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Assessment of the zoonotic potential of the ruminant-associated influenza D virus

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Abstract

Influenza viruses are notorious pathogens that frequently cross the species barrier with often severe consequences for both animal and human health. In 2011, a novel member of the *Orthomyxoviridae* family, Influenza D virus (IDV), was identified in the respiratory tract of pigs with influenza-like symptoms and subsequently also in cattle, a species that previously never was associated with influenza virus infection. Epidemiological surveys among livestock demonstrated that IDV is worldwide distributed among ruminants, but the most striking observation is the detection of IDV-directed antibodies among humans with occupational exposure to livestock. As a first step toward identifying the zoonotic potential of the newly emerging IDV we determined the replication kinetics and cell tropism at the primary site of replication using an *in vitro* respiratory epithelium model of humans. The inoculation of IDV on human airway epithelial cell (hAEC) cultures revealed efficient replication kinetics and apical progeny virus release of IDV at different body temperatures. Intriguingly, the replication characteristics of IDV revealed many similarities to the human-associated Influenza C virus, including the predominant cell tropism for ciliated cells. Moreover, analysis of the host response during IDV infection revealed only a pronounced upregulation of Type III interferon (IFN) transcripts. Nevertheless, viral progeny virus is replication competent and can be efficiently sub-passaged in hAEC cultures from different donors. Highlighting, that there is no intrinsic impairment of IDV replication within the human respiratory epithelium and might explain why IDV-directed antibodies can be detected among humans with occupational exposure to livestock.

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Importance

Influenza viruses are notorious pathogens that frequently cross the species barrier with often severe consequences for both animal and human health. In 2011, a novel member of the *Orthomyxoviridae* family, Influenza D virus (IDV), was identified among pigs with influenza-like symptoms and subsequently also in cattle. IDV infections in humans have not yet been described, although IDV-directed antibodies have been found among people with occupational exposure to livestock. This observation suggests a possible spillover from livestock to humans. Using an *in vitro* human respiratory epithelium model we demonstrate there is no inherent restriction for IDV to replicate within the human respiratory epithelium and this might explain why IDV-directed antibodies are detected among humans with occupational exposure to livestock.

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Introduction

Since the initial discovery of Influenza D virus (IDV) in 2011, among swine with Influenza-like symptoms, knowledge about this new genus in the family of *Orthomyxoviridae* is increasing (1, 2). Epidemiological studies have shown that the virus has a worldwide distribution that can be divided into at least two distinct cocirculating lineages which reassort (3–10). Because of the high seroprevalence the proposed natural reservoir of IDV is cattle, which in an experimental setting is shown to cause a mild respiratory infection (11). In addition to cattle, virus specific-antibodies towards IDV have also been detected in swine, feral swine, equine, ovine, caprine and camelid species, suggesting a broad-host tropism for IDV (3, 9, 12, 13). However, the most striking observation is the detection of IDV-directed antibodies among humans with occupational exposure to livestock (14).

The hemagglutinin-esterase fusion (HEF) glycoprotein of IDV utilizes the receptor determinant 9-*O*-acetyl-*N*-acetylneuraminic acid for cell entry, which is similar to that of the closely related human Influenza C virus (ICV) (15, 16). The fusion of the IDV HEF glycoprotein with the host cell membrane is efficient at both 33°C and 37°C, which is in contrast to the HEF glycoprotein of ICV that is restricted to 33°C (10, 17). This discrepancy between both viruses is mediated by the open receptor-binding cavity in the HEF of IDV (15). Consequently, due to this temperature insensitivity, IDV can replicate efficiently at both 33°C and 37°C in various immortalized cell lines, including those derived from humans (1). Interestingly, like ICV, the HEF glycoprotein of IDV can bind to the luminal surface of epithelium from the human upper respiratory tract (15). However, this does not necessarily imply that cells within the human respiratory epithelium can be infected by IDV. Therefore, it remains unclear whether the ruminant-associated IDV can infect cells within the human respiratory epithelium, and thus whether it has a zoonotic potential.

