

Bovine-derived influenza A virus (H5N1) shows efficient replication in well-differentiated human nasal epithelial cells without requiring genetic adaptation

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Abstract

Highly pathogenic avian influenza H5N1 viruses of clade 2.3.4.4b have caused widespread avian mortality and sporadic mammalian infections, raising concerns about their potential for efficient replication in the human population. Efficient replication in the human upper respiratory tract is considered a key barrier to transmission. Here, we demonstrate that an H5N1 virus isolated from bovine milk in Texas in 2024 (H5N1_{Tex/24}) replicates as efficiently as the 2009 pandemic H1N1 virus (H1N1_{HH4/09}) in well-differentiated human nasal epithelial cells. These cells express both avian- and human-type influenza receptors, indicating receptor adaptation is unnecessary for entry. H5N1_{Tex/24} replicates effectively at 33 °C, reflecting nasal cavity temperature, whereas earlier avian H5N1 strains require 37 °C, suggesting that H5N1_{Tex/24} has acquired another key adaptive feature to the human upper respiratory tract. H5N1_{Tex/24} remains sensitive to interferon-λ (IFN-λ) despite inducing low cytokine levels. Notably, no known mammalian-adaptive mutations such as PB2-E627K were detected. These findings suggest that H5N1_{Tex/24} possesses intrinsic traits enabling efficient replication in the human upper airways, a critical step toward potential airborne transmission, underscoring the need for vigilant surveillance.

Introduction

In recent years, H5N1 highly pathogenic avian influenza (HPAI) viruses of the phylogenetic clade 2.3.4.4b have spread worldwide, causing the death of millions of wild birds and domestic poultry^{1,2}. In addition, viruses of this clade were able to infect various mammalian species including harbor seals, sea lions, foxes, minks, and cats, indicating a significantly expanded host range compared to older clades³. In some mammalian species such as cats and foxes, H5N1 viruses of clade 2.3.4.4b showed a marked neurotropism causing a mostly fatal disease^{4,5}. In addition, clade 2.3.4.4b viruses of genotype B3.13 were able to infect dairy cows in which they replicated in mammary gland tissue, resulting in high titers of infectious virus shed into milk⁶. This “bovine H5N1” virus was further disseminated by milking equipment and animal transport to 17 US states with more than 1000 herds being affected as of March 2025⁷.

Several people who had been in close contact with infected cows or poultry were infected by H5N1 viruses of clade 2.3.4.4b⁸. Most affected persons displayed only mild symptoms such as conjunctivitis⁹, however, one adult person with underlying comorbidities died following infection⁸. In addition, a 13-year-old girl in Canada with a history of mild asthma and elevated body-mass index succumbed to infection by H5N1 of genotype D1.1¹⁰. Since no human-to-human transmission has been observed, it is believed that H5N1 viruses of clade 2.3.4.4b are not replicating efficiently in the human upper respiratory tract¹¹.

There is evidence that the differential glycosylation of appropriate receptors may be an important species barrier for efficient replication of avian influenza viruses in the human respiratory tract¹². Avian influenza viruses are known to preferentially bind to α 2,3-linked sialic acid residues, which are less abundantly expressed in the human upper respiratory tract than α 2,6-linked sialic acid, the receptor determinant for human influenza viruses¹³. Recently, bovine H5N1 virus has been shown to retain its preference for α 2,3-linked sialic acid residues

as receptor determinant¹⁴⁻¹⁶. However, a single amino acid substitution in HA is sufficient to switch the preference to the human-type receptor^{17,18}. Another factor that affects the replication of avian influenza viruses is temperature. While the physiological temperature in the nasal cavity ranges from 25 °C (at the nares) to 33 °C (in the nasopharynx), avian influenza viruses are typically adapted to replication at 41 - 42 °C - the normal body temperature of avian species¹⁹. However, avian influenza viruses have the capacity to adapt to replication at reduced temperature²⁰⁻²⁷. Finally, the interferon (IFN)-stimulated MxA protein can effectively limit the replication of avian influenza viruses in the human respiratory tract^{28,29}. A recent study suggests that bovine H5N1 virus remains susceptible to the antiviral effects of human MxA³⁰.

In this study, we examined the replication of a clade 2.3.4.4b H5N1 virus isolated from bovine milk in 2024 (H5N1_{Tex/24}) in well differentiated human nasal and bronchial epithelial cells cultured at the air-liquid interface, comparing its replication with that of the 2009 pandemic H1N1 virus (H1N1_{HH4/09}) and avian H5N1 virus strains. Multi-step replication kinetics were assessed at 33 °C and 37 °C, and potential genomic changes arising during replication in these cells were analyzed. We also evaluated the presence of avian- and human-type influenza virus receptors in differentiated human nasal epithelial cells, as well as the induction and secretion of IFNs following infection. Our results show that H5N1_{Tex/24} replicates as efficiently as H1N1_{HH4/09} without requiring genetic adaptation. Notably, its robust replication in human nasal epithelial cells suggests that H5N1_{Tex/24} already possesses traits that may facilitate infection of the human upper respiratory tract, posing a potential risk to human populations lacking adequate immunity to this zoonotic virus.

Results

Replication of H5N1_{Tex/24} in primary human nasal epithelial cells

To assess the ability of influenza A viruses to replicate in the human upper respiratory tract, we used fully differentiated primary human nasal epithelial (HNE) cell cultures derived from a pool of 14 donors and maintained at an air-liquid interface (ALI). The epithelium comprised ciliated and non-ciliated epithelial cells as well as goblet and basal cells³¹. The HNE cultures were infected with A/cattle/Texas/24-029328-02/2024 (H5N1_{Tex/24}) or A/Hamburg/4/2009 (H1N1_{HH4/09}). H5N1_{Tex/24} was isolated in 2024 from the milk of an infected dairy cow in Texas, USA, and belongs to the phylogenetic clade 2.3.4.4b, genotype B3.13³², while H1N1_{HH4/09} was isolated from a human patient during the 2009 pandemic by swine-origin H1N1 virus³³. Here, we used reverse genetics to synthetically produce H1N1_{HH4/09} (rH1N1_{HH4/09})³⁴.

HNE cultures were infected with H5N1_{Tex/24} or rH1N1_{HH4/09} using a multiplicity of infection (MOI) of 0.001 TCID₅₀/cell and incubated at 37 °C for 72 h. Virus released at the apical surface was sampled at 2, 24, 48, and 72 h post infection (p.i.) and titrated on MDCK cells. H5N1_{Tex/24} replicated as efficiently as rH1N1_{HH4/09}, reaching a maximum titer of 10⁸ TCID₅₀/mL at 48 h (**Fig. 1a**). Because the H5N1_{Tex/24} virus stock had been propagated on MDCK cells, we investigated whether adaptive mutations might have facilitated its replication in HNE cells. Viral genome sequencing revealed only a few nucleotide changes relative to the published sequence, most of which were non-synonymous (**Table 1**). Notably, a G754T mutation in segment 4 (HA-D236Y, H5 numbering) was present in 17.17% of reads in the virus stock and persisted at a lower frequency (5.82%) at 72 h p.i.. A PA-I38M substitution emerged at 72 h p.i. but was present in only 5.48% of reads (**Table 1**). We did not detect mutations associated with enhanced mammalian replication (e.g. PB2-E627K, PB2-T271A and PB2-Q591R³⁵) or altered receptor specificity¹⁷ (**Table 2**). To further exclude effects of cell culture adaptation, we generated a recombinant H5N1 virus (rH5N1_{Tex/24}) based on the published sequence of

A/bovine/Texas/24-029328-02/2024 (H5N1)³⁶. We also constructed a mutant virus (rH5N1_{Tex/24}-HA_{mb}) with a modified HA cleavage site that cannot be processed by furin³⁷. Analysis of these two recombinant viruses showed that rH5N1_{Tex/24} replicated in HNE cells with kinetics comparable to the original isolate, peaking at 48 h p.i., while rH5N1_{Tex/24}-HA_{mb} exhibited delayed replication kinetics, reaching its maximum titer at 72 h p.i. (**Fig. 1b**). These findings indicate that bovine H5N1 virus efficiently replicates in primary human nasal epithelial cells without requiring prior adaptation.

