H5 influenza virus mRNA-lipid nanoparticle (LNP) vaccination elicits adaptive immune responses in Holstein calves

Carine K. Souza[1#], Jefferson J. S. Santos[2#], Paola Boggiatto[1], Haley Sterle[1], Bailey Arruda[1], Mitchell V. Palmer[1], Alessandra Campos[1], Jiaojiao Liu[2], Naiqing Ye[2], Drew Weissman[3,4], Scott E. Hensley[2,*], Amy L. Baker[1,*]

[1]National Animal Disease Center – United States Department of Agriculture, Agricultural Research Service, Ames, IA, USA.
[2]Department of Microbiology, [3]Infection Disease Division, [4]Penn Institute of RNA Innovation, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[#]Authors equally contributed to this manuscript.
*Correspondence to hensley@pennmedicine.upenn.edu and Amy.L.Baker@usda.gov

Abstract

Highly pathogenic avian influenza (HPAI) clade 2.3.4.4b H5N1 is circulating widely in lactating cows in the United States. Due to the critical need for intervention strategies for this outbreak, we evaluated antibody and cellular immune responses of a clade 2.3.4.4b H5 mRNA-LNP vaccine in calves. We found that the H5 mRNA-LNP vaccine induced a robust antibody and $CD8^+$ T cellular-mediated immune response and conferred protection against clade 2.3.4.4b H5N1 infection.

Main

Highly pathogenic avian influenza (HPAI) H5 viruses threaten wild birds, poultry and represent a human pandemic risk. In March 2024, an outbreak of HPAI clade 2.3.4.4b H5N1 virus was reported for the first time in dairy cattle[1]. Since then, over 950 HPAI H5N1 (2.3.4.4b) cases in livestock herds of dairy cattle have been confirmed, and the virus has spread to 16 states in the U.S. (https://www.aphis.usda.gov/livestock-poultry-disease/avian/avian-influenza/hpai-detections/hpai-confirmed-cases-livestock). In the

field, high quantities of H5N1 have been detected in raw unpasteurized milk, although detection also occurred in conjunctivital, nasal, and urine samples of infected dairy cows[1, 2]. In experimentally intramammary inoculated cows, viral RNA was primarily detected in mammary glands and milk, although RNA was detected in a small subset of nasal and ocular swabs[3]. Most human infections associated with HPAI H5N1 clade 2.3.4.4b reported in the U.S. have been relatively mild[4, 5]. However, a severe case of HPAI H5 clade 2.3.4.4b resulted in the first U.S. H5N1-related human death (https://ldh.la.gov/news/H5N1-death). Furthermore, there is an increased concern about mammalian adaptation of the HPAI H5 clade 2.3.4.4b and the risk of human-to-human transmission[6].

It is possible that vaccines might be able to control H5 spread in livestock and prevent the emergence of novel viruses that pose a pandemic risk to humans. Bovine H5 vaccines are needed but are currently not available. We developed a nucleoside-modified mRNA-lipid nanoparticle (LNP) vaccine expressing the hemagglutinin (HA) protein of a clade 2.3.4.4b H5 virus and found that this vaccine was immunogenic in mice and ferrets[7]. Further, the vaccine reduced morbidity and mortality in vaccinated compared to unvaccinated ferrets[7]. Here, we assessed the antibody and T cell-mediated immune responses of calves vaccinated with this clade 2.3.4.4b H5 mRNA-LNP vaccine.

For these experiments, we tested a monovalent mRNA-LNP vaccine encoding HA from the clade 2.3.4.4b A/Astrakhan/3212/2020 virus. We administered the vaccine via the intramuscular route to calves in two doses of 50 and 500 ug in a prime-boost vaccine regimen. Both H5 mRNA-LNP vaccine doses elicited antibodies that bound to