The respiratory epithelium is the main entry port for respiratory pathogens and is therefore an important first barrier towards intruding viruses. During the past 15 years, the human airway epithelial cell (hAECs) culture model has been applied as an *in vitro* surrogate model of the *in vivo* respiratory epithelium to study a wide range of respiratory viruses (18–26). Including the assessment of the zoonotic potential of Middle East Respiratory Syndrome coronavirus (MERS-CoV) and a novel dromedary camel virus related to the common cold virus human coronavirus 229E (HCoV-229E) (27, 28). Although the novel dromedary camel virus replicates in an immortalized human cell line, it failed to efficiently replicate in the hAEC culture model (28). Indicating that immortalized cell lines do not always reflect the *in vivo* situation and therefore such results are to be interpreted carefully.

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As a first step towards identifying the zoonotic potential of IDV we determined the replication kinetics, cell tropism, and host response at the primary site of replication using the hAEC culture model. We first inoculated the prototypic D/Bovine/Oklahoma/660/2013 strain on hAEC cultures established from different donors to monitor virus replication kinetics. This revealed efficient replication of IDV in hAEC cultures at different body temperatures. To this end we compared the replication kinetics, cell tropism, and host response of ruminant-associated IDV with that of the human-associated ICV. Demonstrating that the replication characteristics of IDV has many similarities to the human-associated ICV, including the cell tropism. To address whether IDV progeny is replication competent, we performed sequential passaging of IDV upon hAEC cultures from different donors. These results emphasize, that there is no fundamental restriction of IDV replication within the human respiratory epithelium. However, our cross-sectional survey, with pooled serum of over a thousand healthy donors, revealed that there is no evidence that IDV is circulating among the general population.

Results

Efficient replication of Influenza D virus in hAEC cultures

There are several factors indicating that IDV has zoonotic potential. This includes the broad *in vivo* and *in vitro* host tropism, utilization of the same receptor determinant as human ICV, and binding of the HEF glycoprotein to the luminal surface of human respiratory epithelium (1, 15, 16). Although, this does not necessarily imply that cells within the human respiratory epithelium can be infected by IDV. Therefore, it remains unclear whether the ruminant-associated IDV indeed can infect cells within the human respiratory tract and thus whether it has a zoonotic potential. However, the detection of IDV specific antibodies in individuals whom have occupationally exposure to livestock suggest that there is spillover from livestock to humans.

Therefore we first addressed the question whether IDV can infect cells within the human respiratory tract. For this we inoculated hAEC cultures from three different donors with the prototypic D/Bovine/Oklahoma/660/2013 strain. Because the IDV HEF glycoprotein previously showed binding to the luminal surface of human respiratory epithelium we inoculated our hAEC cultures accordingly. We monitored viral progeny release for a duration of 72 hours by collecting washes with 24 hour intervals. This was done at both 33°C and 37°C, as IDV has shown to replicate at these ambient temperatures that correspond with those of the human upper and lower respiratory tract, respectively. This would reveal whether there is any temperature restriction for IDV in an *in vitro* surrogate model of the human

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respiratory epithelium. The release of viral progeny from the apical washes was analyzed by qRT-PCR for viral transcripts and virus titration for infectious virus. Here we observed that the first viral transcripts could be detected at 24 hours post-infection (hpi) among all donors, independently of the incubation temperature **(Figure 1A and B)**. However, some temperature dependent differences were observed when we analyzed the apical washes for infectious virus. For the hAEC cultures incubated at 33°C, viral titers were detected for every donor at 48 and 72 hpi, but only for one donor we could detect viral titers at 24 hpi **(Figure 1C)**. In contrast, for the IDV infection at 37°C we observed viral titers as early as 24 hpi for every donor that increased over time **(Figure 1D)**. These results indicate that IDV kinetics seems to be more efficient at ambient temperatures corresponding to the human lower respiratory tract. This, most likely, reflect the necessity for IDV to replicate at the body temperature of cattle, which is between 37 - 39°C.

