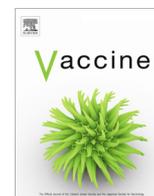


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# Vaccine

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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- $\gamma$ -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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**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<sub>50</sub>, 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<sub>50</sub>, 50% tissue culture infectious dose; TMB, tetramethylbenzidine; TPCK, L-(tosylamido-2-phenyl) ethyl chloromethyl ketone.

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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

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<sub>2</sub>. 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<sub>50</sub> PR8 or 40 MLD<sub>50</sub> 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<sub>50</sub> 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

the following modifications: TMB substrate (Sigma-Aldrich, St. Louis, MO) was used, the reaction was stopped using 1 M HCl and plates were read on a V-max plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 450 nm. Data was analyzed using Softmax Pro software.

### 3. Results

#### 3.1. M2SR virus is restricted in normal cells

We modified the previously described M2 knock-out influenza A virus [15] by deleting additional nucleotides after the two M2 protein stop codons inserted downstream of the PR8 M1 open reading frame (Supplementary Fig. 1). The resulting M2SR virus encodes the M2 ectodomain, but lacks the coding capacity for the M2 transmembrane domain and cytoplasmic tail. The replication restriction and genetic stability for 20 passages of the M2SR virus, its ability to express influenza proteins and attenuation in mice are described in Supplementary Figs. 2–4.

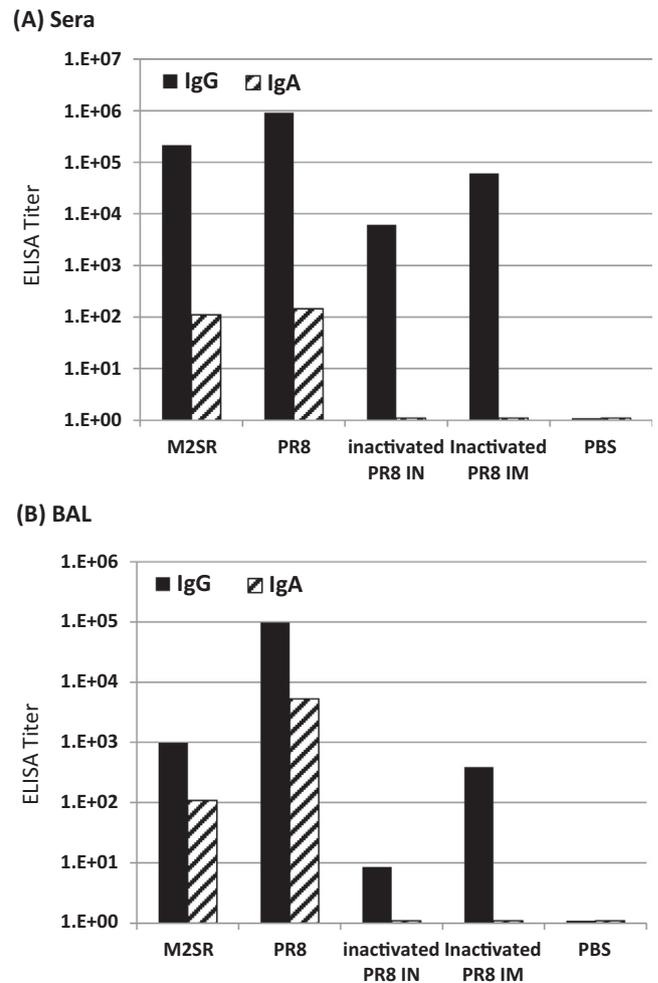
#### 3.2. M2SR virus elicits both systemic and mucosal immune responses in mice

The immunogenicity of M2SR-PR8 virus in mice was compared to that of live wild-type PR8 virus, both administered IN, or to inactivated whole PR8 virus, administered either IM or IN. Three weeks after immunization, serum and BAL were collected from the mice and anti-PR8 IgG and IgA levels were measured by ELISA. Mice in the immunized groups showed higher levels of anti-PR8 antibodies in serum and BAL compared to the control group (Fig. 1). Serum anti-PR8 IgG levels for the M2SR vaccinated group were higher than the inactivated PR8 vaccinated groups and similar to the live PR8 virus vaccinated group. More importantly, anti-PR8 IgA antibodies were present only in the live PR8 and M2SR immunized mice in both sera and BAL. These data demonstrate that M2SR virus elicits systemic and mucosal immune responses in mice.

