A bivalent live-attenuated influenza vaccine for the control and prevention of H3N8 and H3N2 canine influenza viruses

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ABSTRACT

Canine influenza viruses (CIVs) cause a contagious respiratory disease in dogs. CIV subtypes include H3N8, which originated from the transfer of H3N8 equine influenza virus (EIV) to dogs; and the H3N2, which is an avian-origin virus adapted to infect dogs. Only inactivated influenza vaccines (IIVs) are currently available against the different CIV subtypes. However, the efficacy of these CIV IIVs is not optimal and improved vaccines are necessary for the efficient prevention of disease caused by CIVs in dogs. Since live-attenuated influenza vaccines (LAIVs) induce better immunogenicity and protection efficacy than IIVs, we have combined our previously described H3N8 and H3N2 CIV LAIVs to create a bivalent vaccine against both CIV subtypes. Our findings show that, in a mouse model of infection, the bivalent CIV LAIV is safe and able to induce, upon a single intranasal immunization, better protection than that induced by a bivalent CIV IIV against subsequent challenge with H3N8 or H3N2 CIVs. These protection results also correlated with the ability of the bivalent CIV LAIV to induce better humoral immune responses. This is the first description of a bivalent LAIV for the control and prevention of H3N8 and H3N2 CIV infections in dogs.

KEYWORDS: Influenza A virus (IAV); canine influenza virus (CIV); inactivated influenza vaccine (IIV); live-attenuated influenza vaccine (LAIV); bivalent vaccine; reverse genetics; temperature sensitive (ts); cold adapted (ca); attenuated (att); protection efficacy.

INTRODUCTION

Influenza A virus (IAV) is an important pathogen that can infect a number of different hosts, including fowl, humans, pigs, horses and dogs, causing respiratory disease, provoking an elevated number of deaths, and resulting in substantial economic impact [1]. Canine influenza is a contagious respiratory disease produced by two subtypes of canine influenza viruses (CIVs): H3N8 that was transmitted from horses to dogs around 1999 in the United States (US) [2], and the avian-origin H3N2 that was transferred to dogs around 2005 in China and which has been circulating in Asia since then [3, 4]. In 2015, the first outbreak of CIV H3N2 occurred in the US [5]. While H3N8 CIV infections were only reported in dogs [6, 7], CIV H3N2 has also been isolated from cats in South Korea and more recently in the US [8-10]. Both H3N8 and H3N2 CIVs are able to rapidly spread among dogs in some geographical regions of the USA [2, 10-13], representing an important hazard for the health of the canine population in the US and worldwide. Natural and experimental dog infections with human IAVs have been reported [14, 15] and reassortant viruses between the H3N2 CIV and the 2009 human pandemic H1N1 (pH1N1) viruses were isolated from infected dogs in Korea [16-18]. Two human IAV pandemics have initiated around 1918 and 2009 when novel viruses are introduced in the human population, while others derived from the introduction of novel gene segments into the pre-existing human viruses [19, 20]. Because dogs live in close contact with humans, the emergence of new viruses in dogs that can infect humans represents a zoonotic

risk, which makes the control of CIV infections in the dog population important not only for canine health, but also likely for human wellbeing.

Currently, only inactivated influenza vaccines (IIVs) are available for the prevention of CIV infections in dogs [10]. Historically, live attenuated influenza vaccines (LAIVs) have been shown to induce better immunogenicity and protection than IIVs [21, 22]. This is due by the fact that IIVs produce humoral immunity, mostly by the induction of neutralizing antibodies against the viral hemagglutinin (HA) protein, but limited induction of cellular immune responses, while LAIVs are able to induce both humoral and cellular immune responses [23-25]. Human LAIVs contain three (trivalent) or four (quadrivalent) reassortant viruses that contain the six internal genes of a temperature-sensitive (ts), cold-adapted (ca) and attenuated (att) master donor virus (MDV) along with the HA and neuraminidase (NA) viral segments from strains recommended by national and international public health agencies [26-29]. Previously we have described the generation, using reverse genetics techniques, of individual LAIVs for the prevention of either H3N8 [30] or H3N2 [31] CIVs. The H3N8 CIV LAIV [30] contained four mutations that were responsible for the ts, ca and att phenotype of the human MDV A/Ann Arbor/6/60 H2N2 LAIV [32, 33] in the PB2 (N265S) and PB1 (K391E, E581G, A661T) viral segments (D34N is natively present) of H3N8 CIV. The H3N2 CIV LAIV was prepared as a recombinant virus containing the internal genes of the MDV H3N8 CIV LAIV [30] and the HA and NA viral segments of the newly introduced H3N2 CIV [31]. In a mouse model of infection, both LAIVs were safe and able to induce, upon a single intranasal immunization,

complete protection against homologous challenges with their respective parental form of the virus [30, 31]. Both the H3N8 and H3N2 LAIVs induced better immune B cell responses and detectable T cell responses than IIVs, suggesting that they would provide better vaccine protection for the control of the H3N8 or H3N2 CIV infections [30, 31]. However, each individual vaccine did not confer protection against challenge with the heterologous CIV [30, 31].

Here, we report the development of a bivalent LAIV for the prevention of H3N8 and H3N2 CIVs based on our previously described individual LAIVs. Our results demonstrate that the bivalent CIV LAIV is safe and able to induce, upon a single intranasal administration, protective immunity against exposure to both H3N8 and H3N2 CIVs.

MATERIALS AND METHODS

Cells and viruses

Madin-Darby canine kidney cells (MDCK; ATCC CCL-34) were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS), and 1% PSG (penicillin, 100 units/ml; streptomycin 100 µg/ml; L-glutamine, 2 mM) at 37ºC with 5% CO_2 [30, 31].

Influenza A/Ca/IL/41915/2015 H3N2 wild-type (CIV H3N2 WT) was provided from the Baker Institute for Animal Health at Cornell University. Influenza A/Ca/NY/dog 23/2009 H3N8 wild-type (CIV H3N8 WT) [34, 35] and LAIV (CIV H3N8 LAIV) [30]; and CIV H3N2 LAIV [31] were described previously. Viruses were propagated in MDCK cells at 33ºC [30, 31, 36, 37]. Viral titers were

115 determined by immunofocus assay (fluorescent forming units, FFU/ml) in MDCK
116 cells at 33ºC [30, 31, 37] using monoclonal antibodies (mAbs) against the viral
117 NP: HB-65 (ATCC, H16-L10-4R5) for CIV H3N8 (WT and LAIV) and CIV H3N2
118 LAIV; and HT-103 [38] for CIV H3N2 WT.

