Journal of Infection 92 (2026) 106722

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Letter to the Editor

Clade 2.3.4.4b H5N1 influenza A virus exhibits high infectivity in human respiratory tract models

Dear Editor,

We read with great interest the recent letter by Li and colleagues reporting the emergence of mammalian-adapted clade 2.3.4.4b H5N1 viruses carrying the PB2-E627K mutation in migratory birds at Qinghai Lake.[1] The identification of this canonical mammalian-adaptation marker in avian reservoirs has further intensified concerns regarding progressive viral adaptation and the potential for increased human-to-human transmission, thereby elevating pandemic risk.

The expanding host range of clade 2.3.4.4b viruses, now including poultry, dairy cattle and other mammals since early 2024, underscores their dynamic evolution and increasing interface with human populations, providing repeated opportunities for cross-species transmission and human exposure.[2–4] Although most human infections have been mild, severe and fatal cases have been reported,[2] underscoring the need for refined, functionally informed risk assessment approaches. In mammalian isolates, adaptive substitutions in the viral polymerase, most prominently PB2-E627K but also alternative changes such as PB2-M631L, have been associated with enhanced replication efficiency and pathogenicity.[5] Experimental infection studies in nonhuman primates further demonstrate that clade 2.3.4.4b viruses can cause severe and sometimes lethal respiratory disease.[4] However, whether currently circulating clade 2.3.4.4b viruses require the acquisition of PB2-E627K to exhibit substantial pathogenicity in human respiratory tissues remains unresolved.

To address this question, we investigated the replication competence, cellular tropism, tissue damage, and host transcriptional responses of a contemporary bovine clade 2.3.4.4b H5N1 isolate (H5N1bov, A/Cattle/Texas/063224-24-1/2024)[3] in three physiologically relevant primary human respiratory models representing the nasal (nasal air-liquid interface epithelium; nALI), bronchial (organoid-derived bronchial epithelium; brEp), and alveolar (*ex vivo* human lung tissue; aHuLu) compartments. Comparative infections were performed using a zoonotic clade 1 H5N1/2004 (A/Thailand/1(KAN-1)/2004), a seasonal H3N2/1999 virus (A/Panama/2007/1999) and an early clade 2.3.4.4b H5N8/2016-HPAIV (A/tufted duck/AR8/444/2016) known to replicate poorly in aHuLu.[6] H5N1bov replicated efficiently across all models, at levels comparable to H5N1, whereas human-adapted H3N2[7] showed the highest and H5N8 the lowest replication (Fig. 1A). Viral titers were highest in brEp, exceeding those in nALI and aHuLu by approximately ~2 orders of magnitude. While aHuLu and brEp supported rapid viral growth, replication in nALI was delayed. At 24 h post-infection (hpi), H5N1bov and H5N1 titers were ~10^3-fold lower than H3N2 but increased up to 72 hpi, reaching 10^7 PFU/ml for H5N1bov. These findings warrant attention, as efficient replication and shedding from nasal tissue are key determinants of IAV transmissibility. The delayed replication may provide the host immune system a window to mount an effective defense and limit disease progression. However, this advantage could be lost if further viral adaptations, particularly polymerase mutations, accelerate early replication and transmission in the upper respiratory tract, potentially leading to more severe clinical outcomes.[8]

To determine cellular tropism in the gas-exchanging alveolar compartment, we performed microscopic analysis of aHuLu at 24 hpi and 72 hpi. At 24 hpi, H5N1bov predominantly infected alveolar type II (ATII) cells with occasional alveolar macrophages (AM) infection and additional involvement of ATI cells at 72 hpi (Fig. 1B, upper panel). At this time, progressive epithelial damage, including ATII detachment, occludin loss, and increased caspase-3 activation (Fig. 1B, lower panel), indicated substantial alveolar injury and a potential for severe pneumonia.[2] Comparable patterns were observed for other strains depending on infection extent (Fig. S1). Characterization of the host immune response at 24 hpi by bulk RNA-sequencing followed by differential gene expression analysis revealed robust innate immune activation in aHuLu and brEp following H5N1bov infection, with induction of proinflammatory chemokines (*CXCL9-11*, *CCL5/RANTES*, *CCL8*), cytokines (*IFNB1*, *IFNL1-3*, *TNFSF13B*), and interferon-stimulated effector and regulatory genes (e.g., *RSAD2*, *IFIT-*, *MX-*, *OAS*-genes, *USP18*, *BATF2*, *AIM2*) (Fig. 1C), indicating coordinated antiviral and immunoregulatory responses.[9] In contrast, H5N8 elicited weak responses, consistent with limited replication and zoonotic potential.[6] Across strains, brEp showed the strongest transcriptional changes, including upregulation of metabolic and structural pathways at high replication levels. In contrast no significant immune gene induction ($\log_2$ fold change) was detected in nALI at 24 hpi, except for H3N2 (Fig. 1C), suggesting that early replication did not reach the threshold required for immune activation. To confirm this hypothesis, we correlated expression of chemokine and cytokine genes with viral titers across all models and strains at 24 hpi. All genes exhibited significantly positive Spearman correlation coefficients with viral titers, indicating that the magnitude of immune gene induction is closely linked to individual replication levels (Figs. 1D, S2). Accordingly, we observed positive correlation between virus titers (infectious virions released) and the abundance of virally encoded transcripts (normalized counts per million) (Figs. 1D, S3). These findings imply that in nALI, robust immune responses may occur at later time points (e.g., 48 or 72 hpi), following the delayed replication kinetics.

