

The bat-borne influenza A virus H9N2 exhibits a set of unexpected pre-pandemic features

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Additional Declarations: Yes there is potential Competing Interest. The Icahn School of Medicine at Mount Sinai has filed patent applications relating to influenza virus vaccines, SARS-CoV-2 serological assays and SARS-CoV-2 vaccines which list Florian Krammer as co-inventor. Viviana Simon is also listed as coinventor on patent applications for SARS-CoV-2 serological assays. Mount Sinai has spun out companies, Kantaro and Castlevax, to market the SARS-CoV-2 related technologies. Florian Krammer has consulted for Merck and Pfizer (before 2020), and is currently consulting for Pfizer, Seqirus, 3rd Rock Ventures, GSK and Avimex. The Krammer laboratory is also collaborating with Pfizer on animal models of SARS CoV 2 and with Dynavax on universal influenza virus vaccines. All other authors declare no competing interests.

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1 The bat-borne influenza A virus H9N2 exhibits a set of 2 unexpected pre-pandemic features

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35 Abstract

- 36 An Old World bat H9N2 influenza A virus (IAV) identified in Egypt exhibits high replication and
- 37 transmission potential in ferrets, efficient infection of human lung explant cultures and marked
- 38 escape from the antiviral activity of MxA. Together with low antigenic similarity to N2 of
- 39 seasonal human strains, bat H9N2 meets key criteria for pre-pandemic IAVs.

41 Main

42 Influenza A viruses (IAVs) are highly infectious viral pathogens that can cross interspecies 43 barriers and infect a wide range of avian and mammalian species¹. Although bats have long been known to be reservoirs for a variety of viruses², they were only recently found to also 44 harbor IAVs^{3,4}. While H17N10 and H18N11 strains were first identified in Central and South 45 46 American bat species^{3,4}, H9N2 viruses have recently been isolated from Egyptian fruit bats 47 (*Rousettus aegyptiacus*) in the Nile Delta region⁵. Phylogenetic analyses suggest that this Old 48 World bat H9N2 virus is distinct from New World bat IAVs and emerged as a reassortant from 49 an ancestral bat backbone and avian IAV H9 and N2 segments⁶. Avian H9N2 viruses were 50 first isolated from turkeys in North American poultry farms in 1966⁷ and subsequently became 51 endemic in poultry farms in many countries in the Middle East and Asia^{8,9}. Since then, avian 52 H9N2 viruses have become widespread and have undergone extensive reassortment with 53 other circulating avian IAVs, resulting in at least 74 different lineages¹⁰. Over the past two 54 decades, avian H9N2 infections have been recorded in swine populations and mink farms^{11,12}. 55 Furthermore, since 1998, the WHO has reported 82 human spill-over infections with avian IAVs 56 in China or Cambodia, resulting in mild to severe disease¹³. Interestingly, sero-epidemiological 57 data from Ghanaian straw-colored fruit bats showed a high prevalence of H9-specific 58 antibodies (30%)¹⁴, and bat H9N2 was also recently detected in South African bats¹⁵, 59 suggesting widespread circulation of bat H9N2 in African bat populations. Similar to avian 60 H9N2, bat H9N2 initiates infection by utilizing avian IAV-like α 2,3 sialic acid receptors, and 61 replicates in mice, but not in adult chickens⁵. Here, we investigated whether bat H9N2 is of 62 zoonotic concern.

63

64 As H9N2 viruses were originally isolated from turkeys⁷, we first determined the replication 65 properties of bat H9N2 in one-day-old turkeys. Following oro-nasal inoculation, bat H9N2 replicated efficiently to 10⁵ to 10⁷ copies mL⁻¹ at 1 day post infection (dpi; Extended Data Fig. 66 67 1a). Thereafter, viral loads rapidly decreased but again reached titers of 5x10⁵ copies mL⁻¹ 68 between 5 to 8 dpi. Infectious virus was isolated from oral swabs collected at 5 dpi (Extended 69 Data Fig. 1a). At 11 dpi, all but one oral swab was negative for viral RNA (Extended Data Fig. 70 1a) and no viral RNA was detected in cloacal swabs at any time point measured. All turkey 71 hatchlings seroconverted with antibodies targeting the viral nucleoprotein (NP) at 21 dpi 72 (Extended Data Fig. 1b,c), demonstrating that bat H9N2 maintained its ability to replicate in 73 turkeys. In contrast, and in agreement with previous reports⁵, bat H9N2 failed to replicate 74 efficiently in one-day-old chicken and did not elicit an antibody response (Extended Data Fig. 75 1d,e).

