Analysis of SARS-CoV-2 replication in explant cultures of the human upper respiratory tract reveals broad tissue tropism of wild-type and B.1.1.7 variant viruses

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Summary

This communication reports widespread and similar replication competence of early SARS-CoV-2 and its B.1.1.7 variant in explanted tissues of the human upper respiratory tract and primary nasal epithelial cells. B.1.1.7 induced a reduced type III IFN response in nasal cells.

Abstract

The upper respiratory tract (URT) is the primary entry site for SARS-CoV-2 and other respiratory viruses, but its involvement in viral amplification and pathogenesis remains incompletely understood. Here we investigated primary nasal epithelial cultures, as well as vital explanted tissues to scrutinize the tropism of wild-type SARS-CoV-2 and the recently emerged B.1.1.7 variant. Our analyses revealed a widespread replication competence of SARS-CoV-2 in polarized nasal epithelium as well as in the examined URT and salivary gland tissues, which was also shared by the B.1.1.7 virus thereby highlighting the active role of these anatomic sites in COVID-19.

Keywords:

COVID-19, SARS-CoV-2, Upper Respiratory Tract, Influenza, Variants of Concern

Background

The emergence of the highly pathogenic human Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) lead to a global pandemic which caused more than 4.6 million deaths worldwide so far. SARS-CoV-2 is the causative agent of Coronavirus Disease 2019 (COVID-19) and is transmitted via infected droplets and aerosols [1].

The upper respiratory tract (URT) is believed to have essential roles in person-to-person spread of SARS-CoV-2 and its dissemination to the lung, but its precise role in COVID-19 pathogenesis remains incompletely understood. A variety of clinical symptoms including ageusia and anosmia, dry mouth, oral lesions, sore throat, dry cough, sinusitis or rhinorrhea indicate that cells of the oropharynx and the URT are targeted by SARS-CoV-2 [2]. High SARS-CoV-2 viral loads in clinical specimens of URT fluids and in saliva of a majority of COVID-19 patients consistently detected at illness onset suggest active viral replication, which is further supported by isolation of infectious virus and detection of viral replicative RNA intermediates from throat-swabs of COVID-19 patients and in salivary glands. This indicates that tissues of the URT and salivary glands are not just sites of infection but also of viral replication [2-4] However, knowledge on the precise tissue source of transmissible SARS-CoV-2 remains limited. Productive viral replication has been demonstrated for nasal turbinates [5], but the permissiveness of other tissues lining the surface of the URT has yet to be investigated.

To date, a number of single cell RNA sequencing studies confirmed expression of SARS-CoV-2 entry factors including angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine subtype 2 (TMPRSS2) in different cell types of the URT [4, 6, 7]. Here we report an ex vivo approach to characterize SARS-CoV-2 replication and extended tropism in available tissues of the human URT and salivary glands. We utilized surgical specimens freshly derived from paranasal sinuses, adenoids, tonsils as well as salivary glands to establish short-term tissue cultures, which were infected with SARS-CoV-2. For a comparison, we employed seasonal H3N2 influenza A virus (IAV), that is also shed from the URT in naïve individuals over a period of 5-7 days [8]. The newly established models were further used to address the hypothesis that increased transmission and rapid worldwide spread of

the recently emerged SARS-CoV-2 lineage B.1.1.7 is caused by more productive replication compared to earlier variants. The B.1.1.7 viruses were first detected in the UK in September 2020 and accumulate higher viral RNA loads in nasopharyngeal swabs of COVID-19 patients, which may also be associated with increased mortality [9, 10].

Methods

Cell culture. Vero E6 and Calu-3 cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 10% (Vero E6) or 15% (Calu-3) fetal bovine serum (FBS), respectively, supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1x non-essential amino acids and 1 mM sodium pyruvate and incubated at 37°C with 5% CO2 in a humidified atmosphere. Calu-3 cells were seeded in ThinCert tissue culture inserts (0.4µm pore size) and were cultivated under Air-Liquid-Interface (ALI) for 14 days prior to infection. Primary human nasal epithelial cells (phNECs) were collected from healthy donors by nasal brushings. Nasal cells were expanded using the conditionally reprogrammed cell (CRC) culture method and then then differentiated on porous Transwell or Snapwell (Corning) supports in UNC-ALI medium for at least 3 weeks prior to infection.

