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Supplementary Information for

Heterosubtypic influenza protection elicited by double-layered polypeptide nanoparticles in mice

Lei Deng[1], Timothy Z. Chang[2], Ye Wang[1], Song Li[2], Shelly Wang[3], Shingo Matsuyama[1], Guoying Yu[4], Richard W. Compans[3], Jian-Dong Li[1], Mark R. Prausnitz[2], Julie A. Champion[2], Bao-Zhong Wang[1,*]

[1] Center for Inflammation, Immunity & Infection, Georgia State University Institute for Biomedical Sciences, Atlanta, GA, USA

[2] School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA, USA

[3] Department of Microbiology and Immunology and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA, USA

[4]State Key Laboratory of Fibrosis Biology, College of Life Science, Henan Normal University, Xinxiang, Henan, P. R. China

[*] Corresponding author: Bao-Zhong Wang (E-mail: bwang23@gsu.edu)

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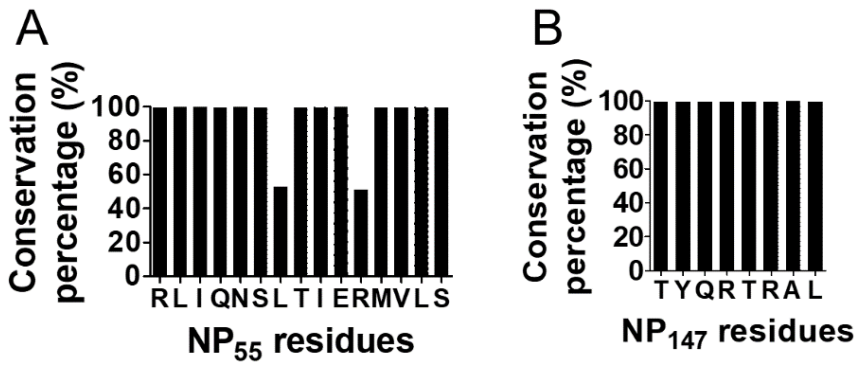

Fig. S1. Conservation rate of NP epitopes. (*A*) and (*B*), Homogeneity of amino acid residues in NP_{55} and NP_{147} epitopes based on NP sequences from 18540 human influenza isolates deposited in Influenza Research Database, IRD website: www.fludb.org).

