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# Reduction of influenza virus transmission from mice immunized against conserved viral antigens is influenced by route of immunization and choice of vaccine antigen

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ABSTRACT

Transmission of influenza virus between susceptible hosts mediates spread of infection in the population and can occur via direct-contact or airborne routes. Mathematical models suggest that vaccines that reduce viral transmission from infected individuals could substantially reduce viral spread in an epidemic or pandemic, even if they do not completely protect against infection. Vaccines targeting conserved nucleoprotein (A/NP) and matrix 2 (M2) antigens of influenza virus do not completely prevent infection upon influenza virus challenge, but reduce viral replication, morbidity, and mortality. Using a mouse model of influenza virus transmission, we have previously shown that immunization with recombinant adenovirus vectors expressing the combination of A/NP and M2 can reduce viral transmission to unimmunized contacts. Here we demonstrate that transmission reduction is more effective when mice are immunized against A/NP and M2 intranasally than via the intramuscular route. We show that immunization against the combination of A/NP and M2 is more effective at reducing transmission than either antigen alone, with a clear hierarchy of effectiveness (A/NP + M2 > A/NP > M2). Transmission reduction is seen to a similar degree under both direct-contact and airborne transmission conditions. Finally, using seroconversion as an indicator of infection, we show that immunizing contact mice against A/NP and M2 prevents a significant fraction (~50%) from becoming infected under direct-contact conditions. These findings suggest that when strain-matched vaccines are unavailable, conserved antigen vaccines could not only reduce severity of disease in vaccinated individuals but also limit the spread of virus during influenza epidemics or pandemics.

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## 1. Introduction

Emergence of transmissible, antigenically novel influenza viruses remains a substantial public health concern. Conventional vaccines induce antibodies against hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, and require strain-matching against circulating viruses. This approach has significant disadvantages: antigenic mismatches between vaccine strains and circulating viruses cause ineffective protection, necessitating continual viral surveillance and frequent vaccine updates [1]. Importantly, vaccine derivation and manufacture take several months [2], meaning strain-matched vaccines are unavailable when new viruses emerge.

In contrast, immune responses against conserved antigens are cross-reactive between influenza viruses regardless of their HA and NA, forming the basis for heterosubtypic immunity [3,4] and thus "universal" influenza vaccines. Heterosubtypic immunity can protect animals from influenza virus challenge [5–8], with evidence mounting for heterosubtypic protection in humans [9–12].

We previously demonstrated that a candidate universal influenza vaccine based on recombinant adenovirus (rAd) vectors expressing conserved A/NP and M2 antigens significantly reduces respiratory tract virus titers and protects mice from lethal challenge after a single dose [13,14]. However, a perceived weakness of such vaccines is that because they permit limited infection, vaccinated individuals who become infected might still transmit infection to others.

We addressed this issue using a mouse model of influenza virus transmission, showing that intranasal (i.n.) A/NP + M2-rAd-immunization significantly reduced transmission to naïve animals placed in direct-contact with vaccinated and subsequently infected mice [15]. However, vaccination route and antigen selection are critical parameters in vaccine development, and that earlier study

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did not examine parenteral immunization or dissect contributions of individual component antigens (A/NP and M2) in transmission prevention. Also, influenza spread between humans is thought to occur by both direct-contact and airborne routes, so assessing whether vaccination reduces transmission via both routes is important. To further explore these issues we examined the contribution of immunization route and vaccine antigen choice. We tested transmission reduction for direct-contact and airborne routes, and demonstrated that vaccination of contacts can protect them from becoming infected under direct-contact conditions.

## 2. Materials and methods

### 2.1. Animals and housing

Female BALB/cAnNCR (BALB/c) and outbred CFW [Crl:CFW (SW)] mice were purchased from Charles River Laboratories and housed as previously described [15]. One day post-infection, 2 infected BALB/c donors were re-caged with 3 naïve CFW contacts and allowed to mix freely (direct-contact conditions) or were separated from contacts by a perforated barrier as described previously [15] to allow only airborne transmission. Nasal wash and lung virus titers were assessed four days post-infection (three days post-contact). Contacts were deemed positive for transmission if virus was detected by $TCID_{50}$ assay in either lung homogenate or nasal wash, or both. Animal experiments were conducted at ABSL2 in facilities accredited by the AALAC, with all animal experiments and procedures approved by the FDA White Oak Campus IACUC.

### 2.2. Nasal wash, BAL, and lung sampling

Mice were euthanized by ketamine (300 mg/kg)-xylazine (60 mg/kg) overdose. Nasal wash, lungs, and bronchioalveolar lavage (BAL) were collected as described [15,16].