H5N1_{Tex/24} infection of HNE cells triggers IFN-λ secretion

Epithelial cells can sense viruses, triggering the transcription of IFN-λ genes. These cytokines are important components of the innate immune system of epithelial cells³⁸. Binding of IFN-λ to cognate receptors on epithelial cells triggers intracellular signaling pathways that result in the transcription of multiple genes that code for proteins with antiviral activity such as human myxovirus resistance protein 1 (MxA). MxA is known to restrict the replication of HPAI H5N1 viruses both *in vitro* and in MxA transgenic mice³⁰. Given that H5N1_{Tex/24} replicates efficiently in well differentiated HNE cultures, this virus may either evade detection by cellular pattern recognition receptors (PRRs) or actively suppress innate immune responses. To distinguish between these possibilities, we performed RT-qPCR analysis of HNE cells 48 h after infection with rH1N1_{HH4/09}, H5N1_{Tex/24}, or rH5N1_{Tex/24}-HA_{mb} (**Fig. 2a-g**). Infection with rH1N1_{HH4/09} did not significantly induce transcription of genes encoding type III IFN (IFN-λ1, IFN-λ2 and IFN-λ3) relative to mock-infected controls (**Fig. 2a, b**). In contrast, H5N1_{Tex/24} infection resulted in a robust upregulation of IFN-λ transcripts, with 43-fold and 100-fold increases for IFN-λ1 and IFN-λ2/3, respectively, as well as a sevenfold induction of IFN-β mRNA (**Fig. 2c**). Consistent with this response, expression of the IFN-stimulated genes (ISGs) CXCL10, Mx1 (MxA), and DDX58 (RIG-I) was significantly increased exclusively in H5N1_{Tex/24}-infected cells (**Fig. 2d–**

f). By contrast, the transcription of the proinflammatory cytokine IL-6 was not induced by H5N1_{Tex/24} infection (**Fig. 2g**).

Despite 7-fold induction of IFN- β gene transcription in H5N1_{Tex/24}-infected HNE cells (**Fig. 2c**), ELISA measurements revealed no significant increase in IFN- β protein levels in the basolateral compartment of HNE air-liquid interface cultures infected with H5N1_{Tex/24} at 24 or 48 h p.i. (**Fig. 2h**). On the other hand, IFN- λ 1-3 protein levels reached mean concentrations of 910 and 500 pg mL⁻¹ in rH1N1_{HH4/09}- and H5N1_{Tex/24}-infected cultures, respectively, at 48 h p.i. (**Fig. 2i**). These findings indicate that infection with H5N1_{Tex/24} triggers IFN- λ gene transcription in HNE cells resulting in the concomitant IFN- λ secretion.

H5N1_{Tex/24} is sensitive to the antiviral effects of IFN- λ

To assess the sensitivity of H5N1_{Tex/24} and rH1N1_{HH4/09} to IFN- λ -induced antiviral activity, well differentiated HNE ALI cultures were pretreated for 24 h at 37 °C with IFN- λ 1 (3, 30, or 300 ng.mL⁻¹) added to the basolateral compartment or were left untreated. Cultures were subsequently infected from the apical side with H5N1_{Tex/24} or rH1N1_{HH4/09} at an MOI of 0.001. Apical washes were collected at 24, 48, and 72 h p.i., and infectious virus titers were determined. Pretreatment with 30 ng.mL⁻¹ IFN- λ 1 significantly inhibited H5N1_{Tex/24} replication at all time points, resulting in reduction of infectious titers by 2.0 log₁₀ at 24 h, 3.1 log₁₀ at 48 h and 2.4 log₁₀ at 72 h p.i.. Increasing the IFN- λ 1 concentration to 300 ng.mL⁻¹ led to an even more pronounced suppression of viral replication, with titer reductions of 2.9, 4.5 and 4.1 log₁₀ at 24, 48 and 72 h, respectively (**Fig. 3a**). By contrast, replication of rH1N1_{HH4/09} was only modestly affected by IFN- λ 1 pretreatment, showing reductions of 1.0 log₁₀ and 2.0 log₁₀ at 24 h p.i. with 30 and 300 ng.mL⁻¹ IFN- λ 1, respectively, whereas differences at later time points were not statistically significant (**Fig. 3b**). To corroborate these findings in an additional

cellular system, A549 cells were pretreated for 24 h with IFN- λ 1 (up to 300 ng.mL⁻¹) and subsequently infected with H5N1_{Tex/24} or rH1N1_{HH4/09} at an MOI of 0.01. Quantification of infectious virus released into the supernatant at 24 h p.i. revealed a dose-dependent inhibition of H5N1_{Tex/24} replication by IFN- λ 1, whereas replication of rH1N1_{HH4/09} remained largely unaffected (**Fig. 3c**). Together, these results demonstrate that the bovine-derived H5N1_{Tex/24} virus is markedly more sensitive to the antiviral effects of IFN- λ 1 than the pandemic rH1N1_{HH4/09} virus.

Replication of H5N1_{Tex/24} in HNE cultures at 33 °C

The human upper respiratory tract is exposed to temperatures below the core body temperature of 37 °C that predominates in the lower respiratory tract. To assess the effects of temperature on influenza A virus replication, differentiated HNE cultures were infected with rH1N1_{HH4/09} and H5N1_{Tex/24} as well as with two other HPAI H5N1 viruses. H5N1_{BE/22}, like H5N1_{Tex/24}, belongs to clade 2.3.4.4b but was isolated in 2022 from a diseased Dalmatian pelican in Bern Animal Park, Switzerland. On the other hand, rH5N1_{Yam/04} is the recombinant version of a clade 2.5 H5N1 virus that was isolated in 2004 from infected chickens in Japan. H1N1_{HH4/09} replicated efficiently at 37 °C on HNE cultures, reaching a maximum titer of 2.8 x 10⁸ TCID₅₀/mL at 48 h p.i.. The titer subsequently decreased, dropping to 2.2 x 10⁷ TCID₅₀/mL at 72 h p.i., a 12-fold decrease in titer (**Fig. 4a**). At 33 °C, H1N1_{HH4/09} showed slightly delayed kinetics compared to 37 °C and only reached its highest titer at 72 h p.i. (**Fig. 4a**). The replication of H5N1_{Tex/24} was more significantly impaired at 33 °C. At 24 h and 48 h p.i., H5N1_{Tex/24} reached infectious titers that were 100 and 50 times lower than those at 37 °C, respectively (**Fig. 4b**). However, after 72 h p.i., the replication curve of H5N1_{Tex/24} reached similar values at 33 °C and 37 °C. Remarkably, H5N1_{BE/22} was even more attenuated at 33 °C than H5N1_{Tex/24}, reaching titers after 48, 72, and 96 h p.i. that were 959-fold and 3704-fold, and 79-fold lower, respectively,

compared to the corresponding titers at 37 °C (**Fig. 2c**). Thus, at 33 °C, this virus never reached the titers measured at 37 °C. The replication kinetics of rH5N1_{Yam/04} showed that this virus replicates poorly even at 37 °C and is further attenuated at 33 °C, producing significantly lower titers at 24, 48, and 72 h p.i. (**Fig. 2d**). These results indicate that, among the three different H5N1 HPAI viruses tested, H5N1_{Tex/24} is already well adapted for efficient replication in HNE cells at temperatures characteristic of the upper respiratory tract.