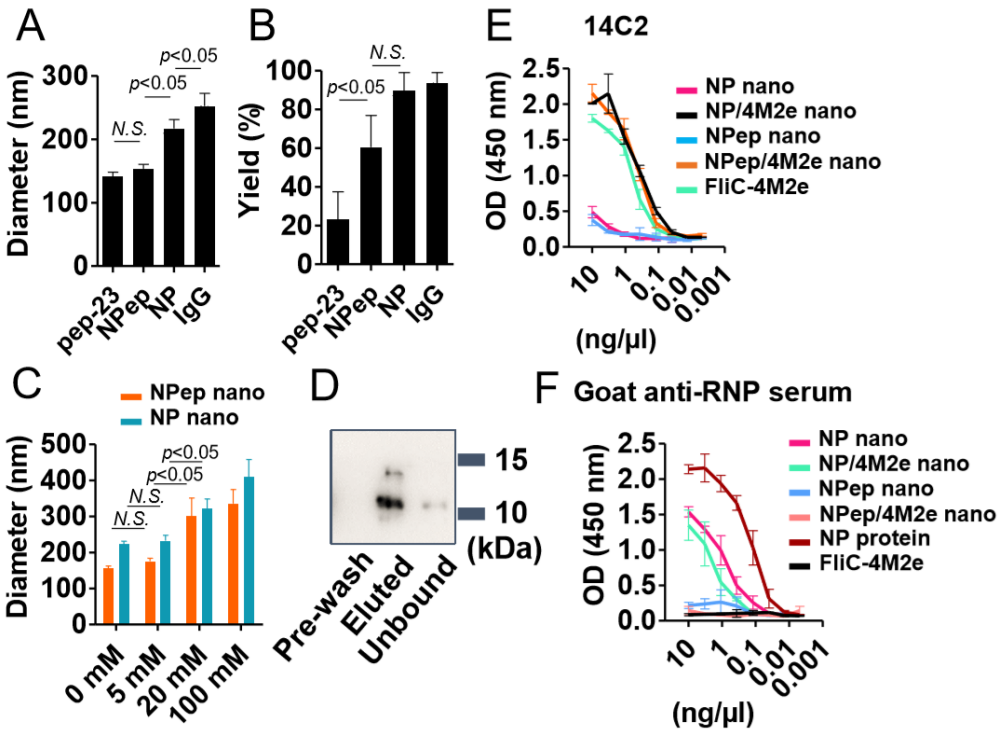

Fig. S2. Characterization of fabricated nanoparticles. (*A*) and (*B*) Bar chart depicting desolvated nanoparticle (*A*) size and (*B*) yield variation with different materials including peptides containing 23 a.a., NP peptide (NPep), NP protein and IgG. Desolvated nanoparticles were fabricated in 4 volumes of absolute ethanol and in the absence of a crosslinker. (n=3) (*C*) Bar chart depicting the size variation of desolvated NPep- and NP-nanoparticles with increasing amounts of DTSSP. (n=3) (*D*) Western blotting analysis of Pre-wash, Unbound fraction and Eluent samples in pull-down assay. (*E*) and (*F*) Sandwich ELISA of desolvated nanoparticles using capture antibodies (*E*) 14C2 and (*F*) goat anti-mouse ribonucleoprotein serum. (n=3)

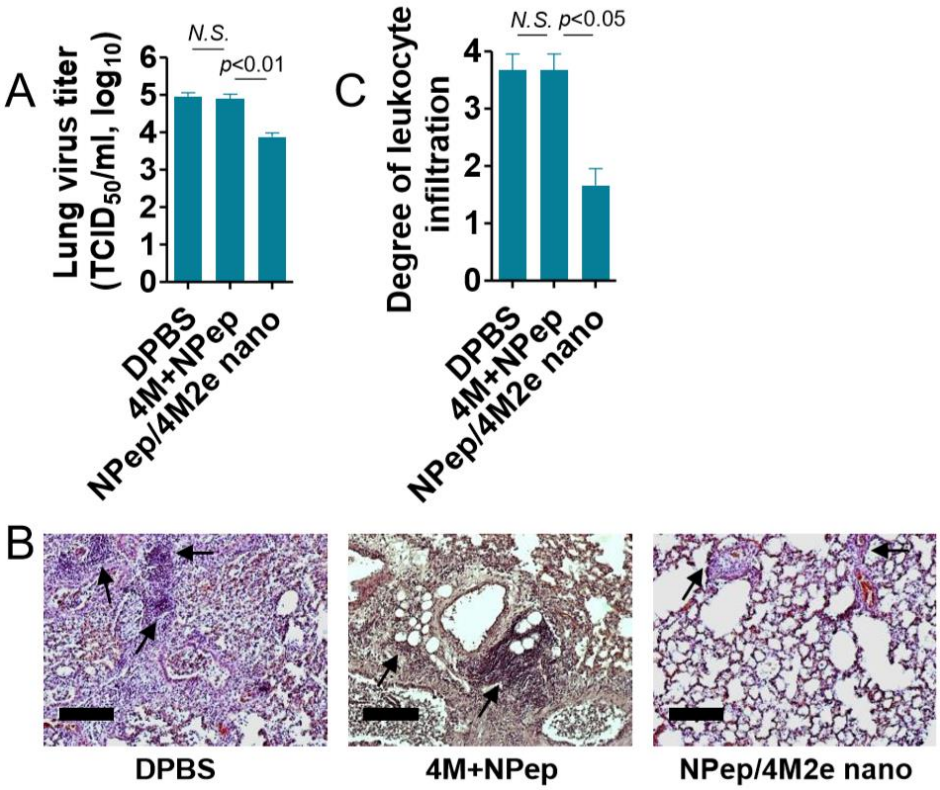

Fig. S3. Lung virus pathology analysis. (*A*) Determination of mouse lung virus titers at day 5 post a sublethal dose infection with H5N1. (*B*) Histological pathology analysis. Black arrows in images indicate leukocyte infiltration in lung sections from mice infected with H5N1. (*C*) Bar chart showing the scores of leukocyte infiltrations degree after infection with H5N1. Data are presented as mean ± s.d. Statistical significance was analyzed by t-test for (*A*) and (*C*). *P* values shown in bar charts and N.S. indicates no significance between two compared groups. The experiments were repeated twice with similar results.