After having demonstrated that IDV is able to replicate in hAEC cultures from different donors at both 33°C and 37°C, we wanted to confirm these results through visualization of IDV-infected cells via immunofluorescence analysis. However, because commercial antibodies against IDV are currently unavailable, we ordered a custom generated antibody directed against the nucleoprotein (NP) of the prototypic D/Bovine/Oklahoma/660/2013 strain. With this antibody, we performed microscopic analysis of IDV-infected hAEC cultures from three different donors 72 hpi. This revealed clusters of NP-positive cells in IDV-infected hAEC cultures incubated at both 33°C and 37°C, whereas no fluorescence signal could be observed in the respective control hAEC cultures **(Figure 1E-H)**. The majority of fluorescence signal from the NP-positive cells has a cytoplasmic distribution pattern, however some of those also appeared to have a nuclear staining pattern. Suggesting that, like other orthomyxoviruses, the NP of IDV is actively translocated to the nucleus during viral replication (29, 30).

These results combined demonstrate that IDV is able to efficiently replicate in hAEC cultures from different donors at temperatures corresponding to both the upper and lower respiratory tract of humans.

Comparison of Influenza C and Influenza D virus infection

Influenza C virus (ICV) is a well-known common cold virus that is able to cause mild upper respiratory tract infections in humans (31). Because of the structural similarity of the HEF of IDV and ICV, and the fact that we showed that IDV is able to replicate in hAECs, we wondered how this relates to replication efficiency in our hAEC cultures. To address this question, we inoculated hAECs with equal amounts of

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hemagglutination units for ICV (C/Johannesburg/1/66) and IDV and incubated the cultures at 33°C. This due to the previous reported temperature restriction of the ICV HEF glycoprotein fusion efficiency. The replication kinetics were monitored as before, by collecting apical washes every 24 hours for a duration of 72 hours. Here, we observed similar replication kinetics for both viruses, although the viral RNA yield for ICV was higher compared to IDV **(Figure 2A and B)**. However, the replication kinetics of the IDV-infected hAEC cultures were similar compared to the previous experiment at 33°C **(Figure 1A)**. Revealing that the replication kinetics for IDV in hAEC cultures is robust and independent from the donor that is used. More importantly, we show that the replication kinetics of IDV are almost identical to that of well-known common cold ICV.

In addition to the replication kinetics analysis for ICV and IDV in hAEC cultures, we wanted to determine their respective cell tropism as both viruses utilize the 9-*O*-acetyl-*N*-acetylneuraminic acids as receptor determinant. For this we formalin-fixed the previous infected hAEC cultures to analyze the cell tropism for both viruses via immunostaining. To discriminate between the ciliated and non-ciliated cell types we stained the cultures with well-defined antibodies to visualize the cilia (β-tubulin IV) and tight junction borders (ZO-1) at the apical surface between the different cells, while the nucleus was visualized using DAPI. For detection of IDV infected cells we used our previous generated NP-antibody, whereas for ICV there are unfortunately no commercial antibodies available. Therefore, we used intravenous immunoglobulins (IVIg) that contains polyclonal immunoglobulin G from over a thousand of healthy donors, as most people encounter one or multiple ICV infections during their life and generate antibodies directed against ICV (31, 32). By overlaying the different cellular marker stains with that of the virus antigen we observed that for both ICV and IDV the virus-positive signal overlaps with that of the ciliated cell marker **(Figure 2C and 2D)**.

To accurately define the cell tropism, we counted all cell types among ten random fields per donor, with the criteria of at least having one virus-positive cell. For the IDV-infected hAEC cultures we counted a total of 2273 cells of which 94 were NP-positive, while for ICV a total of 2526 cells were counted with a total of 84 ICV antigen-positive cells. The majority of antigen-positive cells for both IDV and ICV overlapped with the ciliated cell marker and corresponds with an overall percentage of 97.3 and 95.5, respectively **(Figure 2E)**. This is in line with our initial observation, and shows that IDV and ICV both have a predominant preference for ciliated cells. This is most likely due to the usage of the same 9-*O*-acetyl-*N*-acetylneuraminic acids as receptor determinant to enter the host cell. In addition to the cellular tropism, we also calculated the overall infection rate for IDV and ICV, which is 4.1 and 3.3

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percent, respectively **(Supplementary table 1)**. Showing that the overall infection rate of IDV and ICV are almost identical, which is in accordance with the previous observed replication kinetics.

These results establish that the ruminant-associated IDV has almost identical replication kinetics and overall infection rate characteristics in hAEC cultures to that of the human-associated ICV. Furthermore, both viruses show to exhibit a predominant preference for ciliated cells, which is most likely due to the usage of the same receptor determinant for cell entry.