55 the HA from the homologous vaccine strain (Fig. 1a) and the cattle 2.3.4.4b strain (Fig.
56 1b). Antibody levels were significantly higher in animals receiving the 500 ug dose
57 compared to the 50 ug dose, and antibodies were boosted by the second vaccine dose
58 in all vaccinated animals. We were also able to detect antibodies at high levels using a
59 commercial H5 ELISA (ID Screen® Influenza H5 Antibody Competition ELISA,
60 Innovative Diagnostics) (Fig. 1c). We found that the vaccine elicited antibodies that
61 inhibited agglutination of a representative wild bird 2.3.4.4b strain (Fig. 1d) and
62 neutralized both the homologous vaccine strain (Fig. 1e) and the cattle 2.3.4.4b strain
63 (Fig. 1f).
64 We next measured cell-mediated immune (CMI) responses elicited by the vaccine
65 pre-boost at 21- and post-boost at 35-days post-vaccination (dpv) after stimulating
66 peripheral blood mononuclear cell (PBMC) with recombinant H5 protein or virus. The
67 500 µg H5 mRNA-LNP vaccine dose elicited a significantly higher percentage of
68 proliferating $CD8^+$ T cells compared to the 50 µg H5 mRNA-LNP vaccine dose at 21-
69 and 35dpv or unvaccinated, following *in vitro* stimulation with either H5 protein (Fig. 1g,
70 red dots) or inactivated 2.3.4.4b H5 virus (Fig. 1g, blue dots). We found that $CD8^+$ T
71 cells from animals receiving the 500 ug vaccine dose produced IFN-γ^+ after stimulation;
72 however, $CD8^+$ T cells from animals receiving the 50 ug vaccine dose only sporadically
73 produced IFN-γ^+ after stimulation (Fig. 1h). The vaccine also elicited low but detectable
74 levels of proliferating $CD4^+$ T cells and $\gamma\delta$ T cells (Extended data Fig. 1) that produced
75 low IFN-γ^+ production.
76 We evaluated vaccine protection by feeding vaccinated and unvaccinated calves
77 with milk from lactating cows experimentally infected with H5N1 virus via the

78 intramammary route. Virus titers in the milk used as the inoculum ranged from $10^{2.25}$ -
79 $10^{7.5}$ $TCID_{50}$/mL (Supplementary Table 1). Presence of viral RNA in nasal secretions of
80 the calves were assessed via qPCR (Fig. 2a) and viral titers were detected in nasal
81 secretions at 1-, 2-, 3-, 4- and 5-days post-infection (dpi) in the unvaccinated/challenged
82 group. In contrast, all vaccinated calves had minimal amounts of virus detected in nasal
83 secretions from 1 to 6 dpi (Fig. 2a). None of the calves developed a fever (Fig. 2b) or
84 clinical signs associated with influenza virus infection. Each lung lobe homogenate was
85 tested for presence of viral RNA by qPCR. Viral RNA was only weakly detected in one
86 calf in the H5 mRNA-LNP 50 ug vaccine group (Supplementary Table 2). Tracheal swab
87 and broncho-alveolar lavage fluid (BALF) samples collected at necropsy (6 dpi)
88 contained undetectable or minimal amount of virus (Supplementary Table 2). The
89 histopathology scores and immunohistochemistry (IHC) results from lung lobes,
90 turbinate, and trachea samples were minimal in both vaccinated and control groups;
91 only one calf from the unvaccinated/challenged group was positive via IHC in a single
92 bronchiole in the caudal left lobe (Supplementary Table 3).
93 In summary, our study demonstrates that our H5 mRNA-LNP vaccine is
94 immunogenic in calves and reduces the amount of virus shedding following H5N1
95 2.3.4.4b virus infection. Future studies will evaluate this vaccine in lactating dairy cattle
96 to determine if the vaccine provides protection against intramammary H5N1 infections.
97 While similar levels of protection were observed for the 50 and 500 ug doses, additional
98 experiments will be needed to determine the minimum amount of vaccine required for
99 eliciting protective immune responses, and it will also be important to carefully
100 determine the longevity of immune responses elicited by this vaccine. Further

101 development of this vaccine could potentially aid in controlling the widespread H5N1
102 2.3.4.4b outbreak in the U.S. lactating cows.
103

