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RESEARCH PAPER

UniFluVec influenza vector induces heterosubtypic protection in ferrets after intranasal administration despite high attenuation

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ABSTRACT

BACKGROUND: Current influenza vaccines primarily elicit strain-specific immunity, providing limited protection against heterologous influenza strains.
OBJECTIVE: This study aimed to develop a novel live attenuated influenza vaccine candidate with enhanced broad-spectrum protection against heterologous strains.
METHODS: A new live attenuated influenza vector, UniFluVec, was constructed based on the A/Puerto Rico/8/1934 (H1N1) (PR/8/34) strain, incorporating surface antigens from the A/Mississippi/10/2013 (H1N1pdm) strain. The NS genomic segment of UniFluVec was modified to express a truncated NS1 protein (124 amino acids) fused to conserved sequence from the HA2 subunit found in both influenza A and B viruses. To further enhance attenuation, the *nep* gene of PR/8/34 was replaced with its counterpart from the A/Singapore/1/57 (H2N2) strain. The protective efficacy of UniFluVec was tested in ferrets against the heterologous seasonal A/Saint Petersburg/224/2015 (H3N2) strain, following either single or double immunizations, and compared to the reassortant differing from UniFluVec by the presence of an intact NS fragment (WTNS1).
RESULTS: UniFluVec demonstrated full attenuation in ferrets, causing no clinical symptoms, weight loss, or fever when administered intranasally at a dose of 7.8 $\log_{10}EID_{50}$. Replication in the nasal tissues was significantly reduced compared to the control WTNS1 reassortant virus. Although UniFluVec elicited lower hemagglutination inhibition (HAI) antibody titers after a single immunization compared to WTNS1, it significantly accelerated the clearance of the heterologous H3N2 virus from the respiratory tract after challenge. The protective effect of a single immunization was comparable to double vaccination and superior to that observed with WTNS1.
CONCLUSION: The novel UniFluVec vector demonstrated excellent safety in ferrets after intranasal administration and conferred effective protection against a heterologous strain following a single immunization.

Keywords: influenza virus, vaccine, truncated NS1, heterologous protective immunity

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INTRODUCTION

According to the World Health Organization (WHO), approximately one billion people each year are infected with seasonal influenza virus, resulting in 3 to 5 million severe cases annually (https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)). The high mutation rate of influenza viruses, driven by the lack of proofreading activity in their RNA polymerases and the selective pressure of herd immunity, leads to frequent changes in the virus's surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). This antigenic drift results in the emergence of new variants, which are responsible for annual influenza epidemics [1].

The standard method of influenza prevention relies on yearly vaccinations with licensed live attenuated or inactivated vaccines. Inactivated influenza vaccines, which dominate the market, primarily stimulate a strain-specific antibody response and B cell memory but fail to effectively prime cross-reactive $CD8^+$ cytotoxic T lymphocytes (CTLs). As a result, they offer limited protection against emerging homosubtypic or heterosubtypic strains [2]. In contrast, live attenuated influenza vaccines (LAIVs) have been shown to induce broader protection in laboratory animals by stimulating local mucosal IgA production and generating cross-reactive T-cell responses, including the formation of respiratory resident memory T-cells (Trm) [3-6]. However, clinical studies have demonstrated that while LAIVs can protect against new viruses of the same subtype, they are often insufficient to protect against heterosubtypic variants [7, 8].

To enhance the protective efficacy of live influenza vaccines, we developed a novel vaccine platform utilizing viruses with deleted or truncated NS1 proteins [9]. This approach has led to the development of several influenza vaccine candidates and viral vectors [10-14]. Previous research has shown that influenza viruses with shortened NS1 proteins promote the production of polyfunctional $CD8^+$ and $CD4^+$ Trm cells, which recognize a broad range of conserved influenza epitopes [15-16]. Furthermore, vaccine candidates with deleted *ns1* gene have been evaluated in multiple clinical trials, demonstrating excellent safety profiles and robust immunogenicity [17, 18].

In this study, we aimed to further improve the heterosubtypic protective efficacy of live attenuated influenza vaccines by modifying the NS genomic segment. We developed a novel vaccine candidate, UniFluVec, designed for production in embryonated chicken eggs. The vector was engineered to express a truncated NS1 protein consisting of the N-terminal 124 amino acids (NS_{124}). Additionally, the NS_{124} protein was fused with 21 N-terminal amino acid (aa) fragment derived from the HA2 subunit of the influenza B/Lee/40 virus, which contains the highly conserved fusion peptide found in both influenza A and B viruses, as well as a conserved B-cell epitope of the influenza A NP protein [19, 20]. To further improve the safety profile of UniFluVec, the original *nep* gene of the A/Puerto Rico/8/1934 (H1N1) (PR/8/34) virus was replaced with the corresponding gene from the A/Singapore/1/57 (H2N2) virus [21]. This genetic modification increased viral attenuation, allowing for the administration of a high dose (7.8 $\log_{10}EID_{50}$, or 50% embryo infectious dose) of the vector in ferret immunization studies.