Host innate immune response

The previous results revealed that the ruminant-associated IDV and the human-associated ICV exhibit similar infection characteristics in hAEC cultures. Though, whether they share similar characteristics in terms of host response is unclear. Within the respiratory epithelium, the host innate immune system plays a pivotal role in the disease outcome during viral infection. Upon the recognition of a viral pathogen, sensing Pattern Recognition Receptors (PRRs) will bind to their respective signalling proteins that stimulate the expression of pro-inflammatory cytokines as well as Type I and III interferons (IFNs), followed by induction of several hundred IFN-Stimulated Genes (ISGs) (33–36). These ISGs (i) protect the epithelial barrier by altering the intra- and extra-cellular environment, (ii) impair virus propagation, spread and transmission, and finally (iii) shape the host's adaptive immune response and thereby impact respiratory disease outcome (33–36).

To address whether IDV provokes a similar host response as ICV we inoculated hAEC cultures of different donors as described previous. However, for the analysis of the host response we lysed the hAEC cultures at 18, 36, 48 and 72 hpi for relative quantification of interferon (IFN)-β, IFN-λ1 and IFN-λ2/3 mRNA-transcripts. Interestingly, for both IDV and ICV there was no upregulation of IFN-β detected. This is in contrast to the IFN-λ1 and IFN-λ2/3 transcripts that all increase overtime **(Figure 3A and 3B)**. The observed increase was most pronounced for the IDV-infected hAEC cultures, as for ICV the amplitude in IFN-λ1 and IFN-λ2/3 transcripts increase was approximately 10-fold lower **(Figure 3A and 3B)**. This seemed to correlate with the difference in the amount of cellular associated viral transcripts for both viruses (data not shown). Because we observed an upregulation in the type III IFNs mRNA transcripts we wondered whether an induction of downstream ISGs transcripts could be detected. To this end we also monitored the expression levels for several well-known ISGs, namely MxA, 2`-5`OAS and IFIT1. Interestingly, for both viruses we observed only a minor upregulation of 2`-5`OAS and IFIT1 transcripts beyond 48 hpi, while for MxA the transcriptional levels remained constant

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(Figure 3C and 3D). The minor upregulation of 2`-5`OAS and IFIT1 at 48 hpi seems to correlate with the observed increase level of Type III IFN transcripts. However, in which extend this would impair virus propagation and spread remains to be elucidated.

Nonetheless, these results reveal that both the human-associated ICV and ruminant-associated IDV provoke a similar type of host response during infection, which is characterized by an induction of only type III IFNs.

Sequential propagation and seroprevalence of IDV

A requisite for the successfully establish of a zoonotic virus among the human population is the availability of a cellular entry receptor. Followed by, cell entry and efficient replication within the new host that usually coincides with efficient suppression of the host's innate immune response to prevent impairment of virus propagation, spread and subsequent transmission. We are aware that not all of these zoonotic prerequisites can be addressed with the hAEC cultures. Though, thus far, we demonstrated that IDV is able to replicate efficiently in an *in vitro* surrogate model of the human respiratory epithelium and that during this process an innate immune response is induced. Still, whether this host response would impair subsequent virus propagation and spread could potentially be addressed through sub-passaging IDV obtained from one donor onto hAEC cultures from different donors.

We therefore decided to use the individual apical washes from the three donors from our cell tropism experiment to inoculate hAEC cultures of a different donor (P2), of which the 48 hpi apical wash is diluted and used for further sub-passaging upon hAEC cultures of the same donor (P3; **Figure 4A)**. To investigate a role of temperature on subsequent virus propagation and spread we performed this experiment at both 33°C and 37°C. During which we monitored the release of viral progeny from the apical washes at 48 and 96 hpi for each sub-passaging experiment. In the first passaging experiment we observed a viral RNA yield at 48 hpi that increased with one order of magnitude at 96 hpi **(Figure 4B)**. However, we observed that the viral yield at 37°C was approximately one order lower in the first round compared to the viral yield at 33°C, however at 96 hpi this difference was slightly reduced. Interestingly, in the second passaging experiment we did not observe any difference between the different incubation temperatures **(Figure 4B)**. In addition, we observed no pronounced differences in the viral titers between the different temperatures or passage numbers at 96 hpi (**Figure 4C)**. However, at 48 hpi we could only detect infectious virus in the apical wash from the last

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passaging experiment that was performed at 37°C (P3; **Figure 4C**). These results show that the viral progeny from the initial experiments on hAEC cultures is replication competent and that IDV can be sub-passaged on hAEC cultures from different donors at both 33°C and 37°C.