119 **Indirect immunofluorescence**

120 MDCK cells (48-well plate format, $1x10^5$ cells/well, triplicates) were infected
121 with the indicated viruses at a multiplicity of infection (MOI) of 3 [30, 31]. Infected
122 cells were incubated for 12 h at 33°C and then fixed and permeabilized (4%
123 formaldehyde, 0.5% Triton X-100 in PBS) for 15 min at RT. Cells were
124 subsequently incubated in blocking solution (2.5% bovine albumin, BA, in PBS)
125 for 1 h at RT. CIV-infected cells were incubated with 2 µg/ml of mAbs
126 A/equine/Miami/1/63 H3N8 (anti-HA) or HB-65 (anti-NP); with a 1:1,000 dilution
127 of goat polyclonal sera (pAb) anti-NA A/Singapore/1/57 H3N2 (BEI Resources
128 NR-3137); and with a mixture of mAb Equine 7.1 and pAb anti-NA
129 A/Singapore/1/57 H3N2 (2 µg/ml and 1:1,000 dilution, respectively) diluted in
130 blocking solution for 1 h at 37ºC. After washing with PBS, cells were incubated
131 with a 1:200 dilution of a Alexa-Fluor 594-conjugated secondary anti-mouse
132 antibody, a fluorescein isothiocyanate (FITC)-conjugated secondary anti-goat
133 antibody or with a mixture of both secondary antibodies in blocking solution;
134 together with 4',6-diamidino-2-phenylindole (DAPI; Research Organics) for 1 h at
135 37°C. After washing with PBS, cells were examined and photographed using a
136 fluorescent microscope (Olympus IX81) and camera (QIMAGING, Retiga 2000R).

137 **Mouse Infections**

Six- to eight-week-old female C57BL/6 mice were purchased from the National Cancer Institute (NCI) and maintained in the animal care facility at the University of Rochester under specific pathogen-free conditions. All animal protocols were approved by the University Committee of Animal Resources and complied with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council [39]. To evaluate the attenuation of the bivalent LAIV *in vivo*, mice (N = 12) were anesthetized intraperitoneally (i.p.) with 2,2,2-tribromoethanol (Avertin; 240 mg/kg of body weight) and then inoculated intranasally (i.n.) with 30 µl of a virus preparation containing 10^3 FFU of CIV H3N2 LAIV and 10^4 (N = 6) or 10^5 (N = 6) FFU of CIV H3N8 LAIV. Consistent with previous studies, no signs of infection were observed after mouse inoculation with CIV H3N2 or CIV H3N8 WT or LAIV [30, 31, 35],. For this reason virus replication was determined by measuring viral titers in the lungs and nasal mucosa of infected mice at days 2 (N = 3) and 4 (N = 3) p.i. To that end, mice were euthanized by administration of a lethal dose of Avertin and exsanguinated. Virus titers in homogenized lungs and nasal mucosa were determined by immunofocus assay (FFU/ml) [30, 31, 35] using the anti-NA goat pAb A/Singapore/1/57 (CIV H3N2 LAIV) and the anti-HA A/equine/Miami/1/63 mAb Equine 7.1 (CIV H3N8 LAIV). To assess immunogenicity and protection efficacy, mice (N = 12) were anesthetized and vaccinated i.n. with PBS or with a virus preparation containing 10^3 and 10^4 FFU of CIV H3N2 and H3N8 LAIV, respectively. In addition, a group of mice (N = 12) were inoculated intramuscularly (i.m) with a preparation containing 100 µl of a commercial CIV

H3N2 IIV (Zoetis) [31] and 100 µl of a commercial CIV H3N8 IIV (Novartis) [30]. Fifteen days after vaccination, mice were challenged i.n. with 10^5 FFU of WT H3N2 (N = 6) or H3N8 (N = 6) CIVs. After challenge, H3N2 or H3N8 WT viral replication in mice lungs was evaluated at days 2 (N=3) and 4 (N=3) p.i.

Analysis of humoral responses

Mouse sera were collected by submandibular bleeding 24 h prior to viral challenges to evaluate immune responses. The level of virus-specific antibodies present in the sera of vaccinated mice were evaluated by enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with lysates from mock-, H3N2 WT CIV- or H3N8 WT CIV-infected MDCK cells, as previously described [30, 31]. Presence of neutralizing antibodies was analyzed by Virus Neutralization (VN) assays where 100 FFU of H3N2 or H3N8 WT CIVs were incubated with two-fold serial dilutions (starting dilution 1:25) of heat inactivated sera as previously described [31].

RESULTS

CIV H3N2 and H3N8 LAIVs co-infect cells *in vitro*

In order to generate a bivalent LAIV that protects against both CIV subtypes, we blended our previously described individual CIV H3N8 and H3N2 LAIVs (**Fig. 1**) [30, 31]. To analyze the identity of both LAIVs and to demonstrate that both viruses are able to co-infect cells *in vitro*, we infected MDCK cells with CIV H3N8 and H3N2 LAIVs either individually or together and performed an immunofluorescence assay using antibodies that differentiate between the HA

184 and the NA proteins of H3N8 and H3N2 CIVs [31], respectively (**Fig. 2**). H3N8
185 LAIV-infected cells were recognized by the anti-HA A/equine/Miami/1/63 H3N8
186 mAb when cells were infected with H3N8 LAIV alone or mixed with the H3N2
187 LAIV (**Fig. 2**, red fluorescence in the first and third columns). On the other hand,
188 H3N2 LAIV-infected cells were recognized by the anti-NA pAb A/Singapore/1/57
189 H3N2 when they were infected alone or in combination with H3N8 LAIV (**Fig. 2**,
190 green fluorescence in the second and third columns). However, H3N2- or H3N8-
191 infected cells were not recognized with the anti-HA A/equine/Miami/1/63 H3N8
192 mAb or the anti-NA pAb A/Singapore/1/57 H3N2, respectively. All infected cells,
193 independently of the virus, were recognized by the anti-NP mAb HB-65 (**Fig. 2**,
194 last column) included as internal control of infection. These results demonstrate
195 the identity of both H3N8 and H3N2 LAIVs [31] and their ability to co-infect
196 MDCK cells *in vitro*.

