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Targeting of nucleoprotein to chemokine receptors by DNA vaccination results in increased CD8$^+$-mediated cross protection against influenza

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ABSTRACT

Vaccination is at present the most efficient way of preventing influenza infections. Currently used inactivated influenza vaccines can induce virus-neutralizing antibodies that are protective against a particular influenza strain, but hamper the induction of cross-protective T-cell responses to later infections. Thus, influenza vaccines need to be updated annually in order to confer protection against circulating influenza strains. This study aims at developing an efficient vaccine that can induce broader protection against influenza. For this purpose, we have used the highly conserved nucleoprotein (NP) from an influenza A virus subtype H7N7 strain, and inserted it into a vaccine format that targets an antigen directly to relevant antigen presenting cells (APCs). The vaccine format consists of bivalent antigenic and targeting units, linked via an Ig-based dimerization unit. In this study, NP was linked to MIP-1α, a chemokine that targets the linked antigen to chemokine receptors 1, 3 and 5 expressed on various APCs. The vaccine protein was indirectly delivered by DNA. Mice were vaccinated intradermally with plasmids, in combination with electroporation to enhance cellular uptake of DNA. We found that a single DNA vaccination was sufficient for induction of both antibody and T cell responses in BALB/c mice. Targeting of nucleoprotein to chemokine receptors enhanced T cell responses but not antibody responses. Moreover, a single dose of MIP1α-NP conferred protection in BALB/c mice against a lethal challenge with an H1N1 influenza virus. The observed cross-protection was mediated by CD8$^+$ T cells.

1. Introduction

Influenza epidemics annually cause thousands of deaths and severe illness in millions of people worldwide [1]. Conventional prophylactic influenza vaccines typically induce protection [2], but have a limitation in that they only induce strain-specific antibodies. This is a problem since the surface proteins (hemagglutinin, neuraminidase) on influenza virus particles are subject to antigenic drift, and a consequence is that the antigenic determinants may change so that last year's antibodies do not recognize this year's influenza strain. Thus, influenza vaccines have to be updated every year. Furthermore, in the event of a pandemic, the long production time required for conventional vaccines represents an obstacle towards efficient protection of the population.

Natural infection with influenza A viruses induces both humoral and cellular immunity. Unlike neutralizing antibodies against surface proteins, T cell immunity cannot block the initial viral entry into somatic cells. However, activated T cells can selectively kill virus-infected cells [3], and the presence of CD8$^+$ T cells specific for conserved viral epitopes have been shown to correlate with less severe illness during natural infection in humans [4]. T cell epitopes on internal proteins are commonly highly conserved between different strains of influenza viruses [5–7], and a number of studies in mice and nonhuman primates have shown that heterosubtypic immunity is mediated predominantly by cross-reactive cytotoxic CD8$^+$ T cells [8–12]. Such T cells can even confer heterosubtypic

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protection in individuals lacking pre-existing antibodies [6]. Importantly, vaccine-induced T cells could thus confer some protection against pandemic influenza. Moreover, vaccine induced T cells could play a role in protection against influenza in elderly individuals subject to immune exhaustion [13–16].

Long-lasting cellular immunity is directed predominantly against conserved, internal viral proteins, such as nucleoprotein (NP) [17,18]. NP is a major component of the ribonucleoprotein (RNP) complexes. The RNP consists of RNA, multiple copies of NP, and polymerase subunits (PB1, PB2 and PA), together forming a large loop that is twisted into a helical rod-like structure [19,20]. NP plays regulatory roles in transcription, replication, and virus maturation [21], and it is one of the most conserved proteins among influenza A viruses with >90% protein sequence homology [22,23]. Correspondingly, several immunization studies with NP have shown induction of effective immune responses and protection against various influenza A subtypes [5,6,24–27]. However, protection is dependent on large vaccine doses and multiple immunizations [28], and it has been difficult to achieve sufficiently potent immune responses to confer protection against highly pathogenic H5N1-viruses [29,30].

Previously, we have demonstrated that the selective targeting of antigen to APC increases immune responses [31–34]. In fact, such targeting may be a key to overcome the hurdle of low immunogenicity that is often observed with subunit DNA vaccination. Here, we have investigated whether targeting of a conserved influenza antigen, NP, to chemokine receptors can enhance relevant T cell responses against influenza. To this end, NP was inserted into the previously described vaccine format, consisting of bivalent antigenic and targeting units that are linked through an Ig-based dimerization unit [31,33]. As targeting unit, we used macrophage inflammatory protein-1 alpha (MIP-1α) that binds chemokine receptors 1, 3 and 5 (CCR1/3/5), a choice based on previous results demonstrating that MIP-1α is a particularly well suited targeting unit for efficient induction of $CD8^+$/Th1 T cell responses [33]. CCR1/3/5 are expressed on various cell types such as dendritic cells, monocytes, macrophages, NK cells, eosinophils, basophils, neurons, platelets, fibroblasts, microglial cells, and endothelial cells [35]. MIP-1α is a small pro-inflammatory chemokine of the CC subfamily [36], and is involved in recruiting CCR1/3/5^+ cells to sites of infection. Here, we demonstrate that a single vaccination with a dimeric MIP1α-NP vaccine can enhance protection against a lethal hetero challenge with influenza. The vaccine-induced protection is mediated mainly by $CD8^+$ T cells.