In conclusion, our data demonstrate that a contemporary bovine clade 2.3.4.4b H5N1 virus replicates efficiently in primary human respiratory tissues, induces robust antiviral and inflammatory responses, and causes epithelial injury comparable to zoonotic H5N1 and seasonal H3N2 viruses. These findings extend recent reports of

https://doi.org/10.1016/j.jinf.2026.106722

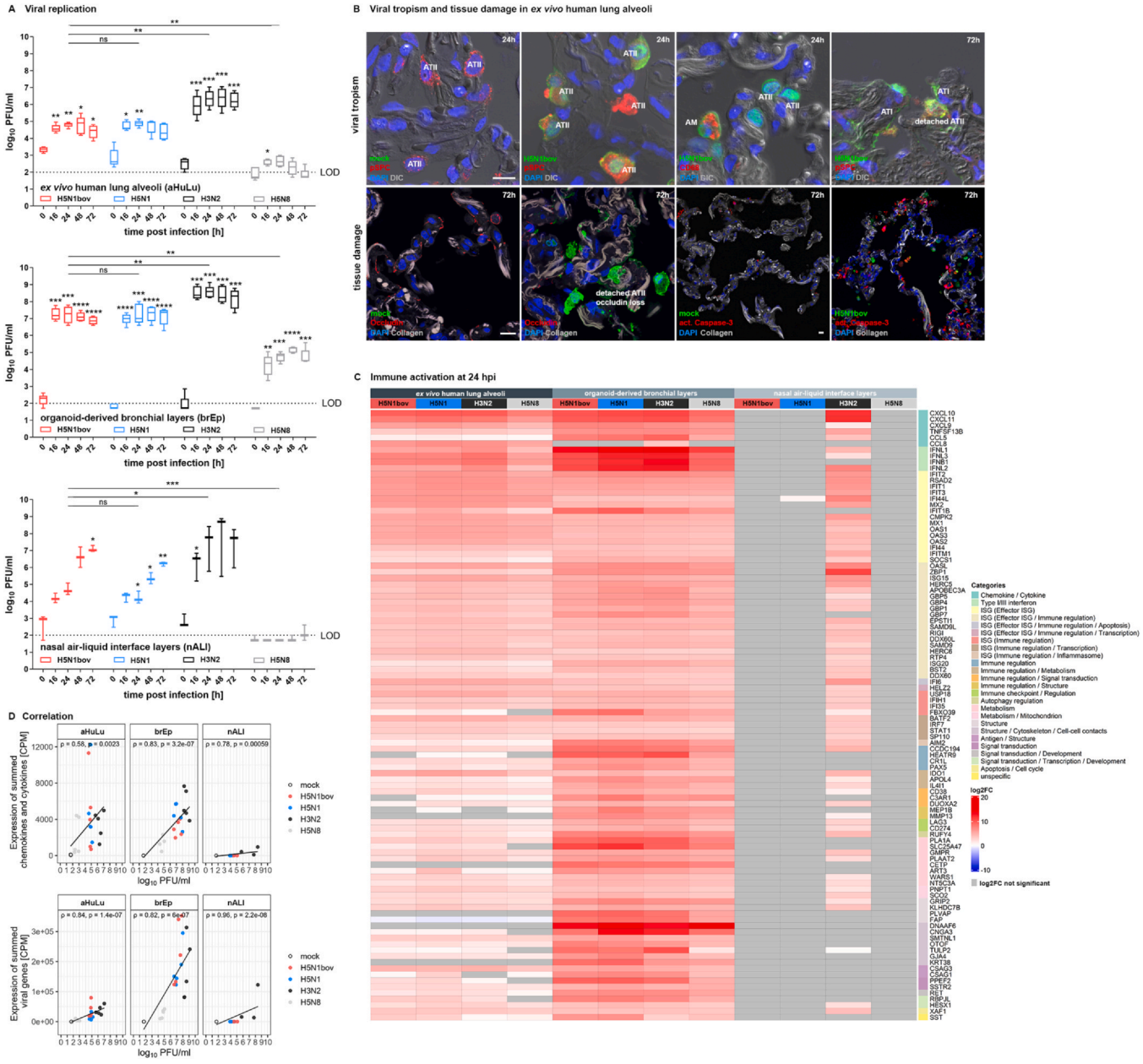

Fig. 1. Replication capacity, tissue tropism, and immune activation of avian and seasonal influenza viruses in human respiratory models. (A) Replication kinetics of H5N1bov (red), H5N1 (light blue), H3N2 (black), and H5N8 (light gray) in three human respiratory models: *ex vivo* human lung alveoli (aHuLu, top, n = 5), organoid-derived 3D bronchial epithelium layers (brEp, middle, n = 5), and nasal air-liquid interface layers (nALI, bottom, n = 3). Viral titers were measured at indicated hours post-infection (hpi) and are presented as $\log_{10}$ PFU/ml. Dashed line represents the limit of detection (LOD). All values below LOD were set to LOD/2, corresponding to 50 PFU/ml. Data are presented as median ± interquartile range; whiskers represent the full data range and are shown only if n > 3. To assess the increase in viral replication over time for each virus strain, viral titers at each post-infection time point were compared to the baseline at 0 hpi using two-way ANOVA followed by Tukey's post-hoc test. To compare overall replication kinetics between different virus strains, the area under the curve (AUC) was calculated for each strain. The AUC of H5N1bov was compared to the AUCs of the other virus strains using one-way ANOVA with Tukey's post-hoc test. ns = not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (B) Viral tropism and tissue damage in aHuLu upon infection with H5N1bov. Upper panels show viral tropism by immunolabeling of influenza antigen localization (green) at 24 hpi and 72 hpi and cellular markers: alveolar type 2 cells (ATII; pSPC, red) and alveolar macrophages (AM; CD68, red). Images were combined with Differential Interference Contrast (DIC) to reveal lung and cell morphology. The two left images show immunolabeling of tight junction protein occludin (red), and the two right images show immunolabeling of an apoptosis marker active caspase-3 staining (red). In each case, the left image represents the uninfected control, while the right image shows *ex vivo* human lung alveoli infected with H5N1bov. Collagen fibers were visualized via their intrinsic autofluorescence, which is shown in gray. Cell nuclei are visualized by 4′,6-diamidino-2-phenylindole (DAPI) stain (blue). Scale bars: 10 µm. (C) Heatmap