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Supplementation of H1N1pdm09 split vaccine with heterologous tandem repeat M2e5x virus-like particles confers improved cross-protection in ferrets

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Abstract

Current influenza vaccines induce strain-specific immunity to the highly variable hemagglutinin (HA) protein. It is therefore a high priority to develop vaccines that induce broadly cross-protective immunity to different strains of influenza. Since influenza A M2 proteins are highly conserved among different strains, five tandem repeats of the extracellular peptide of M2 in a membrane-anchored form on virus-like particles (VLPs) have been suggested to be a promising candidate for universal influenza vaccine. In this study, ferrets were intramuscularly immunized with 2009 H1N1 split HA vaccine (“Split”) alone, influenza split vaccine supplemented with M2e5x VLP (“Split+M2e5x”), M2e5x VLP alone (“M2e5x”), or mock immunized. Vaccine efficacy was measured serologically and by protection against a serologically distinct viral challenge. Ferrets immunized with Split+M2e5x induced HA strain specific and conserved M2e immunity. Supplementation of M2e5x VLP to split vaccination significantly increased the immunogenicity of split vaccine compared to split alone. The Split+M2e5x ferret group showed evidence of cross-reactive protection, including faster recovery from weight loss, and reduced inflammation, as inferred from changes in peripheral leukocyte subsets, compared to mock-immunized animals. In addition, ferrets immunized with Split+M2e5x shed lower viral nasal-wash titers than the other groups. Ferrets immunized with M2e5x alone also show some protective effects, while those immunized with split vaccine alone induced no protective effects compared to mock-immunized ferrets. These studies suggest that supplementation of split vaccine with M2e5x-VLP may provide broader and improved cross-protection than split vaccine alone.

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Keywords

M2e protein; ferret; cross-protection

Introduction

Infection with influenza A virus is a significant cause of morbidity and mortality worldwide [1–3]. Vaccination is the most cost-effective measure to prevent influenza disease and its mortality. Current influenza vaccines are based primarily on immunity to the highly variable hemagglutinin antigen, and induce strain-specific immunity. Consequently, influenza vaccine must be updated frequently to match circulating viruses. In addition, novel influenza A strains such as reassortants with avian or swine strains, which occasionally enter the human population and represent a potential pandemic threat, are not protected against by current vaccines. The emergence of the triple-reassortant 2009 pandemic H1N1 virus (A(H1N1)pdm09) is an example of a new influenza strain with distinct antigenic properties [4]. Although the hemagglutinin (HA) protein from this strain is of the H1 subtype, many decades of separate evolution in swine versus humans led to this strain becoming antigenically distinct from the seasonal A(H1N1) which circulated in humans before 2010 [5]. Accordingly, standard assays such as hemagglutination inhibition (HI) and virus neutralization assays showed no cross-reactive immunity between these strains, and the pandemic of 2009–2010 demonstrated that prior exposure to seasonal H1N1 viruses or vaccines conferred little or no protection against A(H1N1)pdm09 viruses. Therefore, influenza vaccines capable of inducing broad, cross-protection against different influenza variants or strains need to be developed.

The extracellular domain of the influenza A ion-channel protein M2 is well conserved across influenza A subtypes [6, 7] and is one potential target for a universal influenza A vaccine [8–11]. M2 has a small size extracellular domain containing 23 amino acids (M2e) and low immunogenicity even in a conjugate to carrier molecules and in the presence of adjuvants [12]. We have previously shown that influenza M2 full-length proteins incorporated into virus-like particles (VLPs) conferred cross protection against antigenically different influenza viruses but M2 immunogenicity was low [13]. To improve the efficacy of M2e-based VLP vaccines, we generated five tandem repeats of M2e from human, swine, and avian origin influenza A viruses in a membrane-anchored form to be expressed on VLPs (M2e5x VLP), resulting in enhanced cross protection in mice [14].

The ferret remains the most widely accepted small animal model for influenza virus infection and vaccine protection studies [15–18]. In this present study, we demonstrate that M2e5x VLP was immunogenic in ferrets and supplementation of split vaccine with M2e5x VLPs could further enhance cross-protective immunity.

Materials and Methods

Viruses

A/Brisbane/59/2007 (BR/59), an A(H1N1) human seasonal influenza virus, and A/California/08/2009 (CA/08), an A(H1N1)pdm09 2009 pandemic H1N1 virus, were used in these experiments. Although these viruses both are in the A(H1N1) subtype, they are serologically distinct and conventional inactivated vaccines against one do not lead to cross-protection against the other. Virus stocks were propagated in the allantoic cavity of embryonated chicken eggs as previously described [19]. Stocks were titered in a standard plaque assay using Madin-Darby Canine Kidney cells as described [4] and expressed as plaque forming units (pfu) [20]. CA/08 and BR/59 were used as antigens for serological testing in hemagglutination inhibition (HI) assay.

Vaccines

The M2e5X VLP containing heterologous M2e tandem repeat was constructed and produced using the recombinant baculovirus (rBV) expression system as previously described [14]. Briefly, Sf9 insect cells were co-infected with recombinant baculoviruses co-expressing influenza virus M1 matrix protein and a tandem repeat of heterologous M2e (M2e5x). Culture supernatants containing released M2e5x VLP were harvested, and M2e5x VLP vaccine was purified using sucrose-gradient ultracentrifugation. Commercial human A(H1N1)pdm09 split vaccine was derived from the 2009 pandemic strain of A/California/07/2009 virus (CA/07) and kindly provided by Green Cross (South Korea). Potency of the split vaccine was assessed by single radial immunodiffusion (SRID) with CA/07 reference serum and antigen obtained from the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration (Kensington, MD).