The primary goal of this investigation was to evaluate the protective efficacy of the UniFluVec vector against a heterologous H3N2 virus in ferrets following either single or double intranasal immunization. Additionally, we compared the protective efficacy of UniFluVec to a control virus carrying the wild-type (wt) NS1 protein (WTNS1) to assess the impact of NS segment modifications on the immune response.

MATERIALS AND METHODS

Epidemic virus

Epidemic wild-type influenza challenge virus A/Saint Petersburg/224/2015 (H3N2) was obtained from the collection of Smorodintsev Research Institute of Influenza (St. Petersburg, Russia). The virus was grown and titrated on Madin-Darby canine kidney (MDCK) cells in DMEM medium (Gibco, USA) at 37°C and 5% CO_2 with the addition of 1 μg/ml trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone, TPCK-treated, Sigma-Aldrich, USA). The infectious titer was expressed in $\log_{10}TCID_{50}$/ml.

Virus vector

The recombinant influenza virus vector UniFluVec was constructed by the reverse genetic method [22, 23] using synthetic plasmids generated by GeneArt (Germany). The HA and NA fragments originated from A/Mississippi/10/2013 (H1N1pdm) influenza virus and PB2, PB1, PA, NP, M and NS fragments – from PR/8/34 strain. The NS fragment contained a chimeric *ns1* gene encoding the NS_{1-124} protein of PR/8/34 virus fused to 21 aa sequence of the fusion peptide of the influenza B/Lee/1940 virus and the $NP_{243-251}$ peptide of PR/8/34 virus. NEP open reading frame (ORF) was replaced by

its equivalent from A/Singapore/1/57 (H2N2) influenza strain. Viral vector UniFluVec was obtained employing electroporation of Vero cells followed by propagation in specific-pathogen free (SPF) embryonated chicken eggs (CE, Valo BioMedia, Germany) as described earlier [21].

Influenza virus WTNS1 was used as a positive control of immunization. This virus is the 6/2 reassortant containing HA and NA fragments from virus A/Mississippi/10/2013 (H1N1pdm), while PB2, PB1, PA, NP, M, NS fragments originate from PR/8/34 (H1N1). WTNS1 virus was obtained by the same method as UniFluVec using the same set of plasmids except the pHW-PR8-NS to obtain wt NS fragment encoding original NS1 and NEP proteins of PR/8/34 virus.

UniFluVec and WTNS1 viruses were grown in 9-10-day-old CE at 34°C for 48 h. The infectious activity of the viruses was assessed by titration in CE and expressed in $\log_{10}EID_{50}$/ml. The genetic stability of the vector was controlled by consecutive 10 passages in CE with subsequent control of insertion.

Animal experiment

The animal experiment was carried out at MediTox s.r.o. (Czech Republic) in compliance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123), Collection of laws No. 246/1992, inclusive of the amendments, on the Protection of animals against cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Czech Collection of laws No. 419/2012 as amended, on keeping and exploitation of experimental animals. The study was approved by the Institutional Animal Care and Use Committee (IACUC) and the Committee for Animal Protection of the Ministry of Industry and Trade of the Czech Republic (49/2015). Procedures used for experiments were designed to conform to accepted practices and to minimize or avoid causing pain, distress, or discomfort to the animals.

Ferrets were housed individually in cages (MIDWEST Homes for Pets, USA). Environmental conditions (temperature and relative humidity) were monitored and recorded daily. Room temperature was within the range of 15-21°C, relative humidity 20-85%, light/dark regime 12 h. Access to every room was under Biosafety level 2 (BSL-2) conditions.

The animals were fed a standard pelletized diet (Calibra Cat) with monitored quality during the acclimation and study periods. Water of monitored quality was supplied ad libitum during the acclimation and study periods. Ferrets were acclimated for 8 days. Only animals in good health conditions were used for the study.