shows $\log_2$ fold change (FC) of select host genes comparing infected samples to mock-infected controls at 24 hpi. For gene selection, genes were ranked by mean $\log_2$FC across all conditions (aHuLu [n = 5], brEp [n = 5], and nALI [n = 3] infected with H5N1bov, H5N1, H3N2, or H5N8), with non-significant $\log_2$FC set to zero, and the top 100 genes were selected. These genes were manually assigned to functional categories for ease of interpretation, which were then used to group the heatmap. Functional categories include Chemokines/Cytokines, Type I/III Interferons, ISGs, Immune regulation, Autophagy, Metabolism, Structure, Signal transduction, and Apoptosis. Each column represents a different virus strain. Increased expression in infected samples is shown in red, decreased expression in blue. Gray fields indicate adj. p ≥ 0.05 for the condition. (D) Correlation between viral replication (log10 PFU/ml) and the summed of counts per million (CPM; bulk RNA-seq) of cytokine and chemokine genes from the functional categories Chemokines/Cytokines and Type I/III Interferons (*CXCL10*, *CXCL11*, *CXCL9*, *TNFSF13B*, *CCL5*, *CCL8*, *IFNL1*, *IFNL3*, *IFNB1*, *IFNL2*; top) and of viral genes (*M*, *NP*, *NA*, *NS*, *PA*, *PB1*, *PB2*, *HA*; bottom) at 24 hpi.

efficient attachment and replication of clade 2.3.4.4b H5N1 viruses in human respiratory epithelium and highlight compartment-specific differences along the respiratory tract.[10] Importantly, these effects occur in the absence of the canonical mammalian-adapting PB2-E627K mutation, indicating that substantial pathogenic potential in human lower respiratory tissues does not strictly depend on this substitution. Alternative adaptive changes, including PB2-M631L or broader genomic constellation effects, may similarly support virulence in the human respiratory tract.[5] The attenuated replication of H5N1bov and earlier H5N1 viruses in nALI suggests that limited human-to-human transmission may reflect insufficient early shedding from the upper respiratory tract, despite preserved replication competence in bronchial and alveolar compartments.[8] The pronounced compartment-specific differences in viral growth, immune activation, and tissue damage underscore the importance of evaluating emerging H5N1 variants across physiologically relevant human respiratory models. As clade 2.3.4.4b viruses continue to expand their host range and diversify genetically, integrating functional human tissue data with genomic surveillance will be essential for accurately assessing zoonotic risk, refining pandemic preparedness strategies, and informing evidence-based public health decision-making.