Tissue samples. Informed consent was obtained from all patients and the study was approved by the Charité ethics committee (EA2/054/20). Tissue samples of overall 33 patients (17 males, 13 females, 3 unknown; age range, 1–77 years; median age, 30 years) were cut into small pieces (weight approx. 0.05 - 0.1 mg per piece) and incubated overnight in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 250ng/ml Amphotericin B at 37°C with 5% CO2 in a humidified atmosphere.

Virus infection. Cells were infected with SARS-CoV-2 (hCoV-19/Germany/BY-ChVir-929/2020, lineage B.1.153 (GISAID accession: EPI_ISL_406862)), SARS-CoV-2 lineage B.1.1.7 (hCoV-19/Germany/BY-ChVir21652/2020 (GISAID accession: EPI_ISL_802995)) or Influenza A/Panama/2007/1999 virus (NCBI

accession numbers: DQ487333-DQ487340) at MOI of 0.1 (growth curve analysis) or of 1 (qPCR) in supplemented DMEM containing 10% or 15% FBS (SARS-CoV-2) or 0.3% BA (IAV), respectively (Vero E6, Calu-3), or in UNC-ALI medium (phNEC) for 1h at 37°C. For phNECs DPBS washes (100µl) were performed at 37°C for 30min to collect apical samples. Tissue cultures were inoculated with $1x10^6$ pfu of SARS-CoV-2 lineage B.1.153 or lineage B.1.1.7 or IAV per tissue slice, respectively and incubated for 24h in supplemented RPMI 1640 medium containing 10% FBS (SARS-CoV-2) or 0.3% BA (IAV). The primary structures of the stock viruses were verified by whole genome sequencing, indicating a subpopulation of B.1.1.7 genomes (37%) carrying a "T" instead of "G" at nucleotide position 23,607. To quantify infectious virus particles, supernatants were harvested at indicated time points and titrated on Vero E6 cells for SARS-CoV-2 and MDCKII cells for IAV by standard plaque titration assay.

Quantitative RealTime PCR. RNA was isolated using Lysing Matrix D tubes (MP Biomedicals) and RNeasy Mini Kit (Qiagen) according to the manufactures protocol. 1µg of RNA isolated from tissue cultures or 300 ng of RNA isolated from phNECs was converted into cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and cDNA was used in TaqMan Gene Expression Assay (Thermo Fisher Scientific; Assay IDs: Hs 02758991_g1 (GAPDH), Hs 0185333_m1 (ACE2), Hs 00965485_g1 (Furin), Hs 01122322_m1 (TMPRSS2), Hs 00942302_m1 (CD147), Hs00265051_s1 (IFNα2), Hs01077958_s1 (IFNβ1), Hs00820125_g1 (IFNλ2), Hs01911452_s1 (IFIT1)). To estimate relative gene expression of host genes in ENT tissues $2^{-\Delta Ct}$ was calculated between gene of interest and GAPDH as reference gene. For determination of immune factor gene expression in phNECs $2^{-\Delta\Delta Ct}$ was calculated between ΔCt values of infected samples and mock-infected ones.

Results

We used Vero E6 cells to demonstrate comparable replication capabilities of SARS-CoV-2 and seasonal IAV in a common cell culture model, in which both viruses replicated to titers of about 10^8 pfu/ml (Fig. 1a). Differentiated ALI cultures of human Calu-3 cell line and phNECs were employed to evaluate infectivity of SARS-CoV-2 in models more closely mimicking the conditions of the URT (Fig. 1a). In both settings, SARS-CoV-2 isolated early in 2020 (lineage B.1.153) replicated to high viral titers about 10^8 pfu/ml (Calu-3) and 10^7 pfu/ml (phNEC), respectively. Propagation of SARS-CoV-2 proceeded with a slightly delayed kinetic compared to IAV reaching peak titers at 72h p.i.

In all URT and salivary gland explant cultures, SARS-CoV-2 replicated to similar titers up to 10^4 pfu/ml at 24h p.i. (Fig. 1b). This is in line with comparable expression of host factors in these tissues supporting SARS-CoV-2 growth including ACE2, TMPRSS2, furin and CD147 as analyzed by quantitative real-time PCR (Fig. 1c). In the URT explant cultures as well as phNECs, expression of the viral entry receptor ACE2 was lower compared to expression levels of host proteases TMPRSS2 and furin and the recently suggested alternate entry receptor CD147 [11]. In contrast to SARS-CoV-2, replication of IAV that uses sialic acid conjugates as receptors was more restricted. This becomes apparent by failure of IAV to productively infect tonsillar and salivary gland tissue, which contrasts with very efficient replication in paranasal sinuses (up to 10^8 pfu/ml) and adenoids (up to 10^5 pfu/ml) (Fig. 1b).