SPGN buffer (6% w/v Sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM L-glutamate, 75 mM NaCl, Sigma-Aldrich , USA) served as the vehicle for the challenge, immunization, and nasal washings. At day 1 the group of sedated (isoflurane inhalation) ferrets (n=9) was immunized intranasally (i.n.) with 0.5 ml of UniFluVec at a dose of 7.8 $\log_{10}EID_{50}$/animal. At D22 the group of UniFluVec primed animals was boosted again with UniFluVec (7.8 $\log_{10}EID_{50}$/animal, referred to as UniFluVec x2) whereas the remaining three groups of ferrets received only a single i.n. immunization either with WTNS1 virus (n=9) (7.8 $\log_{10}EID_{50}$/animal), UniFluVec (7.8 $\log_{10}EID_{50}$/animal, referred to as UniFluVec x1) (n=9) or with SPGN (n=8) buffer in a volume of 0.5 ml/ferret. After another three weeks (D43), all ferrets received light sedation and were subsequently inoculated with the challenge virus A/Saint Petersburg/224/2015 i.n. in a dose of 4.2 $\log_{10}TCID_{50}$/animal.

At D24, D26, and D28 after immunization and D45, D47, and D49 after the challenge infection, nasal washings were collected from all ferrets. Clinical observation was determined on days D21-33 and D42-53. Fever was determined at days D18-33 and at days D39-53. Body weight measurements were conducted at days D -8, D1 (UniFluVec x2 group), between days D20-33 and D41-53. Serum samples were collected at days D -8, D21 (UniFluVec x2 group) and D42.

Assessment of clinical symptoms

All animals underwent weighing procedures at D-8 and during two intervals: from D20 to D33, and from D41 to D53. The animals of the UniFluVec x2 group were weighed at D1 before the primary immunization with UniFluVec. All animals were monitored daily for mortality and general health status (D -8-53). Clinical symptoms including sneezing, nasal discharge, activity status, neurological symptoms, and inappetence were monitored twice a day and the daily score was assigned as the sum of morning and evening scores. All scores were summed for each ferret over the period of observation (D21-33 and D42-53), divided by the number of days (13 and 12 respectively), and averaged per group to get a mean daily score. Neurological symptoms of animals (e.g., ataxia, limb paresis, torticollis, abnormal behavior, tonic movements, stereotypes, etc.) were also assessed as well as inappetence (yes/no). The activity was monitored for 30 min, with scores as follows: alert and playful (0), alert but playful only when stimulated (1), alert but not playful when stimulated (2), and neither alert nor playful when stimulated (3). Sneezing signs were recorded over a 10-minute period, with scores as follows: no sneezing (0), sneezing 1-10 times (1), sneezing more than 10 times (2),

and respiratory distress (3). Nasal discharge was assessed as follows: no discharge (0), moderate serous discharge (1), severe serous discharge (2), moderate mucopurulent discharge (3), severe mucopurulent discharge (4). Temperature was measured twice a day (at 7-9 a.m. and 8 h after the first measurement) 15 min after awaking the ferret in the periods D18-33 and D39-53).

Determination of viral load in ferret nasal wash samples

Nasal washes were collected at days D24, D26, D28 and days D45, D47, D49. Viral load in nasal wash samples was determined by titration in MDCK cells. Cells were cultivated in DMEM medium supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, USA). For the assay, cells were seeded in 96-well plates in a volume of 100 µl/well and cultivated at 37°C and 5% CO_2 until the confluent monolayer was formed. Then the growth medium from the cells was removed and the cells were washed twice with PBS. Frozen nasal wash samples were thawed under cold running water, vortexed and kept on ice. Serial 10-fold dilutions of nasal washes were prepared in OptiPro medium (Invitrogen, USA), supplemented with 4 mM L-Glutamin, 1% antibiotic-antimycotic (Invitrogen), 2 µg/ml fungizone (Invitrogen), 25 µg/ml gentamycin, and 5 µg/ml trypsin. Then 100 µl of the diluted samples were added to the wells of a 96-well plate (in 6x repeats) with freshly washed cells. Plates were incubated at 37°C and 5% CO_2 for one week and then scored microscopically for the presence of a cytopathic effect. Virus titers were calculated by the Reed and Muench method and expressed as $\log_{10}TCID_{50}$/ml.