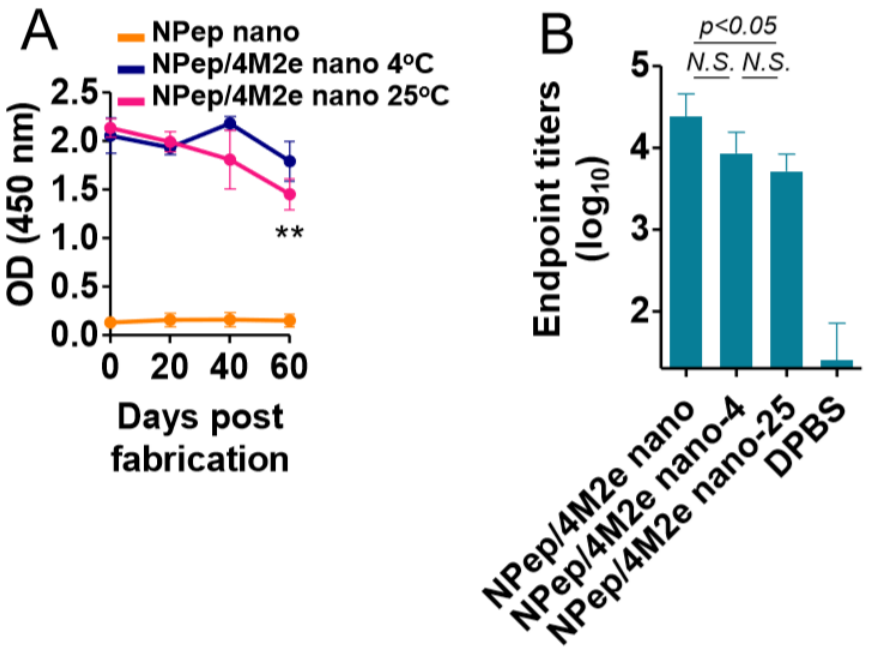

Fig. S4. Nanoparticle antigenicity and immunogenicity after long-term storage. (*A*) Sandwich ELISA of freshly fabricated NP-peptide/4M2e layered nanoparticle (NPep/4M2e nano) and those stored at 4 °C and 25 °C for 20, 40 and 60 days using capture antibody 14C2. NP-peptide nanoparticle (NPep nano) was used as negative control. (*B*) HuM2e-specific serum antibody endpoint titers in BALB/c mice that were IM immunized with freshly fabricated or aged NPep/4M2e nano stored at 4 °C (NPep/4M2e nano-4) and 25 °C (NPep/4M2e nano-25) for 60 days. Mock immunization with DPBS as negative control.