Having established the URT and phNEC culture as suitable infection models we used them to compare replication of the early reference virus and lineage B.1.1.7 SARS-CoV-2. However, no significant differences in viral growth were identified in either of the primary models (Fig. 2a, b). COVID patients upregulate genes for antiviral type I and III IFN in their nasal mucosa [12] and this was recapitulated in the phNEC (Fig. 2c). Interestingly, we observed a significantly reduced upregulation of IFN-λ transcripts as well as a trend for lesser IFN-β induction by the B.1.1.7 virus in comparison to the reference virus, whereas similar levels of IFN-α and the IFN-inducible gene IFIT1 were found in cells infected with either virus (Fig. 2c).

Discussion

SARS-CoV-2 replicated to similar titers in various URT and salivary gland explant cultures that expressed similar levels of host factors important for viral propagation. SARS-CoV-2 was amplified to high titers in the range of 10^8 PFU/ml in phNEC ALI cultures, which closely reflects high viral loads in nasal tissues in COVID-19 patients [3].

Tissue of the nasal cavity has been reported to be permissive for SARS-CoV-2 propagation due to expression of ACE2 and TMPRSS2 with highest levels of ACE2 expression detected in ciliated and secretory cells [7, 13]. Interestingly, SARS-CoV-2 accumulated to lower titers in whole tissue samples of paranasal sinuses compared to phNEC cultures, which was unexpected as both tissues originate from the nasal cavity and IAV propagated equally well in both systems. Nevertheless, the observed replication phenotypes are in line with previous findings by Alfi et al. infecting nasal turbinates and Hou et al. performing infection experiments in human nasal epithelial cultures [5, 13]. Differences in replication efficiency may be caused by different levels of ACE2 expression in freshly excised nasal tissue compared to nasal epithelial cells. The latter were differentiated ex vivo for 28 days under ALI conditions during which they acquire high ACE2 levels throughout the cultivation period [6]. In fact, slightly but significantly enhanced expression of ACE2 in phNECs compared to nasal tissue could be confirmed by qPCR in our study. Our observations show substantial replication of SARS-CoV-2 in cells and tissues of the nasal cavity thereby confirming and extending findings from a study conducted in primary human airway epithelial cultures from the upper and lower airways [13].

In contrast to tissue samples of paranasal sinuses and adenoids, SARS-CoV-2 viral titers in tonsils and salivary glands exceeded that of IAV. The reason for this discrepancy in the tonsillar tissue is not readily apparent. Previous data suggested that interactions between immune cells and epithelial cells during SARS-CoV-2 infection can increase susceptibility, which may explain the observed robust replication in lymphoid tissues such as adenoids and tonsils [7]. Due to high expression of ACE2 and

TMPRSS2 along with detection of viral RNA in COVID-19 autopsies, salivary glands are considered potential amplification sites for the virus [4] which is supported by active replication in parotid salivary glands available in our study.

SARS-CoV-2 B.1.1.7 is associated with higher viral loads in COVID-19 patient swabs and has a capability for enhanced spread, therefore increased replication was expected in the investigated model systems of the URT. Nevertheless, the B.1.1.7 virus replicated to similar titers in the URT explant and phNEC cultures compared to the reference virus. Analysis of innate immune response factors revealed a reduced induction of IFN-λ in samples infected with the B.1.1.7 lineage compared to the reference virus from 2020. These observations are in line with a reduced production of IFN-stimulated gene products in human Calu-3 adenocarcinoma cells infected with a B.1.1.7 virus compared to earlier virus lineages as reported by Thorne et al. [14]. Hence, our findings give initial support for a model, in which the phenotype of the B.1.1.7 lineage involves the capacity to distinctively target the host innate immune response, which may impact on the efficacy of viral transmission as has been observed for HIV-1 [15] but further analyses is required to substantiate this hypothesis for SARS-CoV-2.