Hemagglutination inhibition assay (HAI)

Collection of serum samples was performed at D -8, D21 and D42. Blood samples were drawn under anesthesia (Midazolam 0.5 mg/kg + Medetomidine 0.08 mg/kg, intamuscularly), centrifuged (6000 rpm for 10 min) and serum was removed and frozen at -20°C. To destroy unspecific inhibitors serum samples were mixed with 3 volumes of receptor-destroying enzyme (RDE; Denka Seiken, Japan) and treated according to the manufacturer's instructions. The starting sample dilution was 1:4. Each ferret serum (50 µl) was added to the first well of the 96-well plate. To the rest of the wells, 50 µl of PBS (Gibco, USA) was added. Serial twofold dilutions of each serum sample including a negative control sample were made. Then, 50 µl of the prepared A/Mississippi/10/2013 (H1N1pdm) virus (4 HA units/50 µl solution) was added to the 96-well plate following incubation for 40 min at room temperature. After that 100 µl of 0.8% chicken erythrocytes (freshly prepared) were added and plates were incubated for 60-70 min at room temperature. The highest dilution of serum, which completely inhibits hemagglutination of erythrocytes, was considered as the HAI titration endpoint. The reciprocal of the serum dilution in the last well with no hemagglutination was considered as serum HAI titer. Antibodies to the challenge virus A/Saint Petersburg/224/2015 (H3N2) were assessed by the same.

Statistical analysis

The parameters of distribution were assessed with the Shapiro-Wilk normality test. Group means and standard deviations (SD) were calculated. For group comparisons Kruskal-Wallis One-Way ANOVA test for non-parametric data was used. The differences were considered significant at $p<0.05$. GraphPad Prism program version 10.2.0 was used for analyses.

RESULTS

Vaccine vector structure

The UniFluVec vector and the control WTNS1 virus were both constructed as 6/2 reassortants, incorporating surface glycoproteins HA and NA from an influenza virus of the H1N1pdm lineage. Specifically, the HA and NA segments were sourced from the A/Mississippi/10/2013 (H1N1pdm) influenza virus, while the remaining genomic segments were derived from the PR/8/34 strain.

The primary distinction between the two viruses is that UniFluVec carries a chimeric NS fragment encoding an NS1 protein truncated to 124 aa. This truncated NS1 protein is further modified by the addition of the N-terminal sequence from the HA2 subunit of the influenza B virus, as well as a conserved B-cell epitope, $NP_{243-251}$ (RESRNPGNA), from the influenza A virus (Fig. 1). Moreover, the *nep* gene of PR/8/34 was replaced with the *nep* gene from A/Singapore/1/57 (H2N2), resulting in a temperature-sensitive (ts) phenotype and enhanced attenuation, as previously demonstrated [21].

Safety of UniFluVec vector and WTNS1 virus for ferrets

Groups of ferrets were immunized intranasally with the vector UniFluVec at a dose of 7.8 $\log_{10}EID_{50}$/animal once (UniFluVec x1) or twice (UniFluVec x2) with an interval of three weeks between immunizations. A positive control group was immunized with an analogous 6/2 reassortant WTNS1 (H1N1) possessing an intact NS genomic segment at a dose of 7.8 $\log_{10}EID_{50}$/animal. The negative control group received SPGN buffer. The experimental scheme is presented in Fig. 2.

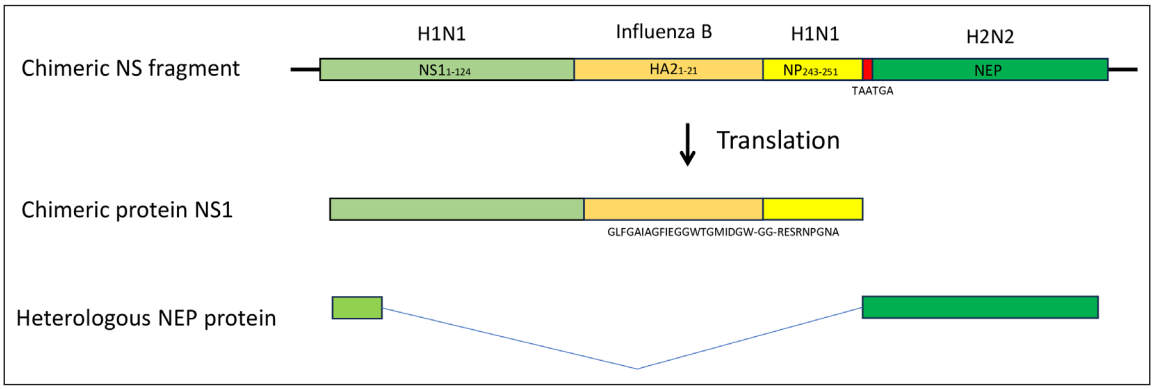

Fig. 1. Schematic representation of the chimeric NS segment. The chimeric NS segment includes the truncated *ns1* gene encoding the $NS1_{1-124}$ protein, along with the $HA2_{1-21}$ fusion peptide of influenza B/Lee/40 linked to the $NP_{243-251}$ peptide of PR/8/34 virus. The gene coding NEP protein originates from A/Singapore/1/57 (H2N2) virus.