197 **The bivalent CIV LAIV is attenuated *in vivo***

198 Since H3N8 and H3N2 LAIVs were attenuated in a mouse model of infection
199 when compared with their respective CIVs WT [30, 31], we analyzed the
200 replication of both LAIVs in the lower (lungs) and upper (nasal mucosa)
201 respiratory tract of mice when they were administered together (**Fig. 3**). Two
202 groups of mice were inoculated i.n. with viral preparations containing a constant
203 amount of 10^3 FFU of H3N2 LAIV, and two different amounts (10^4 or 10^5 FFU) of
204 H3N8 LAIV. We used these combinations based on the ability of H3N2 LAIV to
205 replicate *in vitro* and *in vivo* more efficiently than H3N8 LAIV [30, 31]. In the lungs
206 of mice vaccinated with 10^3 FFU of H3N2 LAIV and 10^4 FFU of H3N8 LAIV (**Fig.**

207 **3A**), we were not able to detect either LAIV at days 2 or 4 p.i. On the other hand,
208 in the nasal mucosa (**Fig. 3B**), we were able to observe similar viral titers of
209 ~10^3-10^4 FFU/ml for both H3N8 and H3N2 LAIVs at days 2 and 4 p.i. Similarly,
210 we could not detect either LAIV in the lungs of mice immunized with 10^3 FFU of
211 H3N2 LAIV and 10^5 FFU of H3N8 LAIV at any day p.i. (**Fig. 3C**). However, in the
212 nasal mucosa (**Fig. 3D**), H3N8 LAIV reached similar viral titers at both times p.i.,
213 while the viral titers of H3N2 LAIV were ~1 log lower at day 2 p.i. in relation to
214 those obtained by H3N8 LAIV, or were not detected at day 4 p.i. These results
215 suggest that replication of H3N8 LAIV impeded the efficient replication of H3N2
216 LAIV and that our blended CIV LAIV is safe since we were not able to detect any
217 of the LAIVs in the lungs of infected mice (**Figs. 3A** and **3C**). Also, these results
218 indicate that a dose of 10^3 FFU of H3N2 LAIV and 10^4 FFU of H3N8 allows
219 similar level of replication of both viruses in the nasal mucosa of this mouse
220 strain, which is important for efficient induction of protective immune responses
221 (**Figs. 3B** and **3D**). Notably, these results are comparable to those obtained
222 previously using individual H3N8 [30] or H3N2 [31] LAIVs.

223 **The bivalent CIV LAIV is immunogenic and induces protection against**
224 **challenge with H3N2 and H3N8 CIVs**

225 To evaluate the humoral responses and protection efficacy induced when both
226 LAIVs were blended, we vaccinated mice i.n. with a virus preparation containing 10^3
227 and 10^4 FFU of H3N2 and H3N8 LAIV, respectively; or mock-vaccinated with PBS (**Figs.**
228 **4** and **5**). Additionally, and in order to compare our bivalent LAIV approach with the
229 commercial available blended IIV, a group of mice was vaccinated i.m. with 100μl of a

H3N2 IIV and 100μl of a H3N8 IIV. Mice were then subdivided in two groups for subsequent challenges with H3N2 (**Fig. 4**) or H3N8 (**Fig. 5**) WT CIVs. Humoral immune responses were evaluated by ELISA using cell extracts from H3N2-infected MDCK cells (**Figs. 4B** and **5C**) or H3N8-infected MDCK cells (**Figs. 4C** and **5B**) [30, 31]. Antibodies against total H3N2 (**Figs. 4B** and **5C**) or H3N8 (**Figs. 4C** and **5B**) CIV proteins were detected in the sera from all LAIV or IIV vaccinated mice. Notably, humoral responses elicited by the bivalent LAIV against both CIVs were greater than in mice vaccinated with the bivalent IIV against either H3N2 (**Figs. 4B** and **5C**) or H3N8 (**Figs. 4C** and **5B**) CIVs. Neutralizing antibody responses, as determined by VN assay, against H3N2 (**Fig. 4D**) and H3N8 (**Fig. 5D**) CIVs were similar in both LAIV- and IIV-vaccinated mice.

To evaluate the protection efficacy of the bivalent LAIV against H3N2 or H3N8 WT CIV challenges, vaccinated mice were challenged with 10^5 FFU of H3N2 WT (**Fig. 4E**) or H3N8 WT (**Fig. 5E**) CIVs. In mock-vaccinated mice, we observed H3N2 WT viral titers of ~ 1 x 10^6 FFU/ml at days 2 and 4 post-challenge. On the other hand, in mice vaccinated with the bivalent LAIV, H3N2 WT was only detected in one mouse at day 2 post-challenge and it was not detected at day 4 p.i. All mice vaccinated with the bivalent IIV showed H3N2 WT titers at day 2 post-challenge, although virus was not detected in any of the vaccinated mice at day 4 p.i. (**Fig. 4E**). Mock-vaccinated mice challenged with H3N8 WT (**Fig. 5E**), exhibited viral titers of ~ 10^6 and ~ 10^5 FFU/ml at days 2 and 4 post-challenge, respectively; while mice vaccinated with the bivalent LAIV showed viral titers that were reduced ~ 4 logs (two mice) or not detected (one mouse) at day 2 post-challenge. Notably, H3N8 WT virus was not detected at day 4 p.i. in mice vaccinated with the bivalent LAIV. All mice vaccinated with the bivalent IIV had detectable H3N8