Ferret immunization and viral challenge

All animal experiments were performed under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Male Fitch ferrets (*Mustela putorius furo*), about 6 months of age (Triple F Farms, Sayre, PA), serologically negative for currently circulating influenza viruses, were used in this study.

Briefly, groups of five ferrets were immunized with M2e5X VLPs alone; conventional monovalent split vaccine against A(H1N1)pdm09; a combination of M2e5X VLP and split vaccine; or mock immunized with PBS. Ferrets were injected intramuscularly with immunogens (5 µg of HA or 100 µg of M2e5X VLP total protein) diluted in a total of 0.25 mL sterile phosphate-buffered saline (PBS). Immunizations with split vaccine were repeated so that the split vaccine alone group elicited HI titers that were similar and greater than 40 (see Figure 1 for timing of immunizations). After immunization, ferrets were challenged with the seasonal human influenza strain BR59. Although CA/08 and BR/59 are both in the A(H1N1) subgroup, these strains have diverged extensively and conventional split vaccines do not confer cross-protection between these strains [5]. After challenge, clinical signs of infection (changes in body weight and temperature), concentration of virus

shed in nasal washes, and changes in peripheral blood leukocyte frequencies were measured for 14 days.

Viral challenge

Fourteen days after the last immunization, all animals were challenged intranasally with 0.5 ml PBS containing 1×10^6 PFU of BR/59. Ferrets were monitored for changes in body weight and temperature as well as clinical signs of illness (sneezing, lethargy, nasal discharge, diarrhea and neurological dysfunction) on a daily basis for two weeks. Body temperatures were measured using an implantable subcutaneous temperature transponder (BioMedic Data Systems, Inc., Seaford, DE). On days 0–7, 9, 11, and 13 post-challenge, animals were anesthetized and blood samples of 200–250 μ l taken from the cranial vena cava as previously described [19]. Nasal washes were collected with 1 ml of PBS on days 0–7, and 9 (Fig. 1). Virus titers were determined by a plaque assay in MDCK cells and expressed as log₁₀ PFU in 1 ml of nasal washes. The limit of virus detection was 100 PFU per 1 ml of nasal washes.

Serology

HI assays were performed as previously described [21]. HI titers against CA/08, and BR/59 were assessed and expressed as the reciprocal of the highest dilution of the samples inhibiting hemagglutination.

M2e-specific serum antibody responses were determined by ELISA using synthetic human peptides (2 μ g/ml) as a coating antigen as previously described [13, 14]. Briefly, horseradish peroxidase-conjugated goat anti-ferret IgG (KPL, Inc Gaithersburg, Maryland USA) was used as the secondary antibody to determine total IgG antibodies. The substrate TMB (eBioscience, San Diego, CA) was used to develop color and 1 M H₃PO₄ was used to stop developing color reaction. The optical density at 450 nm was read using an ELISA reader.

Antibodies, preparation of leukocytes and flow cytometry

Flow cytometry assays were performed as previously described [19]. Briefly, peripheral blood was collected in tubes containing EDTA and red blood cells lysed with erythrocyte lysing solution (0.15M NH₄Cl, 10mM KHCO₃, and 1mM EDTA pH 7.3). Cells were stained with monoclonal antibodies recognizing CD4 (clone 02, Sino Biological Inc., Beijing, China), CD8 (clone OKT8, eBioscience, San Diego, CA) CD11b (clone M1/70, eBioscience), CD3 (clone PC3/188A, Santa Cruz Biotechnology, Santa Cruz, CA), and CD79a (clone HM47, eBioscience). Data were acquired on a FACSCanto II flow cytometer (BD Bioscience, San Jose, CA), and analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Statistical significance and 95% confidence intervals were calculated using a linear mixed model with repeated measures, implemented in the SAS program. Compound symmetry was used for the covariance structure for flow cytometry values; unstructured covariance was used for the HI titers. Confidence intervals were calculated using compound symmetry covariance to pool variability between groups. A p value < 0.05 was used as the cutoff for

statistical significance. The experiments were repeated twice. Post-challenge data were analyzed both as separate experiments and as merged data, with very similar results; figures shown here are from merged data.

Results

Immunogenicity

Groups of ferrets were intramuscularly immunized with M2e5x VLP alone (M2e5x), split vaccine alone (Split), or supplemented split vaccine with M2e5x VLP (Split+M2e5x) as summarized in the Figure 1. Ferrets showed detectable anti-M2e antibody responses 21 days even after a single immunization with M2e5x VLP, with or without split vaccine (M2, Fig. 2A). Second and third immunizations with M2e5x VLP further at day 21 and 35 respectively increased antibodies specific for M2e antigens (days 35 and 47, M2e5x, Fig. 2A). After the 2nd boost immunization (day 35), there was no more M2e5x VLP immunization. Although M2e antibody titers dropped slightly in the 9 weeks (day 61, Fig. 2A), levels of anti-M2 antibodies remained high and easily detectable at the time of challenge.

Ferrets immunized with split vaccine alone showed moderate levels of HI antibodies titers that ranged from <10 to 40 (geometric mean titer [GMT] = 30) only after the 3rd immunization (Fig. 2B) and were further boosted with a 4th immunization with split vaccine, resulting in HI titers of 40 to 320 (GMT = 139).