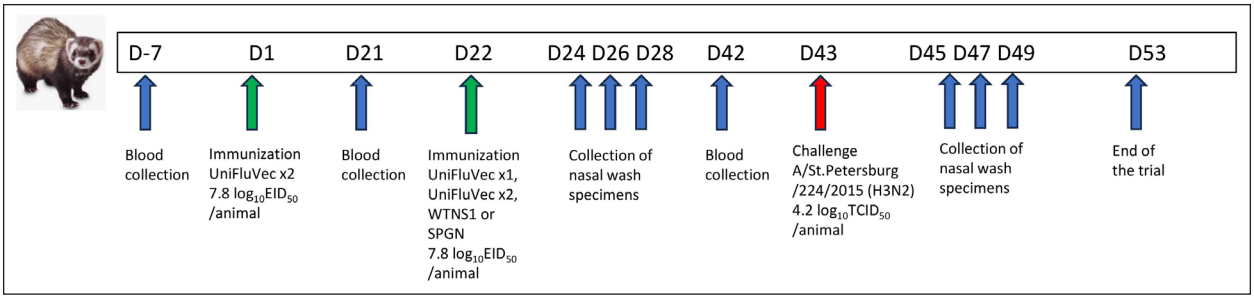

Fig. 2. The scheme of the experiment to assess the protective potential of the UniFluVec vector in the ferret model. The days of the experiment are designated as D.

No clinical findings were observed in animals after immunization with any of the viruses. Nasal discharge, sneezing, and activity symptom scores of immunized animals did not differ from those in the control group (SPGN) during the observation period after the first or second immunization (data not shown). Furthermore, there was no decrease in weight or significant increase in temperature observed in ferrets during the postimmunization period (Fig. 3A, B). Thus, the resulting viral vector was attenuated in terms of clinical manifestations upon administration at a high dose, regardless of the modification of the NS genomic fragment.

Despite this, the virus with the wt NS segment showed significantly higher titers in the respiratory tract of ferrets on days 2 and 4 post immunization, with detectable nasal titers remaining 6 days after immunization. In contrast, titers in ferrets immunized with the vaccine vector UniFluVec peaked on D2 after immunization, and the virus was undetectable by D6 (Fig. 3C). These data confirm the additional attenuation of the vaccine vector UniFluVec in direct comparison with the WTNS1 virus.

Ferrets immunized once with the WTNS1 virus developed significantly higher HAI antibody titers (GMT=439) against the H1N1pdm virus when compared to animals immunized once with UniFluVec (GMT=94), although no statistically significant difference was revealed when compared to group UniFluVec x2 (GMT=277; Fig. 3D). Antibodies to influenza virus A/Saint Petersburg/224/2015 (H3N2) were not detected in any group (data not shown).

Protective efficacy of the UniFluVec vector against heterologous A/Saint Petersburg/224/2015 (H3N2) influenza virus in ferrets

On D43, all animals were challenged with the seasonal epidemic influenza virus A/Saint Petersburg/224/2015 (H3N2). Animals in the control group immunized with SPGN buffer experienced a transient decrease in body weight until day 46, while ferrets immunized with the WTNS1 virus and UniFluVec twice showed body weight loss until day 45 (Fig. 4A). In contrast, ferrets in the UniFluVec x1 group (immunized once) were protected from transient body weight loss and began gaining weight immediately after the challenge infection. A significant difference in percent of body weight increase for both groups of vector-immunized animals compared

