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Multi-subtype influenza virus-like particles incorporated with flagellin and granulocyte-macrophage colony-stimulating factor for vaccine design

Wen-Chun Liu [a, 1], Ying-Yu Liu [a, 1], Ting-Hsuan Chen [a], Chia-Chyi Liu [b], Jia-Tsrong Jan [c], Suh-Chin Wu [a, d, *]

[a] Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan
[b] National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Zhunan, Taiwan
[c] Genomics Research Center, Academia Sinica, Taipei, Taiwan
[d] Department of Medical Science, National Tsing Hua University, Hsinchu, Taiwan

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ABSTRACT

Virus-like particle (VLP) technology is an attractive platform for seasonal and pandemic influenza vaccine development. We previously showed that influenza VLPs can be modified using M2 fusion with molecular adjuvants such as *Salmonella typhimurium* flagellin (FliC) to enhance VLP immunogenicity. For this study, three types of chimeric VLPs were incorporated with FliC, granulocyte-macrophage colony-stimulating factor (GM-CSF), or both GM-CSF and FliC (GM-CSF/FliC) to enhance anti-influenza immunogenicity. Our results indicate that immunizations with the chimeric FliC VLPs and GM-CSF/FliC H5N1 VLPs elicited more potent and broadly neutralizing antibodies and neuraminidase-inhibiting antibodies in sera, and induced higher numbers of hemagglutinin-specific antibody-secreting cells and germinal center B cell subsets in splenoctyes. Immunization with the chimeric GM-CSF H5N1 VLPs induced stronger Th1 and Th2 cellular responses. The chimeric GM-CSF/FliC H5N1 VLP constructs were further obtained to include H7 or H1H7 bi- or tri-subtype. It is our hope that these findings provide useful information for developing multi-subtype influenza vaccines.

1. Background

Existing and newly emerging highly pathogenic avian influenza (HPAI) H5N1 viruses are raising global concerns about a potential human pandemic. The first human HPAI H5N1 virus case occurred in Hong Kong in 1997, re-emerged in 2003, and has periodically caused human infections in Asia, the Middle East, Europe, and Africa, with an overall mortality rate of 53%. Antigenic shifts have led to further HPAI H5N1 evolution, resulting in 10 distinctive clades (numbered 0 through 9); most of the recently identified subclades are mostly within clades 1 and 2 (Sonnberg et al., 2013; WHO, 2014). Currently there is great interest in developing a universal influenza vaccine against different HPAI H5N1 clades/subclades, and in some cases further against other heterosubtypic avian or human influenza viruses such as H7N9, pH1N1 and H3N2 (Krammer and Palese, 2015).

Influenza virus-like particles (VLPs) are non-infectious nanoparticles that do not contain genomic RNAs but contain the native HA and NA oligomeric structures that support maximum vaccine immunogenicity without compromising safety or tolerability (Haynes, 2009; Lopez-Macias, 2012). Influenza VLP assembly consists of (i) initiating virus budding by HA and/or NA viral proteins targeted at lipid rafts, (ii) assembly by M1 viral protein polymerization, and (iii) membrane scission with M2 ion channel proteins (Rossman and Lamb, 2011). Influenza VLP production entails the overexpression of HA, M1 with or without NA and/or M2 viral proteins (D'Aoust et al., 2010; Giles et al., 2012; Ross et al., 2009; Wei et al., 2011; Wu et al., 2012). Once established, VLPs can elicit protective immune responses following either single- or double-dose injections mostly by intramuscular (i.m.) inoculation (Lin et al., 2013; Ross et al., 2009; Wei et al., 2011), although recently

* Corresponding author. Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan.
E-mail address: scwu@mx.nthu.edu.tw (S.-C. Wu).
[1] These authors contributed equally to this work.

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some success has been reported with intranasal (i.n.) (Hossain et al., 2011; Perrone et al., 2009; Richert et al., 2012; Wang et al., 2010) or intradermal (i.d.) immunization (Pearton et al., 2010; Quan et al., 2010).

We previously reported that high-immunogenicity influenza VLPs can be obtained via the overexpression of four viral proteins (HA, NA, M1, M2) using M2 fusion with the TLR5 ligand, *Salmonella typhimurium* flagellin (FliC) (Wei et al., 2011; Lin et al., 2013). For this study, we obtained chimeric VLPs further incorporated with the molecular adjuvant of granulocyte-macrophage colony-stimulating factor (GM-CSF), FliC, or a GM-CSF/FliC combination. All three forms of chimeric H5N1 VLPs were examined for the elicitation of total IgG and virus-neutralization antibodies in sera, B and T cell responses in splenoctyes, and protective immunity against live virus challenges. The GM-CSF/FliC H5N1 VLPs were further obtained to include H7 or H1H7 antigens for developing multi-subtype influenza vaccines.