Interestingly, ferrets that received supplemented split vaccination with M2e5x VLP induced significant levels of vaccine virus specific antibody responses even after primary immunization, reaching HI titers of 40 to 80 (GMT = 53, day 21, Split+M2e5x, Fig. 2B). After two immunizations, the M2e5x VLP supplemented ferret group reached high levels of HI titers, ranged from 160 to 320 (GMT = 184 day 35, Fig. 2B). These results suggest that M2e5x VLPs appear to act as an adjuvant role in increasing HI titers of vaccine, reaching higher levels at early time points. The adjuvant effect of the M2e5x VLP was balanced by repeated administration of the split vaccine alone (Fig. 1). As HA neutralizing antibody is the best established correlate of protection for influenza, animals receiving split vaccine only were immunized until CA/08 HI titers reached HI levels comparable to those in animals receiving Split+M2e5x (Fig. 2B). Prior to challenge, ferrets in both the split and Split+M2e5x groups demonstrated CA/08 HI titers ranging from 80 to 320 with GMTs of 139 and 160, respectively, which is expected to confer protection against homologous virus.

Clinical signs after viral challenge

The major focus of this study was to determine the efficacy of M2e5x VLP and supplemented vaccination (Split+M2e5x) in conferring cross protection. As expected, all naïve and vaccinated animals were seronegative for the challenge virus BR/59 prior to challenge (Fig. 2C), as well as to currently circulating B and H3 strains (not shown). Clinical signs of lethargy, sneezing, and nasal discharge were minor and did not differ between groups. All ferrets exhibited a spike in body temperature one day post-challenge with BR/59, followed by a reduction in temperature by day two (Fig. 3A). Although body

temperatures fluctuated throughout the course of the experiment, as is typical of ferrets [22], no consistent trends were observed after day one (Fig. 3A).

All treatment groups lost at least 5% of their body weight over the first 2 days of infection. Beyond 2 days post-challenge, ferrets immunized with supplemented Split+M2e5x demonstrated significantly less weight loss and more rapid recovery than all other groups (Fig. 3B).

Virus replication in the upper respiratory tract was determined by titrating nasal washes. Peak viral shedding was observed at day 1 post-challenge for all animals (Fig. 4). All groups shed similar titers of virus during the first 3 days. However, from days 4 on, ferrets immunized with Split+M2e5x had significantly lower viral nasal wash titers than other groups (Fig. 4), and from day 5 on, ferrets immunized with M2e5x alone shed significantly lower viral titers than did ferrets immunized with split vaccine alone or with PBS. Of eight ferrets included in both experiments, only three immunized with Split+M2e5x shed virus by day 6 post-challenge, and all completely cleared virus by day 7 post-challenge. In the group immunized with M2e5x alone, six ferrets shed virus by day 6, and all ferrets were clear of virus by day 7 post-challenge. All ferrets receiving PBS or split vaccine alone shed virus at day 6 post challenge, while one ferret immunized with split vaccine shed virus by day 7 post-challenge and three ferrets receiving PBS still shed virus at this time point (Fig. 4). All groups completely cleared the virus by day 9 postchallenge.

Serological changes after challenge

Following challenge, animals immunized with vaccines containing M2e5x increased M2e-specific antibody responses (M2e5x, Split+M2e5x, Fig. 2A), while anti-M2e titers in animals vaccinated with split vaccine only, or mock immunized, showed little or no serological response to M2 (Fig. 2A). HI antibody titers to BR/59 also increased for all groups by days 7 and 14 post-challenge (Fig. 2C). Ferrets that received vaccines containing the M2e5x VLP produced significantly higher HI titers at days 7 and 14 post challenge than the split alone group (Split+M2e5x, M2e5x, Fig. 2C). Ferrets receiving vaccines containing split CA/08 modestly increased HI titers to CA/08 upon BR/59 challenge; in the other groups, anti-CA/08 titers remained unchanged after challenge (Fig. 2B).

In addition, we tested all sera collected after the full immunization regimen (challenge day 0) as well as on days 7 and 14 post-challenge for HI activity against a number of H3, H5 and H7 subtype viruses (data not shown). As expected, neither the combination of M2 and inactivated vaccine (challenge day 0), nor exposure to challenge virus (days 7 and 14 post-challenge) induced HI cross-reactivity with any of these distantly-related strains.

Changes in leukocyte subsets after challenge

A marked, transient lymphopenia and granulocytosis was apparent in all treatment groups 1 and 2 days post-challenge (Fig. 5A–D). Both groups receiving M2e5x VLPs exhibited an earlier decline in peripheral CD4⁺ and CD8⁺ T cells, with their nadir occurring one day postinfection rather than 2 days (Fig. 5B–C). Similarly, these groups showed earlier granulocytosis (CD11b⁺ve; presumably mainly neutrophils), with its peak on day 1 rather than day 2 (Fig. 5D). B cells (CD79A⁺) also showed moderate changes after the challenge

(data not shown). However, as described previously [19], B cells showed significant fluctuation and variability throughout the post-challenge period and did not demonstrate consistent differences among the groups.

From day 7 on, vaccinated animals with M2e5x alone and Split+M2e5x had a faster recovery of T cells (Fig. 5A). Significant recovery in percentage of total counts was observed for both CD4 and CD8 T cells (Fig. 5B–C). We have previously shown that total peripheral leukocyte counts remain approximately constant after viral challenge, so that the granulocytosis and lymphopenia are both relative and absolute changes in numbers [19].