#### 3.3. M2SR virus protects mice from lethal homosubtypic and heterosubtypic challenge

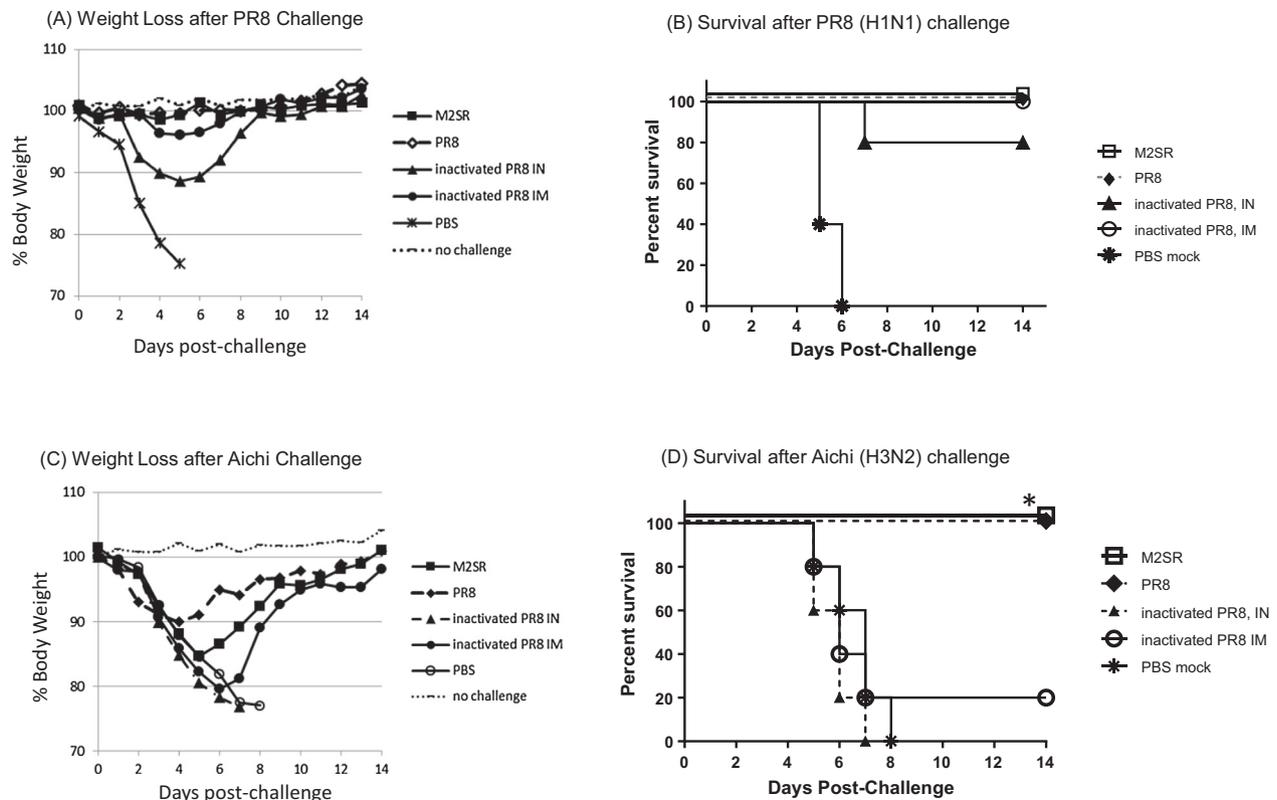
The protective efficacy of M2SR virus was evaluated by challenging groups of mice, immunized as described above, with lethal doses of the wild-type PR8 (H1N1; homosubtypic challenge) or Aichi (H3N2; heterosubtypic challenge) six weeks post-immunization. None of the mice immunized with either M2SR or live wild-type PR8 and subsequently challenged by wild-type PR8 showed any clinical symptoms, including weight loss (Fig. 2A & B). In contrast, control mice lost more than 20% of their body weight and died or were euthanized due to severe illness by day 5 (Fig. 2A & B). No virus was detected in the lungs or nasal turbinates of M2SR or live PR8 immunized mice, 3 days after homosubtypic challenge (Fig. 3A), indicating that M2SR induced sterilizing immunity similar to wild-type PR8 infection. In contrast, challenge PR8 virus was recovered from the lungs of the inactivated PR8-immunized and control groups.

All M2SR-immunized mice survived the heterosubtypic challenge with Aichi virus, as did mice immunized with wild-type PR8 (Fig. 2C & D). Mice in both groups displayed ~10–15% weight loss but recovered fully. In contrast, mice that had received inactivated PR8, or PBS alone, continued to lose weight and succumbed to infection (Fig. 2C & D). Although M2SR vaccinated mice recovered from heterosubtypic challenge, virus was detected in the lungs and nasal turbinates at day 3 after challenge (Fig. 3B), indicating that heterosubtypic sterilizing immunity was not elicited.



**Fig. 1.** M2SR induces strong humoral and mucosal antibody responses. BALB/c mice (21 per group), anesthetized with isoflurane, were vaccinated with M2SR ( $1.2 \times 10^4$  PFU), live wildtype PR8 (10 PFU), inactivated PR8 (1  $\mu$ g) or vehicle (PBS) intranasally (IN) or inactivated PR8 (1  $\mu$ g) intramuscularly (IM). Three weeks after vaccination, serum and bronchoalveolar lavage (BAL) were collected from mice (N = 5). For BAL collection, mice were anesthetized with isoflurane and exsanguinated via the femoral artery. The trachea was cannulated via a small incision and two lavages were performed with a total volume of 1 ml PBS. BAL was harvested after low speed centrifugation to pellet cells. Anti-PR8 IgG and IgA levels were measured by ELISA for pooled sera for each group. 96 well high protein-binding plates (Nunc MaxiSorp, Thermo Fisher Scientific, Waltham, MA) were coated with 200 ng/well purified inactivated influenza PR8 antigen (Charles River Avian Vaccine Services, North Franklin, CT). After incubation of the plates with test samples, bound antibody was detected using horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-mouse IgA followed by development with SureBlue TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). Endpoint antibody titers are given as the reciprocal of the dilution determined by least squares method giving an OD reading of 0.3 at 450 nm and three standard deviations above the mean background. Panel A. Antibody titers in sera. Panel B. Antibody titers in BAL.