2. Materials and methods

2.1. Mice and cell lines

Six to eight weeks old female BALB/c mice were purchased from Taconic (Ry, Denmark) and housed under minimal disease conditions. All animal experiments were approved by the Norwegian Animal Research Authority (NARA). The HEK293E [37] cell line was purchased from ATCC (Manassas, VA, USA). MDCK cells were a kind gift from Tone Fredsvik Gregers at the University of Oslo.

2.2. Construction of vaccine molecules

Nucleoprotein from the influenza virus A/whooper swan/Norway/10 438/2006 (H7N7) was amplified by RT-PCR using NP-specific primers [38] and inserted into a pCR4-TOPO vector (Invitrogen). The gene was picked up by PCR with flanking SfiI-sites (underlined): 5′ NP: GGC CTC GGT GGC CTGGCG TCT CAA GG; 3′ NP: CCG GCC CTG CAG GCCTCA CTT TAA TTG TC, and the gene inserted into the restriction sites of previously described pLNOH2 expression vectors [31,33]. To allow for a comparison of responses in the absence of the bivalent vaccine format, a vaccine containing only the antigenic unit (NP) was prepared using the 3′NP primer described above and 5′: CAC AGG TGT GCA TTC CGC GTC TCA AGG C (AleI and BsmI restriction sites are underlined). Molecular cloning was verified by restriction enzyme digest and sequencing.

2.3. Western blot

Supernatants of HEK293E cells transiently transfected with plasmids encoding the different vaccine molecules were up-concentrated and run on a Novex 4–12% Tris–glycine gel (Invitrogen) under non-reducing conditions, together with Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). Following blotting (Immuno-Blot PVDF membrane, BioRad), the membrane was blocked overnight at 4 °C (2% Membrane Blocking agent, RPN212SV, GE Healthcare) and incubated with biotinylated mAb against IgG Fc (1:10,000) (HP-6017, Sigma Aldrich, Germany). After 3 washes with PBS/0.1% Tween 20, the membrane was incubated with Streptavidin-HRP (1:10,000) (GE Healthcare), developed with ECL (Amersham ECL Select, GE Healthcare), and analyzed on G:Box (SYNGENE, UK) with GeneSys software. For detection of NP alone, 1 μg of purified protein was run on a Novex 4–12% Tris–glycine gel under reducing conditions, blotted and blocked as above. Next, the membrane was incubated with αNP HRP-conjugated C43 mAb (1:10,000) (Abcam, UK), and developed as above.

2.4. Chemotaxis assay

The chemotactic integrity of the vaccine proteins was assessed as previously described [39]. Briefly, one million Esb/MP cells were added to the upper chamber of a 24-well Transwell® plate (5.0 μm pore size) (Corning Laboratories, Corning, NY, USA), while the lower chambers were filled with serially diluted supernatants from HEK293E cells transiently transfected with 1 μg of plasmids encoding the different vaccine molecules, or recombinant LD78β (20 ng/ml). The plates were incubated for 2 h at 37 °C in 5% CO_2, and cell count determined with a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

2.5. ELISA for detection of NP vaccine proteins

Ninety-six well plates (Coastar 3590) were coated with either anti-NP mAb (1 μg/ml) (C43, Abcam, UK) or mouse anti-human IgG (CH3 domain) (2 μg/ml) (MCA878G, AbD Serotec, Oxford, UK), and blocked with PBS/BSA for 1 h at room temperature (RT). Following washing, supernatants of HEK293E cells transiently transfected with 1 μg vaccine encoding plasmids were added into wells in triplicates, and incubated for 2 h at RT. Plates were washed, and vaccine proteins detected by incubation with either biotinylated anti-NP mAb (1 μg/ml) (H16-L10-4R5, ATCC: HB-65) or biotinylated mAb against IgG Fc (1 μg/ml) (HP-6017, Sigma Aldrich, Germany) for 2 h at RT, and then Streptavidin alkaline phospatase (1:3000) (GE Healthcare, USA) for 45 min at RT. Development was performed with Phosphatase substrate (P4744-10G, Sigma Aldrich, Germany), and plates were read at 405 nm with a Tecan reader using the Magellan v5.03 program.

2.6. ELISA for detection of serum anti-NP antibodies

Blood samples were collected from mice and sera isolated. Ninety-six well plates were coated overnight with recombinant NP-H7N7 (0.5 μg/ml in PBS/azide) (ESB-EP325878IMI, CUSABIO, China) or inactivated influenza A/Puerto Rico/8/1934 (H1N1) (Charles River Laboratories), blocked as above, and incubated over night at