Discussion

In 2009, a novel A(H1N1) influenza virus entered the human population, after decades of circulating in swine. Extensive antigenic drift between this strain and human influenza viruses meant that vaccines against contemporary human A(H1N1) strains did not confer protection against the swine-origin virus, leading to a global pandemic and the complete replacement of human seasonal A(H1N1) viruses by the A(H1N1)pdm09 strain. Although seasonal A(H1N1) strains no longer circulate in humans, viruses that are antigenically related to these strains still circulate in North American swine. There now exists a population of children, under about 6 years of age, who have not been exposed to or vaccinated against seasonal A(H1N1) strains, although they may be immune to A(H1N1)pdm09 viruses, so that there is a growing possibility of a new pandemic or pseudopandemic if swine A(H1N1) viruses enter this susceptible population. Here we show that, while ferrets immunized with conventional split vaccine against H1N1pdm09 had no protection against infection with seasonal H1N1, supplementing the split vaccine with M2e5x VLPs conferred significant cross-protection against the seasonal virus.

Ferrets that were challenged with the seasonal A(H1N1) strain BR/59 after immunization with a combination of split A(H1N1)pdm09 vaccine and M2e5x VLPs had significantly less weight loss than ferrets that received split vaccine alone, or naïve ferrets. Ferrets that received M2e5x VLPs only also showed some improvement over naïve ferrets, with a more rapid weight recovery. Similarly, immunization with M2e5x VLPs appeared to reduce inflammation, as measured by changes in peripheral blood leukocytes. Due to the normal variations in temperature in ferrets, alterations in leukocytes can provide a more detailed view of the inflammatory impact of influenza virus infection and vaccine protection than fever measurement. In particular, ferrets receiving M2e5x VLPs showed a more rapid and complete recovery of T cells (both CD4 and CD8) following the initial period of lymphopenia. Interestingly, both groups that received M2e5x VLPs showed a more rapid initial loss of peripheral lymphocytes, with the lowest levels of peripheral T cells occurring on day 1 instead of day 2 in these groups. This peripheral loss may reflect rapid trafficking of primed influenza-specific T cells from the periphery to local sites of infection, leading to earlier reduction of viral replication through both humoral and cellular immune responses. Although assessment of ferret cellular immunity is still in its infancy due to a lack of ferret-reactive reagents, experiments are under way to test the specific cellular immune response in vaccinated versus unvaccinated ferrets.

We have previously shown significant efficacy of M2e vaccines against influenza in mice, in which intramuscular vaccination with M2e5x VLP induced M2e-specific humoral and cellular immune responses and conferred cross-protection as evidenced by lower weight loss and higher survival rates [14]. Conventional split inactivated vaccines confer protective immunity mainly through virus-neutralizing antibodies targeting HA. In contrast, M2 immunity does not induce virus-neutralizing antibodies and typically permits infection, even when the severity of the disease and the extent of virus shedding are reduced. As an alternative approach, supplementing conventional vaccination with M2e5x VLPs showed significantly improved cross-protection compared to either vaccine alone in mice.

In previous experiments in BALB/c mice, M2e5x VLP addition also demonstrated adjuvant effects in conjunction with split vaccine, increasing IgG antibody responses specific for split vaccine virus by 2 to 4 fold compared to the split vaccine alone [23]. In those experiments, we observed that mice with M2e5x VLP-supplemented split vaccination shifted the virus-specific immune responses toward T helper type 1 (Th1) IgG2a isotype antibodies, compared to the predominately Th2-associated IgG1 isotype antibodies induced by split vaccine alone. In addition, M2e5x VLP-supplemented split vaccination of mice induced Th1 type IFN- γ cytokine producing splenocytes and lung cells at significantly higher levels in response to *in vitro* stimulation with M2e peptide or virus, than split alone vaccination [23]. While this adjuvant effect is a further potential benefit to the combined M2e5x VLP-split vaccine approach, in the present experiments we offset the adjuvant effects of M2e5x VLP by repeated vaccination with the split vaccine alone.

Although mice are a convenient animal model to demonstrate cross-protection after influenza M2e-based vaccination, ferrets are considered to be the best animal model for predicting influenza pathogenesis and vaccine efficacy in humans, since the clinical signs and immune response in these animals closely mimics those in humans. Both seasonal A(H1N1) viruses, which circulated in humans before 2010, and the pandemic variant of A(H1N1), which is now prevalent in humans, infect ferrets without preadaptation to this species, and it has been shown that in ferrets as in humans conventional split vaccines do not confer cross-protection between these strains [24], due to the many decades of separate antigenic evolution in humans versus swine these strains experienced. As with mice, ferrets vaccinated with both split vaccine and M2e5x VLPs showed significant cross-protection, recovering body weight and clearing the virus from the upper respiratory tract significantly faster than animals immunized with naïve and split vaccine alone. The M2e5x VLP alone group also showed some protective effects on lowering and clearing virus in the upper respiratory tracts compared to split alone or naïve ferrets, but did not confer clinical benefits in terms of fever, weight loss, or markers of systemic inflammation. Interestingly, we found that inclusion of M2e5x VLPs in the split vaccination resulted in significantly increasing the immunogenicity of split vaccine in ferrets compared to split vaccine alone, as measured by serological assays (HI assays). Based on the effectiveness of M2e5x VLPs + split vaccine against divergent H1N1 strains, further experiments are under way to test this combination against more pathogenic and distantly-related influenza strains.