This observation raises the question if IDV is potentially circulating among the general population. To address this question, we performed a cross-sectional serological survey using IVIg in a hemagglutination inhibition (HI) assay to determine whether IDV-directed antibodies can be detected among pooled immunoglobulin G from thousands of healthy donors. For the HI assay we used ICV as a positive control, as the majority of adults have antibodies towards this virus and we could previously detect ICV-positive cells by immunofluorescence. Here we could readily observe that IVIg inhibited red blood cell hemagglutination by ICV, however for IDV no inhibition was observed (**Figure 4D and 4E)**. Indicating that, unlike ICV, no IDV-directed antibodies can be detected among the general population and that pre-existing antibodies against ICV are not cross-reactive against IDV.

Combined these results highlight that there is no intrinsic barrier for IDV to replicated within the human respiratory epithelium. However, thus far, there is no evidence that IDV is circulating among the general population.

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Discussion

In this study, we demonstrate that IDV replicates efficiently in an *in vitro* surrogate model of the *in vivo* respiratory epithelium at different ambient temperatures corresponding to the human upper and lower respiratory tract. Intriguingly, the replication characteristics of the ruminant-associated IDV revealed many similarities to the human-associated ICV, including the tropism for ciliated cells. Moreover, analysis of the host response during IDV infection revealed only a pronounced upregulation of Type III IFN transcripts. Nonetheless, viral progeny virus showed to be replication competent as it could be efficiently propagated onto hAEC cultures from different donors at both 33°C and 37°C. Emphasizing that there is no intrinsic impairment of IDV propagation within the human respiratory epithelium.

For inter-species transmission a virus needs to overcome several barriers before it can efficiently replicate in the new host species (37). These barriers can be classified into three major groups; (i) viral entry through availability of the cellular receptor and proteases, (ii) viral replication and subversion of the host innate immune system followed by (iii) viral egress and release of infectious progeny virus. Our results clearly demonstrates that IDV fulfils most of these criteria, as there is no fundamental restriction for viral replication and sequential propagation of IDV within hAEC cultures from different donors. Altogether, this might explain why IDV-directed antibodies are detected among individuals whom have occupationally exposure to livestock (14). However, we cannot assess whether IDV can be transmitted among humans with our model. Nonetheless, it is interesting to note that, IDV can be transmitted among infected and naïve ferrets, an animal model often used as surrogate model to assess transmission potential of influenza A viruses among humans (1, 38, 39). However, besides individuals whom have occupationally exposure to livestock there is currently no epidemiological evidence that IDV is circulating among the general population. Suggesting that beyond the spillover from livestock to humans virus transmission might be restricted due to unknown host factors.

Within the respiratory epithelium, the host innate immune system plays a pivotal role in the disease outcome during viral infection. Interestingly, in our study we observed a pronounced upregulation for Type III IFNs, but not for Type I IFN. This data suggest that Type III IFNs play a more pronounced role in comparison to Type I IFNs in the context of IDV infection in the human respiratory epithelium. In contrast to the IFN expression, we only detected a mild upregulation for some ISGs transcripts during IDV infection. This suggests that certain proteins of IDV, such as the non-structural protein 1 (NS1), might efficiently antagonize the induction of ISGs in the human airway epithelium.

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Something we have observed previously for another respiratory virus with zoonotic potential (27). Conversely, thus far, we only determined the host response up to 72 hpi and therefore it might be that a more pronounced ISGs induction can be observed during later time points. Therefore expanding our knowledge on the dynamics of the innate immune response at multiple stages during IDV infection and the role of host and viral proteins herein remains warranted.