H5N1_{Tex/24} replication in HNE cultures leads to loss of ciliated cells

Ciliated epithelial cells are essential for airway defense, as they mediate mucociliary clearance of mucus and inhaled particles. To assess whether H5N1_{Tex/24} infection affects ciliated cells, we infected differentiated HNE ALI cultures with H5N1_{Tex/24} or rH1N1_{HH4/09} at an MOI of 1. At 24 h p.i., both viruses had infected approximately 60% of the apical epithelial cell layer, as determined by the proportion of NP antigen-positive cells relative to total DAPI-positive nuclei (**Fig. 5a, b**). In mock-treated cultures, about 70% of HNE cells were ciliated. However, following infection with H5N1_{Tex/24}, the proportion of ciliated cells dropped to 27%, while rH1N1_{HH4/09} infection led to a more moderate reduction to 49% (**Fig. 5c**). These findings indicate that H5N1_{Tex/24} infection is associated with a pronounced loss of ciliated epithelial cells, suggesting a greater potential to impair mucociliary clearance compared to rH1N1_{HH4/09} infection.

HNE cells express both avian- and human-type influenza virus receptors

The receptor-binding specificity of influenza virus hemagglutinin (HA) is a key determinant of host range and tissue tropism¹². Previous studies have shown that bovine-derived H5N1 viruses preferentially bind α 2,3-linked sialic acids, characteristic of avian-type receptors¹⁴, although low affinity binding to α 2,6-linked sialic acid, the predominant human-type receptor, has also

been reported³⁹. To define the distribution of influenza virus receptors in differentiated HNE and human bronchial epithelial (HBE) ALI cultures, we took advantage of biotin-labeled *Sambucus nigra* agglutinin (SNA), which recognizes α 2,6-linked sialic acids⁴⁰, and biotin-conjugated MAL-I/MAL-II lectins from *Maackia amurensis* which selectively bind α 2,3-linked sialic acids⁴¹. In HNE cultures, both receptor types were abundantly expressed, with 58% of cells positive for α 2,3-linked sialic acids and 72% positive for α 2,6-linked sialic acids (**Fig. 6a, b**). Similarly, HBE cultures exhibited widespread receptor expression, with α 2,3-linked sialic acids detected in 71% of cells and α 2,6-linked sialic acids in 96% of cells (**Fig. 6c, d**). Given the high prevalence of both receptor types, a substantial proportion of epithelial cells is likely to co-express α 2,3- and α 2,6-linked sialic acids. Together, these findings indicate that receptor availability in the human upper respiratory tract is unlikely to constitute a major barrier to infection by avian influenza A viruses.

Discussion

H5N1 HPAI viruses of clade 2.3.4.4b have spread globally in recent years, causing mass mortality in wild birds and significant losses in domestic poultry⁴². These viruses have also crossed species barriers and infected a growing number of mammalian species, including humans³. Most human cases have occurred after close contact with infected poultry or, more recently, infected dairy cows^{6,9,43,44}. Reported human disease has been mild in most instances⁹, and human-to-human transmission has not been documented to date^{3,45}. One proposed explanation is that these avian-origin viruses cannot replicate efficiently in the human upper respiratory tract, owing to factors such as limited availability of compatible receptors, suboptimal temperature for replication, or insufficient adaptation to evade innate immune responses¹¹. Using primary, well differentiated HNE cells cultured at the air-liquid interface, we show here that a bovine-derived H5N1 virus replicates as efficiently as the 2009 pandemic

H1N1 virus in the human nasal epithelium. Furthermore, it is noteworthy that this efficient replication occurred in the absence of hallmark mammalian-adaptive mutations, raising the question of which viral or host factors enable viral growth in the human upper airway.

Recent studies indicate that bovine H5N1 viruses retain a largely avian receptor-binding profile and preferentially recognize α 2,3-linked sialic acids^{14,46}. Although a single mutation at HA position 222 (H5 numbering) can shift binding toward α 2,6-linked sialic acids¹⁷, we did not detect this mutation in the H5N1_{Tex/24} isolate (**Table 2**), neither before nor after replication for 72 h on HNE cells (**Table 1**). Lectins staining demonstrated that differentiated HNE cells display both α 2,3- and α 2,6-linked sialic acids, consistent with the previous finding that the human respiratory tract has potential binding sites for human and avian influenza viruses⁴⁷. These findings also align with a recent immunohistochemical study showing efficient HA binding of bovine H5N1 viruses to human conjunctival, tracheal, and mammary tissues³⁹. Together, these observations indicate that receptor distribution in nasal epithelium does not impose a strong barrier to infection and thus provides limited selective pressure for adaptation toward α 2,6-linked receptors.

Temperature represents another potential barrier to mammalian adaptation^{22,26}. Avian influenza viruses typically replicate optimally at 41-42 °C, the body temperature of birds¹⁹, but typically show restricted replication at the lower temperatures of the human upper airways. Here, bovine H5N1_{Tex/24} replicated efficiently at both 33 °C and 37 °C, whereas H5N1_{BE/22}, despite belonging to the same clade, replicated poorly at 33 °C. The PB2-E627K substitution, known to facilitate replication at lower temperatures⁴⁸, is absent in H5N1_{Tex/24} (**Table 2**), suggesting that other genomic determinants for efficient replication of this virus at 33 °C. Prior studies indicate that HA and NA proteins also require temperature adaptation²⁶. H5N1_{BE/22} and H5N1_{Tex/24} differ at

only four amino-acid positions in HA and several positions in NA. Identifying which of these residues enhance replication at 33 °C will be an important direction for future work.

Innate immune restriction represents an additional barrier to mammalian infection by animal influenza viruses⁴⁹. However, influenza viruses can evade the antiviral activity of ISGs. For example, certain mutations in the nucleoprotein (NP) can overcome the restriction imposed by MxA and BTN3A3^{50,51}. It is noteworthy that the NP proteins of H5N1_{Tex/24} and H5N1_{BE/22} but not of H5N1_{Yam/04} have the NP-Y52H substitution (**Table 2**), which confers resistance to the restriction factor BTN3A3⁵². However, H5N1_{Tex/24} lacks known MxA resistance mutations such as NP-F313Y and NP-N319K^{52,53}, and remains susceptible to the antiviral activity of human MxA³⁰. H5N1_{Tex/24} induces only low levels of type I and type III IFNs in HNE cells, perhaps allowing the virus to tolerate some sensitivity to ISGs. In contrast, rH1N1_{HH4/09} has characteristic mutations in the NP protein that confer profound resistance to MxA⁵⁰.

The most important viral factor known to contribute to the prevention of IFN induction is the NS1 protein^{54,55}. H5N1_{Tex/24} harbors the mutations NS1-L103F and NS1-I106M that are associated with enhanced binding of NS1 to the pre-mRNA processing factor CPSF30⁵⁶, however, these substitutions are also present in H5N1_{BE/22} and H5N1_{Yam/04} (**Table 2**), and therefore do not uniquely explain the phenotype observed with H5N1_{Tex/24}. The PA-X protein contributes to immune evasion through its host shut-off activity⁵⁷⁻⁵⁹. Compared to H5N1 viruses that circulated in 1996-97, current H5N1 avian influenza viruses exhibit increased PA-X host shut-off activity, which is primarily due to the T118I and I127V substitutions⁶⁰. These two amino acid exchanges are found in all three H5N1 viruses analyzed here and thus do not explain their differential replication efficacy. There are numerous other amino acid differences between the PA-X proteins of H5N1_{Tex/24} and H5N1_{Yam/04}, but it is currently unknown whether any of these would enhance shut-off activity in human cells.

Adaptation of avian influenza viruses to mammals often involves changes in the viral RNA polymerase complex^{35,61}. The PB2-E627K substitution, a canonical marker of mammalian adaptation, enhances polymerase activity via improved interaction with ANP32 proteins^{49,62,63}. H5N1_{Tex/24} lacks PB2-E627K as well as other mammalian-adaptive PB2 substitutions such as PB2-Q591R⁶⁴, PB2-D701N^{23,65}, and PB2-T271A⁶⁶ (**Table 2**). Consistent with this, only a minority of human-derived clade-2.3.4.4b viruses sequenced to date carry PB2-E627K^{67,68}. However, alternative compensatory mutations may enhance polymerase activity in the absence of PB2-E627K. PB2-M631L, which is present in H5N1_{Tex/24} but absent in H5N1_{BE/22} and H5N1_{Yam/04} (**Table 2**), improves polymerase activity in human cells⁴⁴. The substitutions PB2-L89V, PB2-G309D, PB2-T339K and PB2-R477G, which can also compensate for the lack of PB2-E627K⁶⁹, are present in all three H5N1 viruses. On the other hand, known human adaptive mutations in PB1 such as PB1-L472V and PB1-L598P⁷⁰, and in PA such as PA-L336M, PA-T552S, and PA-K615N⁷¹⁻⁷³ are absent in all three viruses analyzed (**Table 2**). Thus, we have so far only been able to identify the PB2-M631L mutation as a possible molecular determinant for the particularly efficient replication of H5N1_{Tex/24} in differentiated HNE cells. However, the actual significance of this mutation needs to be confirmed in further studies.