253 WT viral titers (~ 10^4 FFU/ml) at day 2 p.i. and were not protected as those vaccinated
254 with the bivalent LAIV (**Fig. 5E**).
255
256 **DISCUSSION**
257 Here we combined our previously described CIV H3N8 and H3N2 monovalent LAIVs
258 [30, 31] (**Fig. 1**) to generate a bivalent CIV LAIV that confers protection against both
259 subtypes of CIVs. Both CIV LAIVs were able to co-infect cells *in vitro* (**Fig. 2**). Moreover,
260 in mice immunized with 10^3 of H3N2 and 10^4 FFU of H3N8 LAIVs both CIV LAIVs
261 replicated similarly in the nasal mucosa (**Fig. 3**). However, neither CIV LAIV replicated
262 in the lungs of intra-nasally vaccinated mice (**Fig. 3**), further demonstrating our initial
263 results on the safety profile of the CIV LAIVs [30, 31]. In order to directly compare the
264 immunogenicity and protection efficacy induced by the CIV bivalent LAIV with those
265 induced by the CIV H3N8 and H3N2 monovalent LAIVs [30, 31], we utilized the same
266 vaccination/challenge regimes as those previously used with the monovalent vaccines
267 [30, 31]. The bivalent CIV LAIV induced greater humoral responses and better
268 protection against H3N8 and H3N2 CIVs than those observed with a bivalent CIV IIV
269 (**Figs. 4** and **5**) corroborating our previously results on the CIV LAIV superiority in terms
270 of protection efficacy.
271 Vaccination of dogs with CIV IIVs requires two i.m. injections separated a few weeks
272 apart with 1ml of vaccine containing ~ 10^7 FFU equivalent of viral antigen. Taking into
273 consideration that the average weight for a mouse (~ 20 g) is 500 times lower than the
274 average weight of a beagle dog (~ 10 Kg), we used a 50 times higher (100 µl) dose of
275 each CIV IIV in our mice experiments, the equivalent to ~ 10^6 FFU. Even under these

conditions the CIV bivalent LAIV containing only 10^3 and 10^4 FFU of H3N2 and H3N8 LAIVs, respectively, conferred better protection than the one obtained with the bivalent CIV IIV in mice immunized with a single dose of each bivalent vaccine. Since the induction of neutralizing antibodies were only slightly higher in the case of CIV LAIVs versus IIVs, this better protection efficacy is probably linked to the ability of the CIV LAIVs to induce CD8 T-cell responses, which are not observed using the CIV IIVs [30, 31].

Compared to the current blended IIV the bivalent LAIV approach provides the following advantages: it is administered i.n., therefore mimicking the natural route of influenza infection; requires significantly less virus to induce superior protection against WT CIV infections; stimulates more robust humoral response; elicits CD8 T-cell immunity which allows a more effective control of influenza infection with a single immunization; and as it is based on reverse genetics the system allows the generation of new LAIVs against other or newly introduced viruses. This blended CIV LAIV therefore likely represents an excellent option for the prevention and control of CIV infections if the results are translated to dogs.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

FIGURE LEGENDS

Figure 1. Schematic representation of a CIV H3N8 and H3N2 blended LAIV: We have previously developed and characterized monovalent CIV H3N8 (black) and H3N2 (red) LAIVs (top left). Both monovalent CIV LAIVs protected against homologous challenge with WT CIVs but not against heterologous CIV challenges (top right). A bivalent CIV LAIV, made of H3N8 and H3N2 LAIVs, (bottom left) was used to evaluate its ability to confer protection against H3N8 and H3N2 CIV challenges (bottom right).

Figure 2. Characterization of the bivalent CIV blended LAIV: MDCK cells were mock-infected or infected at high multiplicity of infection (MOI = 3) with the CIV H3N8 (black) or H3N2 (red) LAIVs, or co-infected with both LAIVs and incubated at 33ºC. At 12 h p.i., cells were fixed and stained with the indicated monoclonal or polyclonal antibodies against HA (H3N8 LAIV), NA (H3N2 LAIV) or NP (both H3N8 and H3N2 LAIVs). DAPI was also included for nuclear staining. Representative images are shown. Scale bars, 200 µM.

Figure 3. Attenuation of the bivalent CIV H3N8 and H3N2 blended LAIV *in*

322 ***vivo*:** Female 6-to-8-week-old C57BL/6 mice (N = 6) were infected intranasally
323 (i.n.) with the bivalent CIV LAIV using 10^3 FFU of H3N2 LAIV and 10^4 FFU of
324 H3N8 LAIV (**A**-**B**), or 10^3 FFU of H3N2 LAIV and 10^5 FFU of H3N8 LAIV (**C**-**D**).
325 Presence of CIV H3N8 and H3N2 LAIVs in the lungs (**A** and **C**) and the nasal
326 mucosa (**B** and **D**) of infected mice were evaluated at days 2 (N = 3) and 4 (N =
327 3) p.i. by immunofocus assay (FFU/ml) using a monoclonal antibody against
328 A/equine/Miami/1/63 H3N8 HA (CIV H3N8 LAIV), or a polyclonal antibody
329 against A/Singapore/1/57 H3N2 NA (CIV H3N2 LAIV) (**Figure 1**). Symbols
330 represent data points for individual mice. ND, not detected. Bars, geometric
331 mean viral titers. Dotted black lines indicates the limit of detection (200 FFU/ml).
332 **Figure 4. Immunogenicity and protection efficacy of the bivalent CIV LAIV**
333 **against CIV H3N2: A)** Schematic representation of the protocol used to assess
334 humoral response and protection efficacy. Female 6- to-8-week-old C57BL/6
335 mice (N = 6) were vaccinated with the bivalent CIV LAIV (10^3 FFU and 10^4 FFU
336 of H3N2 and H3N8 LAIVs, respectively). Mice were also mock (PBS) vaccinated
337 (N = 6) or vaccinated (N = 6) i.m. with a bivalent CIV IIV (100 μl/mice of H3N2 IIV,
338 Zoetis, and 100 μl/mice of H3N8 IIV, Nobivac). **B**-**C) Induction of humoral**
339 **responses:** 14 days post-vaccination, mice were bled and the sera were
340 collected and evaluated individually by ELISA for IgG antibodies against total
341 viral proteins using cell extracts of MDCK cells infected with H3N2 (**B**) or H3N8
342 (**C**) WT CIVs. Mock-infected cell extracts were used to evaluate the specificity of
343 the antibody response. OD, optical density. Data represent the means +/- SDs of
344 the results for 6 individual mice. **D) Virus neutralization (VN) titers against CIV**

H3N2: sera from vaccinated mice were evaluated for the presence of neutralizing antibodies using 100 FFU of CIV H3N2 WT and 2-fold serial dilutions of the indicated pooled mice sera. Data represent the means +/- SDs. ND, not detected. **E) Protection efficacy against CIV H3N2:** 15 days post-vaccination, mice (N = 6) were challenged with 10^5 FFU of CIV H3N2 WT and viral titers at days 2 (N = 3) and 4 (N = 3) p.i. from lung homogenates were evaluated by immunofocus assay (FFU/ml) using the anti-NP mAb HT-103. Symbols represent data points for individual mice. &, virus not detected in 2 mice; ND, not detected. Bars, geometric mean lung viral titers. Dotted black lines indicate the limit of detection (200 FFU/ml).