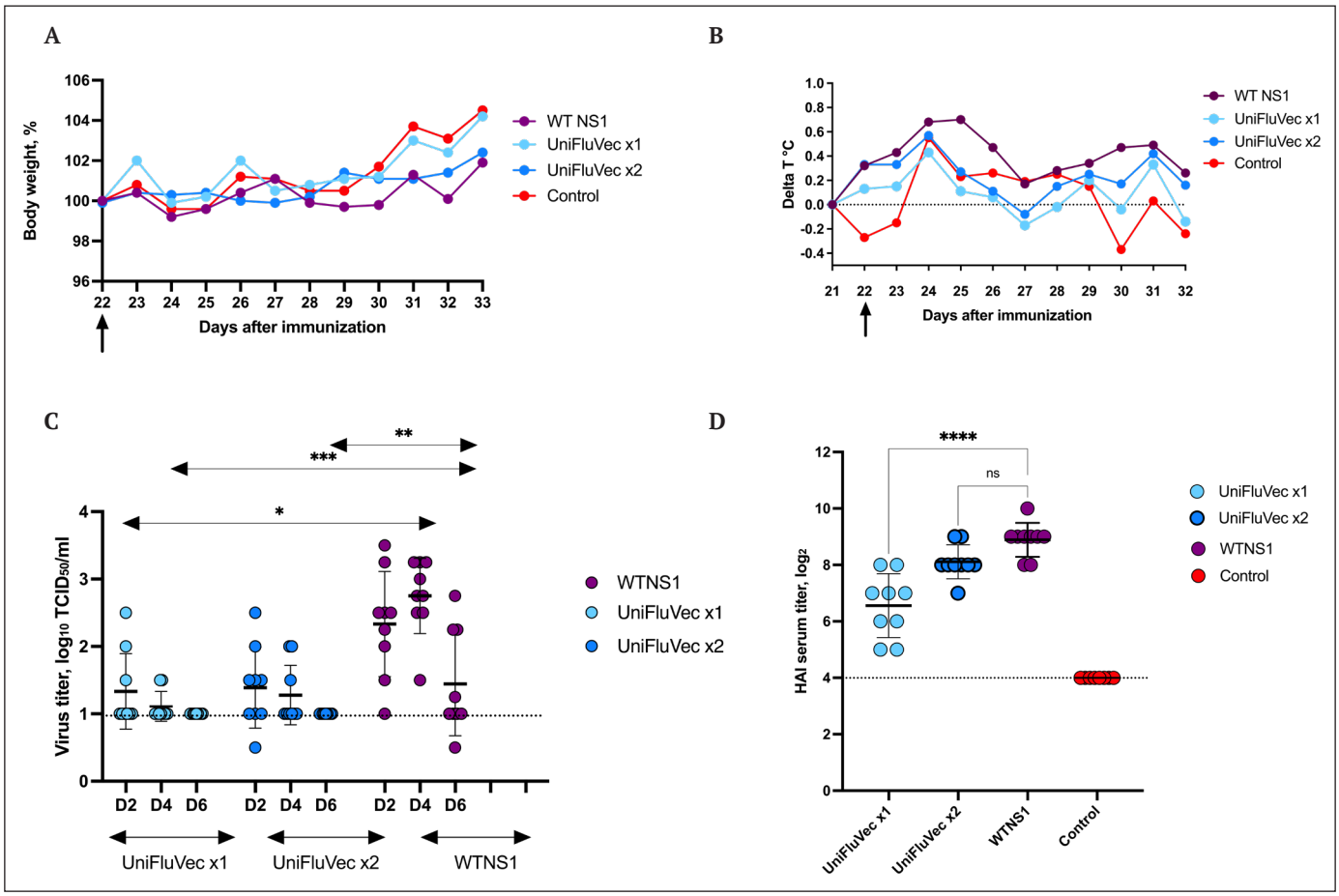

Fig. 3. Body weight, temperature, individual virus titers in ferrets' nasal washes, and HAI antibody titers post immunization with WTNS1, UniFluVec x1, UniFluVec x2 or SPGN. **A.** Body weight of animals expressed as % of change from weight on D22. **B.** Body temperature of ferrets after immunization expressed as group-specific mean body delta temperatures. **C.** Individual nasal wash titers measured 2-, 4-, and 6-days post immunization expressed in $\log_{10}TCID_{50}$/ml. **D.** Individual serum HAI titers of ferrets (against A/Mississippi/10/2013 (H1N1pdm)) three weeks after the last immunization. The arrow indicates the day of immunization; the horizontal line indicates Mean±SD; the dotted line indicates the detection limit of titration; n=8-9 animals per group; (*) p≤0.05, (**) p≤0.01, (***) p≤0.001, (****) p≤0.0001, Kruskal-Wallis One-Way ANOVA test for non-paramrtric data.

to animals from the control group was observed on D46 ($p<0.05$, $p<0.01$).

On the next day after challenge infection (D44) mean body temperature in ferrets of the control group (SPGN) and ferrets immunized with the WTNS1 virus increased on average by about 0.7°C to 0.8°C. In contrast, ferrets of both groups immunized with the UniFluVec showed an average increase of less than 0.3°C at this time point, although the difference was not statistically significant (Fig. 4B).

The peak virus replication of the A/Saint Petersburg/224/2015 (H3N2) strain in the respiratory tracts of ferrets immunized with SPGN occurred on day 2 (Fig. 4C). At this time point, only ferrets immunized twice with UniFluVec x2 showed a statistically significant decrease in virus titers in nasal washes compared to the control group ($p<0.001$). By D4 postchallenge, a significant decrease was observed in ferrets immunized once with UniFluVec.