One mutation that did arise during replication in HNE cells was PA-I38M (**Table 1**), which confers resistance to baloxavir^{74,75}. Although present in only ~5% of reads, its rapid emergence and lack of an apparent fitness cost in human cells is concerning, underscoring that clade 2.3.4.4b viruses can readily acquire antiviral-resistance mutations.

Overall, our results show that bovine-derived H5N1_{Tex/24} replicates at high titers in primary human nasal epithelial cells even though it lacks many canonical markers of adaptation to mammals. This observation contrasts with the currently low number of confirmed human infections. There are several possible explanations for this discrepancy. First, the sensitivity of

H5N1_{Tex/24} to the antiviral effects of MxA may limit virus dissemination to the lower respiratory tract and thus disease severity. Second, human infections may go undetected because they are completely asymptomatic or because they are associated with only mild symptoms. Third, efficient airborne transmission of H5N1 requires that HA induces membrane fusion at pH values significantly lower than pH 6.0^{76,77}. However, the HA protein of bovine H5N1 retains typical avian characteristics, with fusion triggered at approximately pH 6.0^{46,78}. Finally, pre-existing immunity to influenza viruses may provide some partial protection against bovine H5N1⁷⁹. Cross-reactive antibodies against the NA protein of human H1N1 viruses can particularly inhibit avian N1 sialidase activity⁸⁰, thereby potentially limiting replication of clade-2.3.4.4b viruses. The recent fatal human case caused by a H5N5 HPAI virus in the United States is alarming⁸¹, as there is probably no pre-existing immunity to the N5 antigen in the human population. Overall, our results show that H5N1_{Tex/24} has a remarkable ability to replicate in primary human nasal epithelial cells. Since well-differentiated nasal epithelial cells represent a relevant model for the human upper respiratory tract, it is not unlikely that the virus can also replicate efficiently *in vivo in humans*.

Methods

Cells

Primary human nasal epithelial (HNE) and human bronchial epithelial (HBE) cells were prepared at Epithelix Sàrl (Geneva, Switzerland) with cells isolated from a pool of 14 human donors. The cells were tested negative for mycoplasma, HIV-1, HIV-2, hepatitis B, and hepatitis C virus. The airway epithelial cells were grown on porous filter supports (6.5 mm diameter, pore size: 1 µm) using MucilAir culture medium (Epithelix Sàrl). Differentiation of the cells was initiated by culturing them at an air-liquid interface (ALI) with 700 µL of MucilAir culture

medium placed in the basolateral compartment and was evidenced by the formation of beating cilia. Each reconstituted epithelium contained about 500,000 cells.

Madin-Darby canine kidney (MDCK) type II cells were provided by Georg Herrler (University of Veterinary Medicine, Hannover, Germany) and were maintained with minimum essential medium (MEM, Thermo Fisher Scientific, Basel, Switzerland; cat. no. 31095-029) supplemented with 5% and 10% of fetal bovine serum (FBS; Pan Biotech, Aidenbach, Germany; cat. no. P30-3033), respectively. Human embryonic kidney (HEK) 293T cells (American Type Culture Collection (ATCC), Manassas, USA; cat. no. CRL-3216) were maintained with Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific; cat. no. 32430-027) supplemented with 10% FBS. A549 human lung carcinoma cells (ECACC, cat. no. 86012804), were grown in Ham's F12 nutrient mixture (Merck KGaA, Darmstadt, Germany; cat. no. N6658) supplemented with 10% FBS. All cell lines were routinely monitored for contamination by mycoplasma.

Viruses

A/cattle/Texas/063224-24-1/2024 (H5N1_{Tex/24}) was kindly provided by Diego Diel (Cornell University, Ithaca, NY, USA). H5N1_{Tex/24} was isolated from the milk of infected dairy cows in March 2024 in Texas, USA. The virus belongs to clade 2.3.4.4b, genotype B3.13 (GISAID accession no.: EPI_ISL_19155861)³². H5N1_{Tex/24} virus stock was produced by two passages in MDCK cells. The complete genome sequence of this virus stock was determined and deposited in the GenBank database (accession nos.: PX831491-PX831498). A/Dalmatian pelican/Bern/1/2022 (H5N1_{BE/22}) was isolated in February 2022 from a Dalmatian pelican in Bern, Switzerland. H5N1_{BE/22} virus stock was produced by passaging the virus three times in MDCK cells. The complete genomic sequence of H5N1_{BE/22} was deposited at GenBank (accession nos.: PX700809 - PX700813, PX149233 - PX149235).

Recombinant influenza viruses A/Hamburg/4/2009 (rH1N1_{HH4/09}), A/chicken/Yamaguchi/7/2004 (rH5N1_{Yam/04}), and A/bovine/Texas/24-029328-02/2024 (rH5N1_{Tex/24}) were generated using the eight-plasmid reverse genetics system³⁴. cDNAs encoding the 8 genomic segments of H1N1_{HH4/09} (Genbank accession nos.: GQ166207, GQ166209, GQ166211, GQ166213, GQ166215, GQ166217, GQ166219, GQ166221) were provided by Martin Schwemmler (University of Freiburg, Germany). cDNAs encoding the eight genomic segments of H5N1_{Yam/04} (GenBank accession nos.: GU186708 - GU186715) were obtained from Yoshihiro Sakoda (Hokkaido University, Sapporo, Japan). The cDNAs of the eight genomic segments of A/bovine/Texas/24-029328-02/2024 (H5N1) (GenBank accession nos.: PP599470 - PP599477) were synthesized by Genscript Biotech (Piscataway, New Jersey, USA) and cloned into the pHW2000 plasmid³⁴ using the In-Fusion cloning system (Takara Bio, Saint-Germain-en-Laye, France, cat. no. 638948). rH5N1_{Tex/24}-HA_{mb} encodes a modified HA protein in which the polybasic cleavage site (P₃₂₁LREKRRKR↓GLF) was replaced by the monobasic motif (P₃₂₁RETR↓GLF). The nucleotide sequence of each cloned segment was verified by Sanger sequencing.

Virus replication kinetics in differentiated human airway ALI cultures

Well differentiated HNE or HBE cells grown at ALI were washed three times with Hank's Balanced Salt Solution (HBSS; Gibco) to remove mucus prior to infection. The cells were then inoculated apically for 2 h at either 33 °C or 37 °C with 200 µL of the respective influenza A virus. Thereafter, the inoculum was removed, and the apical surface washed three times with HBSS to remove unbound viruses. The third wash was collected and kept at -70 °C (2h-sample). For subsequent time points, apical wash samples were collected by incubating the apical domain of the cells for 10 min with 200 µL of HBSS and stored at -70 °C until virus titration.

Infectious virus titers were determined on MDCK cell monolayers in 96-well culture plates by limiting dilution. To this end, cells were incubated in quadruplicates with serially diluted virus in MEM without FBS (100 μ L/well) for 48 h at 37 °C. Viruses with a monobasic HA cleavage site (rH1N1_{HH4/09}, rH5N1_{Tex/24}-HA_{mb}) were incubated in the presence of 1 μ g/mL of acetylated trypsin (Merck KGaA, Darmstadt, Germany, cat. no. 4370285). After three days, the cells were fixed for 30 min at room temperature with 4% buffered formalin containing 0.1% (w/v) crystal violet. The plates were washed with tap water and dried. Virus titer was calculated using the Spearman-Kärber method and expressed as tissue culture infectious dose 50% per mL (TCID₅₀/mL)⁸².