Figure 5. Immunogenicity and protection efficacy of the bivalent CIV LAIV against CIV H3N8: A) Schematic representation of the protocol used to assess the humoral response and protection efficacy induced by the bivalent CIV LAIV against CIV H3N8. Female 6- to-8-week-old C57BL/6 mice (N = 6) were vaccinated with the bivalent CIV LAIV (10^3 FFU and 10^4 FFU of H3N2 and H3N8 LAIVs, respectively), mock-vaccinated or vaccinated i.m. with a bivalent IIV (as previously indicated in **Figure 4**). **B-C) Induction of humoral responses:** 14 days post-vaccination, mice were bled and the sera were collected and evaluated individually by ELISA for IgG antibodies against total viral proteins using cell extracts of MDCK cells infected with H3N8 (**B**) or H3N2 (**C**) WT CIVs. **D) Virus neutralization (VN) titers against CIV H3N8:** mice sera were also assessed for the presence of neutralizing antibodies using 100 FFU of CIV H3N8 WT and 2-fold serial dilutions of the indicated sera. Data represent the means +/- SDs. ND,

not detected. **E) Protection efficacy against CIV H3N8:** 15 days post-vaccination, mice (N = 6) were challenged with 10^5 FFU of CIV H3N8 WT and viral titers from lung homogenates were evaluated by immunofocus assay (FFU/ml) at days 2 (N = 3) and 4 (N = 3) post-challenge, using the anti-NP mAb HT-103. Symbols represent data for individual mice. *, virus not detected in 1 mouse; &, virus not detected in 2 mice; ND, not detected. Bars, geometric mean lung viral titers. Dotted black lines indicate the limit of detection (200 FFU/ml)**.**