Serum collected from all challenge survivors was evaluated for HAI titers against both PR8 and Aichi challenge viruses. All vaccinated mice survived and had high HAI titers to PR8, fourteen days after homologous challenge with a lethal dose of the virus (Fig. 3C), which likely reflected responses induced by the vaccines that were boosted on subsequent homologous challenge. The fact that the Aichi-challenged mice that survived had much lower HAI titers to PR8 at the same time-point supports this idea. HAI titers to Aichi (heterologous challenge) were much lower for M2SR-PR8 and PR8 vaccinated mice (Fig. 3C). As all of these mice survived, it is probable that other mechanisms, for example anti-viral T cell responses, contributed to heterologous protection. Only 1 out of



**Fig. 2.** M2SR protects against homologous and heterologous challenge. Mice (remaining 16 of 21) that were vaccinated as described in the legend to Fig. 1 were challenged 6 weeks later ( $N = 8$  per virus) with a lethal dose of homologous virus (PR8, H1N1); (A & B) or heterologous virus (Aichi, H3N2); (C & D). Weight loss (A & C) and survival (B & D) were monitored for 14 days for 5 mice per challenge virus. Differences in the survival curves were statistically significant:  $P = 0.002$ . The survival analysis was performed using the Log-rank test (Mantel-Cox method) in Graphpad Prism software.  $P$ -value less than 0.05 is considered significant.

5 mice vaccinated with inactivated PR8 IM survived (Fig. 2) and this mouse had an HAI titer of 40 (Fig. 3C), which may not be representative of the group as a whole, as most did not survive challenge, but probably explains the survival of this individual.

#### 3.4. M2SR elicits influenza-specific T cell responses that are recalled upon heterologous challenge

As an initial step in evaluating the possibility that T cell responses may mediate heterosubtypic protection, influenza specific responses were evaluated and compared to responses induced by a sub-lethal dose of PR8. Mice vaccinated with M2SR or wild-type virus had similar total numbers of cells and similar proportions of  $\alpha\beta$  TCR+ve cells in the BAL at days 4 and 7 after challenge (Supplementary Fig. 5). The relative proportions of CD4 and CD8 T cells were similar at day 4 after challenge, whereas for both M2SR and wild-type virus vaccinated groups, CD8 T cells predominated by day 7 after challenge (Supplementary Fig. 5). Both groups had significant populations of NK cells in the BAL at day 4 after challenge, although this cell type had dispersed by day 7. Very few CD19+ B cells or  $\gamma\delta$  TCR+ve cells were observed (Supplementary Fig. 5).

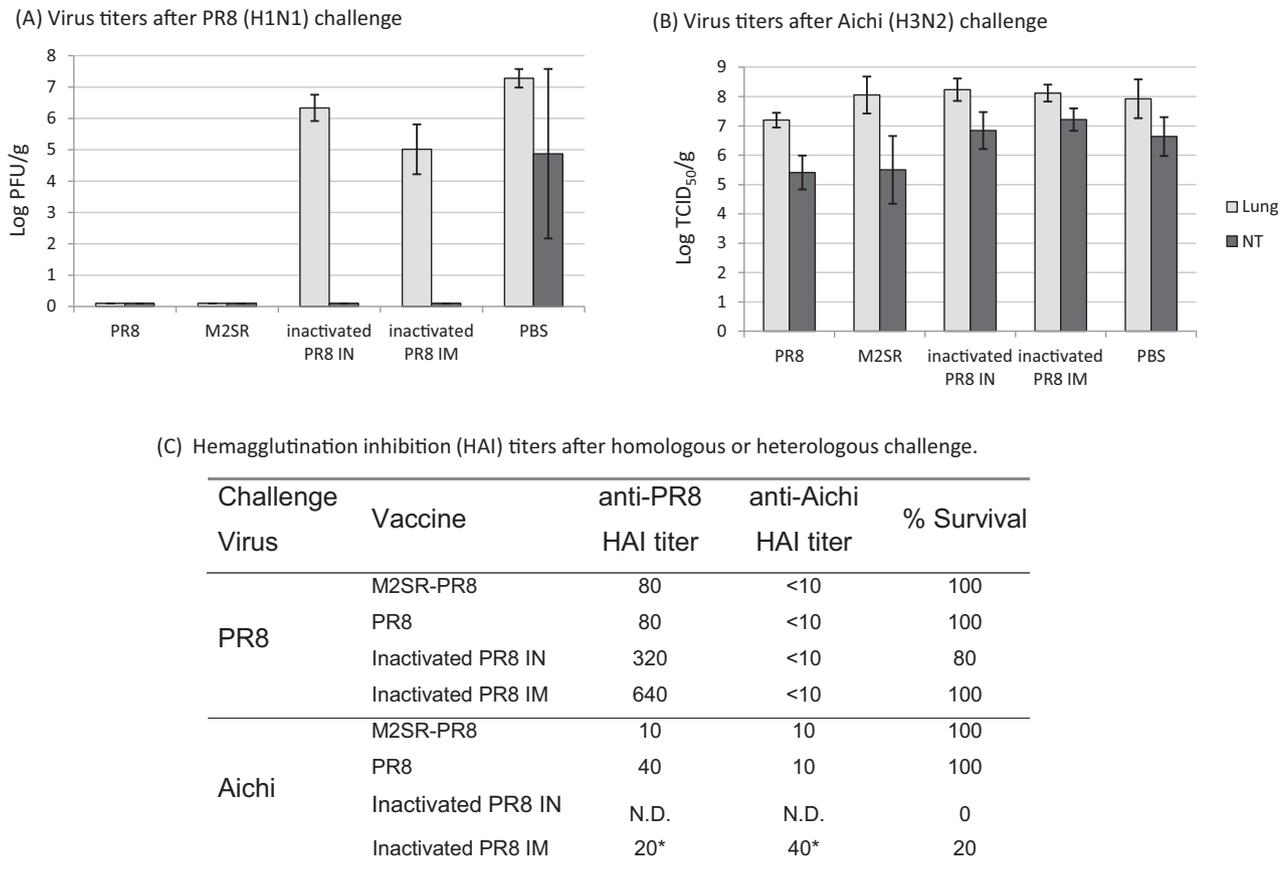
In contrast to mice vaccinated with M2SR or live virus, unvaccinated mice had very few CD8 or CD4 T cells in the BAL at day 4 (Fig. 4) confirming that the observed responses in mice vaccinated with M2SR or live virus were not primary, but recalled secondary responses. BAL cells from mice vaccinated with M2SR or wild-type virus were restimulated *in vitro* with CD8 epitope peptides NP<sub>366-374</sub> or PB1<sub>703-711</sub> or CD4 epitope peptides HA<sub>211-225</sub> or NP<sub>311-325</sub> and stained for CD4 or CD8 and IFN- $\gamma$ . M2SR elicited peptide-specific responses to similar levels as the wild-type PR8