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Conflict of interest statement

Sang-Moo Kang and Min-Chul Kim are inventors in the pending patents on universal influenza vaccine based on heterologous multiple M2e proteins. The authors (SMK, MCK) are listed as inventors in the following patent filed. US Patent 61/738,139: "Universal Influenza Vaccine based on heterologous multiple M2e proteins". US Application No.: 61/722,602 (Filing Date: November 5, 2012). US Application No.: 61/738,139. The authors confirm that this does not alter their adherence to all Vaccine policies on sharing data and materials, as detailed online in the guide for authors.

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Highlights

- High immunogenicity of VLP containing five tandem repeats of M2 proteins in ferrets
- M2e5x VLP with or without split vaccine improves cross protection in ferrets
- Adjuvant-like effects of M2e5x VLP on split vaccine in ferrets.
- Ferrets with Split + M2 VLP show significantly improved cross-protection.

		Immunization phase					Challenge phase																				
Days post-challenge															-3	0	1	2	3	4	5	6	7	9	11	13	14
Days post-prime		0	21	35	47	50	61	64	65	66	67	68	69	70	71	73	75	77	78								
Immunization	M2e5x	√	√	√																							
	Split	√	√	√		√																					
	Split+M2e5x	√	√	M2e5x only																							
	Naïve (PBS)	√	√	√																							
Serology	ELISA	√	√	√	√	√	√	√												√				√			
	HI	√	√	√	√	√	√	√												√				√			
Challenge	BR/59							√																			
Clinical signs	Nasal wash							√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√			
	PBL analysis							√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√			
	Body weight																										
	Temp																										
	Temperature																										

Figure 1. Outline of experimental protocol

Male Fitch ferrets (N = 5 per group) were immunized with M2e5X-VLPs alone, conventional monovalent split vaccine against H1N1pdm09, a combination of M2e5X and split vaccine, or mock immunized with PBS, and received booster immunizations as indicated. On day 64 after priming, all animals were challenged with 10^6 PFU of influenza A/Brisbane/59/2007 (BR/59). Body weight and temperature was monitored as indicated. Blood samples were collected for analysis of peripheral blood leukocytes (PBL) on days 0–7, 9, 11 and 13 relative to day of challenge. To assess antibody responses (HI and ELISA assays), serum were collected at intervals as shown.