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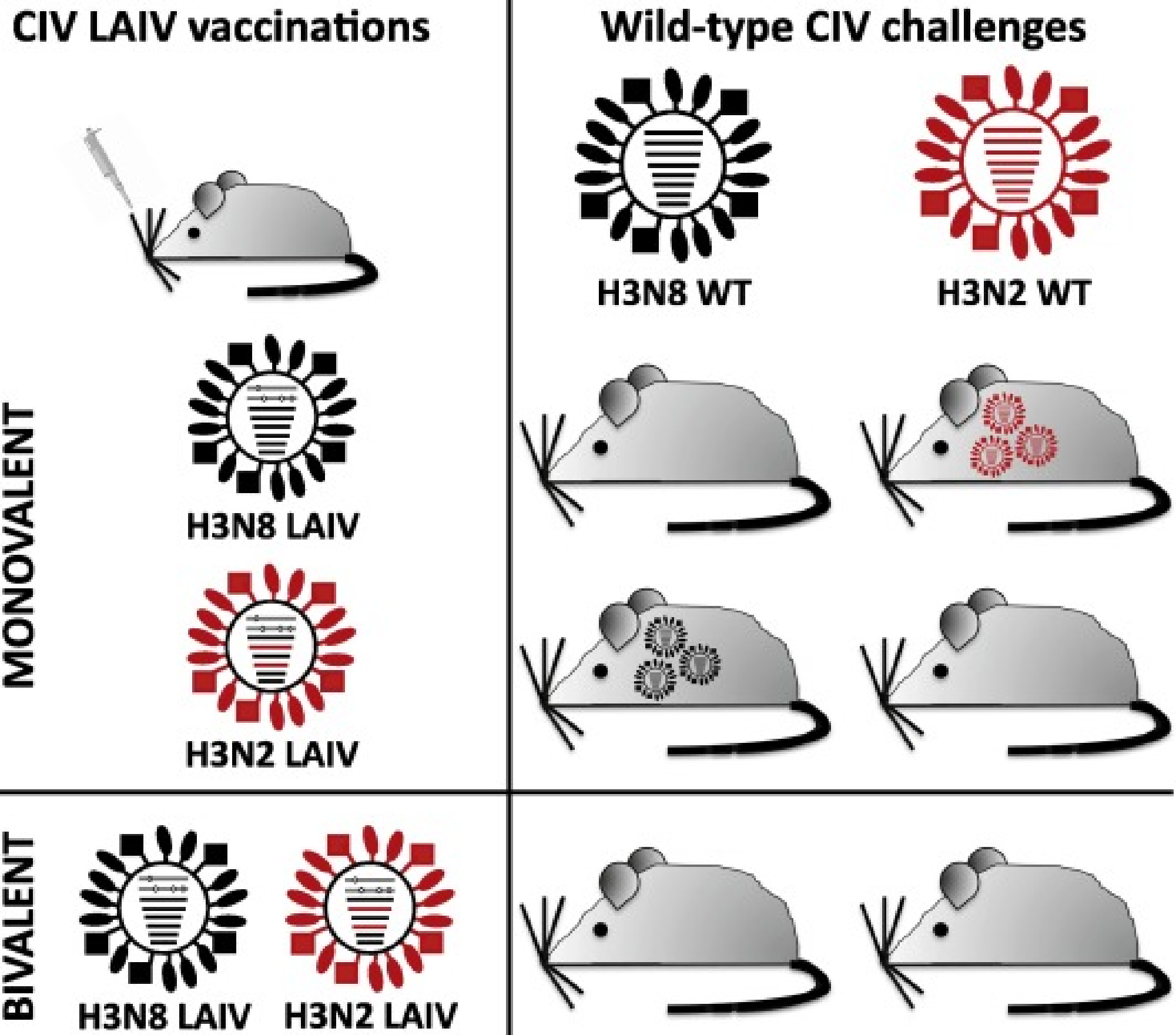
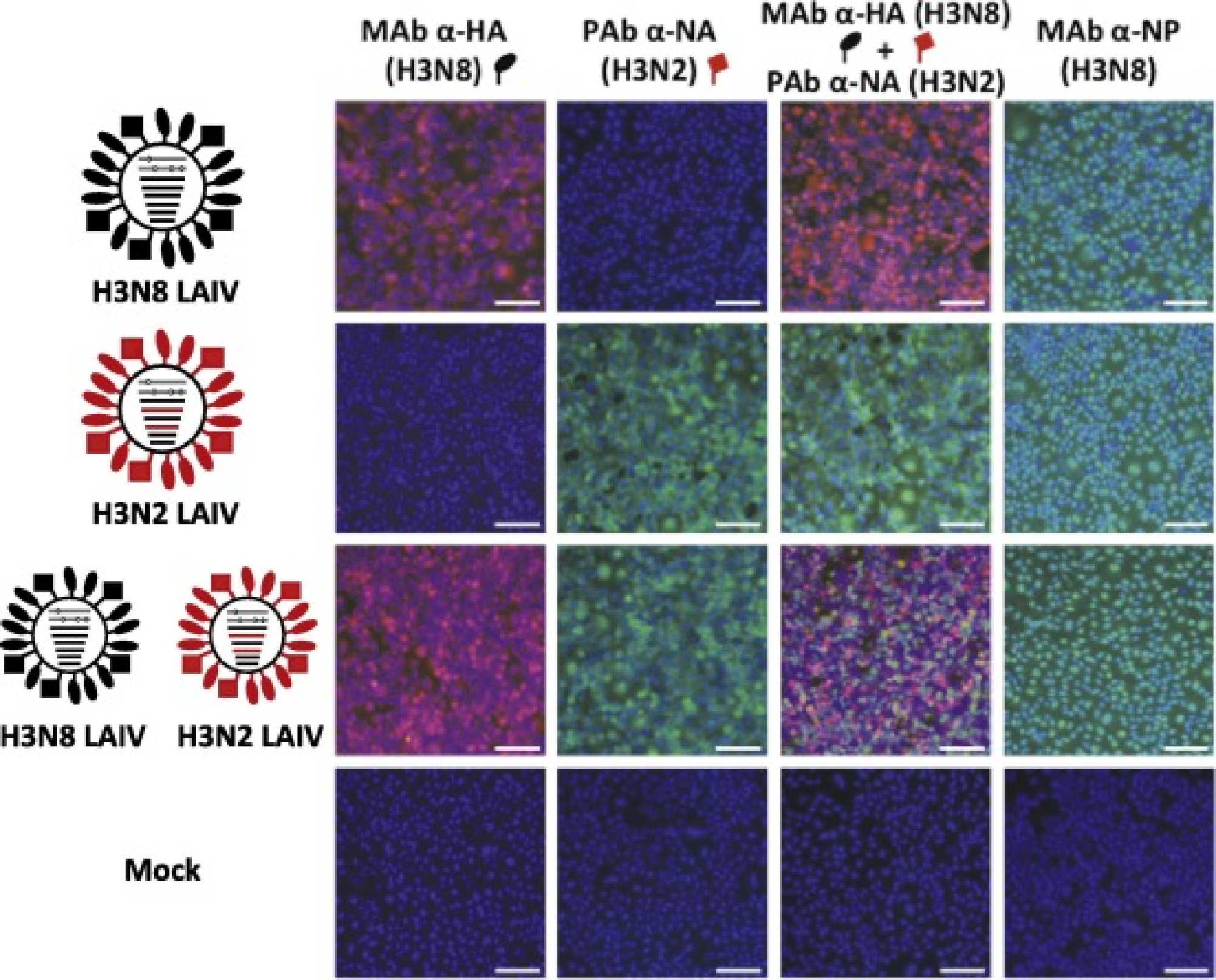