In summary, we show that propagation and shedding of infectious SARS-CoV-2 in body fluids of the URT can be modelled in an ex vivo approach using primary organ cultures and an advanced reconstituted polarized nasal epithelium. Widespread replication competence of SARS-CoV-2 in various URT and salivary gland tissues, as shown in this study, very likely contributes to high viral titers and efficient transmission of SARS-CoV-2 between people. High viral loads in URT further allow seeding of virus to the lower respiratory tract as seen in the pulmonary phase of COVID-19. We expect these newly established infection models to be highly useful in determining the replicative capacity of new emerging viral variants, to investigate their potential virulence in the URT and salivary glands, and to evaluate the effectiveness of new antiviral compounds.

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References:

1. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature **2020**; 579:270-3.

2. Chen L, Zhao J, Peng J, et al. Detection of SARS-CoV-2 in saliva and characterization of oral symptoms in COVID-19 patients. Cell Prolif **2020**:e12923.

3. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. Nature **2020**; 581:465-9.

4. Huang N, Perez P, Kato T, et al. SARS-CoV-2 infection of the oral cavity and saliva. Nat Med **2021**.

5. Alfi O, Yakirevitch A, Wald O, et al. Human nasal and lung tissues infected ex vivo with SARS-CoV-2 provide insights into differential tissue-specific and virus-specific innate immune responses in the upper and lower respiratory tract. J Virol **2021**.

6. Sungnak W, Huang N, Bécavin C, et al. SARS-CoV-2 entry factors are highly expressed in nasal epithelial cells together with innate immune genes. Nat Med **2020**; 26:681-7.

7. Chua RL, Lukassen S, Trump S, et al. COVID-19 severity correlates with airway epithelium-immune cell interactions identified by single-cell analysis. Nat Biotechnol **2020**; 38:970-9.

8. Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE. Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. J Clin Invest **1998**; 101:643-9.

9. Davies NG, Abbott S, Barnard RC, et al. Estimated transmissibility and impact of SARS-CoV-2 lineage B.1.1.7 in England. Science **2021**:eabg3055.

10. Davies NG, Jarvis CI, Group CC-W, et al. Increased mortality in community-tested cases of SARS-CoV-2 lineage B.1.1.7. Nature **2021**.

11. Wang K, Chen W, Zhang Z, et al. CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. Signal Transduct Target Ther **2020**; 5:283.

12. Sposito B, Broggi A, Pandolfi L, et al. The interferon landscape along the respiratory tract impacts the severity of COVID-19. Cell **2021**; 184:4953-68 e16.

13. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. Cell **2020**; 182:429-46 e14.

14. Thorne LG, Bouhaddou M, Reuschl AK, et al. Evolution of enhanced innate immune evasion by the SARS-CoV-2 B.1.1.7 UK variant. bioRxiv **2021**.

15. Sumner RP, Thorne LG, Fink DL, Khan H, Milne RS, Towers GJ. Are Evolution and the Intracellular Innate Immune System Key Determinants in HIV Transmission? Front Immunol **2017**; 8:1246.

Figure 1: Replication of SARS-CoV-2 in tissues of the human upper respiratory tract (URT) and salivary glands (SG) ex vivo. (A) Replication analysis of SARS-CoV-2 and influenza A virus (IAV) was performed for n=3 in duplicates (Vero E6, Calu-3) or n=6 donors of primary human nasal epithelial cells (phNECs). For ALI cultures solid lines represent samples taken from the apical site, dashed lines samples from the basolateral compartment. (B) SARS-CoV-2 and IAV kinetics were conducted in technical duplicates for n=3 (SG), n=4 (paranasal sinuses), n=4 (adenoids) and n=5 (tonsils) donors. Level of detection is shown by dotted lines (A,B). (C) For qPCR, tissues samples or phNEC of n= 5 (phNEC), n=4 (adenoids), n=3 (SG, paranasal sinuses and tonsils) donors were analyzed. Statistical analysis was performed using Mann-Whitney U test, * $p<0.05$.