Determination of influenza virus genome sequences

Total RNA was extracted from apical wash samples collected at 72 h p.i. using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany; cat. no. 52904), according to the manufacturer's instructions. RNA concentration was determined using a Qubit 4.0 Fluorometer with the Qubit RNA Broad Range and High Sensitivity Assay Kits ((Thermo Fisher Scientific, cat. nos. Q10211 and Q32855), and RNA integrity was assessed on an Advanced Analytical Fragment Analyzer System using the Fragment Analyzer RNA Kit (Agilent, cat. no. DNF-471).

The eight influenza A virus genomic segments were reverse-transcribed and amplified using the SuperScript IV One-Step RT-PCR System (Thermo Fisher Scientific; cat. no. 12594100) with the Opti primer set⁸³. Resulting amplicons were purified and quantified using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific; cat. no. Q32854) on a Qubit 4.0 Fluorometer. Amplicon size distributions were verified using an Agilent FEMTO Pulse System with the Genomic DNA 165 kb Kit (Agilent, FP-1002-0275).

Each sample PCR reaction was used as input to generate barcoded SMRTbell libraries according to the following Procedure & checklist: Preparing multiplexed amplicon libraries

using SMRTbell prep kit 3.0 Procedure & checklist (PacBio, 102-359-000 REV04. Instructions in SMRT Link Sample Setup were followed to prepare the SMRTbell libraries for sequencing (PacBio SMRT Link v25) using the Revio SPRQ polymerase kit (PacBio, cat no.103-496-900) and clean-up beads (PacBio, cat. no. PN 102-158-300). The libraries were loaded at an on-plate concentration of 225 pM using adaptive loading with a 24 h movie time. All steps post RNA extraction were performed at the Next Generation Sequencing Platform, University of Bern.

Immunofluorescence

Cells were fixed with 4% formalin for 30 min at room temperature and washed three times with PBS containing 0.1 M glycine. Following permeabilization with 0.25% (v/v) Triton X-100 (15 min, 21 °C), the cells were incubated overnight at 4 °C with a monoclonal antibody directed to influenza NP (1:50 in PBS; American Type Culture Collection, Manassas, Virginia, USA, cat. no. ATCC HB-65) along with either Alexa Fluor-594-labeled mouse monoclonal antibody directed against ZO-1 (1:500; Life Technologies, Zug, Switzerland, cat. no. 339194) or with a rabbit monoclonal antibody directed against acetyl- α -tubulin (1:300; Cell Signaling, Danvers, USA, cat. no. 5335S). The unlabeled primary antibodies were detected by incubation of the cells for 60 min with goat anti-mouse IgG conjugated with Alexa Fluor-488 (1:500; Life Technologies, cat no. A11001) or anti-rabbit IgG conjugated to Alexa Fluor-647 (1:500, Life Technologies, cat. no. A21244). For detection of glycoconjugates containing α 2,3-linked or α 2,6-linked sialic acids, formalin-fixed, non-permeabilized cells were incubated overnight at 4 °C with biotinylated *Sambucus nigra* agglutinin (SNA, 1:200; Vector Labs, Newark, CA, USA, cat. no. B-1305-2) or combined biotinylated *Maackia amurensis* lectins I and II (Mal-I, Mal-II, 1:200 each; Vector Labs, cat nos. B-1315-2 and 1265-1). Thereafter, the cells were washed three times with PBS and incubated for 60 min with streptavidin conjugated with Alexa Fluor-488 (1:250, Life Technologies, cat. no. S11223). For staining of nuclei, cells were incubated for 5 min at room temperature with 0.1 μ g/mL of 4',6-diamidino-2-phenylindole (DAPI, Merck

KGaA, Darmstadt, Germany) and mounted in ProLong Gold Antifade Mountant (Thermo Fisher). Cells were imaged using a confocal laser scanning system mounted on an ECLIPSE Ti inverted microscope (Nikon Instruments) and data processed with ImageJ software.

Production of recombinant human IFN- λ 1

The expression plasmid pcDNA3.1-IFNL1 encoding human IFN λ 1 (GenBank accession no. NM_172140.2) was obtained from GenScript Biotech (Rijswijk, The Netherlands). HEK 293T cells grown in 10-cm cell culture dishes (approximately 10^7 cells/dish) were transfected with 10 μ g of plasmid DNA and 20 μ l of Lipofectamin 2000 (Life Technologies, cat. no. 11668019), according to the manufacturer's instructions. At 20 h post transfection, the cells were washed once with 10 mL of PBS and further incubated in FBS-free DMEM medium for 24 h at 37 °C. The amount of IFN λ 1 in the conditioned cell culture medium was determined by ELISA (see below).

ELISA

Levels of IFN- β and IFN- λ 1/3 proteins released into the basolateral medium of HNE cells were measured with the Human IFN- β DuoSet ELISA (R&D Systems, Minneapolis, MN, USA, cat. #DY814-05) and the human IL-29/IL-28B (IFN λ 1/3) DuoSet ELISA (R&D Systems, cat. no. DY1598B) according to the manufacturer's instructions.

Quantitative PCR (qPCR)

RNA was extracted from cells by using the NucleoMag® VET kit (Macherey-Nagel, Düren, Germany, cat. no. 744200.4) according to the manufacturer's instructions. qPCR reactions were carried out with the TaqMan™ Fast Universal PCR Master Mix (2X) (Thermo Fisher Scientific, cat. no. 4352042) with primers listed in **Supplementary Table 1**. qPCR reactions were performed on the QuantStudio 5 Real-Time PCR System (Thermo Fisher) according to the

following protocol: 95 °C for 20 s, 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method using β -actin RNA as internal control.

Biosafety

Work with H5N1 highly pathogenic avian influenza viruses has been approved by the Swiss Federal Office of Public Health (license no. A230074-01) and was performed at IVI Mittelhäusern in laboratories complying with biosafety level 3 standards.

Statistics

Statistical analysis was performed with GraphPad Prism 10, version 10.4.1. Unless otherwise noted, results are expressed as arithmetic means and standard deviations (SD). Specific statistical tests such as the two-way ANOVA test were employed to assess significant differences in the multi-step replication of different viruses and are indicated in the figure legends. *P* values < 0.05 were considered significant.

Data availability

All data generated or analyzed during this study are included in this article and its supplementary information files. Source data will be accessible at Zenodo at the time of publication. The nucleotide sequences of all eight genomic segments of A/Dalmatian Pelican/Bern/1/2022 (H5N1) are available at GenBank (accession nos.: PX149233 - PX149235 and PX700809 – PX700813). Genomic sequences of A/cattle/Texas/063224-24-1/2024 (H5N1) that was propagated on MDCK cells are available at GenBank (accession nos.: PX831491 - PX831498). The PacBio sequencing reads are available in the ENA archive under project accession PRJEP106468.

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Author contributions

Conceptualization: E.A.M., M.P.A., and G.Z. Investigation and data curation: E.A.M., L.B., T.D., M.W., P.G. and G.Z. Funding acquisition: M.P.A. and G.Z. Resources: C.C., S.C., C.B., V.T., M.P.A. and G.Z. Writing – original draft: E.A.M. and G.Z. Writing – review and editing: E.A.M., C.C., S.C., M.W., P.G., C.B., V.T., M.P.A., and G.Z.

Competing interests

The authors have declared no competing interests.

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Table 1 | Mutations identified in the H5N1_{Tex/24} genome before and after replication in HNE cells.

Viral RNA segment	Protein	Nucleotide change ^{a,b}	Virus stock ^c		Passage in HNE ^d	
			Freq. (%)	AA subst.	Freq. (%)	AA subst.
1	PB2	T165C	16.29	-	22.31	-
		G525A	20.18	-	35.89	-
2	PB1	-	-	-	-	-
3	PA	A114G	-	-	5.48	I38M
		T1545C	19.97	-	5.68	-
4	HA	G754T	17.17	D236Y	5.82	D236Y
5	NP	A1140G	22.75	-	33.9	-
6	NA	A1305C	20.47	-	19.69	-
7	M1, M2	-	-	-	-	-
8	NS1, NEP	-	-	-	-	-

^a The sequence of the passaged virus was compared with the sequence of A/cattle/Texas/063224-24-1/2024 (H5N1) (GISAID accession number: EPI_ISL_19155861)

^b The nucleotide position is based on the start codon.