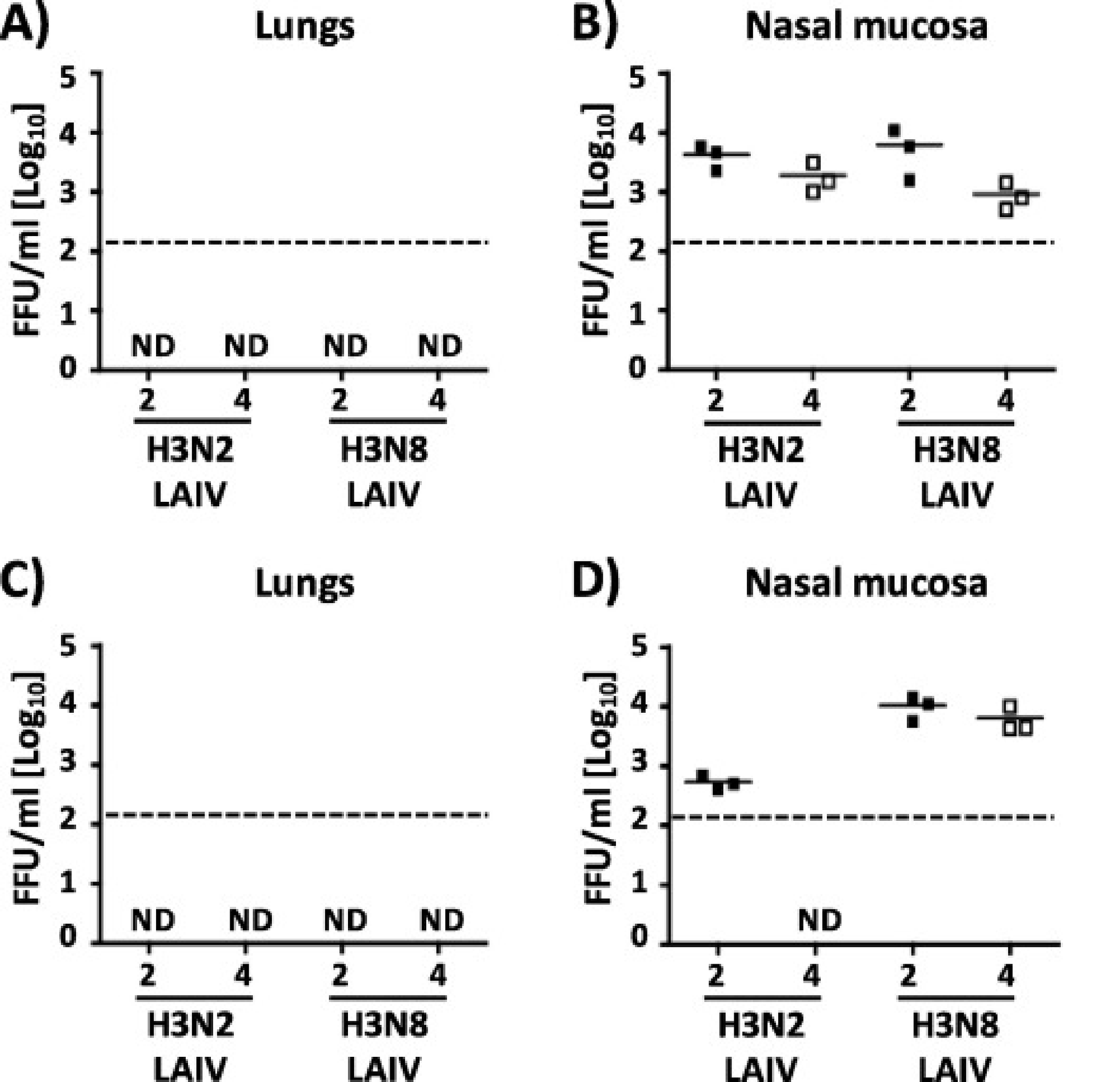
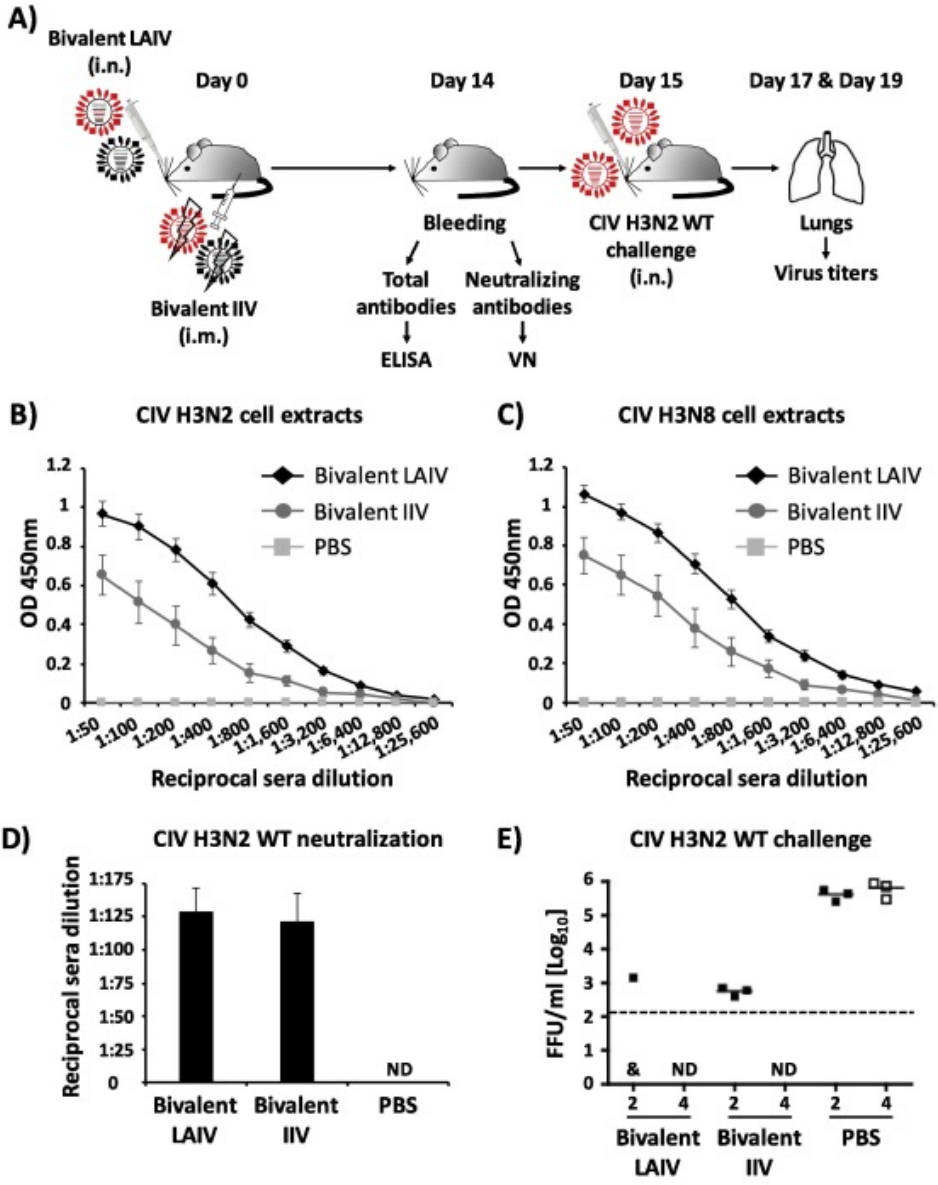
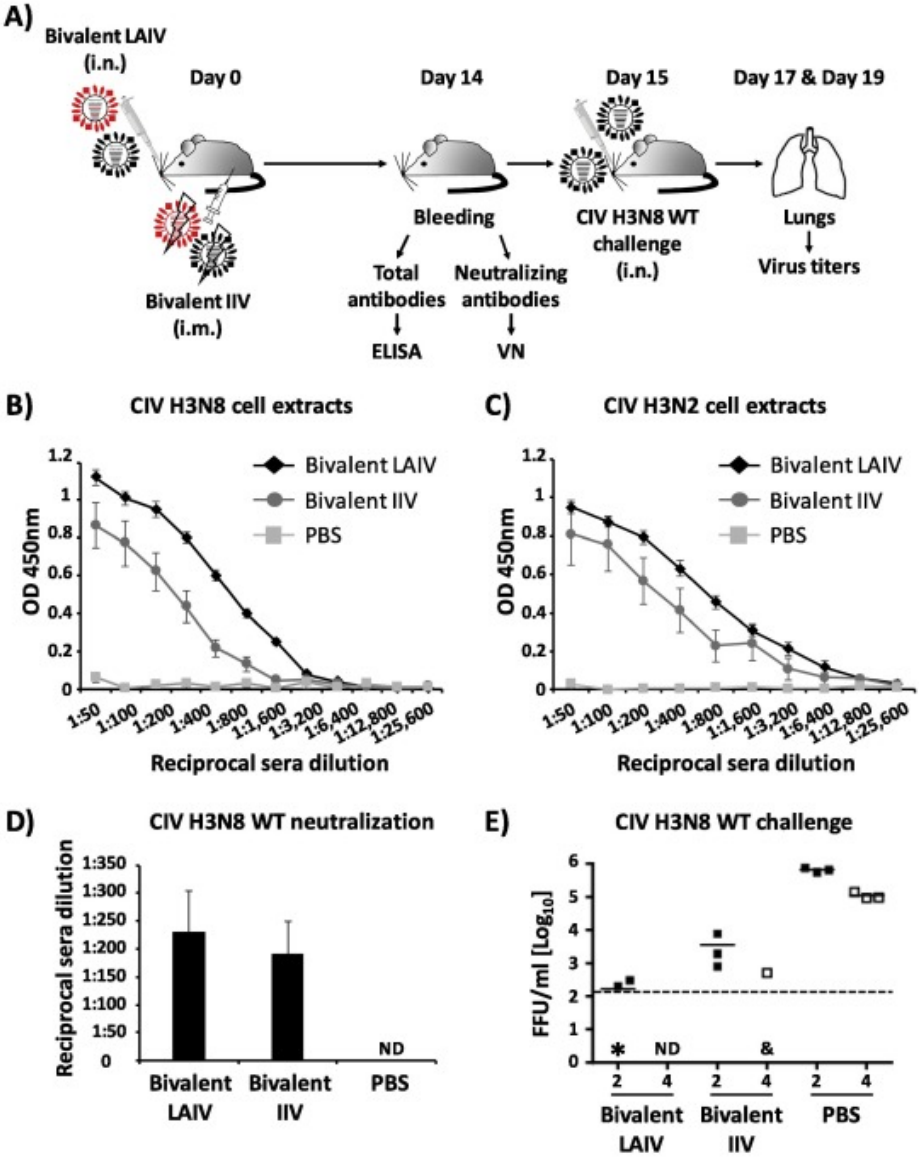