104 **Methods**

105 Study Design

106 Twelve Holstein castrated male calves at 4 months of age were given 3.5 mL of
107 Draxxin (Zoetis, Kalamazoo, MI) and 3 mL of Noromectin (Norbrook Inc., Lenexa, KS)
108 upon arrival. At the beginning of the study, serum samples were collected and tested for
109 Influenza A virus (IAV) antibody nucleoprotein (NP) by a commercial ELISA (MultiS
110 ELISA, IDEXX, Westbrook, Maine) to ensure the calves were seronegative to IAV. Ten
111 calves were split into two groups (*n*=5) and vaccinated with 50 or 500 ug of
112 A/Astrakhan/3212/2020 (H5N8) HA mRNA-LNP vaccine intramuscularly, and two calves
113 were kept as a non-vaccinated control group. The vaccine regimen comprised two
114 doses of mRNA-LNP vaccine with an interval of three weeks between doses. Blood
115 samples were collected at 0, 21, 35, 42, and 49 dpv to evaluate immune responses
116 using serological and *in vitro* recall response assays to measure CMI responses. At 49
117 dpv, we challenged 4 calves from each vaccinated group and two calves as
118 unvaccinated/challenged control group. We kept one calf from each vaccinated group
119 as a vaccinated/non-challenged group. The challenge was performed by feeding calves,
120 twice daily for three days, approximately 1 L of milk from cows experimentally
121 inoculated with A/dairy cattle/Texas/24-008749-002/2024 (H5N1) virus via the
122 intramammary route (unpublished data) [8]. Milk samples from the inoculum were

123 collected twice daily for three days to check virus titers by $TCID_{50}$/mL virus titration was
124 performed according to [9] and *Ct* values by qPCR (Supplementary Table 2). Two
125 vaccinated/non-challenged calves were fed with non-infected milk from healthy cows.
126 FLOQ nylon-tipped nasal swabs (Copan, Murrieta, CA) and rectal temperatures
127 were collected daily from 1 to 6 days post-inoculation (dpi). During the three days of
128 challenge, nasal swabs were collected before the calves were fed with H5N1 infected
129 milk. At 6 dpi, all calves were euthanized with a lethal intravenous dose of pentobarbital
130 sodium (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI). Lung tissue, tracheal
131 swab, and broncho-alveolar lavage fluid were collected at necropsy to evaluate viral
132 RNA detection by qPCR. Lung, turbinate and tracheal tissues were also collected for
133 histologic analysis. The animal study was carried out in an ABSL2 facility during the
134 vaccination stage and a ABSL3-Ag facility during the challenge stage of the study in
135 compliance with the Institutional Animal Care and Use Committee of the USDA-ARS
136 National Animal Disease Center (NADC).
137
138 Recombinant HA protein expression
139 The extracellular domain of A/Astrakhan/3212/2020(H5N8) HA and A/dairy
140 cattle/Texas/24-008749-002-v/2024(H5N1) HA were synthetically fused to the trimeric
141 FoldOn of T4 fibritin, the Avitag sequence, and a hexahistidine affinity tag [10], and cloned
142 into the pCMV Sport6 expression vector. HA expression plasmids were co-transfected
143 with plasmid encoding cell surfaced expressed A/Puerto Rico/8/1934 neuraminidase
144 (NA) into FreeStyle 293F cells (Thermo Fisher, Waltham, MA) with 293fectin reagent
145 (Thermo Fisher, Waltham, MA) following the manufacturer's instructions. After 4 days,

146 the supernatant was clarified by centrifugation. HA proteins were purified using Ni-NTA
147 agarose resin (Qiagen) and gravity flow chromatography columns (Bio-Rad). Purified
148 HA was buffer-exchanged and concentrated using centrifugal filter units with a 30 kDa
149 molecular weight cutoff (Millipore). Purified HA proteins were stored at -80°C.
150
151 ELISAs
152 ELISAs were performed on 96-well Immulon 4HBX extra high binding flat-bottom
153 plates (Thermo Fisher, Waltham, MA). Plates were coated with HA protein at 2 µg/mL in
154 DPBS at 4 °C overnight. Plates were washed three times with 1X PBS+0.1% Tween-20
155 and then blocked for 1 h at room temperature using blocking buffer (1X TBS, 0.05%
156 Tween-20, and 1% bovine serum albumin). Bovine serum samples were heat-
157 inactivated at 56 ˚C for 1 h and then serially diluted four-fold in dilution buffer (1X TBS,
158 0.05% Tween-20, and 0.1% bovine serum albumin). After blocking, plates were washed
159 three times with 1X PBS+0.1% Tween-20. Diluted sera were then transferred to ELISA
160 plates and incubated for 2 hr at room temperature (RT). After washing three times with
161 1X PBS+0.1% Tween-20, peroxidase-conjugated mouse anti-bovine IgG monoclonal
162 antibody (clone IL-A2; Bio-Rad) was added to all plates at a concentration of 1:5000 in
163 dilution buffer for 1 hr at RT. After washing three times with 1X PBS+0.1% Tween-20, all
164 plates were developed by adding SureBlue TMB peroxidase substrate (SeraCare,
165 Gaithersburg, MD) for 5 minutes at RT, followed by stopping the reaction with 250 mM
166 HCl solution. The absorbance was measured at 450 nm using a SpectraMax ABS Plus
167 plate reader (Molecular Devices, San Jose, CA). Endpoint titers were calculated as the
168 reciprocal sera dilution that generated an equivalent optical density (OD) of 0.5.