^c For stock production H5N1_{Tex/24} was propagated for two passages on MDCK cells.

^d HNE cultures were infected at an MOI of 0.001 TCID₅₀ units per cell. Apical washes were collected at 72 h p.i. and viral genome sequences were determined.

Table 2 | Mutations in H5N1 viruses adapted to mammalian hosts.

Segment	Viral protein	Amino acid substitution	Biological effect	Absence/presence		
				H5N1 _{Tex/24}	H5N1 _{BE/22}	H5N1 _{Yam/04}
1	PB2	L89V	Compensation for lack of E627K ⁶⁹	+	+	+
		T271A	Enhances polymerase activity ⁶⁶	-	-	-
		G309D/T339K/R477G	Compensation for lack of E627K ⁶⁹	+/+/+	+/+/+	+/+/+
		Q591R	Enhanced binding to ANP32 family proteins ⁶⁴	-	-	-
		E627K	Enhanced polymerase activity at lower temperature ⁸⁴ Enhanced binding to NP ⁸⁵ Enhanced binding to ANP32 family proteins ⁸⁶	-	-	-
		M631L	Enhanced polymerase activity in human cells ⁴⁴	+	-	-
		D701N/S714R	Enhanced polymerase activity ²³ Promotion of nuclear import of vRNPs ⁶⁵	-/-	-/-	-/-
2	PB1	L472V/L598P	Compensation for lack of E627K ⁷⁰	-/-	-/-	-/-
3	PA	L336M/T552S/K615N	Enhanced viral protein production ⁷¹ and polymerase function ^{72,73}	-/-/-	-/-/-	-/-/-
4	HA	Q222L/N220K ^a	Promotion of binding to α 2,6-linked sialic acids ¹⁷	-/-	-/-	-/-
5	NP	Y52H	Escape from BTN3A3 restriction ⁵²	+	+	-
		F313Y	Escape from MxA and BTN3A3 restriction ⁵²	-	-	-
		N319K	Enhanced binding to importin- α ⁵³	-	-	-
6	NA	S369I/I396M/V	Disruption of the second sialic acid binding site ⁸⁷	-/-	-/-	-/-
		Long stalk	Increased virus transmission ^{88,89}	+	+	-
7	M1	N30D/T215A	Increased lethality in mice ⁹⁰	+/+	+/+	+/+
	M2	S31N	Amantadine resistance ⁹¹	-	-	-
8	NS1	L103F/I106M	Increased binding to CPSF30 ^{56,92}	+/+	+/+	+/+
	NEP	M16I/Y41C/E75G	Increased polymerase activity ^{93,94}	-/-/-	-/-/-	-/-/-

^a Numbering of the amino acids corresponds to the mature H5 hemagglutinin.

Supplementary Table 1 | Oligonucleotide sequences of primers and probes used for RT-qPCR.

Human gene	Oligo	Sequence (5'-3')
Beta-actin	FW ^a	CCACCATGTACCCTGGCA
	RV ^b	CACATCTGCTGGAAGGTGGA
	P ^c	FAM-AAGATCAAGATCATTGCTCCTCCTGAG-BHQ1
MxA	FW	CAGCACCTGATGGCCTATCAC
	RV	CATGAACTGGATGATCAAAGG
	P	FAM-AGGCCAGCAAGCGCATCTCCAG-TAMRA
CXCL-10/IP-10	FW	CCATTCTGATTTGCTGCCTTATC
	RV	GCAGGTACAGCGTACAGTTCT
	P	FAM-CTGACTCTAAGTGGCATTCAAGGAGTACCTCTCTC-TAMRA
IFN- β	FW	CGCCGCATTGACCATCTA
	RV	TTAGCCAGGAGGTTCTCAACAATAGTGCA
	P	FAM-TCAGACAAGATTCATCTAGCACTGGCTGGA-BHQ1
IFN- λ 1/IL-29	FW	GGACGCCTTGGAAGAGTCACT
	RV	AGAAGCCTCAGGTCCCAATTC
	P	FAM-AGTTGCAGCTCTCCTGTCTTCCCCG-BHQ1
IFN- λ 2/3/IL-28	FW	CTGCCACATAGCCCAGTTCA
	RV	AGAAGCGACTCTTCTAAGGCATCTT
	P	FAM-TCTCCACAGGAGCTGCAGGCCTTTA-BHQ1
IL-6	FW	CCAGGAGCCCAGCTATGAAC
	RV	CCCAGGGAGAAGGCAACTG
	P	FAM-CCTTCTCCACAAGCGCCTTCGGT-TAMRA
RIG-I	FW	CCAAGCCAAAGCAGTTTTCAA
	RV	CACATGGATTCCCCAGTCATG
	P	FAM-TTGAAAAAAGAGCAAAGATATTCTGTGCCCGAC-TAMRA

^a FW (forward) primer; ^b RV (reverse) primer; ^c P (probe).

Figures

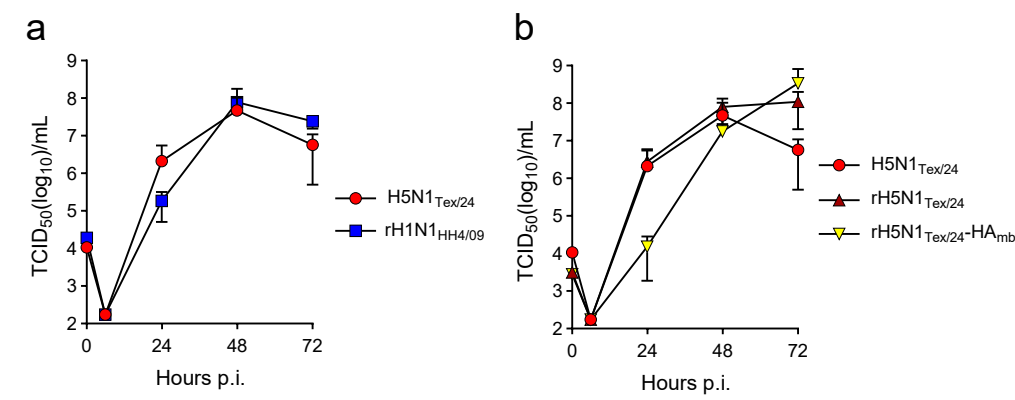


Fig. 1 | Replication kinetics of H5N1_{Tex/24} in HNE cells grown at air-liquid interface. a, b

ALI cultures were infected with H5N1_{Tex/24} and rH1N1_{HH4/09} (a) or with H5N1_{Tex/24}, rH5N1_{Tex/24}, and rH5N1_{Tex/24}-HA_{mb} (b) using an MOI of 0.001. At the indicated times, the cells were washed at the apical site and virus titer in the wash fluid titrated by end point dilutions on MDCK cells. Mean values $\pm$ SD from three biological replicates are shown. Statistical analysis was performed using the two-way ANOVA test with Sidak's multiple comparisons. $P < 0.05$ were considered significant. Only significant p values are depicted in the graphs.

