International Immunopharmacology 63 (2018) 119–128

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Influenza virus-like particles composed of conserved influenza proteins and GPI-anchored CCL28/GM-CSF fusion proteins enhance protective immunity against homologous and heterologous viruses

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ARTICLE INFO

Keywords:
Influenza cVLPs vaccine
GPI-CCL28
GPI-GM-CSF
Broad-spectrum
Intranasal administration

ABSTRACT

Influenza viruses cause significant morbidity and mortality and pose a substantial threat to public health. Vaccination represents the principle means of preventing influenza virus infection. Current vaccine approaches are hindered by the need to routinely reformulate vaccine compositions in an effort to account for the progressive antigenic changes that occur as influenza viruses circulate in the human population. In this study, we evaluated chimeric virus-like particle (cVLP) vaccines containing conserved elements of influenza proteins (HL5M2e (HA stem gene with 5M2e gene inserted) and NP), with or without glycosylphosphatidylinositol-anchored CCL28 (GPI-CCL28) and/or GM-CSF (GPI-GM-CSF) fusion proteins as molecular adjuvants. cVLPs elicited strong humoral and cellular immune responses against homologous and heterologous viruses, and improved survival following lethal challenge with both homologous and heterologous viruses. Inclusion of GPI-anchored adjuvants in cVLP vaccines augmented the generation of influenza-specific humoral and cellular immune responses in mice in comparison to the non-adjuvanted cVLP vaccines. VLPs containing GPI-anchored adjuvants reduced morbidity and improved survival to lethal challenge with homologous and heterologous influenza viruses. This work suggests that VLP vaccines incorporating conserved influenza virus proteins and GPI-anchored molecular adjuvants may serve as a platform for a broadly protective “universal” influenza vaccine.

1. Introduction

Influenza viruses are highly contagious and can cause serious respiratory disease during seasonal epidemics and occasional pandemics [1, 2]. Annually, approximately 3–5 million people are infected with influenza virus and 250,000–500,000 die as a result of influenza virus infections (http://www.who.int/mediacentre/factsheets/fs211/en/). Seasonal epidemics occur as a result of influenza virus antigenic drift in which viral variants that circumvent pre-existing immune responses emerge and become dominant in humans. The plasticity of influenza viruses can result in low vaccine effectiveness, especially in seasons with poor antigenic alignment between vaccine strains and predominant circulating strains [3, 4]. In addition to antigenic drift, novel influenza viruses can emerge as a result of genetic reassortment between two distinct influenza virus subtypes to cause pandemics, a process commonly referred to as antigenic shift. The potentially severe

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https://doi.org/10.1016/j.intimp.2018.07.011
Received 8 March 2018; Received in revised form 1 July 2018; Accepted 10 July 2018

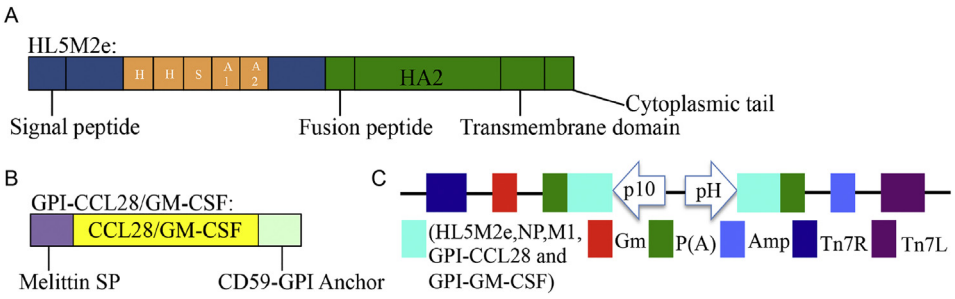

Fig. 1. (**A**) Schematic of HL5M2e constructs. HL5M2e: HLHA gene with 5M2e gene inserted, the HLHA from [41], H: Human influenza A type M2e, S: Swine influenza A type M2e, A1: Avian influenza A type I M2e, A2: Avian influenza A type II M2e. (**B**) Schematic of GPI-CCL28/GM-CSF constructs. CCL28/GM-CSF: the carboxyl terminal GPI anchored signal peptide sequence of mouse CCL28/GM-CSF. (**C**) Schematic of the recombinant baculovirus expressing HL5M2e, NP, M1, GPI-CCL28 and GPI-GM-CSF genes. The Tn7 regions, gentamicin resistance gene (Gm), HSV tk polyadenylation signal [p(A)], p10 promoter (p10), polyhedrin promoter (pH), Ampicillin antibiotics (Amp) and interest proteins HL5M2e, NP, M1, GPI-CCL28 and GPI-GM-CSF.

consequences of pandemic influenza are underscored by the 1918 H1N1 Spanish flu pandemic which was responsible for an estimated 50 million deaths and the more recent 2009 H1N1 'swine flu' pandemic [5, 6].

Vaccination is the most effective and economical means to prevent and control influenza infection. Current influenza vaccination strategies typically elicit strain-specific immune responses with limited cross-reactivity to heterologous viruses, and are therefore poorly equipped to protect against antigenically drifted viruses and newly emergent influenza viruses. New approaches to influenza vaccination that elicit long-lasting protective immunity against diverse influenza subtypes and strains are needed [3, 4].