CRediT authorship contribution statement

Conceptualization, A.L., M.B., S.H., T.W. and A.C.H.; Methodology, A.L., M.B., A.B., T.R., A.E.P, K.H., M.F., M.M., Z.D., E.W. and A.C.H.; Software, V.S., and M.M.; Validation, A.L., and A.C.H.; Formal analysis, A.L., V.S., and M.M.; Investigation, A.L., M.B., V.S., and M.M.; Resources, D.G.D., A.L. and A.B.; Lung Tissue Explants and Meta-data, M.T., T.T.B., M.G., J.N. and J.C.R.; Data curation, V.S., M.M. and L.B.; Writing – original draft, A.L., S.H. and A.C.H.; Writing – review & editing, A.L., M.B., S.H., and A.C.H.; Visualization, A.L., and A.C.H.; Supervision, L.B., S.H., T.W. and A.C.H.; Project administration, A.C.H.; Funding acquisition, A.B., L.B., E.W., M.L., M.A.M., A.D.G., S.H., T.W. and A.C.H. All authors have read and agreed to the published version of the manuscript.

Funding

A.L., S.H., T.W., and A.C.H. were supported by the German Federal Ministry of Research, Technology and Space (BMFTR) through the project FLU-PREP (grant no. 01KI2508A). A.L., A.D.G., S.H., and A.C.H. were supported by Einstein Foundation (EC3R EZ-2020-597-2). A.L. and A.C.H. were funded by Charité 3R/Replace – Reduce – Refine. M.A.M was supported by the German Research Foundation (DFG) (CRC 1449 - project #431232613 and project #450557679) and the German Federal Ministry of Education and Research (grants 82DZL009C1 and 01GL2401A). This work was supported by intramural funding of the Robert-Koch-Institute to T.W. This work was supported in part by USDA-NIFA grant no. 2021-68014-33635 (to D.G.D.) and APHIS NAHLN Enhancement grant no. AP21VSD&B000C005 (to D.G.D.).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Toralf Kaiser, Jenny Kirsch, and Kerstin Heinrich from Flow Cytometry & Cell Sorting, German Rheumatology Research Center (DRFZ), Berlin, Germany, for FACS sorting. We are also grateful to Sandra Kunder and Anne Voß from Department of Veterinary Pathology, Freie Universität Berlin, Berlin, Germany, for their support with sample processing, including paraffin embedding and section preparation for immunohistochemical analysis. Furthermore, we acknowledge Jeannine Wilde, Elisabeth Kirst, and Tatiana Borodina from the MDC/BIH Genomics Technology Platform for their contributions to sequencing.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2026.106722.