77 In order to assess the zoonotic potential and transmissibility of bat H9N2 in the model most 78 relevant to humans, we infected 15 donor ferrets and co-housed three naïve contact animals 79 from 1 to 12 dpi (Fig. 1a). Quantification of viral RNA obtained from nasal lavages revealed substantial viral replication (10⁶ to 10⁸ copies mL⁻¹) within the first two days after infection and 80 81 continuous shedding of viral genomes up to 10 dpi (Fig. 1a). Strikingly, all contact animals 82 immediately acquired a viral infection from donor ferrets after co-housing with peak titers (10⁷ 83 copies mL⁻¹) at 4 days post exposure (dpe) (Fig. 1a). To determine whether bat H9N2 84 replication is limited to the upper respiratory tract, we next measured viral titers in the organs 85 of six donor ferrets euthanized at 6 dpi (Fig. 1b). While all ferrets had substantial viral genome 86 copies in the nasal conchae and five of six animals had moderate levels in the trachea, 1 of 6 87 ferrets had moderate viral genome levels in the cranial lung lobe, 2 in the medial and caudal 88 lung lobes, and 1 ferret even had low viral copies in the colon (Fig. 1b). We did not observe 89 severe body weight loss in most donor and any contact ferrets, although two donor animals 90 exhibited ~15% weight loss at 6 and 12 dpi (Fig. 1c), which was most likely unrelated to 91 infection. Elevated body temperatures ranging from 39 to 41°C were observed at 2 dpi in 14 92 of 15 donor ferrets (Fig. 1d) and all contact animals had elevated body temperatures from 1 93 dpe onwards (Fig. 1d). Seroconversion with NP-specific antibodies was detected as early as 94 6 dpi in donor ferrets, and all donor and contact ferrets examined at 21 dpi exhibited a robust 95 NP-specific antibody response (Fig. 1e). Furthermore, at 21 dpi we determined antibodies with 96 a strong neutralizing capacity against bat H9N2 and some degree of cross-neutralization 97 against the avian H9N2 A/layer chicken/Bangladesh/VP02-plague/2016 isolate (Fig. 1f). 98 Histopathological examination revealed severe purulent to necrotizing rhinitis with viral antigen 99 in the respiratory and olfactory epithelia of all ferrets at 6 dpi (Fig 1g). We observed mild 100 infection-induced changes characterized by focal to oligofocal epithelial necrosis and mild 101 infiltration of the lamina propria in the trachea of four animals. No influenza-associated 102 pathology was detected in the lungs.

103

104 Because severe courses of influenza in humans almost always affect the lower respiratory 105 tract¹⁶, we next infected human ex vivo lung cultures with bat H9N2, a prototypic human 106 seasonal H3N2 isolate (A/Panama/2007/1999) or chicken H9N2 and determined viral growth 107 properties. Intriguingly, bat H9N2 replicated to comparable or even higher viral titers than 108 human H3N2, reaching peak titers of 3×10⁴ plague-forming units (PFU) mL⁻¹ at 48 hours post 109 infection (hpi; Fig. 2a). In contrast, chicken H9N2 showed minimal viral replication in human 110 lung tissue. Immunostaining of lung explants at 24 hpi revealed that all viruses infected alveolar 111 type II cells (Fig. 2b), which is the primary cellular tropism of IAV in the lung¹⁷.

113 Next, we studied whether bat H9N2 is able to escape human MxA, a crucial innate antiviral 114 factor which restricts IAVs by inhibiting their polymerase activity¹⁸. Human-adapted IAVs, such as the pandemic H1N1 virus A/Hamburg/4/2009 (pdmH1N1), acquire characteristic clusters of 115 adaptive mutations in NP that enable escape from MxA^{18,19}, whereas such clusters are virtually 116 117 absent in IAVs of avian origin including the highly-pathogenic H5N1 strain A/Thailand/1(KAN-118 1)/2004 (KAN-1). Bat H9N2 NP also lacks the residues described as conferring MxA resistance 119 (Extended Data Fig. 2a). Thus, as expected, bat H9N2 exhibited a high degree of MxA-120 sensitivity as demonstrated by infecting MDCK cells stably overexpressing either wild-type 121 MxA (MDCK-MxA) or the antivirally inactive MxA_{T103A} variant (MDCK-MxA_{T103A})²⁰. While 122 pdmH1N1, KAN-1 and bat H9N2 replicated efficiently in MDCK-MxA_{T103A} cells to titers between 123 1.3×10^7 and 7×10^8 PFU mL⁻¹ at 48 hpi (Fig. 2c), KAN-1 was nearly completely inhibited in 124 MDCK-MxA cells whereas peak titers of pdmH1N1 decreased only 5-fold. Replication of bat 125 H9N2 was potently restricted in the presence of MxA as illustrated by residual viral titers $\leq 10^2$ 126 PFU mL⁻¹ between 24-48 hpi (Fig. 2c).