169 A commercial ELISA, ID Screen Influenza H5 antibody competition 3.0
170 multispecies (ID Innovative Diagnostics, Grabels, France) was used to measure H5-
171 specific antibody responses in bovine serum.
172
173 Foci Reduction Neutralization assay
174 Foci reduction neutralization (FRNT) assays were carried out using conditionally
175 replicative influenza viruses that encode GFP instead of PB1, as previously described
176 [11]. Conditionally replicative viruses were generated encoding the hemagglutinin (HA)
177 and neuraminidase (NA) from either A/Astrakhan/3212/2020 (H5N8) or A/dairy
178 cattle/Texas/24-008749-002-v/2024 (H5N1). Bovine serum samples were pretreated
179 with receptor-destroying enzyme (RDE) (Denka Seiken, San Jose, CA) for 2 h at 37 ˚C,
180 followed by heat inactivation at 56 ˚C for 30 min. RDE-treated sera were serially diluted
181 in neutralization assay media (NAM, medium 199 supplemented with 0.01% heat-
182 inactivated FBS, 0.3% BSA, 100 U of penicillin/mL, 100 µg of streptomycin/mL, 100 µg
183 of calcium chloride/mL, and 25 mM HEPES), mixed with virus, and incubated at 37 ˚C
184 for 1 h. After incubation, $2.5x10^4$ MDCK-SIAT1-CMV-PB1-TMPRSS2 cells were added
185 to each well. Plates were incubated for 40 hours at 37 °C followed by measurement of
186 GFP fluorescence intensity on an EnVision multi-mode microplate reader (PerkinElmer,
187 Waltham, MA) using an excitation wavelength of 485 nm and an emission wavelength of
188 515 nm. Neutralization titers were expressed as the reciprocal of the highest dilution of
189 sera that inhibited 90% of GFP expression relative to control wells with no sera.
190
191 Hemagglutination inhibition assay

192 The representative wild bird 2.3.4.4b strain used in the HI assay, A/bald
193 eagle/Florida/W22-134-OP/2022 (H5N1) is an engineered influenza viruses carrying a
194 modified HA with a monobasic cleavage site. It is approved for work under BSL-2
195 conditions and was kindly provided by Richard Webby from St. Jude Children's
196 Research Hospital. The virus was propagated in Madin-Darby canine kidney London
197 cell line using OptiMEM (Gibco, Thermo Scientific, Waltham, MA) supplemented with
198 antibiotic-antimycotic (Thermo Scientific, Waltham, MA) and 1 µg/mL L-1-Tosylamide-2-
199 phenylethyl chloromethyl ketone (TPCK, Worthington Biochemicals, Lakewood, NJ).
200 Prior to HI, bovine 50 µL of serum was treated with 150 µL of RDE (Denka Seiken, San
201 Jose, CA) overnight at 37 ^{o}C in a water bath followed by 600 uL of saline solution
202 (0.85%) incubated at 56 ^{o}C for 30 minutes to deactivate RDE. Serum samples were
203 adsorbed with 100% rooster red blood cells for 1 hour at 4 ^{o}C to remove nonspecific
204 hemagglutinin inhibitors and agglutinins. RDE-treated serum samples were run in HA
205 and HI assays using 0.5% rooster red blood, slightly modified from a previously
206 described protocol [12]. The reciprocal titers were divided by 10, $\log_2$ transformed, and
207 reported as the geometric mean.

