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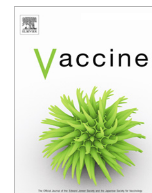

M2SR, a novel live single replication influenza virus vaccine, provides effective heterosubtypic protection in mice

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ABSTRACT

Despite the annual public health burden of seasonal influenza and the continuing threat of a global pandemic posed by the emergence of highly pathogenic/pandemic strains, conventional influenza vaccines do not provide universal protection, and exhibit suboptimal efficacy rates, even when they are well matched to circulating strains. To address the need for a highly effective universal influenza vaccine, we have developed a novel M2-deficient single replication vaccine virus (M2SR) that induces strong cross-protective immunity against multiple influenza strains in mice. M2SR is able to infect cells and expresses all viral proteins except M2, but is unable to generate progeny virus.

M2SR generated from influenza A/Puerto Rico/8/34 (H1N1) protected mice against lethal challenge with influenza A/Puerto Rico/8/34 (H1N1, homosubtypic) and influenza A/Aichi/2/1968 (H3N2, heterosubtypic). The vaccine induced strong systemic and mucosal antibody responses of both IgA and IgG classes. Strong virus-specific T cell responses were also induced. Following heterologous challenge, significant numbers of IFN-γ-producing CD8 T cells, with effector or effector/memory phenotypes and specific for conserved viral epitopes, were observed in the lungs of vaccinated mice. A substantial proportion of the CD8 T cells expressed Granzyme B, suggesting that they were capable of killing virus-infected cells.

Thus, our data suggest that M2-deficient influenza viruses represent a promising new approach for developing a universal influenza vaccine.

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1. Introduction

Seasonal influenza viruses cause between 3000 and 50,000 deaths and an average of 200,000 hospitalizations in the US alone, while historically, global influenza pandemics have resulted in 50–100 million deaths world-wide [1]. The recent emergence of highly pathogenic avian H5N1 and H7N9 strains and the 2009 H1N1 influenza pandemic emphasize the continuing threat to human health posed by this virus. Currently approved vaccines, inactivated split or subunit and live attenuated influenza vaccines (LAIV), must be reformulated annually based on the influenza strains predicted to be prevalent in the next flu season. Thus, these vaccines are not effective if they are poorly-matched to circulating strains, as occurred recently in the 2014/2015 flu season [2]. Although LAIV has previously demonstrated protection in seasons with antigenically mismatched vaccines in children [3–5], accumulating data

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; Aichi, influenza A/Aichi/2/1968; APC, allophycocyanin; BAL, bronchoalveolar lavage; BSA, bovine serum albumin; CPE, cytopathic effect; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HAI, hemagglutination inhibition; IFN, interferon; IN, intranasal; IM, intramuscular; IP, intraperitoneal; LAIV, live attenuated influenza vaccine; LDS, lithium dodecyl sulfate; LOD, limit of detection; M2SR, M2-deficient single replication vaccine virus; M2CK, Madin-Darby canine kidney cells expressing M2 protein; MDCK, Madin-Darby canine kidney; MEM, minimal essential medium; MLD_{50}, 50% mouse lethal dose; MOI, multiplicity of infection; OD, optical density; ORF, open reading frame; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; PFU, plaque-forming unit; PR8, influenza A/Puerto Rico/8/34; RDE, receptor destroying enzyme; RSV, respiratory syncytial virus; $TCID_{50}$, 50% tissue culture infectious dose; TMB, tetramethylbenzidine; TPCK, L-(tosylamido-2-phenyl) ethyl chloromethyl ketone.

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suggest that effective immune responses are mitigated by the pre-existing immunity present in most adults [6–8]. Thus, current vaccines offer little protection against antigenically distinct strains which have the potential to cause influenza pandemics. In addition, most formats are produced by cumbersome procedures in eggs requiring adaptation. The 2009/2010 pandemic vaccine response underscored the urgent need for an effective universal influenza vaccine that can be produced more quickly [9].

To address this need, we developed a novel M2 deficient influenza vaccine that is able to infect cells and therefore induce strong innate, cell mediated and humoral immunity, but does not produce progeny virus.

The influenza A M2 protein has ion channel activity and acts at an early stage in the virus life cycle between viral entry and uncoating [10,11]. The M2 cytoplasmic tail also plays a role in viral assembly and is essential for infectious virus production [12–14]. Our previous studies have demonstrated that M2 cytoplasmic tail mutants can function as live attenuated vaccines and provide effective protection against homologous strains [15–17]. Here we describe a second-generation M2 knockout vaccine format (M2SR), where we abrogated M2 expression by deletion of the M2 transmembrane domain in addition to the insertion of two stop codons downstream of the M1 open reading frame. In the present study, we evaluated the ability of M2SR to induce protective immune responses against both homologous and heterologous influenza virus challenge.