127

128 To assess the importance of MxA in controlling bat H9N2 in vivo, we intranasally infected wild 129 type C57BL/6 (B6), which lack a functional Mx protein, and human MxA-transgenic (hMxA^{tg/tg}) 130 mice with bat H9N2. Surprisingly, lung viral titers were similar in both B6 and MxA-transgenic 131 mice²¹ at 3 dpi (5×10⁵ PFU mL⁻¹; Fig. 2d), as confirmed by comparable NP levels in lung 132 homogenates detected by Western blotting (Fig. 2f). Interestingly, MxA expression was not 133 observed in the lungs of infected hMxA^{tg/tg} mice, but could be potently induced by IFN- α 134 pretreatment 18 h prior to challenge infection with bat H9N2. Under these conditions, we 135 observed induction of MxA (Fig. 2f) and 10-fold lower lung viral lung titers in hMxA^{tg/tg} compared 136 to B6 mice (Fig. 2e), suggesting that MxA, when induced, reduces bat H9N2 replication.

137

Finally, because there is little serological evidence for H9-specific antibodies in the human population^{22,23}, we wondered whether the antibody responses to circulating seasonal H1N1 and H3N2 strains as well as vaccination would be cross-reactive for bat N2. Serum collected from 15 healthy adults before and after seasonal influenza vaccination in 2022/23 revealed no reactivity to bat N2 (Extended Data Fig. 2b,e), but robust reactivity to N2 from the seasonal A/Kansas/14/2017 (H3N2) isolate (Extended Data Fig. 2d).

144

Our study shows that the Old World bat H9N2 virus meets key characteristics of a prepandemic IAV, including replication in and efficient transmission between ferrets, the ability to replicate efficiently in human lung explants and evasion from MxA-mediated restriction. Intriguingly, bat H9N2 exhibits an immediate (at 1 dpe) and highly efficient transmission potential (100%) not previously observed in any avian-derived H9N2 isolate²⁴, which may also allow for spread among and further adaptation to humans. Our data also suggests that bat H9N2 can suppress the expression of MxA, thereby overcoming this important restriction factor for zoonotic spill-over²⁵. This is in strong contrast to zoonotic H5N1 and H7N9 viruses of avian origin that are potently inhibited in hMxA^{tg/tg} mice²¹. Given the ability of bat H9N2 to infect turkey hatchlings, introduction of bat H9N2 into poultry farms and reassortment with avian IAV cannot be ruled out, necessitating increased attention and close monitoring of possible human spillover infections in Africa.

157

A further prerequisite of pre-pandemic viruses is their antigenic novelty to the human immune system. Since the human population is presently exposed only to the currently-circulating H1N1 and H3N2 subtypes, a lack of humoral immunity to bat H9N2 is very likely. Indeed, our serological data demonstrates that seasonal influenza vaccines containing H1N1 and H3N2 do not elicit cross-reactive antibodies to the bat N2 protein, substantiating the general prepandemic features of bat H9N2.

164 Material and Methods

165 Virus

166 The bat-derived H9N2 A/bat/Egypt/381OP/2017 isolate was propagated in embryonated SPF-167 chicken eggs for 5 days at 37 °C. Subsequently, the allantoic fluid was harvested and used as 168 virus stock. The chicken H9N2 isolate A/layer chicken/Bangladesh/VP02-plague/2016 was 169 obtained from the Friedrich-Loeffler-Institut (FLI) virus repository²⁶. Virus stocks of the human 170 seasonal A/Panama/2007/1999 (H3N2) isolate were generated by propagation on MDCKII 171 cells. Recombinant A/Hamburg/4/2009 (pdmH1N1) and A/Thailand/1(KAN-1)/2004 (H5N1) 172 were generated utilizing the eight-plasmid pHW2000-based rescue system²⁷. All recombinant 173 viruses were plague purified and then used for stock generation. Stock titers were determined 174 by a plaque assay on MDCKII cells.

175 **Cells**

176 Madin-Darby Canine Kidney (MDCK) type II cells (Collection of Cell Lines in Veterinary 177 Medicine CCLV RIE1061) were used. Cells were incubated at 37 °C under 5% CO2 178 atmosphere using a mixture of equal volumes of Eagle Minimum Essential Medium (MEM) 179 (Hank's balanced salts solution) and Eagle MEM (Earle's balanced salts solution), 2 mM L-180 Gln, nonessential amino acids, adjusted to 850 mg L⁻¹ NaHCO3, 120 mg L⁻¹ sodium pyruvate, 181 pH 7.2 with 10% FCS (Bio & Sell GmbH) or without FCS in the presence of tosylsulfonyl 182 phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) after virus addition. MDCK-183 MxA and MDCK-MxA_{T103A} were cultured in Dulbecco's modified Eagle's medium (DMEM, 184 Gibco, Thermo Fisher Scientific) containing 10% fetal calf serum (FCS), 100 U penicillin and 185 100 μ g streptomycin mL⁻¹ at 37 °C and 5% CO₂.