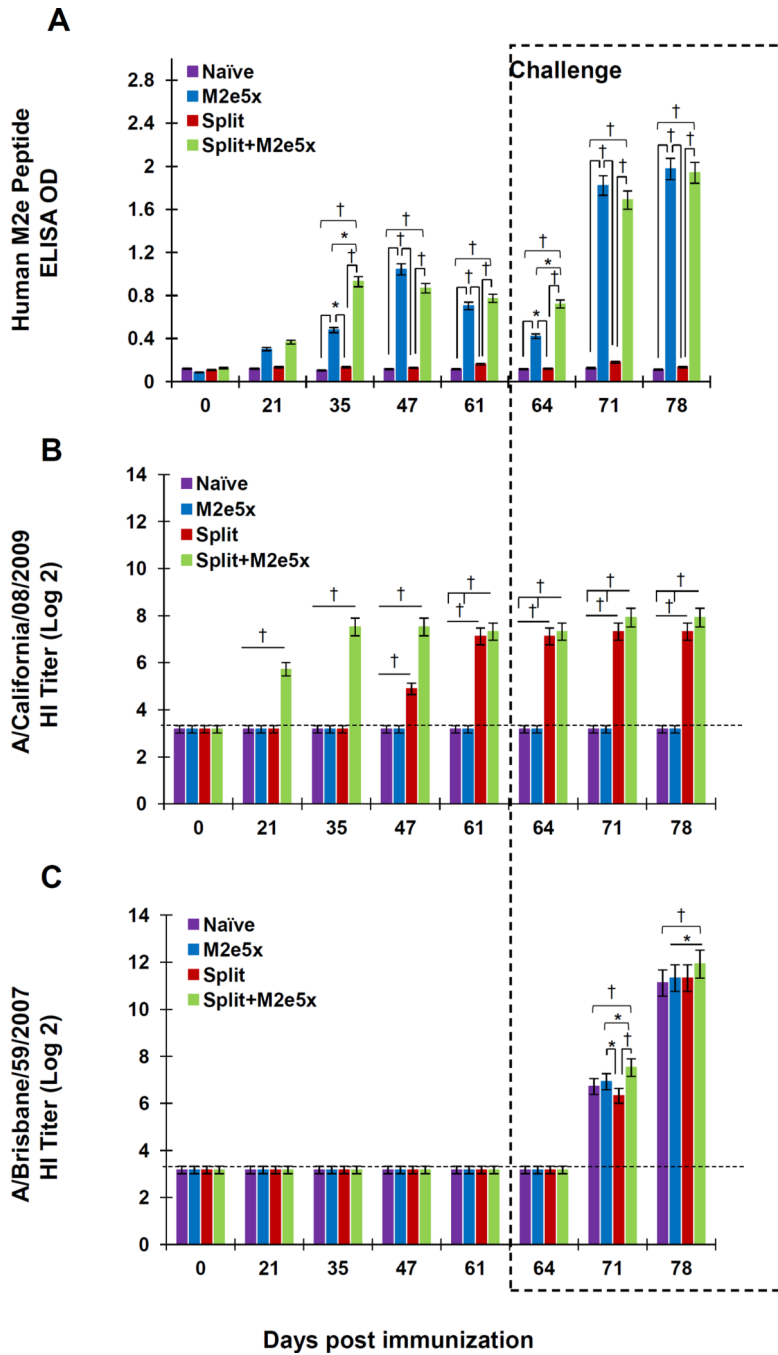


Figure 2. Serological response to immunization

(A) The antibody response against to M2e peptide was measured by an ELISA using synthetic human peptides. (B, C) Hemagglutination inhibition (HI) serum antibody titers were measured using (B) A/California/08/2009 (H1N1pdm), or (C) A/Brisbane/59/2007. “Naïve”, unvaccinated animals; “M2e5x”, vaccinated animals with M2e5x VLPs alone; “Split”, vaccinated animals with monovalent 2009 H1N1 split inactivated virus vaccine alone; “Split+M2e5x”, vaccinated animals with monovalent 2009 H1N1 split inactivated virus vaccine supplemented with M2e5x VLPs. A p value < 0.05 was used as the cutoff for

statistical significance (* $p < 0.05$; † $p < 0.001$). Error bars represent 95% confidence intervals, calculated using compound symmetry covariance to pool variability across groups. The dashed line in panels B and C represents the limit of detection (HI titers = 10).

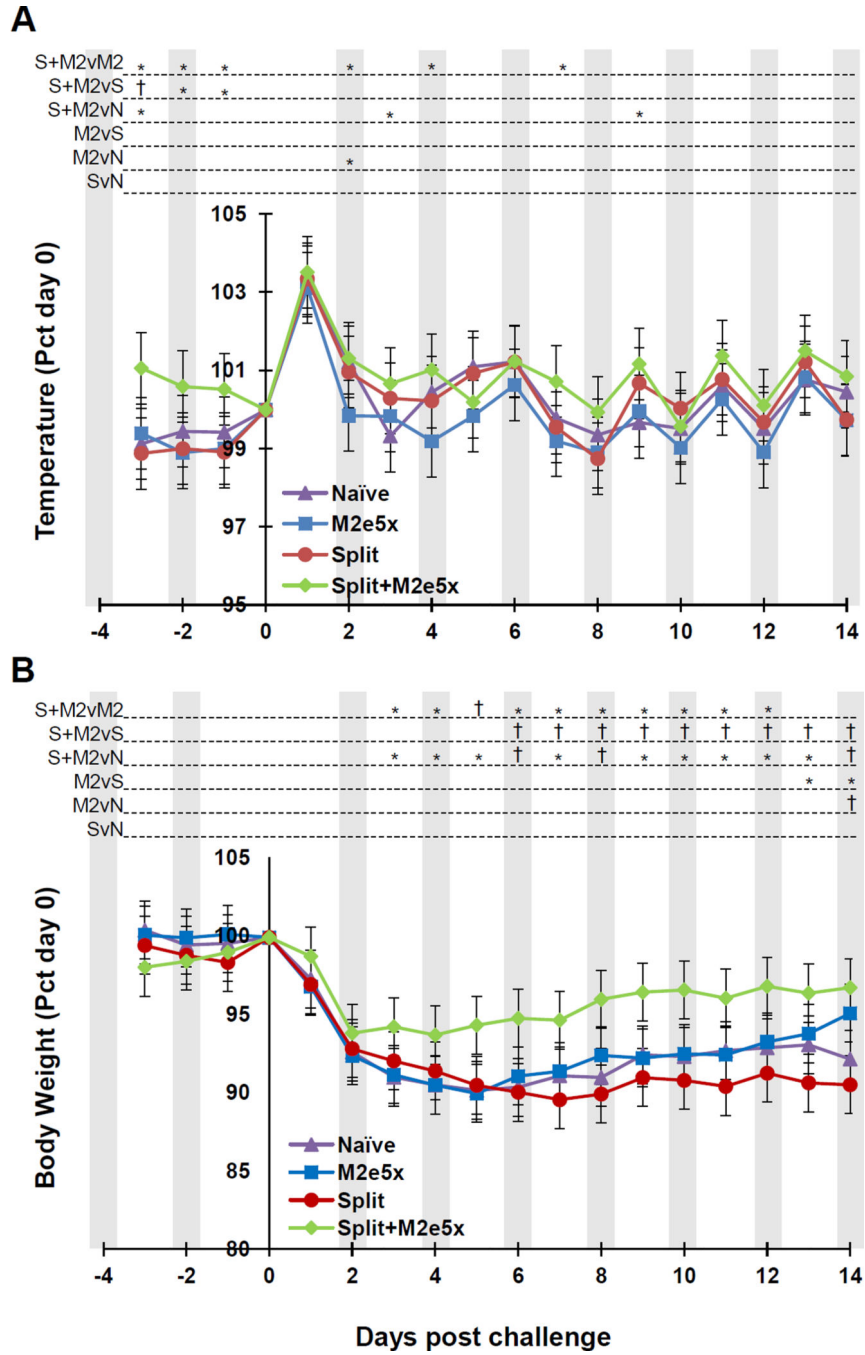


Figure 3. Clinical responses to infection
 Ferrets were challenged with 10^6 PFU of BR/59 intranasally 64 days after the initial immunization. (A) Body temperature and (B) body weight were recorded daily, including three days prior to challenge to establish baseline values. Data shown are normalized to the individual animals' weight or temperature on the day of challenge (day 0), and group averages are shown. "Naive", unvaccinated animals; "M2e5x", vaccinated animals with M2e5x VLPs alone; "Split", vaccinated animals with monovalent 2009 H1N1 split inactivated virus vaccine alone; "Split+M2e5x", vaccinated animals with monovalent 2009

