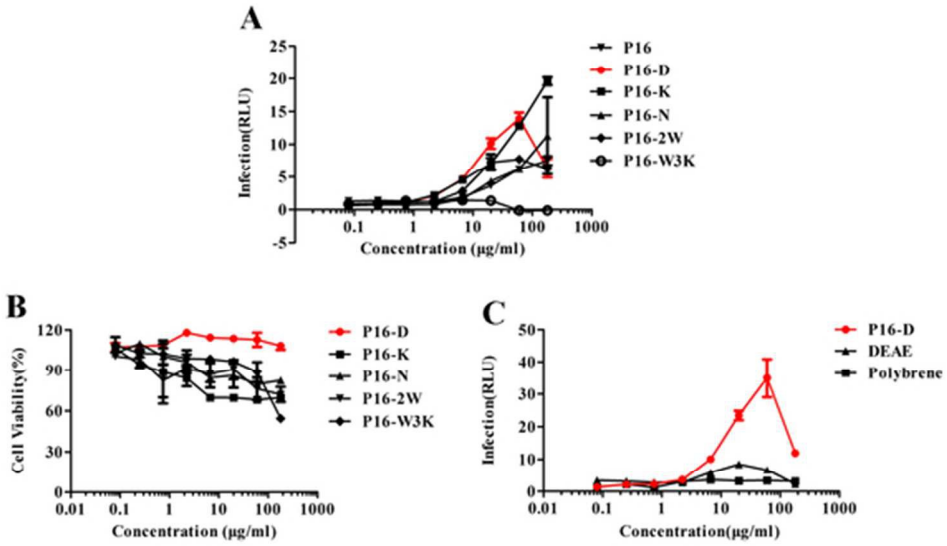

Figure 1. Enhancement of HIV-1 Yu-2 infection by peptides derived from P16. (A) Overview of the activity of analyzed peptides in boosting HIV-1 infection in TZM-bl cells. (B) Effects of peptides on viabilities of TZM-bl cells. Cells were treated with serially diluted peptides for 48 h. Cell viability was determined by the MTT assay. (C) P16-D enhanced HIV-1 Yu-2 infection more efficiently than DEAE and polybrene.
Figure 1
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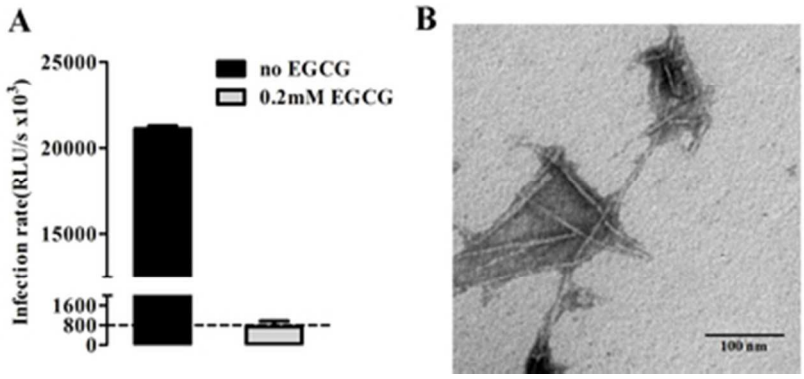

Figure 2. Enhancement of HIV-1 infection by P16-D in the form of amyloid fibrils in TZM-bl cells. (A) EGCG (0.2 mM) abrogated HIV-1 Yu-2 infection enhanced by P16-D in a turbid solution, indicating that P16-D assembled into polymers after agitation. (B) TEM analysis of P16-D exhibiting a typical fibril structure. Bar = 100 nm.
Figure 2
37x16mm (300 x 300 DPI)