186 Virus infections

187 MDCK-MxA and MDCK-MxA_{T103A} cells were seeded and grown in 6-well plates. Prior to 188 infection cells were washed with phosphate buffered saline (PBS) containing 0.2% bovine 189 serum albumin (BSA) and then infected with the indicated virus at an MOI of 0.001 in infection 190 medium (DMEM, containing 0.2% BSA and 100 U penicillin and 100 μ g streptomycin μ L⁻¹). For 191 bat H9N2 and pdmH1N1 1 μ g mL⁻¹ TPCK-treated trypsin was added into the infection medium. 192 Viral titers were determined by plaque assay.

193 Infection of human lung explants

194 Fresh lung explants were obtained from patients suffering from lung carcinoma and undergoing195 lung resection at local thoracic surgeries. Written informed consent was obtained from all

196 patients and the study was approved by the ethics committee at the Charité clinic (project 197 EA2/079/13). Tumor-free peripheral lung tissue was cut into small pieces and incubated 198 overnight at 37°C with 5% CO₂ in Roswell Park Memorial Institute (RPMI) 1640 medium. The 199 next day, lung tissue was infected with 1×10⁶ PFU of either human seasonal H3N2, chicken 200 H9N2 or bat H9N2 for 1.5 h under shaking conditions and excess virus was removed by three 201 washing steps with PBS. Infected lung tissues were incubated at 37°C and 5% CO₂ for up to 202 72 h in RPMI 1640 medium supplemented with 2 mM L-glutamine and 0.3% BA. Viral titers 203 were determined by plaque assay.

204 Western blot

Mouse lung samples were incubated at 95°C in Laemmli buffer and subsequently separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Separated protein samples were blotted onto a nitrocellulose membrane. Proteins were detected using specific antibodies against the highly conserved G domain in MxA (M143)23, NP (Gene Tex, GTX125989, 1:1,000), or actin (Sigma-Aldrich, A3853; 1:1,000), respectively. Primary antibodies were detected using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, 1:5,000).

212 Animal experiment ethics declarations

All ferret and hatchling experiments were evaluated by the responsible ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg–Western Pomerania (LALLF M-V) and gained governmental approval under the registration numbers LVL MV TSD/7221.3-1-029/22 and 7221.3-1-003/22. All mouse experiments were performed in accordance with the guidelines of the German animal protection law and were approved by the state of Baden-Württemberg (Regierungspräsidium Freiburg; reference number: 35-9185.81/G-19/05).

220 Animals

221 One-day-old chickens, one-day-old turkeys, ferrets as well as C57BL/6 (B6) mice and human 222 MxA transgenic (hMxA^{tg/tg}) mice were used for this study. Chicks were bread at the FLI from 223 SPF-chicken eggs (VALOBioMedia, Germany) and one-day-old turkeys were ordered and 224 shipped on hatching day from a local breeding facility (Bösel) to the FLI. The ferrets were 225 obtained from the in-house breeding program at the FLI. B6 mice were obtained from Janvier 226 and hMxA^{tg/tg} mice were bred in-house at the Institute of Virology, Freiburg.

227 One-day-old chicken and turkey studies

228 At the day of hatching, one-day-old turkeys were inoculated with 10⁵ TCID₅₀ per animal and 229 one-day-old chicks were inoculated with 10^{3,9} TCID₅₀ per animal, calculated by back-titration 230 of the original inoculum. All hatchlings were sampled daily via cloacal and oro-pharyngeal 231 swabs until 21 dpi or until the animal samples tested negative in a bat H9N2-specific RT-qPCR. 232 Oro-pharyngeal and cloacal swabs were taken using plain swab sterile paper applicator cotton 233 tips 164C (Copan, Brescia, Italy). The swabs were immediately transferred into 1 mL of cell 234 culture medium containing 1% Baytril (Bayer, Leverkusen, Germany), 0.5% lincomycin (WDT, 235 Garbsen, Germany) and 0.2% amphotericin/gentamycin (Fisher Scientific Waltham, MA, 236 USA). After euthanasia, nasal conchae and colon organ samples were taken for investigation 237 of viral genome loads via RT-gPCR analysis in the respective organs. Clinical status of the 238 animals was checked daily.