H1N1 split inactivated virus vaccine supplemented with M2e5x VLPs. Statistical significance is indicated above each time point (* $p < 0.05$; † $p < 0.001$): S+M2vM2, M2+Split compared to M2 alone; S+M2vS: M2+Split compared to split alone; S+M2vN: M2+Split compared to unvaccinated animals; M2vS: M2 compared to split alone; M2vN: M2 compared to unvaccinated animals; SvN: split alone compared to unvaccinated animals. Error bars represent 95% confidence intervals.

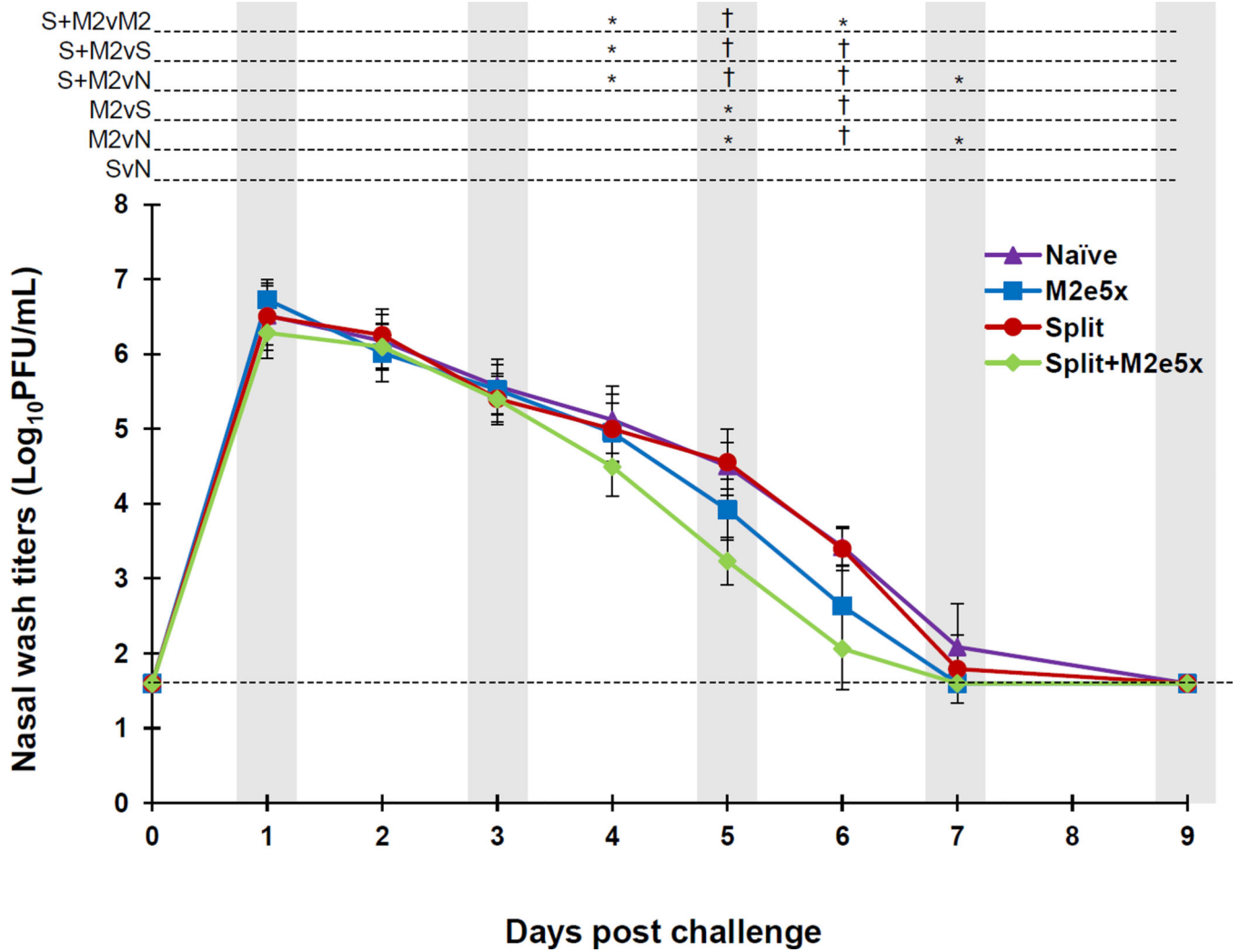


Figure 4. Virus shedding following infection

Virus titers in ferret nasal washes were determined on days 1–7 and 9 post-challenge. “Naïve”, unvaccinated animals; “M2e5x”, vaccinated animals with M2e5x VLPs alone; “Split”, vaccinated animals with monovalent 2009 H1N1 split inactivated virus vaccine alone; “Split+M2e5x”, vaccinated animals with monovalent 2009 H1N1 split inactivated virus vaccine supplemented with M2e5x VLPs. Statistical significance is indicated above each time point (* p = 0.05; † p = 0.001): S+M2vM2, M2+Split compared to M2 alone; S+M2vS: M2+Split compared to split alone; S+M2vN: M2+Split compared to unvaccinated animals; M2vS: M2 compared to split alone; M2vN: M2 compared to unvaccinated animals; SvN: split alone compared to unvaccinated animals. Error bars represent 95% confidence intervals. The limit of detection (100 PFU per 1 ml) is indicated with a dashed line.