208

209 Peripheral blood mononuclear cell (PBMC) isolation and stimulation

210 Whole blood was collected via venipuncture of the jugular vein and placed into
211 tubes containing ethylenediaminetetraacetic acid (EDTA) (Greiner Bio-One, Monroe,
212 NC) to prevent clotting. PBMC were isolated via density centrifugation using Ficoll as
213 described previously [13]. PMBC count and viability were determined using a
214 hemocytometer and trypan blue exclusion, and resuspended to a concentration of $1x10^7$

215 per ml. To assess proliferation and intracellular cytokine production, PMBC were stained
216 with a 1:10 CellTrace Violet (eBioscience, San Diego, CA) solution, according to the
217 manufacturer's recommendations, and resuspended in complete RPMI media. PMBC
218 were plated, $1x10^6$ per well, onto 96-well flat bottom plates and left unstimulated,
219 stimulated with a purified HA recombinant protein of the homologous vaccine strain
220 (A/Astrakhan/3212/2020) at various concentrations (0.5 ug, 1 ug, and 5 ug per well),
221 sucrose purified, 3 x UV-inactivated wild bird representative 2.3.4.4b strain (A/bald
222 eagle/ Florida/W22-134-OP/2022) at three multiplicity of infections (MOI) (0.01, 0.1, and
223 1), or concanavalin A (ConA; 0.5 ug/well; Sigma). Plates were incubated for 5 days at
224 37 °C with 5% CO_2. Approximately 16 hours prior to harvest, all wells were treated with
225 a 1x GolgiStop solution (eBioscience, San Diego, CA), according to the manufacturer's
226 recommendation.
227
228 Flow cytometry staining and analysis
229 Cells were prepared for flow cytometry staining as described previously [13].
230 Briefly, PBMC were transferred to 96-well round bottom plates, centrifuged at 300x g at
231 RT, and then washed in 1x Dulbecco's phosphate-buffered saline (DPBS). PMBC were
232 incubated in a fixable cell viability dye solution (1:2000 dilution) (Thermo Scientific,
233 Waltham, MA) for 20 minutes at 4 °C and then washed once in 1x DPBS and once in
234 FACS buffer (0.5% fetal bovine serum (FBS) in PBS). PMBC were then resuspended
235 with gentle vortexing and incubated with anti-bovine $\gamma\delta$ primary antibody (Washington
236 State University) for 15 minutes at RT and then with rat anti-mouse IgG2b secondary
237 antibody (BUV395-labeled; R12-3) (BD Biosciences, San Jose, CA) for an additional 15

238 minutes at RT. PBMC were washed again twice in FACS buffer and incubated with a
239 cocktail containing antibodies against bovine CD4 (FITC-labeled; CC30) and CD8
240 (AlexaFluor 647-labeled; CC58) (BioRad Antibodies, Hercules, CA) for 15 minutes at
241 RT, followed by two additional washes in FACS buffer. For intracellular staining, PMBC
242 were fixed and permeabilized using a permeabilization kit according to the
243 manufacturer's recommendations (BD Biosciences, Franklin Lakes, NJ). Intracellular
244 cytokine staining was performed using an anti-bovine IFN-γ antibody (PE-labeled;
245 CC302). PBMC were washed twice in wash buffer (BD Biosciences, Franklin Lakes,
246 NJ), once in FACS, and then resuspended in FACS buffer until analysis. Data was
247 collected using the BD Symphony flow cytometer (BD Biosciences, Franklin Lakes, NJ)
248 using the DIVA software and analyzed using FlowJo®.
249
250 Viral RNA detection in clinical samples
251 A piece of tissue approximately 10 mm in size was homogenized in 7.5 mL of
252 MEM (Gibco, Thermo Fisher, Waltham, MA) in M tube using gentleMACS dissociator
253 (Miltenyi Biotec, Auburn, CA). Lung homogenates were spun at 419 x *g* and aliquoted
254 for viral RNA extraction. Viral RNA was extracted from 50 uL of the homogenate using
255 Mag Max Viral RNA Kit (Thermo Fisher Scientific, MA) and reverse transcription
256 quantitative real-time PCR (RT-qPCR) targeting IAV matrix gene, VetMAX™-Gold SIV
257 Detection Kit (Thermo Fisher Scientific, Carlsbad, CA) were performed in the milk from
258 the bucket samples, nasal swabs during the challenge phase and lung lobe
259 homogenates, tracheal swab and BALF from necropsy. An aliquot of 100 uL of each
260 lung lobe homogenate was pooled per animal and submitted to Iowa State University