virus. These data show that M2SR vaccine elicits T cell responses to conserved viral epitopes that are recalled upon heterologous challenge.

#### 3.5. M2SR induces T cells with effector and effector memory phenotypes and cytotoxic potential

T cells from the BAL of M2SR or wild-type virus vaccinated mice were also stained with antibodies to the markers that delineate naïve, effector, central memory, effector memory, effector memory precursor and terminal effector subsets (Supplementary Table 1). For both M2SR and wild-type virus vaccinated groups, the majority of the CD8 T cells after challenge were effector or effector memory cells with lower numbers of effector memory precursor cells (Fig. 5A). A similar profile was observed for CD4 T cells from both M2SR and wild type virus vaccinated mice. These data also suggest that M2SR vaccine induces T cell responses that are recalled upon heterologous challenge.

In order to evaluate their potential to kill virus-infected cells after challenge, T cells from the BAL of wild-type or M2SR vaccinated mice were stained with antibodies to cytotoxic effector molecules, Granzyme B or Fas ligand. A significant proportion of CD8 T cells from both wild-type and M2SR vaccinated mice expressed Granzyme B, although very few CD4 T cells expressed this molecule (Fig. 5B). In contrast, a higher proportion of CD4 T cells than CD8 T cells expressed FasL for both M2SR and wild-type virus vaccinated mice (Fig. 5B). However, the number of CD4 T cells present in the BAL was much lower than that of CD8 T cells. These data show that the T cells that are induced by M2SR are armed with cytotoxic mediators and capable of killing virus-infected cells.



**Fig. 3.** M2SR induces sterilizing immunity to homologous, but not to heterologous, virus challenge. Mouse respiratory organs (lungs and nasal turbinates) were harvested on day 3 after infection from mice (6 of 16 that were challenged;  $N = 3$  per virus) challenged as described in the legend to Fig. 2. In brief, lung lobes were separated from the heart and trachea while the nasal turbinates were harvested after first removing the skin from the mouse skull followed by removal of the skull to expose the intact nasal epithelium. Lung and nasal turbinates were homogenized in MEM containing 0.3% BSA using a mortar and pestle after tissue weights were determined. Supernatants were collected for titration after slow speed centrifugation. Virus titers were determined by  $TCID_{50}$  or plaque assay in MDCK cells. Homologous challenge virus titers are shown in panel A and heterologous challenge virus titers are shown in panel B (mean  $\pm$  SD). The limit of detection (LOD) for the plaque assay is 10 PFU/organ and  $10^{1.67}$   $TCID_{50}$ /organ for the  $TCID_{50}$  using the Reed-Muench method of determining the 50% infectious endpoint. Panel C. Serum samples were obtained from surviving mice 14 days after challenge and HAI titers were determined on pooled samples from groups of 5 mice using turkey erythrocytes. N.D. not determined since all mice died. \*Titer for surviving mouse only and may not be representative of group as 80% of the mice died.

### 3.6. Moderate inflammatory responses are observed in M2SR vaccinated mice after challenge

We examined the cell types and cytokines induced after hetero-subtypic challenge in M2SR or wild-type virus vaccinated groups of mice. At day 4 after challenge, M2SR vaccinated mice had a significantly lower proportion of neutrophils and a significantly higher proportion of lymphocytes in the BAL than unvaccinated mice (Fig. 6A). Wild-type virus vaccinated mice showed intermediate percentages of lymphocytes and neutrophils. The percentage of macrophages in all three groups was similar. However, by day 7 after challenge, similar percentages of macrophages, lymphocytes and neutrophils were found in the BAL of M2SR and wild-type virus-vaccinated mice (Fig. 6B). Extremely low numbers of eosinophils and no basophils or mast cells were observed in the BAL of all three groups of mice at both days 4 and 7 after challenge (Fig. 6A & B).