References

1. Li X, Lv X, Li Y, Fei M, Liu S, Chai H. *Zoonotic threat: emergence of mammalian-adapted H5N1 virus in migratory birds at Qinghai Lake. J Infect* 2025;**91**(5):106623. https://doi.org/10.1016/j.jinf.2025.106623
2. Rolfes MA, Kniss K, Kirby MK, Garg S, Reinhart K, Todd Davis C, et al. *Human infections with highly pathogenic avian influenza A(H5N1) viruses in the United States from March 2024 to May 2025. Nat Med* 2025;**31**(11). https://doi.org/10.1038/s41591-025-03905-2
3. Caserta LC, Frye EA, Butt SL, Laverack M, Nooruzzaman M, Covaleda LM, et al. *Spillover of highly pathogenic avian influenza H5N1 virus to dairy cattle. Nature* 2024;**634**(8034):669–76. https://doi.org/10.1038/s41586-024-07849-4
4. Andersen H, Aid M, Stone JJ, Lyons CE, Berlied A, Nkolola J, et al. *Immunopathogenesis of lethal H5N1 avian influenza virus clade 2.3.4.4b infection in macaques. Immunity* 2025;**58**(9):2157–65. https://doi.org/10.1016/j.immuni.2025.07.020. e5.
5. Capelastegui F, Goldhill DH. *H5N1 2.3.4.4b: a review of mammalian adaptations and risk of pandemic emergence. J Gen Virol* 2025;**106**(6). https://doi.org/10.1099/jgv.0.002109
6. Grund C, Hoffmann D, Ulrich R, Naguib M, Schinkothe J, Hoffmann B, et al. *A novel European H5N8 influenza A virus has increased virulence in ducks but low zoonotic potential. Emerg Microbes Infect* 2018;**7**(1):132. https://doi.org/10.1038/s41426-018-0130-1
7. Yang H, Carney PJ, Chang JC, Guo Z, Villanueva JM, Stevens J. *Structure and receptor binding preferences of recombinant human A(H3N2) virus hemagglutinins. Virology* 2015;**477**:18–31. https://doi.org/10.1016/j.virol.2014.12.024
8. Richard M, van den Brand JMA, Bestebroer TM, Lexmond P, de Meulder D, Fouchier RAM, et al. *Influenza A viruses are transmitted via the air from the nasal respiratory epithelium of ferrets. Nat Commun* 2020;**11**(1):766. https://doi.org/10.1038/s41467-020-14626-0
9. Sohail A, Waqas FH, Braubach P, Czichon L, Samir M, Iqbal A, et al. *Differential transcriptomic host responses in the early phase of viral and bacterial infections in human lung tissue explants ex vivo. Respir Res* 2024;**25**(1):369. https://doi.org/10.1186/s12931-024-02988-8
10. Bauer L, Leijten L, Iervolino M, Chopra V, van Dijk L, Power M, et al. *Attachment and replication of clade 2.3.4.4b influenza A (H5N1) viruses in human respiratory epithelium: an in-vitro study. Lancet Microbe* 2026;**7**(1):101230. https://doi.org/10.1016/j.lanmic.2025.101230

Anna Löwa [1]
Department of Infectious Diseases, Respiratory Medicine and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

Matthias Budt [2]
Unit 17 "Influenza and other Respiratory Viruses", Robert Koch Institute, Berlin, Germany

Anita Balázs
Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Berlin, Germany
German Center for Lung Research (DZL), associated partner site Berlin, Berlin, Germany
German Center for Child and Adolescent Health (DZKJ), partner site Berlin, Berlin, Germany

Victor Sikora, Mark Melzer
Department of Infectious Diseases, Respiratory Medicine and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

Tihomir Rubil
Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Berlin, Germany
German Center for Lung Research (DZL), associated partner site Berlin, Berlin, Germany
German Center for Child and Adolescent Health (DZKJ), partner site Berlin, Berlin, Germany

Annika E. Pfeiffer
Unit 17 "Influenza and other Respiratory Viruses", Robert Koch Institute, Berlin, Germany

Mara Fischer, Maren Mieth, Katharina Hellwig
Department of Infectious Diseases, Respiratory Medicine and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

Emanuel Wyler
Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin Institute for Medical Systems Biology, Berlin, Germany

Diego G. Diel
Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA

Mario Tönnies, Torsten T. Bauer
HELIOS Clinic Emil von Behring, Department of Pneumology and Department of Thoracic Surgery, Chest Hospital Heckeshorn, Berlin, Germany

Mareike Graff
Department for Thoracic Surgery, DRK Clinics, Berlin, Germany

Jens Neudecker, Jens-C. Rückert
Department of General, Visceral, Vascular and Thoracic Surgery, Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

Marcus A. Mall
Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Berlin, Germany
German Center for Lung Research (DZL), associated partner site Berlin, Berlin, Germany
German Center for Child and Adolescent Health (DZKJ), partner site Berlin, Berlin, Germany

Achim D. Gruber
Institute of Veterinary Pathology, Freie Universität Berlin, Berlin, Germany

Markus Landthaler
Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin Institute for Medical Systems Biology, Berlin, Germany
Institute for Biology, Humboldt Universität zu Berlin, Berlin, Germany

Lisa Buchauer, Stefan Hippenstiel
Department of Infectious Diseases, Respiratory Medicine and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

Thorsten Wolff
Unit 17 "Influenza and other Respiratory Viruses", Robert Koch Institute, Berlin, Germany

Andreas C. Hocke *
Department of Infectious Diseases, Respiratory Medicine and Critical Care Medicine, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

*Correspondence to: Charité – Universitätsmedizin Berlin, Department of Infectious Diseases, Respiratory Medicine and Critical Care Medicine, Molecular Imaging of Immunoregulation, Charitéplatz 1, 10117 Berlin, Germany.
E-mail address: andreas.hocke@charite.de (A.C. Hocke),
[1] Contributed equally.
[2] Contributed equally.