### 2.3. rAd vaccines

Recombinant adenovirus (rAd) vectors expressing the consensus M2 sequence, influenza A/PR/8/34 (H1N1) nucleoprotein (A/NP), or influenza B/Ann Arbor/1/86 nucleoprotein (B/NP) were previously described [17,18]. An otherwise identical rAd vector lacking a transgene (empty-rAd) was obtained from ViraQuest (North Liberty, IA). Mice were immunized with $5 \times 10^9$ viral particles (v.p.) each of A/NP-rAd and M2-rAd, $5 \times 10^9$ v.p. of A/NP-rAd or M2-rAd plus $5 \times 10^9$ v.p. of empty-rAd, or $1 \times 10^{10}$ v.p. of B/NP-rAd. All rAds were given i.n. under isoflurane anesthesia in 50 μl, or by intramuscular (i.m.) injection in 100 μl split equally between the quadriceps.

### 2.4. Influenza viruses and virologic analyses

A/Udorn/307/72 (H3N2) (A/Udorn) was propagated in eggs as described [19]. BALB/c mice were infected i.n. with $1 \times 10^4$ $TCID_{50}$ of A/Udorn in 50 μl under isoflurane anesthesia. The 50% mouse infectious dose ($MID_{50}$) of A/Udorn for BALB/c mice under these conditions is 5 $TCID_{50}$ [15]. For virus testing, samples were titrated by $TCID_{50}$ on MDCK cells as described [15], except lungs were homogenized in 1 ml of L-15 medium (CellGro, Manassas, VA) using 2 ml mixed bead tubes (Cayman Chemical Co., Ann Arbor, MI) in a PreCellys 24 system with a Cryolys cooling unit (Bertin Technologies, Atkinson, NH). Samples were homogenized using two 30 s cycles at 6000 rpm separated by a 5 s hold, centrifuged (3000 ×g, 15 min, 4 °C), aliquoted, and stored at −80 °C. Titers were calculated using the method of moving averages and Weil's tables [15]. Assay limits of detection were $10^{2.19}$ $TCID_{50}$/ml.

### 2.5. Peptides and proteins

Influenza A virus peptides $NP_{147–155}$, $NP_{55–69}$, M2-ectodomain$_{2–24}$ consensus sequence (M2e), and adenovirus-5 hexon ($Hex_{486–494}$) peptides have been previously described [13]. Bacterially expressed, N-terminal His-tagged recombinant NP from A/PR/8/34 (H1N1) (A/NP) and influenza B NP (B/NP) from B/Ann Arbor/1/86 were custom produced by GenScript (Piscataway, NJ).

### 2.6. Immunologic assays

Serum and BAL antibody levels against A/NP, M2e, and B/NP were assessed by ELISA [16,20]. Hemagglutination inhibition (HI) assays were as described [15], using 4 HAU/25 μl of A/Udorn as target antigen. Antigen-specific T-cell responses in lungs and spleen were determined by IFN-γ ELISPOT as previously described [18].

### 2.7. Statistical analyses

Statistical analyses were performed using SigmaPlot for Windows version 13 (Systat Software, Inc., San Jose, CA). Virus titers and ELISPOT were analyzed by one way ANOVA with Holm-Sidak *post hoc* comparison. Transmission rate comparisons used the chi-square test or Fisher's exact test.

## 3. Results

### 3.1. Immunization route influences vaccine-mediated transmission reduction

To investigate whether immunization route is critical in reducing transmission, BALB/c mice were immunized i.m. or i.n. with A/NP + M2-rAd or control B/NP-rAd i.n. and one month later examined for mucosal and systemic immune responses, control of challenge virus, and ability to transmit infection to naïve, co-housed CFW contacts. Our previous studies showed that this combination of mice resulted in efficient transmission of A/Udorn [15] and was thus best suited to addressing the requirements for reduction of transmission.

Immune responses were tested in a subset of animals to confirm successful immunization; results are comparable to those in our previous studies [13]. A/NP + M2-rAd induced serum IgG responses against A/NP were strong after i.n. or i.m. immunization (Fig. 1A), but lower in BAL after i.m. than i.n. immunization. M2e-specific IgG responses were detectable in serum and BAL after i.n. immunization, but low in serum and absent in BAL after i.m. immunization. Serum IgA responses were minimal regardless of immunization route, but in BAL were high for i.n. and absent for i.m. immunized mice (Fig. 1B). Serum and BAL IgG and IgA responses against B/NP confirmed antigen specificity and paralleled those for A/NP.

Lung and spleen T-cell responses were assessed by IFN-γ ELISPOT. The total number of $NP_{147-155}$ specific-cells was higher in lung after i.n. than i.m. immunization ($P < 0.05$; Fig. 1C), but much higher in spleen after i.m. than i.n. immunization ($P < 0.05$; Fig. 1D). Subdominant $NP_{55-69}$- and M2e-specific responses were significantly higher in spleens of i.m. than i.n. immunized mice. $Hex_{486-494}$-specific responses were similar between groups in lungs, but significantly greater in spleen after i.m. immunization ($P < 0.05$). Overall, results confirm previous findings that immunization route strongly influences the magnitude of vaccine-specific immune responses at different anatomic sites [13,16].

We next examined whether immunization route affects transmission reduction. One month after A/NP + M2-rAd-immunization, BALB/c mice were challenged with A/Udorn and 24 h later placed

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