261 Veterinary Diagnostic Laboratory in Ames, Iowa, to ensure our calves were free from
262 other respiratory pathogens such as bacteria (*Pasteurella multocida*, *Mycoplasma*
263 *bovis*, *Mannheimia haemolytica* and *Histophilus somni*) or viruses (Bovine
264 parainfluenza-3 virus, Influenza D virus, Bovine coronavirus, Bovine herpesvirus 1,
265 Bovine viral diarrhea virus, Bovine respiratory syncytial virus) by PCR. Additionally,
266 pooled lung homogenates were submitted four routine bacterial culture. All calves were
267 negative for other bovine respiratory pathogens (Supplementary Table 5).
268
269 Histologic assessment of respiratory tissues
270 A section of turbinate, trachea and each lung lobe (cranial part right cranial lobe,
271 caudal part right cranial lobe, cranial part left cranial lobe, caudal part left cranial lobe,
272 middle lobe, accessory lobe, caudal right lobe and caudal left lobe) from each calf was
273 collected and stored in 10% formalin for 48h, then transferred to 70% alcohol. Formalin-
274 fixed tissues were processed routinely and stained with hematoxylin and eosin (H&E).
275 Each lung section was scored as previously described [14]. Turbinate and trachea were
276 scored as previously described [15], with slight modifications (Supplementary Table 3).
277 Additionally, a minimum of two lung sections per animal were evaluated by
278 immunohistochemistry (IHC) [3, 16]. Sections stained by IHC were prioritized based on the
279 severity of the histologic lesion score. If no histologic lesions were noted, sections from
280 the cranial part of the left cranial lobe and middle lobe were selected. In addition, any
281 section with a histologic score greater than 3 was stained by IHC.
282
283 Statistical Analysis

284 Log_2 geometric mean antibody titers and CMI responses were analyzed using
285 analysis of variance (ANOVA), with a *P* value of <0.05 considered significant (GraphPad
286 Prism software version 6.00; San Diego, CA). Response variables shown to have a
287 significant effect by treatment group were subjected to pairwise comparisons using the
288 Tukey-Kramer test, by Graph Pad Prism (10.3.0). In the post-challenge, *Ct* values from
289 qPCR of nasal secretions, lung lobes, tracheal swabs and BALF were interpolated into
290 a Log_{10} $TCID_{50}$/mL standard curve of the A/dairy cattle/ Texas/24-008749-002/2024
291 (H5N1) strain, ranging from $10^{7.75}$ $TCID_{50}$/mL to $10^{-0.7}$ $TCID_{50}$/mL (Supplementary Table
292 4) using a simple linear regression in Graph Pad Prism (10.3.0).
293

294 **Acknowledgements**

295 We thank the USDA NADC and NVSL leadership and personnel from animal
296 resource and facilities and engineering units; Katharine Young, Emily Love and Sarah
297 Anderson for laboratory technical assistance; Tonia McNunn and the NCAH select
298 agent staff for compliance assistance; Jason Huegel, Tiffany Williams, Jonathan
299 Gardner, Jared Peterson, Emma Hay, Emma Pratt and Brian Conrad for assistance with
300 animal studies in the BSL-2 and BSL-3 facilities. This work was supported in part by the
301 USDA ARS (project number 5030-32000-231-000-D); the National Institute of Allergy
302 and Infectious Diseases, National Institutes of Health, Department of Health and Human
303 Services (contract numbers 75N93021C00015). A.C was supported by an appointment
304 to the USDA-ARS Research Participation Program administered by the Oak Ridge
305 Institute for Science and Education (ORISE) through an interagency agreement
306 between the U.S. Department of Energy (DOE) and the USDA under contract no. DE-

AC05-06OR23100. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Government. USDA is an equal opportunity provider and employer.