During our study we used the prototypic D/Bovine/Oklahoma/660/2013 strain representing one of the two distinct cocirculating lineages of IDV (10). The prototypic D/Swine/Oklahoma/1334/2011 is the representative strain of the other lineage (10). Both lineages have greater than 96% identity from which the HEF glycoprotein (96.7 to 99.0% identity) is the most divergent of all 7 segments (2). However, due to the high similarity it is likely that the replication characteristics observations from our experiments are similar between both lineages. Especially, as the previous described IDV replication in human cell lines and the binding of the HEF glycoprotein to the human respiratory epithelium have all been performed in the context of the prototypic D/Swine/Oklahoma/1334/2011 strain (1, 15). Nonetheless, whether both cocirculating lineages of IDV indeed exhibit similar characteristics in human respiratory epithelium remains formally to be elucidated.

Both IDV and ICV utilize the 9-*O*-acetyl-*N*-acetylneuraminic acid as their receptor determinant for host cell entry (15, 16). We have shown that both viruses have a predominant affinity towards ciliated cells, suggesting that the distribution of this type of sialic acid is limited to ciliated cells within our *in vitro* model of the human airway epithelium. This tropism is similar to what we previously observed for the human coronavirus OC43, from which it has been reported to also utilize the 9-*O*-acetyl-*N*-acetylneuraminic acid as receptor determinant (22, 40). Nonetheless, whether this cell tropism for both IDV and ICV corresponds to that of *in vivo* airway epithelium remains to be determined. Although, previous studies have shown that the hAEC cultures recapitulates many characteristics of the *in vivo* airway epithelium, including receptor distribution (18, 22).

In summary, we demonstrate that IDV replicates efficiently in an *in vitro* surrogate model of the *in vivo* respiratory epithelium. Highlighting, that there is no intrinsic impairment of IDV propagation within the human respiratory epithelium. These results might explain why IDV-directed antibodies are detected among individuals whom have occupationally exposure to livestock.

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Material and methods

Cell culture

The Madin-Darby Bovine Kidney (MDBK) cells were maintained in Eagle`s Minimum Essential Medium (EMEM; (Seroglob) supplemented with 7% heat-inactivated fetal bovine serum (FBS, Seraglob), 2mM Glutamax (Gibco), 100 µg/ml Streptomycin and 100 IU/ml Penicillin (Gibco). Whereas the Madin-Darby Canine Kidney (MDCK) cells were maintained in EMEM, supplemented with 5% heat-inactivated FBS, 100 µg/ml Streptomycin and 100 IU/ml Penicillin (Gibco). Both cell lines were propagated at 37°C in a humidified incubator with 5% CO_2.

Viruses

The Influenza D virus (D/Bovine/Oklahoma/660/2013) was kindly provided by Dr. Feng Li, University of South Dakota, USA, and was inoculated on MDBK cells and propagated in infection medium (EMEM, supplemented with 0.5% Bovine Serum Albumin (Sigma-Aldrich), 15 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco), 100 µg/ml Streptomycin and 100 IU/ml Penicillin (Gibco), and 1 µg/mL Bovine pancreas-isolated acetylated trypsin (Sigma-Aldrich)). Infected MDBK cultures were maintained for 96 hours at 37°C. The influenza C virus strain C/Johannesburg/1/66 was inoculated on MDCK cells and propagated in infection medium and maintained for 96 hours at 33°C. Virus containing supernatant was cleared from cell debris through centrifugation for 5 minutes at 500x *rcf* before aliquoting and storage at -80°C.

Human airway epithelial cell culture

Primary human bronchial cells were isolated from patients (>18 years old) undergoing bronchoscopy or pulmonary resection at the Cantonal Hospital in St. Gallen, Switzerland, in accordance with our ethical approval (EKSG 11/044, EKSG 11/103 and KEK-BE 302/2015). Isolation and culturing of primary human bronchial epithelial cells was performed as previously described (41), with the minor modification of supplementing the BEGM with 10µM Rho associated protein kinase inhibitor (Y-27632, Abcam).

Viral replication in hAEC cultures

The hAEC cultures were inoculated with 10000 $TCID_{50}$, or 32 hemagglutination units, of either Influena D virus or Influenza C virus and incubated for 1.5 hours at temperatures indicated elsewhere in a

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humidified incubator with 5% CO_2. Afterwards inoculum was removed and the apical surface was washed three times with HBSS, after which the cells were incubated at indicated temperatures in a humidified incubator with 5% CO_2. The infection was monitored as indicated, during which progeny virus was collected by incubating the apical surface with 100µL of HBSS for 10 minutes prior to the time point. Collected apical washes were stored 1:1 in virus transport medium (VTM) for later quantification (41).