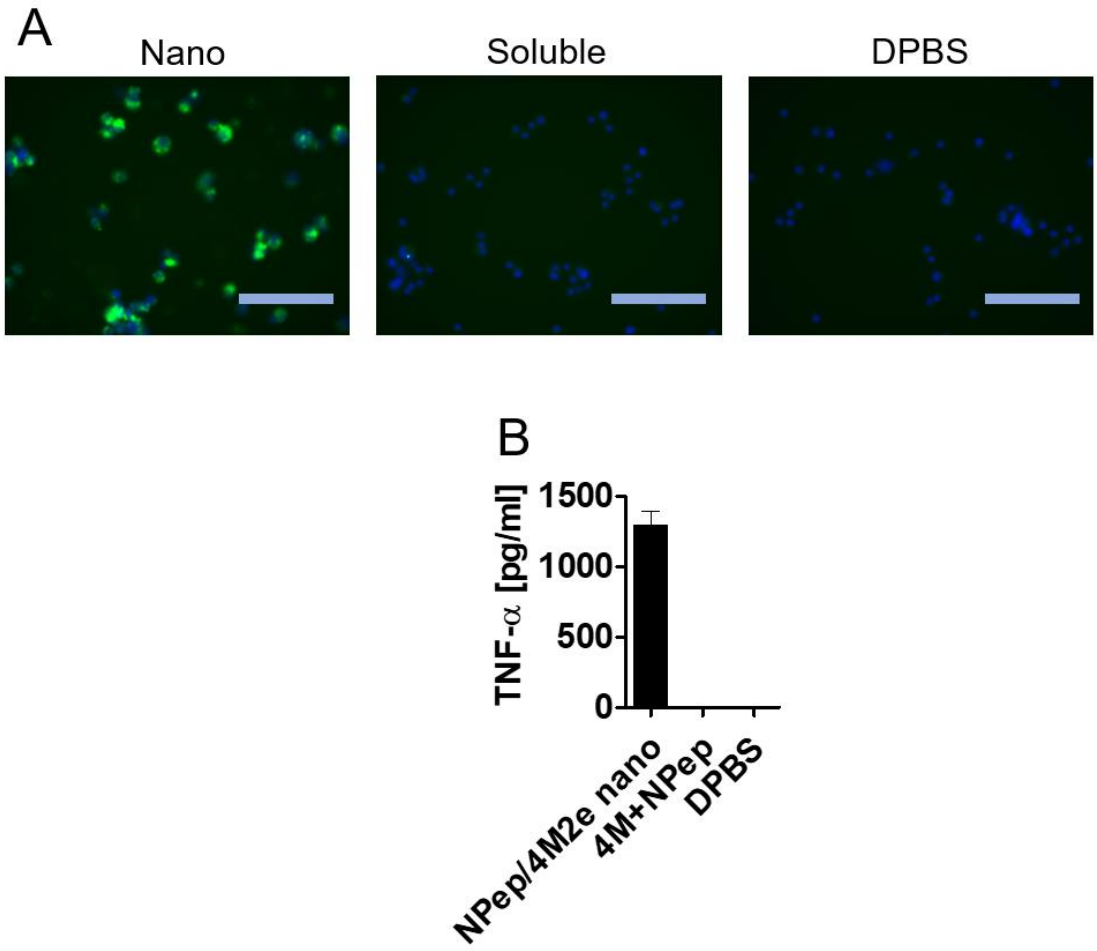

Fig. S5. *In vitro* dendritic cells uptake assay. (*A*) Fluorescent microscopy imaging of JAWS II dendritic cells with green fluorescent NP-peptide nanoparticle (Nano), green fluorescent soluble NP peptide (Soluble) or DPBS as negative control. NP peptide was fluorescently labelled with Alexa Fluor 488 NHS Ester and then desolvated into nanoparticles. After 3 h incubation, the cells were stained with DAPI (blue). (*B*) Dendritic cells TNF-α production. TNF-α secretion by JAWS II dendritic cells after 3 h of stimulation.