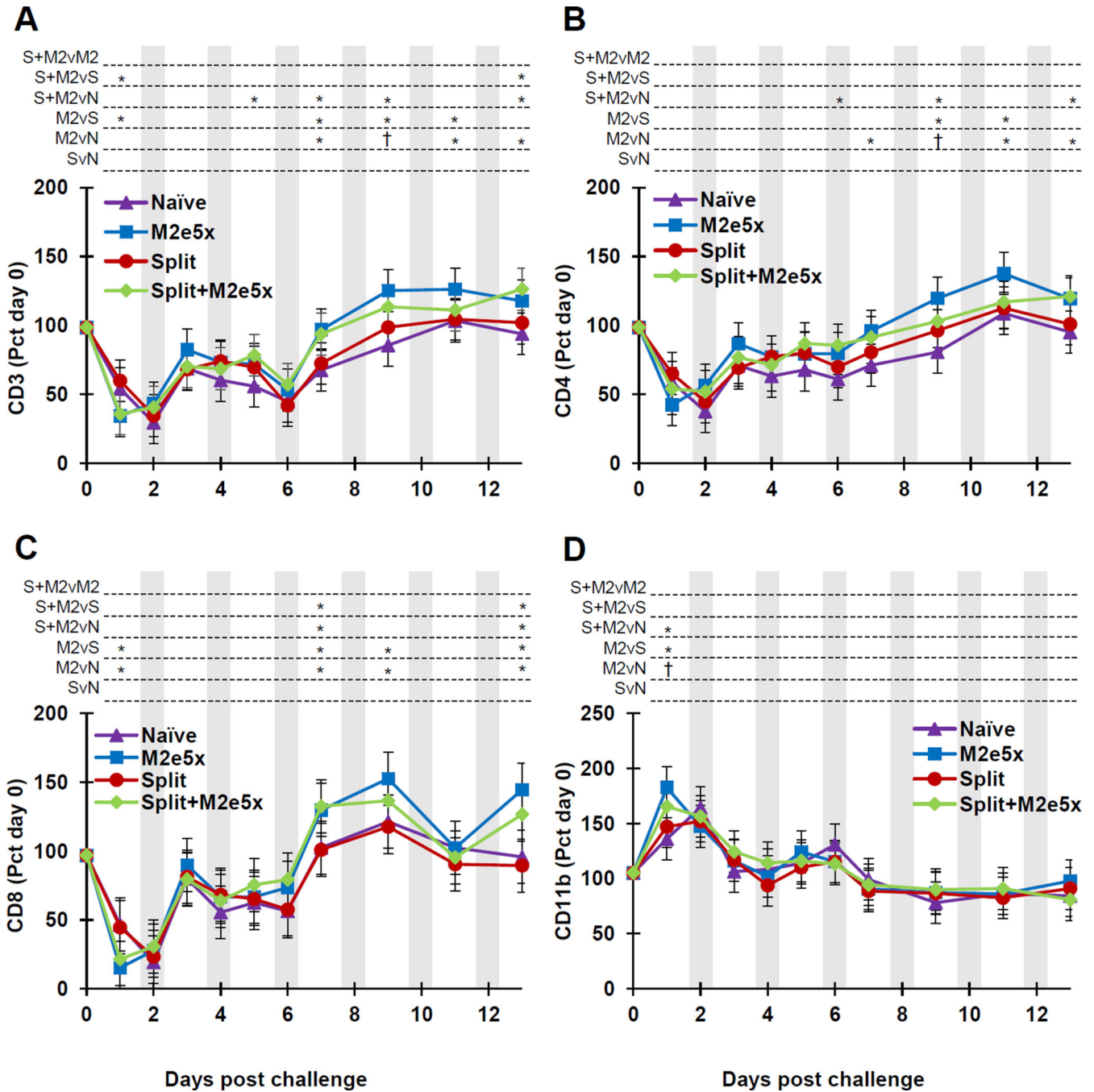


Figure 5. Peripheral blood leukocyte subsets following infection
 Ferrets were bled on days 0–7, 9, 11, and 13 relative to the day of viral challenge, and cells were stained and analyzed by flow cytometry as described in the text. (A) T cells (CD3+), (B) TH cells (CD3+/CD4+), (C) cytotoxic T lymphocytes (CD3+/CD8+), and (D) granulocytes (predominately neutrophils (CD11b+) cells) were measured and expressed relative to the day 0 values as 100%. “Naïve”, unvaccinated animals; “M2e5x”, vaccinated animals with M2e5x VLPs alone; “Split”, vaccinated animals with monovalent 2009 H1N1 split inactivated virus vaccine alone; “Split+M2e5x”, vaccinated animals with monovalent

2009 H1N1 split inactivated virus vaccine supplemented with M2e5x VLPs. Statistical significance is indicated above each time point (* $p < 0.05$; † $p < 0.001$): S+M2vM2, M2+Split compared to M2 alone; S+M2vS: M2+Split compared to split alone; S+M2vN: M2+Split compared to unvaccinated animals; M2vS: M2 compared to split alone; M2vN: M2 compared to unvaccinated animals; SvN: split alone compared to unvaccinated animals. Error bars represent 95% confidence intervals.

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