239 Ferret study

240 Ferrets (Mustela putorius furo) were housed in multiple connected cage units. Before 241 inoculation, blood samples and nasal washings were collected to confirm naivety to IAV of all 242 animals via serological analysis (ELISA) and RT-gPCR. Body weight, body temperature as 243 well as physical condition of all animals was monitored regularly throughout the animal trial. 244 Nasal washing samples were taken under a short-term isoflurane inhalation anesthesia by 245 applying 750 µl of PBS into each nostril and collecting the efflux. Rectal swabs were taken 246 using plain swab sterile paper applicator cotton tips 164C (Copan). The swabs were 247 immediately transferred into 1 mL of cell culture medium containing 1% Baytril (Bayer, 248 Leverkusen, Germany), 0.5% lincomycin (WDT, Garbsen, Germany) and 0.2% 249 amphotericin/gentamycin (Fisher Scientific Waltham, MA, USA). After one week of 250 acclimatization to their new environment (0 dpi), 15 ferrets were intranasally inoculated with 251 10^{4.8} TCID₅₀ per animal in a 200 µL volume (calculated by back-titration of the original material). 252 The inoculum was evenly distributed into each nostril (approximately 100 µl per nostril). At 1 253 dpi, three direct contact animals were co-housed with the donor ferrets. All animals were 254 sampled via nasal washings and rectal swabs daily until 10 dpi and afterwards every second 255 day until 21 dpi or until the samples tested negative via bat H9N2 specific RT-qPCR analysis. 256 Clinical signs of disease (nasal discharge, reduced activity, fever, neurological symptoms and 257 dyspnea), body temperature and body weight were monitored daily. At 6 dpi, in the acute phase 258 of the infection, six donor ferrets were euthanized and subject to necropsy for 259 pathomorphological investigation and analysis of viral genome loads in the upper and lower 260 respiratory organs, as well as in the intestinal tract. The residual animals were kept until the 261 end of the study at 21 dpi to allow for seroconversion. Nasal conchae organ samples from 262 animals euthanized at 21 dpi were analyzed with a bat H9N2-specific RT-qPCR.

263 Mouse study

264 For infection experiments, mice were anaesthetized with a mixture of ketamine (100 mg per g 265 body weight) and xylazine (5 mg per g body weight) administered intraperitoneally and were 266 subsequently inoculated intranasally with 40 µL of the indicated virus dose diluted in Opti-MEM 267 containing 0.3% BSA. For interferon pretreatment 2 μg per 100 μL IFN-α was administered 268 subcutaneously 18 hours prior to challenge with the indicated virus. Throughout the 269 experiment, mice were monitored daily for changes in body weight and other signs of disease. 270 At 3 dpi mice were sacrificed and the lung was dissected. Organs were homogenized in 1 mL 271 PBS by three subsequent rounds of mechanical treatment for 25 s each at 6.5 ms⁻¹. Tissue 272 debris was removed by centrifuging homogenates for 5 min at 5,000 rpm at 4°C and samples 273 were stored at -80°C until further processing. Viral organ titers were determined by plaque 274 assay.

275 **Propagation of bat H9N2 virus isolates from turkey samples**

For isolation of bat H9N2 from turkey hatchlings, swab material was used for inoculation of embryonated chicken eggs. Briefly, 200 µl of selected animal samples were transferred into the allantoic cavity of embryonated SPF-chicken eggs (three eggs per sample), followed by incubation for 5 days at 37°C. Viral genome material was extracted from the allantoic fluid and detected by RT-qPCR analysis.

281 Pathomorphology and immunohistochemistry

282 For the ferret histopathology, nasal conchae, trachea, right cranial, medial and caudal lung 283 lobes as well as the colon were sampled. Tissues were fixed in 10% neutral buffered formalin, 284 embedded in paraffin wax and cut at 3 µm sections. To assess tissue architecture and cell 285 morphology sections were stained with hematoxylin and eosin following standard procedures. 286 For viral antigen detection immunohistochemistry was performed using an in house derived 287 rabbit polyclonal primary antibody directed against the influenza nucleoprotein (NP, 1:750)²⁸. 288 Lesions and cellular vial antigen localization were determined and evaluated by a board-289 certified pathologist (DipIACVP).

To analyze the cellular tropism of IAV infection in human lung tissue samples, tissues were fixed with 4 % paraformaldehyde for 48 h, embedded in paraffin and processed for immunohistochemistry. Lung tissue was then blocked with 5% adequate serum and incubated with primary antibodies direct to CD68 (abcam, Cambridge, UK, 1:50), HT2-280 (terrace biotech, 1:200) and EMP2 (atlas antibodies, 1:50). Viral antigens were stained with polyclonal antibodies to IAV (Serotec, Puchheim, Germany, 1:50) conjugated to a fluorophore (DyLight 488, Thermo Fisher). Primary antibodies were detected using a corresponding secondary 297 labeling kits (OPAL Polaris, Akoyabio) and nuclear counterstaining was performed using DAPI
298 (Sigma, Hamburg, Germany). Finally tissue sections were mounted in Mowiol, and analyzed
299 using a LSM 780 spectral confocal microscope (objectives 63x Plan-Apochromat/oil, NA 1.4,
300 Zeiss, Germany).