By D6 postchallenge infection, the control group was still shedding more than 3.0 $\log_{10}TCID_{50}$/ml of virus, a level significantly higher than the viral titers observed in the other three groups of ferrets immunized with either UniFluVec or WTNS1.

Thus, intranasal immunization with the virus vector in both groups resulted in accelerated clearance of the heterologous influenza virus from the respiratory tract of ferrets. Importantly, the effect of a single immunization with a high dose of UniFluVec was nearly comparable to the double-vaccinated group and even superior to that observed with the WTNS1 virus. This was evidenced by the absence of a temperature rise and the body weight gains in the animals after the challenge infection.

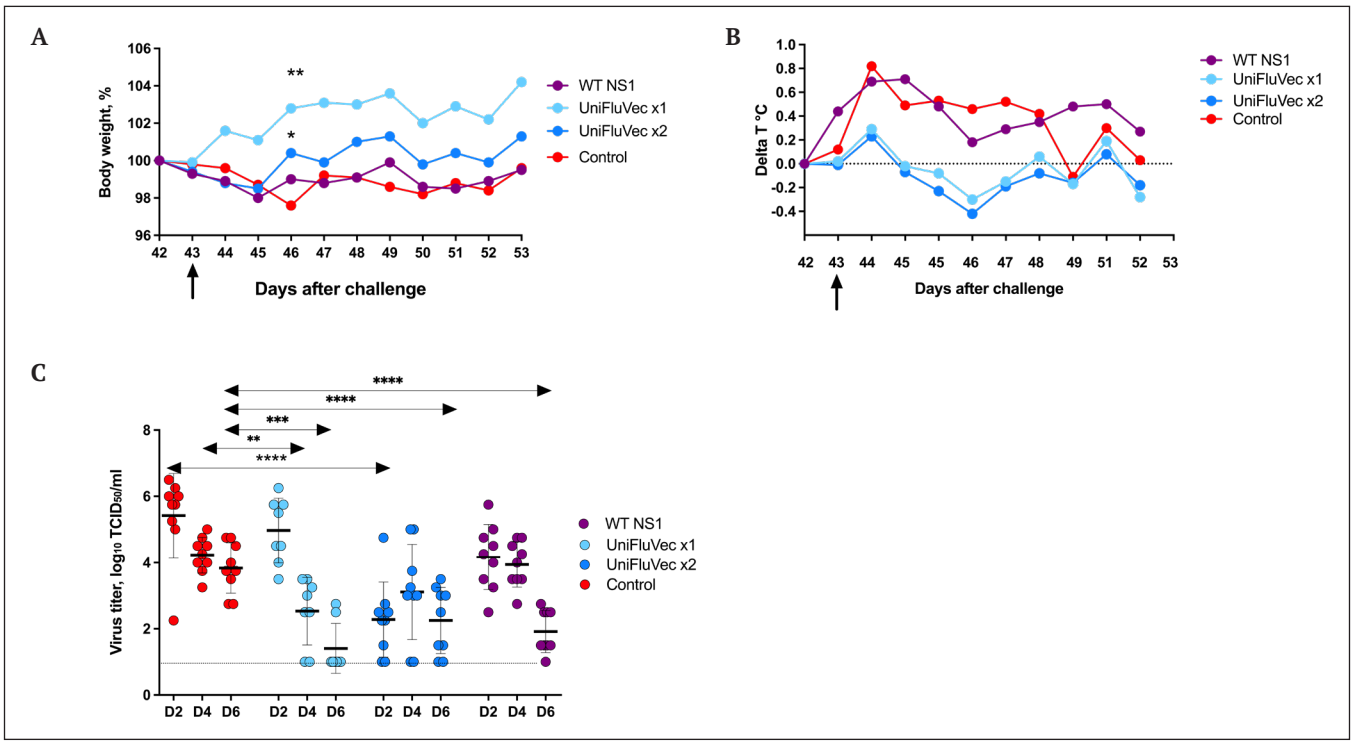

Fig. 4. Body weight, temperature, and individual virus titers in nasal wash samples of ferrets immunized with WTNS1, UniFluVec x1, UniFluVec x2 or SPGN after challenge with A/Saint Petersburg/224/2015 (H3N2) virus. **A.** Body weight of ferrets after challenge. Shown are group-specific mean body weight, %. Stars indicate a significant difference of body weight increase for vector-immunized groups compared to control group. **B.** Body temperature of ferrets after challenge expressed as group-specific mean body delta temperatures. **C.** Individual virus titers in ferrets' nasal washes 2, 4, and 6 days following the challenge infection measured in $\log_{10}TCID_{50}$/ml. The dose of challenge virus was 4.2 $\log_{10}TCID_{50}$/animal; the arrow indicates the day of the challenge infection; the horizontal line indicates Mean±SD; the dotted line indicates the limit of titration; (*) $p<0.05$, (**) $p\leq0.01$, (***) $p\leq0.001$, (****) $p\leq0.0001$, Kruskal-Wallis One-Way ANOVA test for non-parametric data.

