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Virus-derived immunostimulatory RNA induces type I IFN-dependent antibodies and T-cell responses during vaccination

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ABSTRACT

Adjuvants potentiate and direct the type of immunity elicited during vaccination. However, there is a shortage of adjuvants that elicit robust type-1 immunity required for the control of intracellular pathogens, including viruses. RNA derived from Sendai virus defective viral genomes (DVGs) stimulates RIG-I-like receptor signaling leading to type-1 immunity during infection. Here, we investigated whether a 268nt DVG-derived oligonucleotide (DDO) functions as a strong type-1 immunity-inducing adjuvant during vaccination against influenza virus. We show that DDO induces robust IgG2c antibody production when used in an inactivated influenza A virus (IAV) vaccine. Additionally, DDO induces Th1 and $CD8^+$ T-cell responses able to protect against heterosubtypic IAV challenge. Interestingly, DDO synergized with AddaVax and skewed the immune response towards type-1 immunity. The adjuvancy of DDO alone and in synergy with AddaVax was heavily dependent on type I interferon signaling. Our data support a critical role for type I interferon in the induction of type-1 immune responses during vaccination and demonstrate that DDO is a type-1 immunity orienting vaccine adjuvant that can be used alone or in synergy with other adjuvants.

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1. Introduction

Subunit and inactivated vaccines are ideal for vaccine development because they do not revert to virulence and are unlikely to cause disease in immunocompromised individuals. Unfortunately, inactivated and subunit vaccines lack the danger signals required to induce robust adaptive immunity. Thus, adjuvants that boost and shape the immune response towards vaccinated antigens are added to improve vaccine efficacy.

Currently, there are no licensed vaccine adjuvants that induce robust type-1 immunity [1–4]. Type-1 immune responses are important for the control of viruses and other intracellular pathogens and are characterized by the generation of cytotoxic $CD8^+$ T-cells, Th1 $CD4^+$ T-cells, and antibodies of the isotypes IgG2b/c [5,6]. Induction of type-1 immunity is also critical for pathogens where induction of other types of immunity leads to enhanced pathogenesis. For example, infants with type-2 immunity-primed lungs suffer from increased morbidity when infected with respiratory pathogens, such as respiratory syncytial virus (RSV) or rhinovirus [7–9].

Alum, the oldest and most widely used vaccine adjuvant, induces robust type-2 immunity. While effective against extracellular pathogens, type-2 immunity does not protect against most intracellular pathogens [10]. In addition to Alum, the TLR4 ligand monophosphoryl lipid A (MPL) absorbed onto Alum, named adjuvant system04 (AS04) [11], and oil-in-water emulsions, such as MF59, are approved for use in human vaccines [10]. These can induce protective antibodies and mild Th1 responses, but no approved vaccines use these adjuvants to induce protective $CD8^+$ T-cell responses [10].

Many type-1 immunity-inducing adjuvant candidates rely on pathogen associated molecular patterns (PAMPs) recognized by pattern recognition receptors including Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) [12,13]. The use of natural PAMPs more closely mimics the immune responses obtained during infections, such as the induction of type I interferon (IFN). In addition to MPL used in AS04, synthetic PAMPs have been examined as potential type-1 immunity inducing adjuvants. These include the viral RNA mimic polyI:C, which failed to induce strong type-1 immune responses without toxicity [14], and CpG, which is used in a combination adjuvant [15]. Notably, emulsions provide an ideal platform for adjuvant synergy and the combinatorial effects of using multiple adjuvants in a single vaccine can be exploited to generate optimized adjuvants.

We previously identified a Sendai virus PAMP originating from a copy-back defective viral genome (DVG) naturally generated during viral replication [16] and characterized the immunostimulatory

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RNA motif responsible for recognition by RLRs [17]. Subcutaneous injection of a synthetic version of this 546nt DVG (DVG-546) resulted in a local and distinct cytokine profile from polyI:C [18]. Additionally, DVG-546 promoted the accumulation of DCs in draining lymph nodes of mice. Mice vaccinated with DVG-546 and inactivated RSV developed type-1 immunity-associated antibody responses [18]. Additional work indicated an important role for RLRs for the induction of protective immunity in DVG-546-adjuvanted vaccines [19]. These studies suggest that derivatives of this molecule are valuable candidates for type-1 immunity inducing adjuvants.

Here we report a 268nt DVG-derived oligonucleotide (DDO) with enhanced immunostimulatory capabilities *in vitro* [17]. We demonstrate its ability to induce protective type-1 humoral and cellular immune responses during immunization with whole inactivated influenza A virus (inIAV) or a HA-subunit vaccine. Additionally, we show that DDO synergizes with AddaVax (the research version of MF59) to induce potent type-1 polarized immune responses and that both humoral and Th1 responses elicited by vaccines adjuvanted with DDO rely on type I IFN.

2. Methods

2.1. Ethics statement

Studies in mice were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animal of the National Institute of Health. The protocol (804691) was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01.

2.2. Mice and viruses

C57BL/6 mice (6–8 weeks old) were obtained from Jackson Laboratory. *Ifnar1*$^{-/-}$ mice [20] were a kind donation of Dr. Thomas Moran (Icahn School of Medicine at Mount Sinai) and were used with gender and age matched C57BL/6 mice (Jackson Laboratory bred in house). All experiments were performed in male and female mice. Influenza A/X-31 H3N2 (IAV X-31) and A/California/7/2009 H1N1 with D225G HA mutation (IAV-Cal/09-D225G) that allows the wild-type A/California/7/2009 virus to grow in eggs [21] were used as challenge strains. All strains of IAV were grown in 10 day-old embryonated chicken eggs (Charles River Laboratory) at 30,000 medium tissue culture infectious dose ($TCID_{50}$) at 37 °C. Allantoic fluid from infected eggs was collected 40 h later.