301 Experimental sample work-up and analysis

302 Animal organ samples of about 0.1 cm³ size were first homogenized in a 2 mL Eppendorf-tube 303 containing 1 mL of Hank's balanced salts MEM and Earle's balanced salts MEM (2 mM L-304 glutamine, 850 mg L⁻¹ NaHCO3, 120 mg L⁻¹ sodium pyruvate, and 1% penicillin–streptomycin) 305 at 300 Hz using a Tissuelyser II (Qiagen, Hilden, Germany). From each homogenized organ, 306 swab or nasal wash sample, 100 µl was extracted via the NucleoMag Vet kit (Macherey&Nagel, 307 Düren, Germany) according to the manufacturer's instructions on a Biosprint 96 platform 308 (Qiagen). Viral RNA was detected by RT-qPCR using bat H9N2-specific primers and probes²⁹. 309 Absolute quantification was done using a standard of known concentrations, corresponding to 310 the RNA of the original virus used for inoculation. Quantification was established by the QX200 311 Droplet Digital PCR System in combination with the 1-Step RT-ddPCR Advanced Kit for 312 Probes (BioRad, Hercules, CA, US).

313 Human sera collected before and after seasonal influenza vaccination

The observational study protocol IRB-16-00772 was reviewed and approved by the Mount Sinai Hospital Institutional Review Board. All study participants provided written informed consent before biospecimens, and data were collected. Permissions to store and share biospecimen were also obtained from all participants. All specimens were coded before processing and analysis. Whole blood was collected through venipuncture into serum separator tubes and sera were stored at -80 °C until analysis.

320 Serology

321 Serological analysis of blood samples from all animals at respective blood collection time 322 points was performed by using a commercial IAV-specific enzyme-linked immunosorbent 323 assay (ELISA) detecting NP-specific antibodies (ID-Vet, Montpellier, France) according to the 324 manufacturer's instructions. The antibody titers were expressed as "% inhibition", which was 325 calculated as ((OD₄₅₀ negative control – OD₄₅₀ sample) / OD₄₅₀ negative control) × 100.

Neutralizing antibody titers were determined in a virus neutralization test (VNT). Briefly, MDCK cells seeded and grown in 96-well plates 24 hours before infection. Serum samples were serially diluted in DMEM containing 1 μ g mL⁻¹ TPCK-treated trypsin and then and mixed with 100 TCID₅₀ mL⁻¹ of either bat or chicken H9N2. After incubation for two hours at 37°C and 5% CO₂, the serum-virus mixture was transferred onto MDCK and incubated for 72 hours. 331 Neutralization was evaluated by light microscopy for the absence of specific cytopathic effect332 (CPE), and the corresponding VNT titer was determined from the last serum dilution in which

333 no CPE was observed.

334 ELISAs with human sera against a recombinant version of the N2 NA of H9N2 virus 335 A/bat/Egypt/381OP/2017 were performed as described in detail before³⁰. Recombinant NA 336 from human seasonal H3N2 strain A/Kansas/14/2017 was used as to show positive reactivity, 337 recombinant NA from the Wuhan spiny eel influenza virus³¹ (to which humans are naïve) was 338 used as contrast to show negative reactivity. Recombinant proteins were expressed as 339 described previously³². Sera collected from 15 study participants before and after receiving the 340 2022/23 seasonal influenza vaccination were used to determine reactivity to N2 from H9N2, 341 N2 from seasonal H3N2 or to the Wuhan spiny eel influenza virus NA. Monoclonal antibody 342 1G01³³ was used as positive control in all cases.

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361 Author contributions

362 N.J.H., D.H. and M.Be. conceived the study. N.J.H., J.S., L.U. and D.H. designed and 363 performed turkey, chicken and ferret experiments. J.S.E. and L.U. performed ferret pathology. 364 C.M., M.T., M.Bu., S.H., A.C.H. and T.W. designed and performed human lung explant 365 infection experiments. L.H., J.F. and K.C. designed and performed mouse infections. P.B., 366 M.L., A.A., J.M.C., V.S. and F.K. collected serum samples, and designed and performed 367 serological analysis. A.K., R.E.S., M.A.A. and G.K. provided reagents. S.C. performed 368 computational analysis. N.J.H., M.S., K.C. and M.Be. wrote the original draft. N.J.H., J.S.E., 369 L.U., F.K., M.S., K.C., D.H. and M.Be. reviewed and edited the paper. T.W., S.H., A.C.H., M.S., 370 K.C. and M.Be. acquired funds.

372 Competing interests

373 The Icahn School of Medicine at Mount Sinai has filed patent applications relating to influenza 374 virus vaccines, SARS-CoV-2 serological assays and SARS-CoV-2 vaccines which list Florian 375 Krammer as co-inventor. Viviana Simon is also listed as co-inventor on patent applications for 376 SARS-CoV-2 serological assays. Mount Sinai has spun out companies, Kantaro and 377 Castlevax, to market the SARS-CoV-2 related technologies. Florian Krammer has consulted 378 for Merck and Pfizer (before 2020), and is currently consulting for Pfizer, Seqirus, 3rd Rock 379 Ventures, GSK and Avimex. The Krammer laboratory is also collaborating with Pfizer on animal 380 models of SARS-CoV-2 and with Dynavax on universal influenza virus vaccines. All other 381 authors declare no competing interests.