2. Materials and methods

2.1. Cells and viruses

293T human embryonic kidney cells (ATCC CRL-3216), MDCK (Sigma-Aldrich, St. Louis, MO, USA) and M2CK (MDCK cells that stably express the influenza M2 protein) [14] cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and in MEM containing 10% FCS, respectively, at 37 °C in 5% CO_2. M2CK cells were supplemented with hygromycin B (150 μg/mL).

Influenza A PR8 and wildtype A Aichi/2/1968 (Aichi) viruses and M2SR were grown in MDCK and M2CK cells, respectively, in the presence of 1 μg/ml trypsin/TPCK. All viruses were stored at −80 °C until usage.

2.2. M2SR virus generation

M2SR virus (a recombinant PR8 virus with a non-functional M2 protein) was generated using a plasmid rescue system described previously [18]. Briefly, PR8 cDNA (H1N1) was synthesized as described by Hoffmann et al. [19]. Each influenza segment was cloned between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator as previously described [18]. The M2SR M segment was modified by introducing two stop codons in the M2 protein ORF downstream of the M1 ORF followed by deletion of the M2 transmembrane domain. 293T cells were transfected as previously described [15]. M2SR virus in transfection supernatant was amplified in M2CK cells.

2.3. Mice

Six to 8 week-old female mice, 17–20 g in weight (Harlan Laboratories, Livermore, CA or Madison, WI) were used in all experiments. All study protocols were approved by the FluGen or BRISC Institutional Animal Care and Use Committees and all experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.4. Infection and sample collection

Mice were anesthetized with either isoflurane or 2,2,2 tribromoethanol and were infected IN with viruses in 50 μl of sterile PBS. Mock-infected control mice received PBS alone. Additional groups received formalin inactivated whole PR8 virus (1 μg/-mouse; Charles River, North Franklin, CT) either IN or IM. Six weeks post-infection, mice were challenged IN with 100 MLD_{50} PR8 or 40 MLD_{50} Aichi. At specified intervals after immunization or challenge, groups of mice were euthanized and their lungs and nasal turbinates were collected for determination of virus titer. Alternatively, BAL and sera were collected for antibody titer and cytokine analysis.

2.5. Virus titrations from organs

Whole lung or nasal turbinate samples from infected mice were homogenized in 1 mL MEM containing 0.3% BSA (Thermo Fisher Scientific, Waltham, MA). Homogenates were clarified by low speed centrifugation and virus titers were determined on MDCK or M2CK monolayers, as previously described [15] by plaque assay or $TCID_{50}$ assay.

2.6. Virus-specific antibody detection

Immunoglobulin IgG and IgA titers were measured in sera and trachea-lung washes by ELISA as previously described [17] against purified inactivated influenza PR8 antigen (Charles River, North Franklin, CT).

A standard HAI assay was performed to assess functional antibody levels [20]. Serum samples were treated with RDE (Denka Seiken, Tokyo, Japan) overnight at 37 °C followed by heat inactivation for 1 h at 56 °C. Twofold dilutions of RDE-treated serum samples were incubated with influenza viruses (4 hemagglutination units per well) and 50 μL of a 0.5% suspension of turkey red blood cells (Innovative Research, Novi, MN) for 30 min at room temperature. The HAI titer is the reciprocal of the highest dilution of RDE-treated serum that prevented hemagglutination.

2.7. Lymphocyte subsets in the BAL

Lymphocyte or T cell subsets in the BAL were analyzed by staining with fluorochrome-conjugated antibodies to cell surface markers followed by flow cytometric analysis using a BD FACSCalibur flow cytometer and Cellquest Pro software. Subsets of T cells were analyzed by staining with fluorochrome-conjugated antibodies to T cell subsets defined by the markers shown in Supplementary Table 1. Antibodies were obtained from BD Biosciences, Biolegend or E-Biosciences (San Diego, CA).

2.8. Restimulation of CD4 and CD8 T cells with influenza epitope peptides

BAL cells were restimulated *in vitro* for 6 h with 1 μM T cell epitope peptides in the presence of monensin (Golgi-Plug, BD Biosciences, San Diego, CA). Restimulated cells were stained with fluorochrome-conjugated anti-CD4 or anti-CD8 as described above. Cells were then fixed and permeabilized using Cytofix/Cytoperm reagent, stained with fluorochrome-conjugated anti-IFN-γ antibodies (BD Bioscience, San Diego, CA) and analyzed by flow cytometry.

2.9. Cytokine ELISAs

Cytokine concentrations in cell free BAL fluid were determined by sandwich ELISA as described in Sarawar and Doherty [21] with

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