CIV LAIV vaccinations
Wild-type CIV challenges
H3N8 WT
H3N2 WT
MONOVALENT
H3N8 LAIV
H3N2 LAIV
BIVALENT
H3N8 LAIV
H3N2 LAIV


MAb α-HA (H3N8)
PAb α-NA (H3N2)
MAb α-HA (H3N8) + PAb α-NA (H3N2)
MAb α-NP (H3N8)
H3N8 LAIV
H3N2 LAIV
H3N8 LAIV
H3N2 LAIV
Mock


A)
Lungs
FFU/ml [Log10]
5
4
3
2
1
0
ND
ND
ND
ND
2
4
2
4
H3N2
LAIV
H3N8
LAIV
B)
Nasal mucosa
FFU/ml [Log10]
5
4
3
2
1
0
2
4
2
4
H3N2
LAIV
H3N8
LAIV
C)
Lungs
FFU/ml [Log10]
5
4
3
2
1
0
ND
ND
ND
ND
2
4
2
4
H3N2
LAIV
H3N8
LAIV
D)
Nasal mucosa
FFU/ml [Log10]
5
4
3
2
1
0
ND
2
4
2
4
H3N2
LAIV
H3N8
LAIV


A)
Bivalent LAIV
(i.n.)
Day 0
Day 14
Day 15
Day 17 & Day 19
Bivalent IIV
(i.m.)
Bleeding
Total
antibodies
ELISA
Neutralizing
antibodies
VN
CIV H3N2 WT
challenge
(i.n.)
Lungs
Virus titers
B)
CIV H3N2 cell extracts
Bivalent LAIV
Bivalent IIV
PBS
OD 450nm
1.2
1
0.8
0.6
0.4
0.2
0
1:50
1:100
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
Reciprocal sera dilution
C)
CIV H3N8 cell extracts
Bivalent LAIV
Bivalent IIV
PBS
OD 450nm
Reciprocal sera dilution
D)
CIV H3N2 WT neutralization
Reciprocal sera dilution
1:175
1:125
1:100
1:75
1:50
1:25
0
ND
Bivalent
LAIV
Bivalent
IIV
PBS
E)
CIV H3N2 WT challenge
FFU/ml [Log10]
6
5
4
3
2
1
0
&
ND
ND
2
4
2
4
2
4
Bivalent
LAIV
Bivalent
IIV
PBS


A)
Bivalent LAIV (i.n.)
Day 0
Day 14
Day 15
Day 17 & Day 19
Bleeding
CIV H3N8 WT challenge (i.n.)
Lungs
Virus titers
Bivalent IIV (i.m.)
Total antibodies
Neutralizing antibodies
ELISA
VN
B)
CIV H3N8 cell extracts
C)
CIV H3N2 cell extracts
Bivalent LAIV
Bivalent IIV
PBS
OD 450nm
1.2
1
0.8
0.6
0.4
0.2
0
1:50
1:100
1:200
1:400
1:800
1:1,600
1:3,200
1:6,400
1:12,800
1:25,600
Reciprocal sera dilution
D)
CIV H3N8 WT neutralization
Reciprocal sera dilution
1:350
1:300
1:250
1:200
1:150
1:100
1:50
0
ND
Bivalent LAIV
Bivalent IIV
PBS
E)
CIV H3N8 WT challenge
FFU/ml [Log10]
6
5
4
3
2
1
0
*
ND
&
2
4
Bivalent LAIV
Bivalent IIV
PBS