Figure 2: SARS-CoV-2 lineage B.1.1.7 replication phenotype in human upper respiratory tract (URT) and salivary glands (SG) tissues. (A) SARS-CoV-2 lineages B.1.153 and B.1.1.7 were analyzed according to its replication capacity in explant cultures of the URT/SG in technical duplicates for n=4 (SG), n=5 (paranasal sinuses), n=4 (adenoids) and n=3 (tonsils). (B) Replication analysis of SARS-CoV-2 lineages B.1.153 or B.1.1.7 was performed for n=5 donors on primary human nasal epithelial cells (phNECs). Solid lines represent samples taken from the apical site, dashed lines samples from the basolateral compartment. (C) phNEC of n= 5 donors were infected with SARS-CoV-2 lineages B.1.153 or B.1.1.7. Induction of gene expression of IFNα, IFNβ, IFNλ and IFIT1 in infected phNECs was analyzed by quantitative PCR. Statistical analysis was performed using Mann-Whitney U test, * $p<0.05$ (C). Level of detection is shown by dotted lines (A,B).

Footnotes

(1) Potential conflicts of interest: None declared.

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(3) The information has not been presented previously at any meeting.

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Figure 1

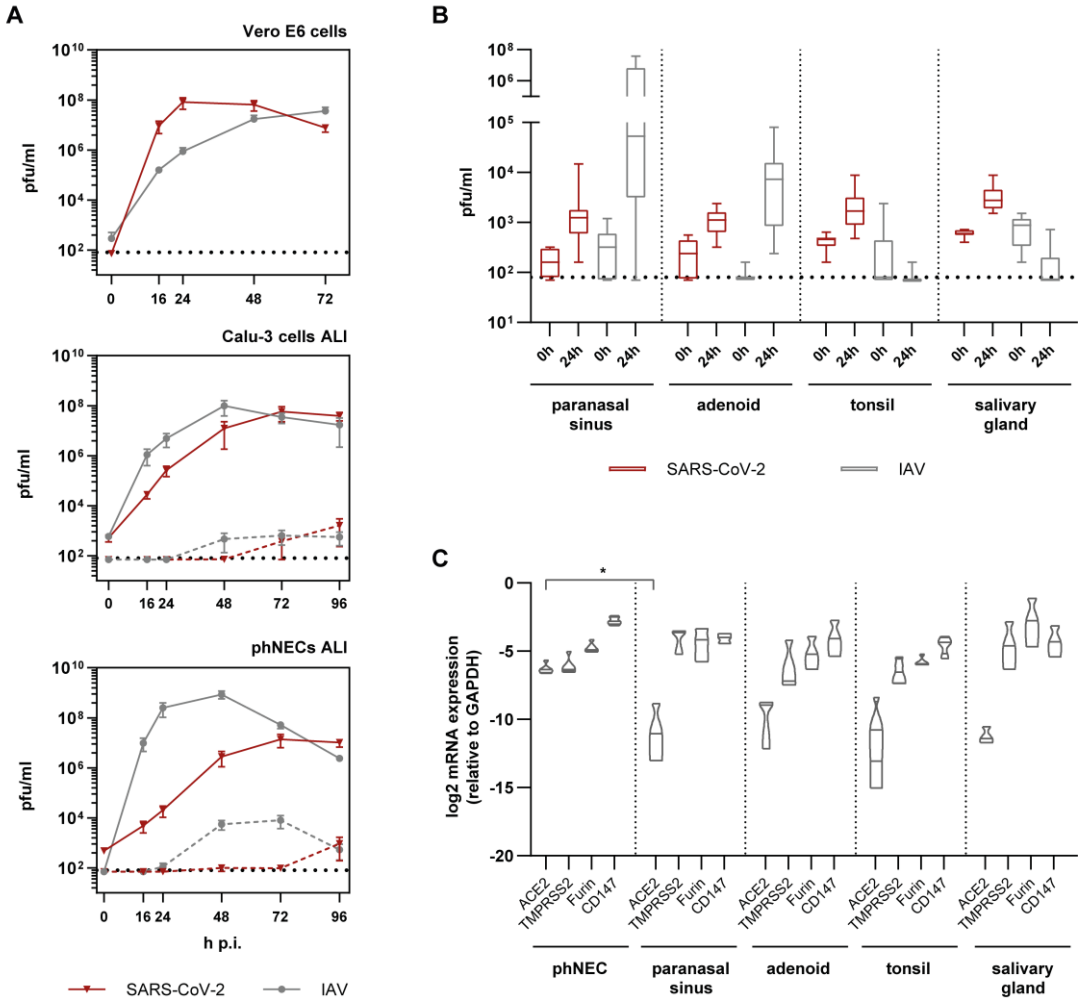

Figure 2