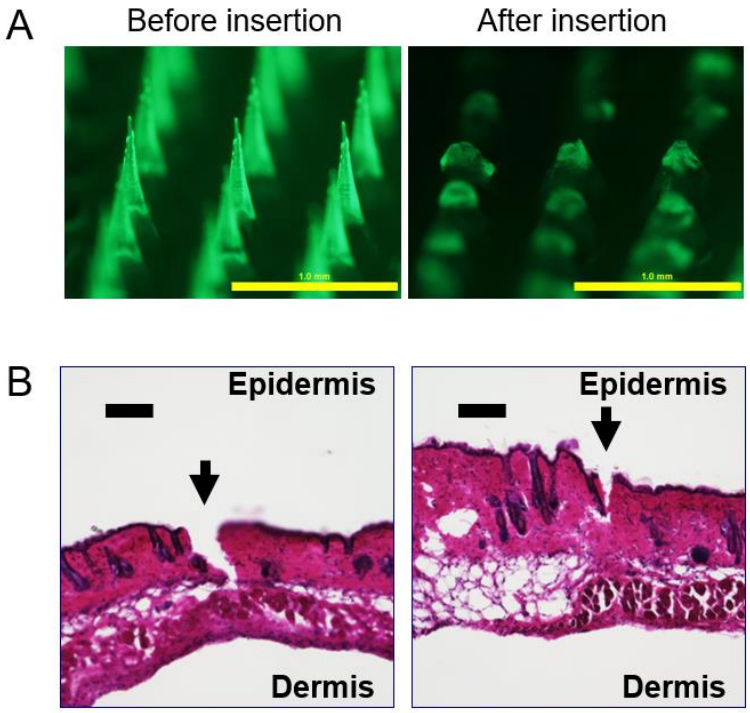

Fig. S6. Characterization of MN patch delivery. (*A*) MN dissolution in BALB/c mice skin *ex vivo*. Top, before insertion; bottom, remaining polymer 20 min after insertion in skin. (Bars represent 1 mm) (*B*) Haemotoxylin and Eosin-staining micrograph of mouse skin histological section after insertion of MN patch *ex vivo*. (Arrows indicate the punctured lesion by MN patch, bars represent 100 µm)

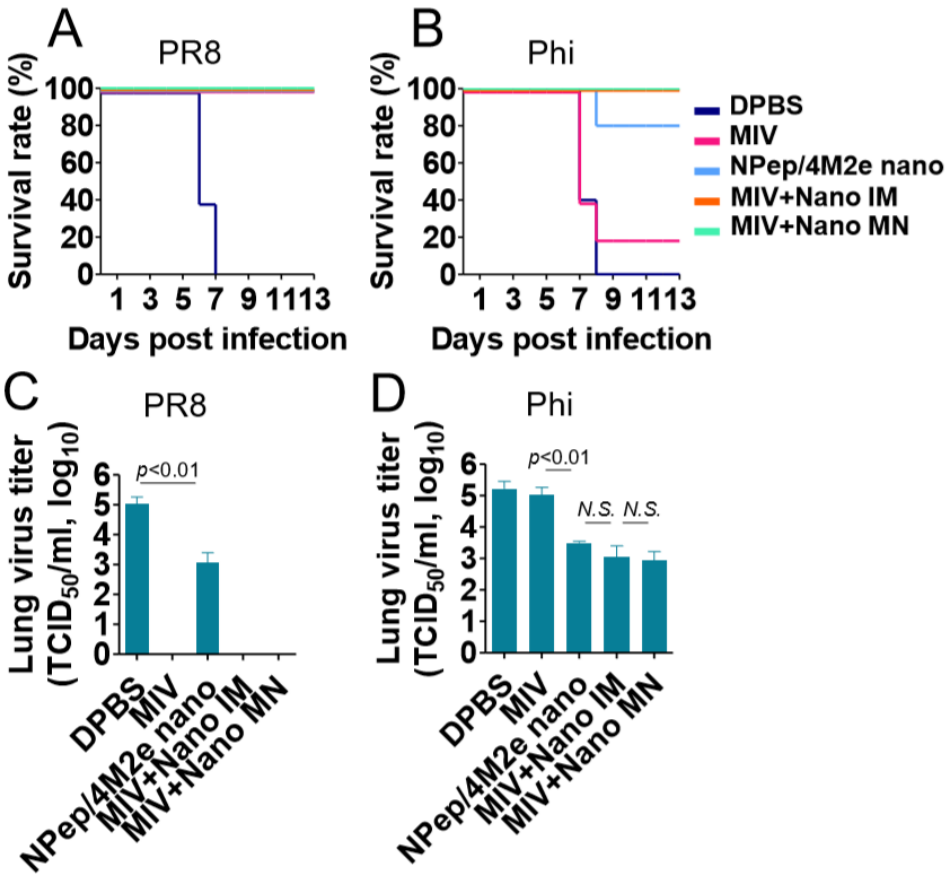

Fig. S7. Mortality and lung viral titers. Mortality of immunized BALB/c after lethal dose 6 × mLD_{50} infection with (*A*) PR8 and (*B*) Phi. Determination of mouse lung virus titers at day 5 post a sublethal dose 0.5 × mLD_{50} infection with (*C*) PR8 and (*D*) Phi.