DISCUSSION

Influenza viruses can confer heterosubtypic protection through natural infection or intranasal vaccination with live vaccines, primarily by inducing T-cell immune response against conserved viral epitopes [6, 7]. Licensed live attenuated influenza vaccines could provide significant level of protection against mismatched strains; however, vaccine efficacy would be reduced in comparison to matched strain [24-26]. This may be attributed to inadequate vaccine doses and inactivation of the vaccine virus in the nasal environment. Safety of cold-adapted influenza viruses depends on specific mutations across viral genomic segments [27], yet these mutations can potentially revert, increasing the virulence of the vaccine virus. Moreover, toxicological concerns associated with LAIVs pose challenges in increasing vaccine dosages [28]. Another factor contributing to LAIVs' efficacy issues could be the presence of immunosuppressive sequences in the NS1 protein, which hinder both innate and specific immune responses [29, 30].

To address these challenges, we developed UniFluVec, a novel vector characterized by high attenuation achieved through extensive modifications in the NS genomic segment. The modifications include replacing the *nep* gene with a counterpart from a heterologous influenza virus and truncating the NS1 protein by 50%. These genetic alterations confer a temperature-sensitive phenotype akin to cold-adapted influenza strains and promote high cytokine production typical of influenza virus NS1 mutants (data not shown). Made modifications enabled safe intranasal administration of UniFluVec in ferrets at doses as high as 7.8 $\log_{10}EID_{50}$. The present study aimed to investigate whether the level of heterologous protection conferred by an intranasal influenza vaccine candidate depends on the vaccine strain's replication in the respiratory tract of immunized animals. Therefore, we constructed an additional reassortant virus, WTNS1, which retained an unchanged NS genomic segment.

Our findings demonstrated that both UniFluVec and WTNS1 viruses were sufficiently attenuated in ferrets,

causing no clinical symptoms when administered intranasally at doses up to 7.8 $\log_{10}EID_{50}$. However, WTNS1, with its wt NS segment, exhibited prolonged replication in the respiratory tract of ferrets at significantly higher titers. To assess immunogenicity and protective efficacy, ferrets were immunized once or twice with UniFluVec or once with WTNS1, followed by a challenge infection with the epidemic seasonal H3N2 strain. WTNS1 induced significantly higher HAI serum antibody responses against vector antigens in ferrets compared to those induced by a single immunization with UniFluVec. As expected, no detectable antibody response to the H3N2 antigen was observed in any group. The highest protective efficacy against heterologous H3N2 strain was observed in ferrets that received a single immunization with UniFluVec, as evidenced by their stable weight gain and minimal temperature reactions post challenge infection. Thus, productive replication of the vaccine virus in the respiratory tract may not be necessary to induce heterosubtypic protection against influenza virus.

In our efforts to enhance UniFluVec's antigenic content, we included conserved influenza regions such as epitopes from the HA stem region, known for cross-clade neutralizing activity [31]. Antibodies against the fusion peptide have shown potent antibody-dependent cellular cytotoxicity (ADCC) against human influenza A and B viruses, leading to complete survival in mouse models after challenge with heterologous influenza viruses [32, 33]. In addition, we incorporated a conserved B cell epitope from the NP protein, specifically the fragment with 243-251 aa residues, at the C-terminus of the insertion. This epitope is highly conserved across various influenza A viruses, showing an identity of 98.4% [34, 35]. Despite these efforts, neither single nor double immunization elicited a detectable immune response to these antigens (data not shown).

We hypothesize that the induction of heterotypic immunity in our experiment is likely independent of the presence of a transgene in UniFluVec, but rather due to T and B cell responses against conserved epitopes within the vector itself. This conclusion is supported by the protective response elicited by the control virus with an unchanged NS fragment. Furthermore, the enhanced protective effect observed with UniFluVec in ferrets suggests a diminished immunosuppressive effect of the NS1 protein, as observed in our previous mouse studies (unpublished results). Importantly, our findings indicate that this protective response, independent of neutralizing antibodies, develops irrespective of vaccine virus replication in the respiratory tract of ferrets. Moreover, even a single immunization with a sufficiently high dose of highly attenuated UniFluVec candidate could induce robust heterotypic protection.

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