2.3. Vaccine formulation

Inactivated IAV (inIAV) vaccine: Influenza A/Puerto Rico/8/1934 H1N1 (IAV PR/8) was harvested from allantoic fluid of 10 day old embryonated eggs and purified through a 35% sucrose cushion. Virus was inactivated with UV light (254 nm at 6-inch distance) for 40 min. Inactivation was confirmed by the inability of the virus to replicate in MDCK cells (Madin-Darby canine kidney cells, gift from Dr. Scott Hensley, University of Pennsylvania) in the presence of 2 mg/ml trypsin. The inIAV vaccine had a total protein concentration of 989 μg/ml, HA titer of 10240U/ml at a 1:100 dilution and an endotoxin level of <1.2 EU/ml. Recombinant IAV-HA protein from IAV-Cal/09-D225G used as a subunit vaccine was obtained from BEI Resource (NR-13691). DDO is a 268nt non-coding, replication-incompetent ssRNA sequence that contains the immunostimulatory DVG motif identified previously [17,18]. DDO was produced, stored, and used as previously described [17]. Purity and integrity of DDO were confirmed using an Agilent Bioanalyzer 2100 and had an OD260/280 ratio of 2.16, an OD260/230 ratio of 2.3, and endotoxin level below 0.1EU/ml/300ug.

2.4. Mouse immunization and challenge

For immunization, mice were anesthetized with isoflurane and injected intramuscularly (i.m.) into the thigh with 10 μg inIAV vaccine or 1 μg recombinant IAV-HA protein diluted in PBS adjuvanted with 5 μg DDO, AddaVax (InVivogen) at 50% v/v, or Alum (Alhydrogel 2%, InvivoGen) at 50% v/v at final volume of 50 μl per dose. Mice were primed and boosted 14 days later with the same vaccine formulation. In some experiments, mice were challenged intranasally with $10^{3.5}$ $TCID_{50}$ of IAV X-31 (heterosubyptic challenge) or 2×10^4 $TCID_{50}$ of IAV-Cal/09-D225G 21 days after boost. All mice were weighed daily post-challenge. Lung tissue was harvested 4 or 10 days post-challenge for viral load quantification or histology.

2.5. Viral load quantification

IAV titration was performed by limiting dilution in MDCK cells as previously described [18]. For quantifying *IAV-NP* transcripts in lung homogenate, 1–2 μg of RNA isolated by TRIzol (Invitrogen)

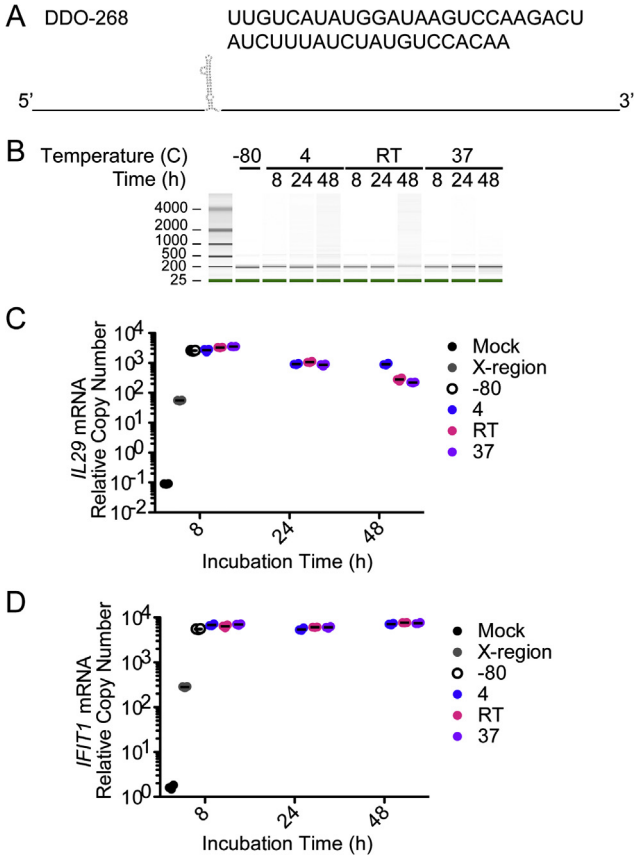

Fig. 1. DDO-268 is a thermostable small RNA with strong immunostimulatory activity. (A) DDO is a 268 nucleotide single-stranded RNA with an immunostimulatory motif shown in its secondary structure together with its sequence. DDO was incubated at 4 °C, room temperature (26 °C/RT), or 37 °C for 8 h, 24 h, or 48 h. (B) Integrity of the RNA was analyzed using electrophoretic analysis on a Bioanalyzer. (C and D) Expression of *IL29* (C) and *IFIT1* (D) mRNA measured by RT-qPCR from A549 cells transfected for 6 h with 4.15pmo DDO used in (B). The experiment was repeated 3 times with a single representative repeat shown here. Data are expressed as copy numbers relative to the housekeeping gene *GAPDH*, value represent mean ± SEM from triplicate technical repeats.

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