Virus-like particle (VLP)-based influenza vaccines represent a promising alternative to current vaccine approaches. VLPs consist of one or more viral structural proteins which self-assemble into structures resembling the organization and conformation of authentic viruses. As such, VLPs are highly immunogenic, but since they lack the viral genome VLPs are replication incompetent [7–9]. Studies have shown that VLPs can stimulate strong specific cellular and humoral immunity [10–14]. Several VLP vaccines have been successfully commercialized or are in pre-clinical development, including vaccines against heptatitis B, human papilloma virus, and a trimeric seasonal influenza vaccine.

The hemagglutinin (HA) stalk protein (headless HA, HLHA), the extracellular region of the matrix protein 2 (M2e), and the nucleoprotein (NP) of influenza virus are highly conserved. Previous studies have confirmed that HLHA protein may serve as a target for the development of cross-protective universal influenza vaccines [15]. Similarly, the conserved M2e protein elicits strong Th1 and Th2 responses and marks influenza virus-infected cells [16], and the NP protein is a target antigen for CTL responses, which collectively may contribute to cross-protective immunity against different subtypes of influenza virus [17]. M1 is a conserved influenza virus protein with approximately 25% amino acid sequence variation. It plays an essential role in structural integrity and stability by forming a matrix layer underneath the lipid bilayer of the viral envelope [18]. However, the vaccine of HLHA, M2e or NP show limited immunogenicity [19, 20].

Vaccine adjuvants serve to enhance immune responses against target antigens. Whereas many adjuvants are pathogen-associated molecular patterns or analogues thereof, cytokines may also have utility as vaccine adjuvants based on their immunomodulatory activities. The chemokine CCL28 is recognized to orchestrate mucosal immune responses, and we have found that there is a high level CCL28 in the mucosal epithelium [21, 22]. It is helpful to increase the chemotaxis on target cells and enhance the cross-protection of conserved epitopes. CCL28 can bind to CCR3 receptors and drives lymphocyte migration, immune priming, and pathogen clearance [23, 24]. Granulocyte-macrophage colony stimulating factor (GM-CSF) enhances immune responses by promoting mononuclear phagocyte activity and has antiviral and anti-tumor effects [25–31]. We sought to study the impact of including GPI-anchored CCL28 and GM-CSF in an influenza VLP-based vaccine consisting of conserved elements of influenza virus HA, M2, and NP proteins. Here we describe the generation of these adjuvanted VLP vaccines and characterize the protective immune responses elicited in mice.

2. Materials and methods

2.1. Viruses,cells and mice

The following influenza viruses were used in this study and were maintained in our laboratory: A/meerkat/Shanghai/SH-1/2012 (H5N1; SH-1; clade 2.3.2.1; group 1), mouse-adapted A/Changchun/01/2009 (H1N1; group 1), mouse-adapted A/baikal teal/Shanghai/SH-89/2013 (H3N2; group 2), and mouse-adapted A/Lesser White-fronted goose/HuNan/412/2010 (H7N7; group 2). The MLD_{50} (mouse lethal dose inducing 50% mortality) and EID_{50} (chicken embryo infectious dose infecting 50% cells) of each virus was determined by infection of mice and chicken embryos with serial viral dilutions using the method of Reed and Muench [32]. *Spodoptera frugiperda* insect cells (Sf9,Invitrogen, Carlsbad, CA, USA) and Madin-Darby canine kidney (MDCK) cells were cultured as described previously [33, 34]. Six- to eight- week old specific pathogen-free female BALB/c mice were purchased from the Changchun Biological Products Co., Ltd. (Changchun, China).

2.2. Construction and expression of HL5M2e,NP,M1,GPI-CCL28 and GPI-GM-CSF

The proteins HL5M2e, NP, M1 genes all from *HPA*I H5N1 virus A/meerkat/Shanghai/SH-1/2012 (H5N1) was used as source material for cloning of the NP, M1, and M2e (Fig. 1A). Using the HA headless area as a vector, the 5M2e gene (two Human M2e, two avian M2e, one swine M2e) was inserted into HA headless region and became the HL5M2e fusion gene as previously described [35–38]. The M1 were expressed as the matrix protein. The membrane binding form of CCL28/GM-CSF was engineered by fusing the honeybee melittin signal peptide to facilitate GPI-CCL28/GM-CSF proteins expression in insect cells, and the mouse CD59 glycolipid-anchoring sequence was used to provide membrane anchoring [39, 40]. The honeybee melittin signal peptide and the mouse CD59 GPI-anchor were fused to the 5′ and 3′ ends of the mouse CCL28/GM-CSF carboxyl terminal GPI anchored signal peptide sequence respectively to obtain the full-length gene encoding GPI-CCL28 and GPI-GM-CSF fusion proteins through gene synthesis [41–43]. The construct was then cloned into the pFastBac-dual plasmid (Invitrogen, USA). The accuracy of the full-length encoding sequence was confirmed by gene sequencing analysis. Recombinant bacmids were generated by site-specific homologous recombination and transformation of plasmid containing influenza genes into *E. coli* DH10Bac competent cells that contain the AcMNPV baculovirus genome (Invitrogen). Purified recombinant bacmid DNA was transfected into Sf9 cells cultured in 6-well plates at 2×10^6 cells/ml using Cellfectin II reagent (Invitrogen), at 27 °C for 4 h. After 3 days of incubation, the supernatant containing recombinant baculovirus was harvested and assessed by immunofluorescence with a fluorescence microscope (Zeiss, Axio, Vert.A1) as previously described [33, 34]. The mock-infected Sf9 cells supernatant