2. Material and methods

2.1. VLP production, purification, and characterization

The pFastBac dual expression plasmids containing HA/M1, M2/NA or FliC-M2/NA were constructed as previously described (Wei et al., 2011; Lin et al., 2013). HA, NA, M1 and M2 cDNAs were obtained from human and avian influenza strains as follows: HA was derived from A/KAN-1/2004, NA from A/Vietnam/1203/2004, and M1 and M2 from A/WSN/1933(H1N1). The FliC gene (GenBank accession number AY649721.1) or the GM-CSF gene (GenBank accession number AK053196.1) was fused in front of the N terminus of M2 gene linked with a six-glycine (GGGGGG). For the bi-subtype and tri-subtype VLPs, the H7HA gene was from the A/Shanghai/02/2013 (H7N9) strain and the H1HA gene was from A/California/07/2009 (pH1N1) strain. Recombinant baculoviruses expressing HA/M1, M2/NA, FliC-M2/NA or GM-CSF-M2/NA were obtained, and the VLPs were obtained from Sf9 cells co-infected with baculoviruses as previously reported (Wei et al., 2011; Lin et al., 2013). Culture supernatants were filtered using a 100 kDa vivaflow filter membrane (Sartorius) and pelleted under 20% sucrose gradient centrifugation (33,000 rpm) for 3 h at 4 °C. Pellets were re-suspended in PBS and ultra-centrifuged for another 4 h at 33,000 rpm (0–60% w/v sucrose gradient sedimentation, 4 °C, Hitachi RPS40ST rotor). Purified VLP fractions were characterized by Western blots using 12% SDS-PAGE analysis. Transmission electron microscopy (TEM) was also conducted followed the conventional procedures and stained with 2% uranyl acetate solution. The stained samples were observed using a JEM-1400 transmission electron microscope.

2.2. Mouse immunization and live virus challenge

Female BALB/c mice (6-to-8 weeks old) were purchased from the Taiwan National Laboratory Animal Center. Five mice per group were immunized with 0.5 μg HA content of purified WT, GM-CSF, FliC, or GM-CSF/FliC VLPs mixed with 300 μg alum phosphate adjuvant (Wei et al., 2011; Lin et al., 2013). The HA content in VLPs was determined using purified HA proteins prepared previously (Lin et al., 2011). Immunizations were by i.m. injection over a three-week interval. Blood samples were collected and isolated 14 days after the booster injections. Sera samples were inactivated by incubation at 56 °C for 30 min and stored at −20 °C until used for analytical purposes. At 3 weeks following second immunizations, mice were i.n. challenged with H5N1 NIBRG-14 viruses (20-fold MLD_{50}, half of a median lethal dose) as previously described (Wang et al., 2009).

2.3. ELISA assay

Individual wells in 96-well plates were coated with 0.2 μg purified H5HA proteins or 1 μg M2 ectodomain (M2e) peptide (MSLLTEVETPIRNEWGCRCNDSSD) and held overnight at 4 °C, washed 3 times with PBST (0.05% Tween-20 in PBS), and blocked with blocking buffer (1% BSA in PBS) for 1 h. Next, 100 μl of two-fold serially diluted sera samples were added and held at room temperature (RT) for 1 h, followed by 3 additional washes with PBST. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (GeneTex) antibodies were added to each well, incubated for another 1 h, and washed 3 times with PBST. Anti-H5HA or anti-M2e IgG titers were determined by adding TMB substrate (Biolegend), holding for 15 min at RT, and stopping reactions with 2N H_2SO_4. End-point titers were determined as the reciprocals of most diluted sera concentrations giving a mean optical density (OD) of 450 nm above 0.2.

2.4. H5N1 pseudotyped particle (H5N1pp) and H7N9pp neutralization assay

HEK293T cells were co-transfected with pNL-Luc-E^-R^-, pcDNA3.1(+) expressing the HA gene of A/Thailand/1(KAN-1)/2004 (H5N1 clade1), A/Indonesia/05/2005 (H5N1 clade 2.1.3.2), or A/bar-headed goose/Qinghai/1A/2005 (H5N1 clade 2.2) plus pcDNA3.1 expressing the NA gene of the A/Viet Nam/1203/2004 strain; or the HA gene of A/Shanghai/02/2013 (H7N9) plus pcDNA3.1 expression the NA gene of A/Shanghai/02/2013 (H7N9). MDCK cells were inoculated with the diluted sera and H5N1pp or H7N9pp for 48 h to measure the luciferase activity using neolite luciferase substrate (PerkinElmer) as previously reported (Lin et al., 2013).

2.5. pH1N1 and H3N2 virus plaque neutralization assay

MDCK cells/well were grown in 6-well plates. Two-fold serially diluted immunized sera were mixed with equal volumes of 100 PFU/mL of pH1N1 (A/California/04/2009) or H3N2 (A/Udorn/307/1972) viruses in MEM-α containing 1.0 μg/mL trypsin-TPCK (Sigma- Aldrich). Plaques were counted and the reduction of plaque numbers was measured for neutralization activity.

2.6. Hemagglutination inhibition assay

Sera were treated with receptor-destroying enzyme (Denka Seiken) for 18 h at 37 °C followed by heating at 56 °C for 30 min to inactivate enzyme activity. Treated sera were two-fold serially diluted (starting from 1:10) and incubated with 4 HA units of H5N1 VLPs, H7N9 VLPs or pH1N1 (A/California/04/2009) viruses. Next, 0.5% turkey red blood cells (RBCs) were added and incubated for another 30 min at RT. Hemagglutination inhibition (HI) titer was measured as the reciprocal of the highest dilution of sera completely inhibiting hemagglutination.

2.7. NA-inhibiting antibodies

NA-inhibiting antibodies were measured using a fetuin-based assay (Liu et al., 2015). Serially diluted serum samples incubated with equal volumes of 1 μg H5N1 VLPs were added to fetuin (Sigma)-coated plates, and then reacted with peroxidase-labeled peanut agglutinin (Sigma). The NA activity was determined by adding TMB substrate and read with an ELISA reader