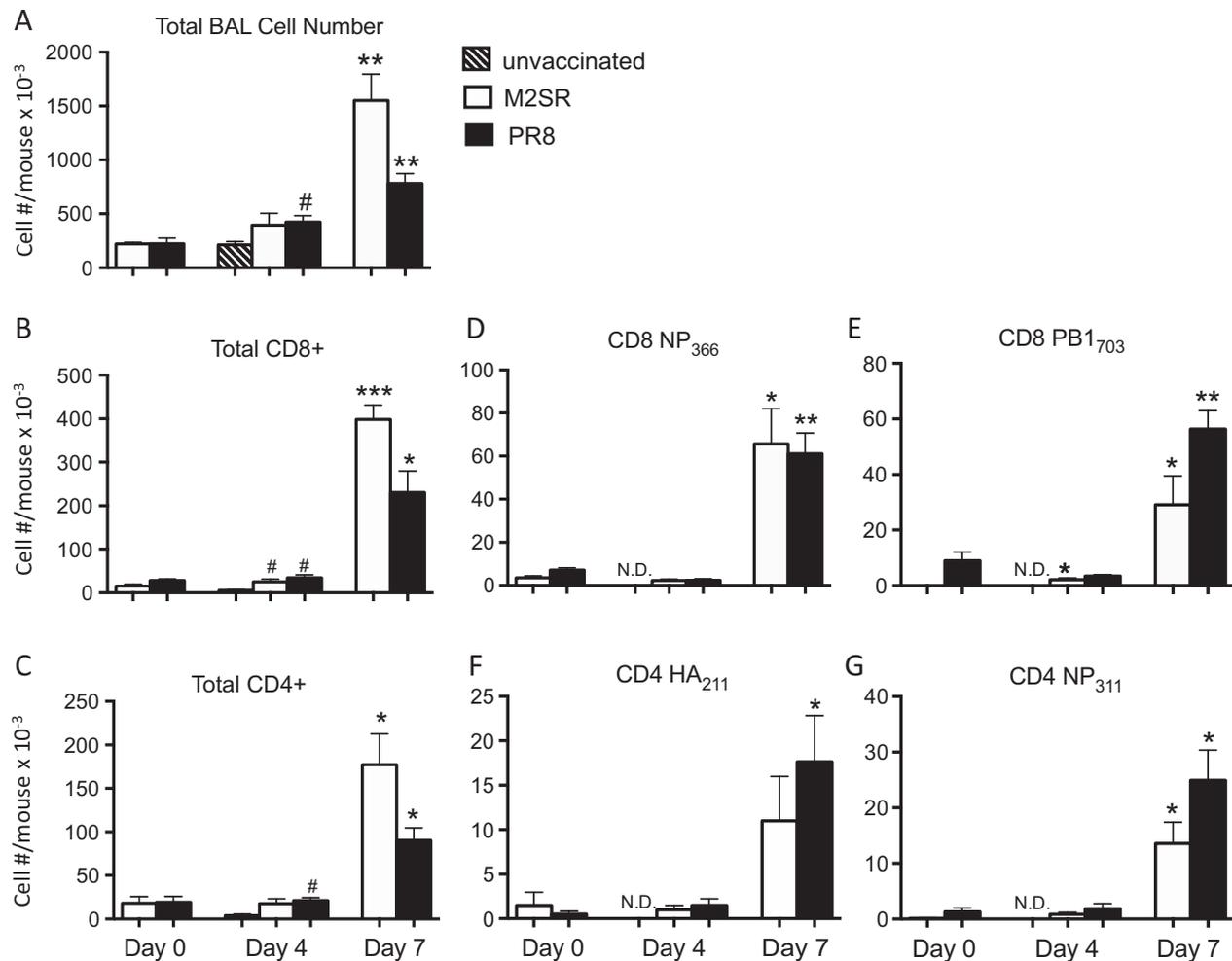
Cytokine levels were determined in cell-free BAL fluid. Very low levels of IL-2, IL-4, IL-17 and IL-10 were detected in the BAL of M2SR or live virus vaccinated mice or unvaccinated controls after challenge (Fig. 6C). Moderate levels of IFN- $\gamma$  and TNF were detected in the BAL of all 3 groups of mice after challenge (Fig. 6C). Levels of IFN- $\gamma$  and TNF in the BAL of the M2SR and wild-type virus

vaccinated groups were similar at day 7 after challenge and somewhat higher than at day 0 or day 4 for both groups.

## 4. Discussion

Our data show that M2SR provides effective protection against both homologous and heterologous strains of influenza. These promising results suggest that M2SR could offer a major advantage over currently approved vaccines, which offer little or no protection against newly emerging or mutated strains [22,23]. Whereas LAIV has provided protection in children in seasons in which the antigenicity of the virus has drifted [3–5,24], recent studies suggest that LAIV may not be as effective in adults due to preexisting immunity [9,25]. M2SR can be produced efficiently in cell culture, avoiding the many disadvantages of production in eggs. The vaccine can be rapidly engineered to match new pandemic strains, without the need for adaptation to growth in eggs. Data from multiple passage studies shows that the vaccine is genetically stable and does not re-acquire the ability to produce M2 or to replicate in cells, other than the M2-expressing producer line.