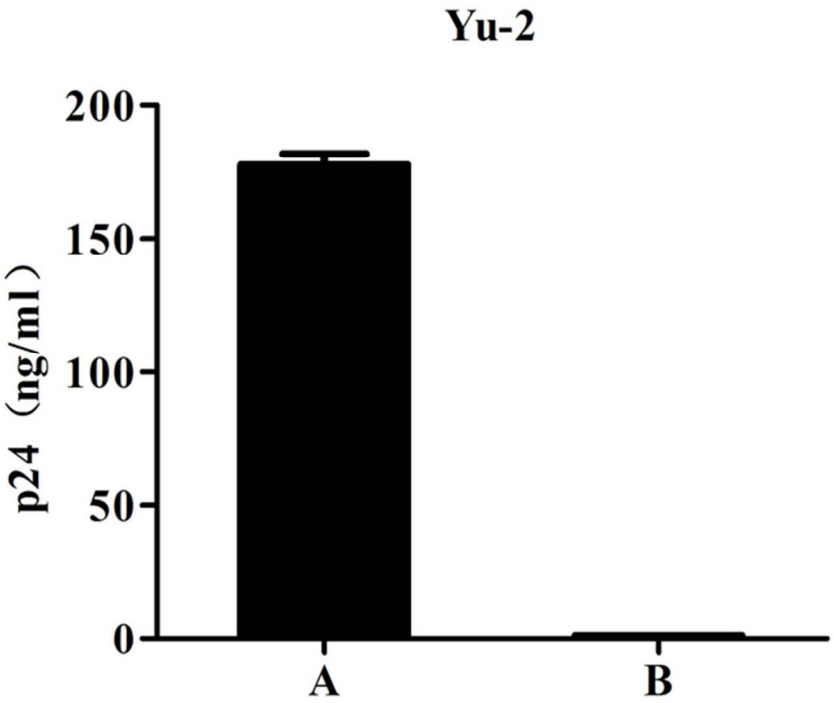

Figure 3. Effective capture and concentration of HIV-1 virions by P16-D fibrils during low-speed centrifugation. Results are shown of p24 ELISA of the original virus stock (A) and the supernatants (B) derived after centrifugation of P16-D-treated virus.
Figure 3
63x49mm (300 x 300 DPI)

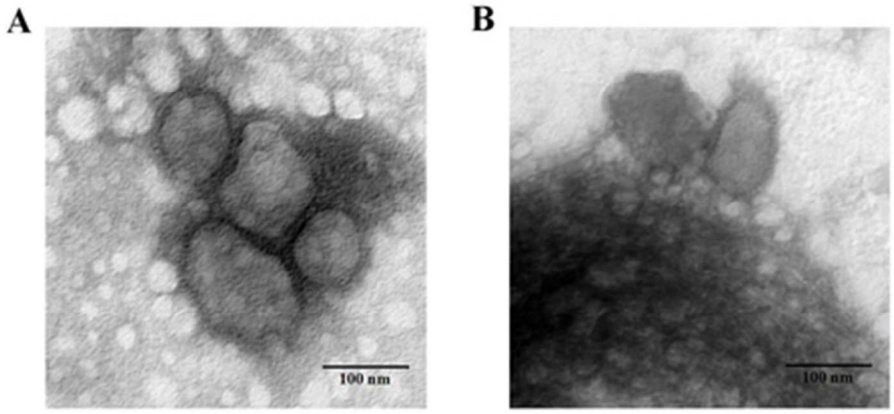

Figure 4. Attenuated H1N1 particles in the absence (A) or presence (B) of P16-D fibrils by TEM. H1N1 was incubated with P16-D fibrils and then subjected to low-speed centrifugation. After removal of the supernatant, the pellet was dissolved in PBS of equal volume for use.
Figure 4
39x18mm (300 x 300 DPI)

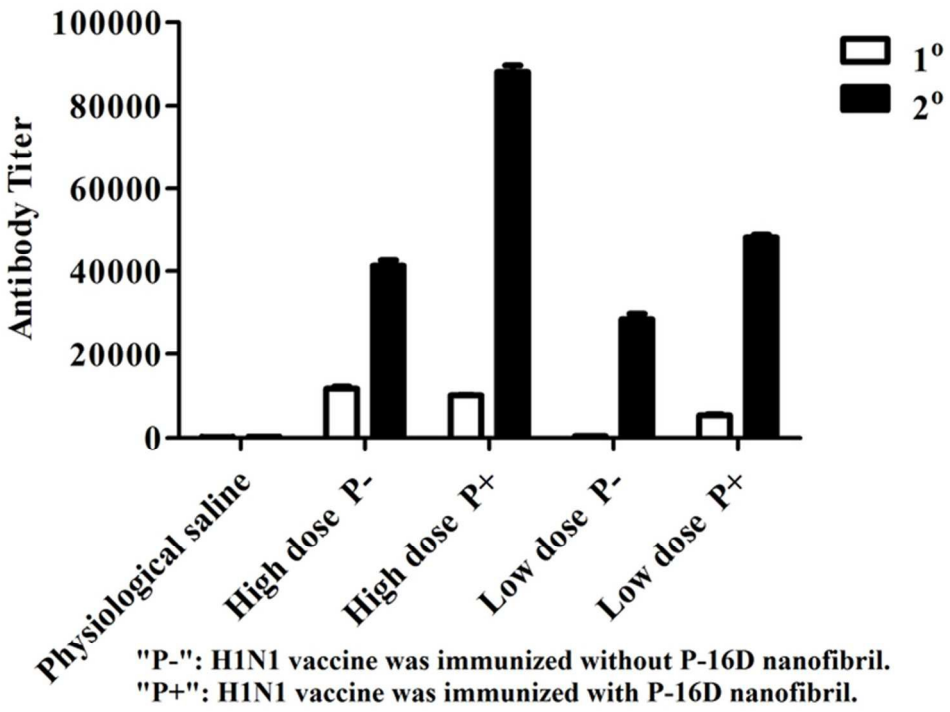

Figure 5. Antibody titer of pooled antisera from Balb/c mice after the second immunization with attenuated H1N1 treated with P16-D fibrils. High dose, 2.5×10^4 $TCID_{50}$ per mouse; low dose, 6.25×10^3 $TCID_{50}$ per mouse.
Figure 5.
65x50mm (300 x 300 DPI)