Virus titration

MDBK cells were seeded at a concentration of 40.000 cells per well in a 96-cluster well plates. The following day medium was removed and cells were washed once with PBS and replaced with 50 µl of infection medium. Virus containing samples were ten-fold serial diluted in infection medium, from which 50 µl was added to the MDBK cells in six technical replicates per sample. The inoculated cells were incubated for 72 hours at 37°C in a humidified incubator with 5% CO_2, after they were fixed by crystal violet to determine the titer according to the protocol of Spearman-Kärber (42).

Hemagglutination assay

Chicken blood for the hemagglutination agglutination (HA) and hemagglutination inhibition (HI) assays was obtained from SPF-bred white Leghorn chickens in compliance with the Animal Welfare Act (TSchG SR 455), the Animal Welfare Ordinance (TSchV SR 455.1), and the Animal Experimentation Ordinance (TVV SR 455.163) of Switzerland. That was reviewed by the ethical committee for animal experiments of the canton of Bern and approved by the cantonal veterinary authorities (Amt für Landwirtschaft und Natur LANAT, Veterinärdienst VeD, Bern, Switzerland) with the agreement BE78/17. The hemagglutination agglutination and hemagglutination inhibition assays were performed using 1% chicken red blood cells diluted in ice-cold PBS as described previous (43). For the HI assay Intravenous Immunoglobulins (IVIg; Sanquin, the Netherlands) was pretreated with receptor-destroying enzyme (Denka Seiken) for 18 hours at 37°C, followed by an inactivation for 30 minutes at 56 °C. The HA- or HI-titer was determined after 30 minutes incubation at room temperature by recording the highest serial dilution that still displayed tear-formation after the plate was tilted 45° for 30 seconds.

Quantitative Real-time PCR (qRT-PCR)

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Total cellular RNA from infected hAECs was extracted using the NucleoMag RNA (Macherey-Nagel) according to manufacturer guidelines on a Kingfisher Flex Purification system (Thermofisher). Reverse transcription was performed with GoScript™ reverse transcriptase mix random hexamers according to the manufacturer's protocol (A2800; Promega) using 200 ng of total RNA. Two microliters of tenfold diluted cDNA was amplified using Fast SYBR™ Green Master Mix (Thermofisher) according to the manufacturer's protocol using primers targeting 18S and MxA, 2'-5'-OAS, IFIT1 as described previously (27). Measurements and analysis were performed using an ABI7500 instrument and software package (ABI). Relative gene expression was calculated using the 2-ΔΔCt method and is shown as fold induction of compared to that of non-infected controls (44).

For quantification of the viral kinetics of IDV and ICV, a total of 50 µL of apical wash was used to extract viral RNA using the NucleoMag VET (Macherey-Nagel) according to manufacturer guidelines on a Kingfisher Flex Purification system (Thermofisher). Two microliters of extracted RNA was amplified using TaqMan™ Fast Virus 1-Step Master Mix (Thermofisher) according to the manufacturer's protocol using the forward primer 5`-AACCTGCTTCTGCTTGCAATCT-3`, reverse 5`-AACAATGAACAGTTACCGCATCA-3` and probe 5`-FAM-AGACCTGTCTAAAACTATTT-BHQ1-3` targeting the P42/M-segment of ICV (AM410042.1). Whereas for the P42/M-segment of IDV (KF425664.1) the forward 5`-ATGCTGAAACTGTGGAAGAATTTTG-3`, reverse 5`-GGTCTTCCATTTATGATTGTCAACAA-3` and probe 5`-FAM-AAGGTTTATGTCCATTGTTTCA-BHQ1-3` were used. A standard curve of the P42/M-segment of Influenza C or D virus, cloned in pHW2000 plasmid, was included to interpolate the amount of genomic equivalents (45). Measurements and analysis were performed using an ABI7500 instrument and software package (ABI).