Competing interest statement

S.E.H. and D.W. are co-inventors on patents that describe the use of nucleoside-modified mRNA as a platform to deliver therapeutic proteins and as a vaccine platform. S.E.H reports receiving consulting fees from Sanofi, Pfizer, Lumen, Novavax, and Merck.

References

1. Burrough, E.R. et al. Highly Pathogenic Avian Influenza A(H5N1) Clade 2.3.4.4b Virus Infection in Domestic Dairy Cattle and Cats, United States, 2024. *Emerg Infect Dis* **30**, 1335-1343 (2024).
2. Guan, L. et al. Cow's Milk Containing Avian Influenza A(H5N1) Virus - Heat Inactivation and Infectivity in Mice. *N Engl J Med* **391**, 87-90 (2024).
3. Baker, A.L. et al. Dairy cows inoculated with highly pathogenic avian influenza virus H5N1. *Nature* **637**, 913-920 (2025).
4. Uyeki, T.M. et al. Highly Pathogenic Avian Influenza A(H5N1) Virus Infection in a Dairy Farm Worker. *N Engl J Med* **390**, 2028-2029 (2024).
5. Garg, S. et al. Outbreak of Highly Pathogenic Avian Influenza A(H5N1) Viruses in U.S. Dairy Cattle and Detection of Two Human Cases - United States, 2024. *MMWR Morb Mortal Wkly Rep* **73**, 501-505 (2024).
6. Taft, A.S. et al. Identification of mammalian-adapting mutations in the polymerase complex of an avian H5N1 influenza virus. *Nat Commun* **6**, 7491 (2015).
7. Furey, C. et al. Development of a nucleoside-modified mRNA vaccine against clade 2.3.4.4b H5 highly pathogenic avian influenza virus. *Nat Commun* **15**, 4350 (2024).
8. Sarlo Davila K., Baker .A.L., Boggiatto P. M. , Palmer M. V. , Putz E. J. , Olsen S. C. , Ciacci Zanella G. , Campos A. , Buckley A. , Arruda B. Transmission of highly pathogenic avian influenza H5N1 to calves fed unpasteurized milk from experimentally infected cows (Preprint at AgriRxiv; 2025).

9. Reed, L.J. & Muench, H. A simple method of estimating fifty per cent endpoints. *American Journal of Epidemiology* **27**, 493-497 (1938).
10. Whittle, J.R. et al. Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. *J Virol* **88**, 4047-4057 (2014).
11. Doud, M.B., Hensley, S.E. & Bloom, J.D. Complete mapping of viral escape from neutralizing antibodies. *PLoS Pathog* **13**, e1006271 (2017).
12. Kitikoon, P., Gauger, P.C. & Vincent, A.L. Hemagglutinin inhibition assay with swine sera. *Methods Mol Biol* **1161**, 295-301 (2014).
13. Boggiatto, P.M., Schaut, R.G. & Olsen, S.C. Enhancing the Detection of Brucella-Specific CD4(+) T Cell Responses in Cattle via in vitro Antigenic Expansion and Restimulation. *Front Immunol* **11**, 1944 (2020).
14. Morgan, S.B. et al. Aerosol Delivery of a Candidate Universal Influenza Vaccine Reduces Viral Load in Pigs Challenged with Pandemic H1N1 Virus. *J Immunol* **196**, 5014-5023 (2016).
15. Gauger, P.C. et al. Live attenuated influenza A virus vaccine protects against A(H1N1)pdm09 heterologous challenge without vaccine associated enhanced respiratory disease. *Virology* **471-473**, 93-104 (2014).
16. Arruda, B. et al. Divergent Pathogenesis and Transmission of Highly Pathogenic Avian Influenza A(H5N1) in Swine. *Emerg Infect Dis* **30**, 738-751 (2024).