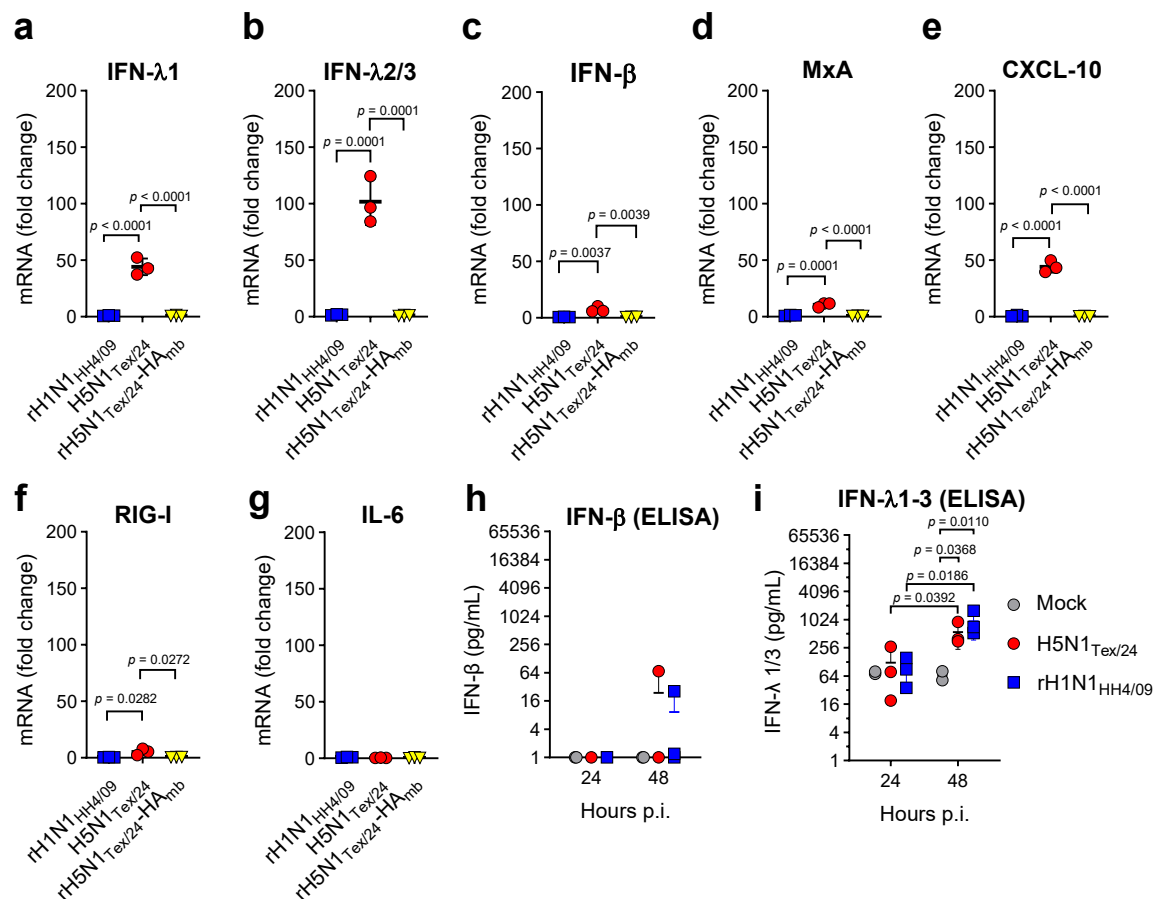


Fig. 2 | Induction of cytokine gene transcription in influenza virus-infected HNE cells. a - g HNE cells grown at the air-liquid interface were infected with either H5N1_{Tex/24}, H5N1_{Tex/24}-HA_{mb} or rH1N1_{HH4/09} (n = 3) using an MOI of 0.001 and incubated for 48 h at 33 °C. Total RNA was extracted and subjected to RT-qPCR analysis of the indicated cellular genes. The increase of the respective transcripts relative to mock-infected cells is shown. Statistical analysis was performed using the one-way ANOVA test with Tukey's multiple comparisons. **h - i** Detection of IFN- β (**h**) and IFN- λ 1-3 (**i**) in the basolateral medium at 24 h and 48 h p.i. by ELISA (n = 3). Statistical analysis was performed using the two-way ANOVA test with Tukey's multiple comparisons.

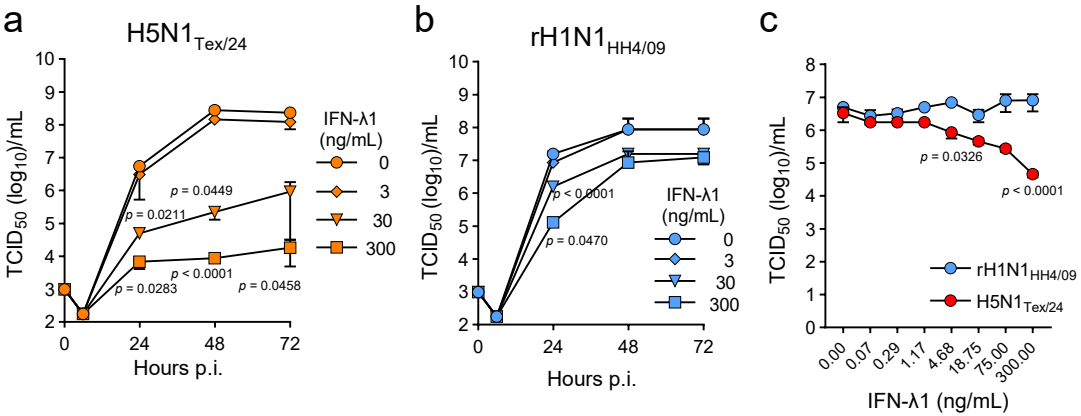


Fig. 3 | H5N1_{Tex/24} is sensitive to the antiviral activity of human IFN-λ1. **a, b** HNE cells grown in the air-liquid interface were incubated for 24 h with medium on the basolateral site containing the indicated concentrations of human IFN-λ1. The cells were subsequently infected from the apical site with either H5N1_{Tex/24} (**a**) or rH1N1_{HH4/09} (**b**) using an MOI of 0.001. At the indicated times, the apical domain of the cells was washed and infectious virus titers in the wash fluid determined. **c** A549 cells were treated for 24 h with human IFN-λ1 using the indicated concentrations. The cells were subsequently infected with either H5N1_{Tex/24} or rH1N1_{HH4} using an MOI of 0.01 and infectious titers in the cell culture supernatant determined at 24 h p.i.. Mean titers and SD of three infection experiments are shown. The two-way ANOVA test with Tukey's multiple comparisons are shown.

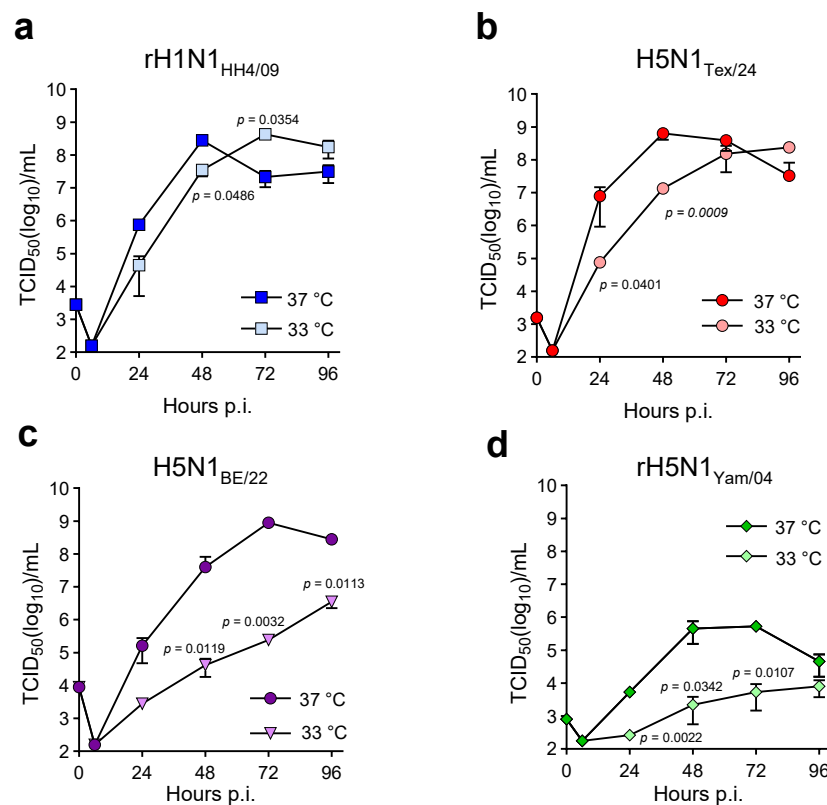


Fig. 4 | Temperature-sensitive replication of influenza A viruses. HNE were grown at the air-liquid interface at either 37 °C or 33 °C. The cells were infected from the apical site with either rH1N1_{HH4/09} (a), H5N1_{Tex/24} (b), H5N1_{BE/22} (c) or rH5N1_{Yam/04} (d) using an MOI of 0.001 and maintained for 96 h at the respective temperatures. At the indicated time points, the cells were washed at the apical site and infectious titers determined in the wash fluid by end point titration on MDCK cells. Mean infectious titers $\pm$ SD of three infection experiment are shown. Statistical comparisons were performed using the two-way ANOVA test with Tukey's multiple comparisons. *P* values are shown in the graphs.