M2SR induces sterilizing immunity to homosubtypic challenge (Fig. 2). This is likely to be mediated by neutralizing antibodies to the head region of the hemagglutinin molecule, since the



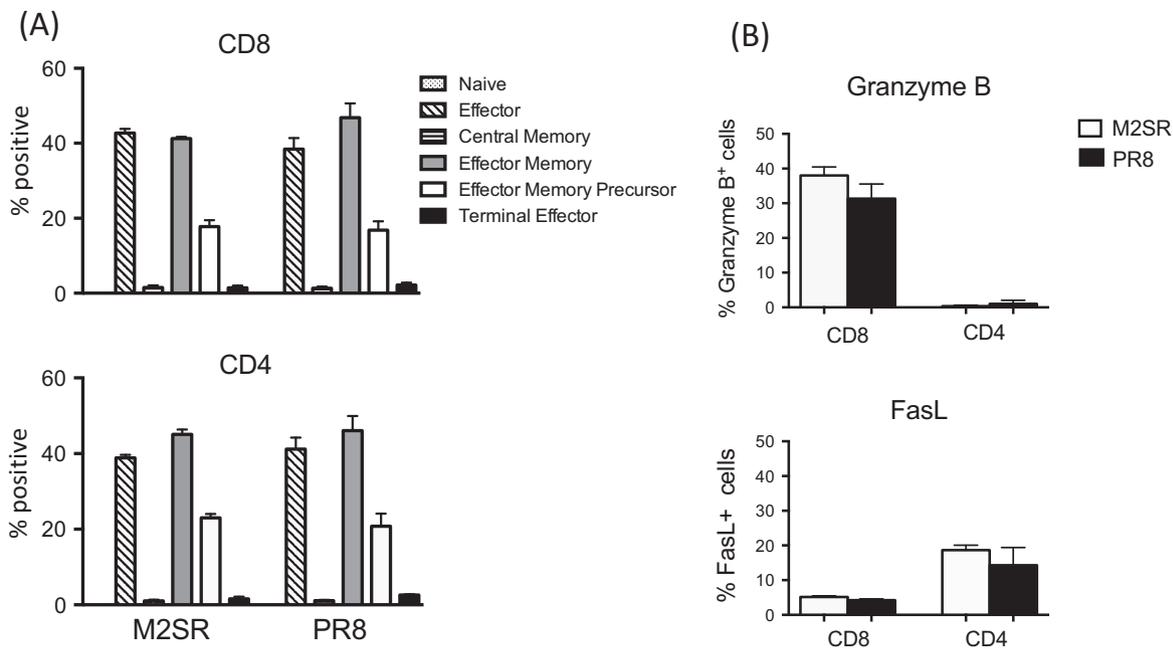
**Fig. 4.** M2SR induces T cell responses to conserved viral epitopes. C57BL/6 mice were vaccinated IN with either M2SR ( $10^6$  PFU) or a sublethal dose of PR8 (30 PFU). Mice were challenged 5 weeks later with a lethal dose of Aichi (40 MLD<sub>50</sub> IN.). For BAL collection, mice were anesthetized with a lethal dose of tribromoethanol and exsanguinated via the axillary vein. The trachea was cannulated via a small incision and three lavages were performed with a total volume of 1 ml PBS. The BAL cellular component was collected by low speed centrifugation. Viable cell (total leukocyte) counts were performed by Trypan Blue exclusion. BAL cells harvested 4 and 7 days after challenge were restimulated *in vitro* with 1  $\mu$ M T cell epitope peptides for 6 h in the presence of monensin. BAL was also harvested from additional groups of mice that were vaccinated but not challenged (designated 'Day 0') and challenged but not vaccinated ('unvaccinated'). BAL was harvested from the latter group at day 4 only. Cells were stained with FITC-conjugated anti-CD4 (RM4-5) or anti-CD8 (53-6-7), fixed, permeabilized and stained with PE-conjugated anti-IFN- $\gamma$  (XMG1.2) antibody and analyzed by flow cytometry. Dead cells, erythrocytes, small vesicles and debris were excluded using forward and side scatter gates. The absolute cell number in each lymphocyte subset was calculated by multiplying the fraction of leukocytes that were positive for markers of that subset by the total number of leukocytes. The mean percentages of CD8 T cells that produced IFN- $\gamma$  in response to the NP366 peptide were 16.6% and 27.3% for M2SR-vaccinated and WT virus- vaccinated groups, respectively. The corresponding percentages for the PB1 703 peptide were 7.2% and 25.6%. The mean percentages of CD4 T cells that produced IFN- $\gamma$  in response to the HA 211 peptide were 5.6% and 18.6% for M2SR-vaccinated and WT-vaccinated groups, respectively. The corresponding percentages for the NP 311 peptide were 7.6% and 27.0%. A. Total BAL cell number per mouse. B. Total BAL CD8<sup>+</sup> cell number per mouse. C. Total BAL CD4<sup>+</sup> cell number per mouse. D. Number of CD8<sup>+</sup> cells producing IFN- $\gamma$  in response to NP<sub>366-374</sub> peptide. E. Number of CD8<sup>+</sup> T cells producing IFN- $\gamma$  in response to PB1<sub>703-711</sub> peptide. F. Number of CD4<sup>+</sup> T cells producing IFN- $\gamma$  in response to HA<sub>211-225</sub> peptide. G. Number of CD4<sup>+</sup> T cells producing IFN- $\gamma$  in response to NP<sub>311-325</sub> peptide. Data shown are means  $\pm$  SEM for 3 groups of 3–5 mice/group. \*, significantly different from day 0 timepoint (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). #, significantly different from unvaccinated group ( $P < 0.05$ ). Statistical analysis was performed using Student's *t* test and Graphpad Prism software (Graphpad Software, La Jolla, CA).

vaccine induces high HAI titers that are boosted on homologous challenge (Fig. 3C). In contrast, immunity to heterosubtypic challenge was not sterilizing. Protective HAI titers against the challenge virus were not elicited suggesting that protection is unlikely to be mediated by neutralizing antibodies to the hemagglutinin. The mouse model is routinely used in the preclinical evaluation of influenza vaccine candidates [26–32]. It remains to be seen how these promising results with M2SR will translate to humans.