Figure Legends

Figure 1. Antibody and cellular-mediated immune responses of H5 HA mRNA-LNP vaccinated calves. The *X* axis represents days post-vaccination (dpv) of the blood collected for serological assays. The *Y* axis of panels a, b, d, e and f represent the antibody titers in a Log_2 scale. The Y axis of the panel c represents the SN%. rHA H5 IgG binding ELISA using the homologous vaccine virus **(a)** rHA H5 IgG binding ELISA using the 2.3.4.4b cattle strain **(b)**, commercial ID.Vet H5 ELISA for H5 antibody detection, with cut-offs of SN%<40 is positive, 40%<SN%<50% is suspect and SN%≥ 50% is negative **(c)**. Hemagglutination Inhibition assay (HI) using the wild bird representative 2.3.4.4b strain **(d)**, Foci reduction neutralization (FRNT) with the homologous vaccine strain **(e)**, and FRNT with the 2.3.4.4b cattle strain **(f)**. Percentage of proliferating $CD8^+$ T cells when PBMC were stimulated with 1 ug of recombinant HA

374 (rHA) protein homologous to the vaccine strain at 21 and 35 dpv or stimulated with
375 m.o.i. of 0.1 of the wild bird representative 2.3.4.4b strain at 35 dpv **(g)** and the
376 percentage of proliferating and IFN-γ^+ CD8$^+$ T cells when PBMC were stimulated with 1
377 ug of recombinant HA (rHA) H5 protein homologous to the vaccine strain at 21 and 35
378 dpv or stimulated with m.o.i. of 0.1 of the wild bird representative 2.3.4.4b strain at 35
379 dpv, represent the Y axis **(h)**. The unvaccinated and H5 mRNA-LNP 50 or 500 μg
380 vaccinated groups are depicted in the *X* axis. The legend shows the PBMC non-
381 stimulated as None in grey dots, stimulated with 1 ug of homologous recombinant HA
382 (rHA) in red dots and stimulated with wild bird representative 2.3.4.4b strain at m.o.i. of
383 0.1 in blue dots. Statistical differences ($p \leq 0.05$) between groups and treatments are
384 indicated by asterisks and the bar indicates group comparisons.
385
386 **Figure 2. Post-challenge virus RNA detection in nasal secretions and rectal**
387 **temperatures**. The *Y* axis represents **(a)** the Log_{10} $TCID_{50}$/mL equivalent Ct detection of
388 viral RNA in nasal swabs and **(b)** rectal temperature higher than 39.7 °C indicates fever.
389 The *X* axis represents the days post-infection (dpi).
390
391 **Extended Data 1. Percentage of proliferating CD4$^+$ and γδ$^+$ T cells and IFN-γ$^+$**
392 **production when PBMC were stimulated with rHA H5 protein homologous to the**
393 **vaccine or the wild bird representative 2.3.4.4b virus**. The unvaccinated and 50 or
394 500 µg H5 mRNA-LNP vaccinated groups are depicted in the *X* axis. The Y axis
395 represents the percentage of proliferating CD4$^+$ **(a)**, IFN-γ$^+$ CD4$^+$ **(b)**, γδ$^+$ **(c)** and IFN-γ$^+$
396 γδ$^+$ **(d)** T cells at 21 dpv when stimulated with rHA H5 protein homologous to the

397 vaccine (red dots) and at 35 dpv when stimulated with rHA H5 protein homologous to
398 the vaccine (red dots) or the wild bird representative 2.3.4.4b virus (blue dots). Non-
399 stimulated T cells (None) are represented with grey dots. Statistical differences ($p \leq$
400 0.05) between groups and treatments are indicated by asterisks and the bar indicates
401 group comparisons.
402
403 **Supplementary files**
404
405
406 Supplementary Table 1. Virus titers ($Log_{10}TCID_{50}$/mL) and *Ct* values of the milk infected
407 with H5N1 used as inoculum to feed calves.
408
409 Supplementary Table 2. $Log_{10}TCID_{50}$/mL equivalent *Ct* in lung tissues, tracheal swabs
410 and BALF samples.
411
412 Supplementary Table 3. Histologic lesion composite scores on turbinate, trachea and
413 lung lobes. IHC positive tissues are marked with an asterisk.
414
415 Supplementary Table 4. $Log_{10}TCID_{50}$/mL equivalent Ct and interpolated Ct values of
416 samples.
417
418 Supplementary Table 5. Bovine respiratory pathogens panel tested from pooled lung
419 lobe homogenate per calf.
420
421