Table S1. Definitions of abbreviations used in article.

Abbreviations	Full names
ADCC	Antibody dependent cellular cytotoxicity
APC	Antigen presenting cells
ASC	Antibody secreting cells
BAL	Bronchial alveolar lavages
CTL	Cytotoxic lymphocyte
DC	Dendritic cells
IFN	Interferon
IIV	Inactivated influenza vaccines
IL	Interleukin
IM	Intramuscular(ly)
IN	Intranasal(ly)
MHC	Major histocompatibility complex
M2e	Maxtrix protein 2 ectodomain
MN	Microneedle
MIV	Monovalent inactivated virus
NP	Nucleoprotein
NPep	Nucleoprotein peptide
OVA	Ovalbumin
Pep-23	Human M2e peptide with 23 amino acids in length
RNP	Ribonucleoprotein

Table S2. Various M2e sequences. *

Codes	Sequence	Origin
huM2e	SLLTEVETPIRNEWGCRCNDSSD	Human consensus
PR8M2e	SLLTEVETPIRNEWGCRCNGSSD	A/Puerto Rico/8/1934, H1N1
p09M2e	SLLTEVETPTRSEWECRCSDSSD	A/California/7/2009, H1N1
PhiM2e	SLLTEVETPIRNEWGCRCNDSSD	A/Philippines/2/1982, H3N2
rVnM2e	SLLTEVETPIRNEWGCRCNGSSD	A/Vietnam/1203/2004, H5N1
rSHM2e	SLLTEVETPIRNEWGCRCNGSSD	A/Shanghai/2/2013, H7N9
4M2e	SLLTEVETPIRNEWGSRSNDSSD PGGSSGGSS SLLTEVETPTRSEWESRSSDSSD PGGSSGGSS Human consensus linker Swine consensus linker SLLTEVETPTRNGWESKSSGSSD PGSGSGSGS SLLTEVETPTRNGWESNSSDSSD Avian consensus linker Domestic fowl consensus	

* Amino acid residues in blue represent differences from the human viral M2e consensus (huM2e). All cysteine (C) residues in M2e consensus sequences in 4M2e construct were mutated into serine (S). Residues in orange are flexible linkers.

Note S1. Nucleotide sequence of NP epitope peptide.

ATGAAATTTCTCGTGAACGTGGCGTTGGTGTTTATGGTAGTATATATAAGTTA
TATCTACGCAGATCGCTTGATTCAGAATAGTCTCACCATTGAGCGTATGGTTC
TTAGTGGTGGAAGCTCTCGCCTCATCCAAAATTCTCTCACAATCGAGCGCATG
GTTCTGAGCGGTGGTAGTTCGCGCCTCATTCAAAACTCTCTTACCATCGAGCG
TATGGTACTTTCAGGTGGTAGCTCCGGAGGAACCTACCAGAGAACTCGTGCA
TTGGTCGGCGGCTCTTCCACGTACCAGAGGACACGTGCGCTGGTGGGCGGAT
CGTCTACCTATCAACGTACCCGTGCGCTGGTGCACCACCATCACCACCACTAA

Note S2. Amino acid sequence of NP epitope peptide. *

MKFLVNVALVFMVVYISYIYADRLIQNSLTIERMVLSGGSSRLIQNSLTIERMVLS
GGSSRLIQNSLTIERMVLSGGSSGGTYQRTRALVGGSSTYQRTRALVGGSSTYQR
TRALVHHHHHH-

* Amino acid sequence in blue indicates the honeybee melittin signal peptide. Magenta sequence indicates the linkers. Yellow sequence means hexa-histidine tag.