Studies in the literature on heterosubtypic immunity to live virus have given divergent results, implicating both humoral and cell-mediated mechanisms of protection [27,33–36]. M2SR induces strong systemic and mucosal antibody responses. It is possible that heterosubtypic protection is mediated by cross-reactive non-

neutralizing antibodies to HA2 (stalk) region of the hemagglutinin molecule, which is more conserved than the head region, or to conserved epitopes in the neuraminidase molecule. Non-neutralizing antibodies might control virus by mechanisms such as antibody-dependent cellular cytotoxicity (ADCC).

Alternatively, protection may be mediated by cross-reactive T cells responding to epitopes shared between the vaccine and challenge viruses. Most of these shared epitopes are in conserved intracellular proteins, such as the nucleoprotein and polymerase. M2SR induces an influenza specific T cell response that is recalled upon heterologous challenge and is similar to that induced by a sublethal dose of live wildtype virus. Large numbers of CD8 T cells, and lower numbers of CD4 T cells producing IFN- $\gamma$  in response to



**Fig. 5.** M2SR induces T cells with Effector and Effector/Memory Phenotypes and Cytotoxic Potential. Groups of mice were vaccinated and challenged as described in the legend to Fig. 4. BAL cells were harvested 7 days after challenge and stained with fluorochrome-conjugated antibodies to CD4 or CD8 and CD62L, CD44, CD127, CCR7, CD27 and KLRG-1 (Panel A) or to CD4 or CD8 and FasL (Panel B), followed by flow cytometric analysis. T cell subsets were defined by the markers shown in Supplementary Table 1. Aliquots of cells stained with anti-CD4 or anti-CD8 were also fixed, permeabilized and stained with a fluorochrome-conjugated antibody to Granzyme B. (A) Percentage of CD4 or CD8 cells expressing Granzyme B. (B) Percentage of CD4 or CD8 cells expressing Granzyme B. Data shown are means + SEM for 3 groups of 3–5 mice per group. Each data point is the mean of 3 group. Each group consists of pooled cells from 3 to 5 mice.

conserved viral epitopes were observed in the BAL of M2SR-vaccinated mice after heterologous challenge. The majority of both CD4 and CD8 T cells had effector or effector memory phenotypes, with lower numbers of effector memory precursors. In a recent study on cellular immune correlates of protection against the 2009 H1N1 influenza in humans, Sridhar et al. [37] reported that, in the absence of cross-reactive neutralizing antibody, the frequency of CD8 T cells specific to conserved viral epitopes correlated with cross-protection against symptomatic influenza. In the latter study, low symptom scores were significantly associated with the presence of IFN- $\gamma$ -producing CD8 T cells with effector memory phenotype responding to conserved epitopes in NP and PB-1 viral proteins. This is the same phenotype that we observe in M2SR-vaccinated mice, following heterosubtypic challenge.

Furthermore, our data shows that a substantial proportion of the CD8 T cells in the BAL of M2SR vaccinated mice express the cytotoxic mediator, Granzyme B, after heterosubtypic challenge. In addition, some CD4 T cells in the BAL expressed FasL, although they were present in much lower numbers than the CD8 T cells expressing Granzyme B. Thus, the T cells in the BAL were armed with cytotoxic mediators, suggesting that they were capable of killing virus-infected cells. Influenza-specific CD4 T cells that presented cytotoxic characteristics were shown to correlate with reduced influenza disease in a human challenge model [38]. High levels of Granzyme B and high IFN- $\gamma$ /IL10 ratios (which we also observed) have been shown to correlate with protection against influenza in vaccinated older individuals [39,40].

Some vaccines to respiratory viruses have been associated with the induction of dysfunctional immune responses upon natural exposure to virus. For example, an early respiratory syncytial virus (RSV) vaccine induced eosinophilia and asthma-like responses in children following community-acquired infection with RSV [41]. In addition, some, but not all, reports have described wheezing in children vaccinated with LAIV (FluMist) [42–44] and enhancement