383 Data and materials availability

384 All data are available in the main text or supplementary materials.

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487 **Figure Legends**

488 Fig. 1: Ferrets are highly susceptible to bat H9N2. a Ferrets (n= 15) were inoculated with 489 $10^{4.8}$ TCID₅₀ of bat H9N2 IAV per animal. At 1 dpi, direct contact animals (*n*= 3) were co-490 housed. Viral shedding was measured by nasal lavage. Dashed line indicates detection limit. 491 **b** Organs collected from euthanized ferrets (n= 6) at 6 dpi with bat H9N2 were tested by RT-492 qPCR to determine viral genome copies. Dashed line indicates detection limit. Data are mean 493 \pm SD. **c** Changes in body weight relative to 0 dpi of bat H9N2-infected (n=15) and contact (n=15) 494 3) ferrets were monitored throughout the course of the experiment. **d** Body temperatures of 495 donor and naïve contact ferrets were monitored from 0 to 14 dpi. Dashed line at 40°C indicates 496 fever. e Ferret serum antibody titers in an IAV NP-specific ELISA at the indicated time points. 497 Dashed lines indicates threshold between 45% and 50% inhibition. Mean antibody titers are 498 indicated. f Ferret neutralizing antibody titers against bat H9N2 and chicken H9N2. g 499 Histopathologic findings with detection of viral antigen in the nasal mucosa of bat H9N2-500 infected ferrets (n= 6) at 6 dpi. Acute severe rhinitis with diffuse necrosis of the olfactory 501 epithelium (arrow) and infiltrating neutrophils (asterisk) (1). Intralesional viral antigen (NP) is 502 abundant in degenerated and desquamated epithelial cells (arrowhead) (2). The inset (3) is a 503 higher magnification of the center of the image (2). Scale bar, 100 μm (main panels), 25 μm 504 (inset).

505

506 Fig. 2: Bat H9N2 replicates in human lung explants and suppresses induction of MxA in 507 **MxA-transgenic mice. a** Human lung tissue explants (*n*= 4) were infected with human H3N2, 508 chicken H9N2 or bat H9N2 with 1×10⁶ PFU, and viral titers were determined at the indicated 509 time points. Error bars indicate standard deviation and statistical analysis was performed using 510 non-paired, non-parametric Kruskal-Wallis test (*p<0.05). Data are mean \pm SE of n=4511 independent experiments. b At 24 hpi, human lung explants were stained for alveolar type I 512 (AT1) (cyan) and type II (AT2) cells (yellow), CD68 indicating alveolar macrophages (green) 513 and IAV antigens (red). Note, in chicken H9N2 and bat H9N2 infected cells, AT2 labeling was 514 omitted for better visualization. White arrows indicate infected cells. Scale bar, 10 µm. c MDCK 515 cells overexpressing MxA or inactive MxA_{T103A} were infected with human-adapted pdmH1N1, 516 avian KAN-1 or bat H9N2 at an MOI of 0.001, and viral titers were determined at the indicated 517 time points. Data are mean \pm SD of n=3 independent experiments; statistical analysis was 518 performed using two-tailed *t*-tests; ***P*=0.01; *****P*=0.0001. Dashed line indicates detection 519 limit. **d** hMxA^{tg/tg} (n=8) or wild-type B6 mice (n=8) were infected with 1×10⁴ PFU. Lung viral 520 titers were determined 3 dpi. **e** hMxA^{tg/tg} (n= 6) or wild-type B6 mice (n= 7) were pretreated 521 with IFN- α 18 h prior to infection with 1×10⁴ PFU. Lung viral titers were determined 3 dpi. Data

522 are mean ± SD; statistical analysis was performed using two-tailed *t*-tests; *****P*=0.0001. **f** 523 MxA, NP and actin protein levels in homogenized lungs from IFN-α pretreated or infected mice 524 from (**d**,**e**) were detected by Western blot.

525

526 Extended Data Fig. 1: Bat H9N2 replicates efficiently in turkey but not chicken 527 hatchlings. a Turkey hatchlings (n=13) were oro-nasally inoculated with bat H9N2 and viral 528 shedding was monitored by RT-gPCR analysis of oro-pharyngeal swab samples. Asterisk 529 indicates successful virus isolation from swab material. Dashed line indicates detection limit. 530 b Serum antibody titers of turkey hatchlings before and after bat H9N2 infection were 531 determined by an IAV NP-specific ELISA. Dashed lines indicates threshold between 45% and 532 50% inhibition. Mean antibody titers are indicated. c Neutralizing antibody titers against bat 533 H9N2. **d** A group of chicken hatchlings (*n*= 13) was oro-nasally inoculated with bat H9N2 and 534 viral shedding was monitored by RT-qPCR analysis of oro-pharyngeal swab samples. Dashed 535 line indicates detection limit. e Serum antibody titers of chicken hatchlings at 21 dpi with bat 536 H9N2 were determined by an IAV NP-specific ELISA. Dashed lines indicate threshold area 537 between 45% and 50% inhibition. Mean antibody titers are indicated.