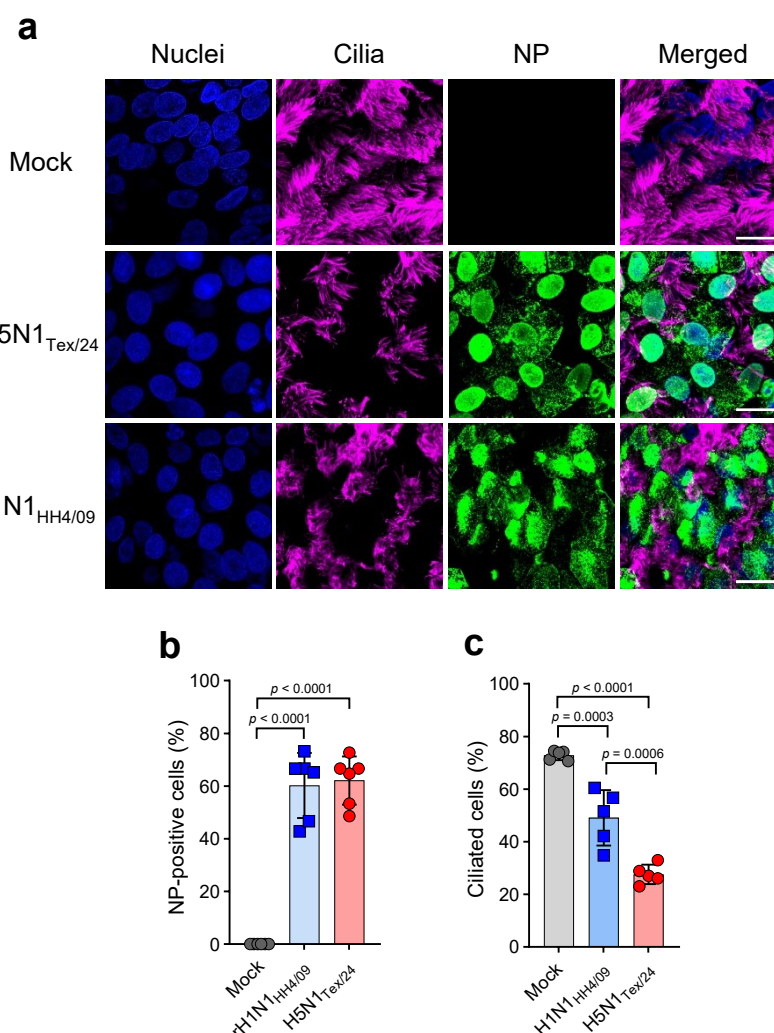


Fig. 5 | Infection of HNE cells causes loss of ciliated cells. **a** HNE cells grown in the air-liquid interface were either mock-infected or infected with H5N1_{Tex/24} or rH1N1_{HH4/09} using an MOI of 1. At 24 h p.i., the cells were fixed, permeabilized, and immunolabeled using a monoclonal antibody directed to the influenza NP antigen. Cilia were labeled with an antibody directed to acetylated tubulin and nuclei were stained with DAPI. Scale bar: 50 μ m. **b, c** Determination of the percentage of influenza virus-infected cells (**b**) and ciliated cells (**c**) using ImageJ. Five to six images per condition, each containing > 40 cells, were analyzed. Statistical analysis was performed using the one-way ANOVA test with Tukey's multiple comparisons.

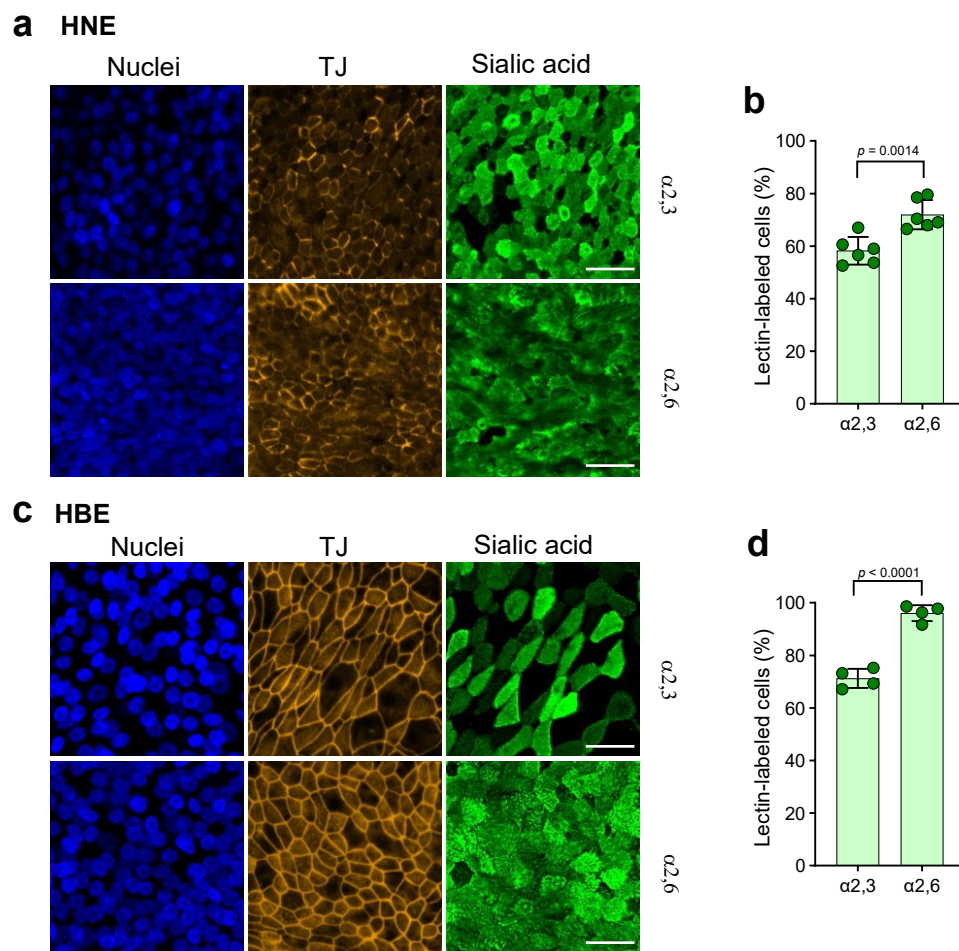


Fig. 6 | Detection of avian- and human-type receptors in HNE and HBE cells. a - d HNE (a) and HBE (c) cells were grown at air-liquid interface at 33 °C and 37 °C, respectively. The cells were labeled using either an antibody directed against the tight junction protein ZO-1 (TJ), lectins MAL-I/MAL-II for staining of $\alpha 2,3$ -linked sialic acid and lectin SNA for detection of $\alpha 2,6$ -sialic acids. Nuclei were stained with DAPI. Scale bar: 100 μ m. Relative number of cells labeled with MAL-I/MAL-II and SNA lectins in HNE (b) and HBE (d) were determined using ImageJ. Four to six images per sample were analyzed, each containing >100 cells. Mean values and SD are shown. Statistical analysis was performed with the unpaired, two-tailed Student's *t*-test.