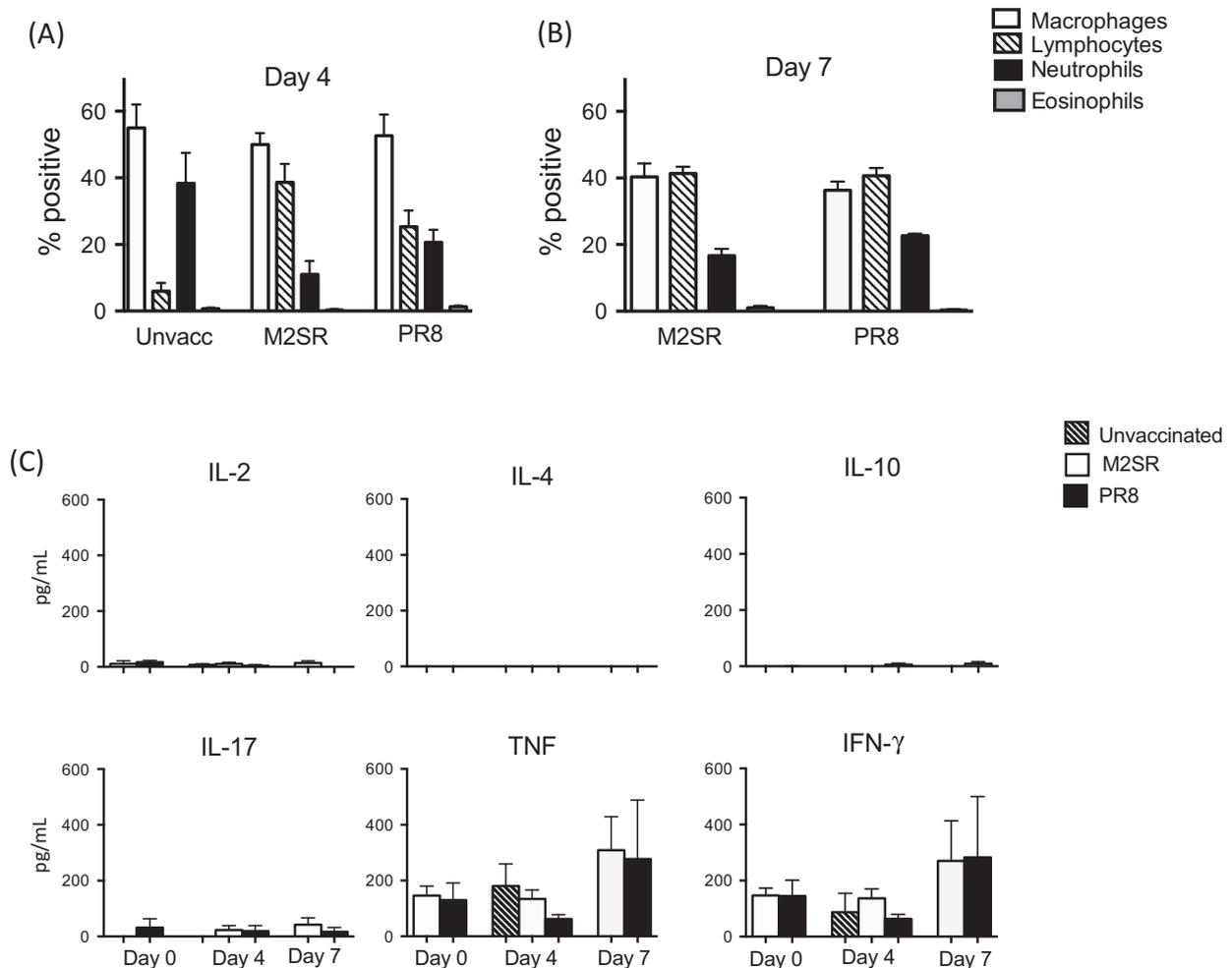
of influenza virus respiratory disease by vaccine induced anti-HA2 antibodies has been reported in pigs [45]. Although M2SR induced sterilizing immunity to homosubtypic challenge, viral replication in the lungs was observed after heterosubtypic challenge and could potentially have induced a dysfunctional inflammatory response in vaccinated mice. Therefore, we examined the cellular infiltrate in the lungs after heterosubtypic challenge in mice vaccinated with M2SR. We observed a moderate cellular infiltrate comprising mainly lymphocytes and macrophages with lower numbers of neutrophils. Very few eosinophils, basophils or mast cells, cell types that are associated with bronchial hyperresponsiveness, wheezing and asthma, were observed.

In general, cytokine concentrations induced by M2SR were low and only moderate levels of IFN- $\gamma$  and TNF were observed in the BAL, with virtually no IL-2, IL-10 or IL-17. This is not consistent with the extremely high levels of cytokines (the so-called ‘cytokine storm’) that are observed during severe or lethal influenza infection and have been postulated to play a role in lung damage [20]. Taken together these data suggest that M2SR induces effective protection without inducing damaging inflammatory responses.

In summary, our data show that M2SR vaccine is a genetically stable vaccine, with the advantage that it can be produced rapidly in cell culture, avoiding the pitfalls of vaccine production in eggs. The vaccine induces strong humoral and cellular immunity that provides effective homosubtypic and heterosubtypic protection in a mouse model and induces known correlates of immune protection in humans.

#### Conflict of interest statements

S.S., P.D. and S.W. have no conflicts of interest. G.N. and Y.K. are founders of FluGen. Y.H. and P.B. are employees of FluGen.



**Fig. 6.** Moderate inflammatory responses in the lungs of M2SR-vaccinated mice after heterologous challenge. Groups of mice were vaccinated and challenged and BAL cells were harvested as described in the legend to Fig. 4. (A & B) Leukocyte subsets in the BAL at days 4 & 7, respectively, after challenge. Aliquots of BAL were cytocentrifuged onto microscope slides and stained with a Hema 3 staining kit to facilitate differential counting of neutrophils, monocytes, lymphocytes, basophils, eosinophils and mast cells. 5–10 high power fields per slide (100–500 cells) were counted. (C) Cytokine production in the BAL before and after challenge. Cytokine concentrations in cell free BAL fluid were determined by sandwich ELISA. The following pairs of antibodies were used in the ELISAs: anti-IL-2, JES5-2A5 and biotinylated JES6-5H4; anti-IL-4, BVD4-1D11 and biotinylated BVD6-24G2; anti-IL-10, JES5-2A5 and biotinylated JES5-16E3; anti-IL-17 ebio17CK15A5 and biotinylated ebio17B7; anti-TNF, G281-2626 and biotinylated MP6-XT3; IFN- $\gamma$ , R46A2 and biotinylated XMG1.2. Data shown are means + SEM for 3 groups of 3–5 mice per group.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2016.08.061>.

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