538

539 Extended Data Fig. 2: No cross-reactive antibodies to bat N2 among individuals 540 vaccinated against seasonal influenza. a Known MxA escape mutations in NP from the 541 1918 and the 2009 pdmH1N1 strains are highlighted in red and the resistance patch of the 542 Eurasian avian-like swine isolate Belzig is shown in blue. Note that the avian-adapted IAV 543 KAN-1 and bat H9N2 do not harbor any of the known MxA-resistance amino acid residues. 544 The NP model was created with PyMol based on the available crystal structure (PDB code: 545 2Q06). b Reactivity of sera from 15 healthy adults taken before the 2022/23 seasonal influenza 546 vaccination to recombinant N2 from bat H9N2. c,d Reactivity of the same sera against the 547 recombinant N2 of a recent seasonal H3N2 strain (c) and the recombinant NA of the Wuhan 548 spiny eel influenza virus (d). e Pre- and post- seasonal vaccination reactivity of sera from 15 549 healthy adults who received the 2022/23 seasonal influenza virus vaccine against recombinant 550 N2 from the bat H9N2 virus. Reactivity was guantified as area under the curve (AUC). A paired 551 *t*-test was used to determine statistical differences. For **b**, **c** and **d**, mAb 1G01 was used as 552 positive control, an irrelevant human mAbs was used as negative control.

Figures



Figure 1

Ferrets are highly susceptible to bat H9N2. a Ferrets (n= 15) were inoculated with 104.8 TCID50 of bat H9N2 IAV per animal. At 1 dpi, direct contact animals (n= 3) were co-housed. Viral shedding was measured by nasal lavage. Dashed line indicates detection limit. b Organs collected from euthanized

ferrets (n= 6) at 6 dpi with bat H9N2 were tested by RT-qPCR to determine viral genome copies. Dashed line indicates detection limit. Data are mean \pm SD. c Changes in body weight relative to 0 dpi of bat H9N2-infected (n= 15) and contact (n= 3) ferrets were monitored throughout the course of the experiment. d Body temperatures of donor and naïve contact ferrets were monitored from 0 to 14 dpi. Dashed line at 40°C indicates fever. e Ferret serum antibody titers in an IAV NP-specific ELISA at the indicated time points. Dashed lines indicates threshold between 45% and 50% inhibition. Mean antibody titers are indicated. f Ferret neutralizing antibody titers against bat H9N2 and chicken H9N2. g Histopathologic findings with detection of viral antigen in the nasal mucosa of bat H9N2-infected ferrets (n= 6) at 6 dpi. Acute severe rhinitis with diffuse necrosis of the olfactory epithelium (arrow) and infiltrating neutrophils (asterisk) (1). Intralesional viral antigen (NP) is abundant in degenerated and desquamated epithelial cells (arrowhead) (2). The inset (3) is a higher magnification of the center of the image (2). Scale bar, 100 μ m (main panels), 25 μ m (inset).



Figure 2

Bat H9N2 replicates in human lung explants and suppresses induction of MxA in MxA-transgenic mice. a Human lung tissue explants (n= 4) were infected with human H3N2, chicken H9N2 or bat H9N2 with 1×106 PFU, and viral titers were determined at the indicated time points. Error bars indicate standard deviation and statistical analysis was performed using non-paired, non-parametric Kruskal-Wallis test (*p<0.05). Data are mean ± SE of n= 4 independent experiments. b At 24 hpi, human lung explants were stained for alveolar type I (AT1) (cyan) and type II (AT2) cells (yellow), CD68 indicating alveolar macrophages (green) and IAV antigens (red). Note, in chicken H9N2 and bat H9N2 infected cells, AT2 labeling was omitted for better visualization. White arrows indicate infected cells. Scale bar, 10 µm. c MDCK cells overexpressing MxA or inactive MxAT103A were infected with human-adapted pdmH1N1, avian KAN-1 or bat H9N2 at an MOI of 0.001, and viral titers were determined at the indicated time points. Data are mean ± SD of n= 3 independent experiments; statistical analysis was performed using two-tailed t-tests; **P=0.01; ****P=0.0001. Dashed line indicates detection limit. d hMxAtg/tg (n= 8) or wild-type B6 mice (n= 8) were infected with 1×104 PFU. Lung viral titers were determined 3 dpi. e hMxAtg/tg (n= 6) or wild-type B6 mice (n= 7) were pretreated with IFN-a 18 h prior to infection with 1×104 PFU. Lung viral titers were determined 3 dpi. Data are mean ± SD; statistical analysis was performed using two-tailed ttests; ****P=0.0001. f MxA, NP and actin protein levels in homogenized lungs from IFN-α pretreated or infected mice from (d,e) were detected by Western blot.