Immunofluorescence of hAEC cultures

The hAEC cultures were formalin-fixed and stained for immunofluorescence as previously described (41). For the detection of IDV-positive cells, hAEC cultures were stained with a custom generated rabbit polyclonal antibody directed against the nucleoprotein (NP) of the prototypic D/Bovine/Oklahoma/660/2013 strain (Genscript). Alexa Fluor® 647-labeled donkey anti-Rabbit IgG (H+L) (Jackson Immunoresearch) was applied as secondary antibody. For the characterization and quantification of IDV cell tropism hAEC cultures were stained with the previous custom generated polyclonal rabbit anti-NP (Genscript), mouse Anti-β-tubulin IV (AB11315, Abcam), goat anti-ZO1

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(AB99642, Abcam). Alexa Fluor® 488-labeled donkey anti-mouse IgG (H+L), Cy3-labeled donkey anti-goat IgG (H+L) and Alexa Fluor® 647-labeled donkey anti-Rabbit IgG (H+L) (Jackson Immunoresearch). In the case of ICV, hAEC cultures were stained with human IVIg (Sanquin, the Netherlands), mouse Anti-β-tubulin IV (AB11315, Abcam), rabbit anti-ZO1 (617300, Thermofisher). Using Alexa Fluor® 488-labeled donkey anti-mouse IgG (H+L), Alexa Fluor® 594-labeled donkey anti-human IgG (H+L) and Alexa Fluor® 647-labeled donkey anti-Rabbit IgG (H+L) (Jackson Immunoresearch) as secondary antibodies. All samples were were counterstained using 4',6-diamidino-2-phenylindole (DAPI, Thermofischer) to visualize the nuclei. The immunostained inserts were mounted on Colorforst Plus microscopy slides (Thermofischer) in Prolong diamond antifade mountant (Thermo Fischer) and overlaid with 0.17 mm high precision coverslips (Marienfeld). The Z-stack images were acquired on a DeltaVision Elite High-Resolution imaging system (GE Healthcare Life Sciences) using a step size of 0.2 µm with a 60x/1.42 oil objective. Images were deconvolved and cropped using the integrated softWoRx software package and processed using Fiji (ImageJ) and Imaris version 9.1.3 (Bitplane AG, Zurich, Switzerland) software packages.

Data presentation

Data was plotted using GraphPad Prism 7 and figures were assembled in Adobe InDesign CS6.

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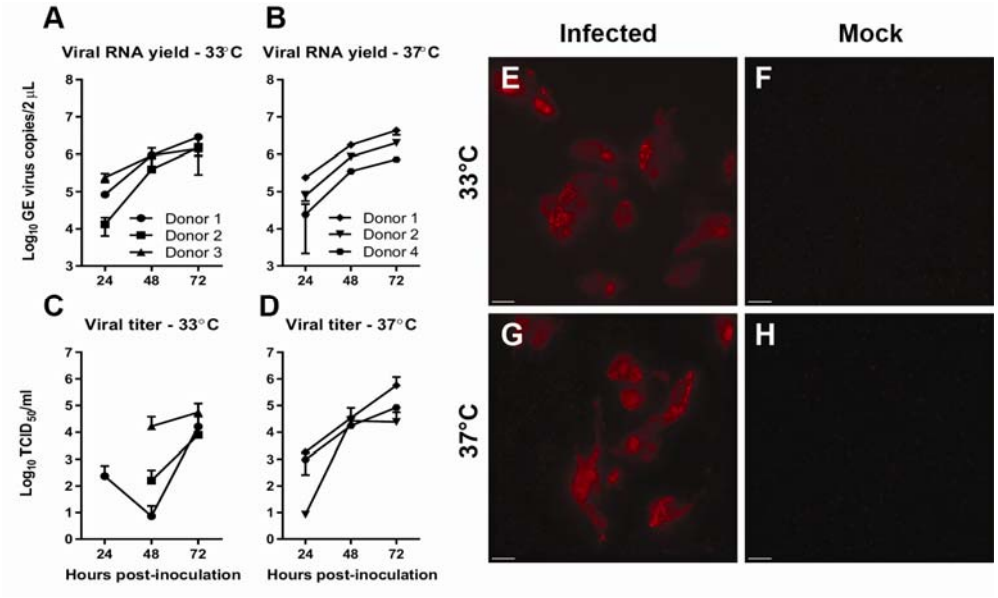

Figure 1. Efficient replication of Influenza D virus in hAEC cultures.

Human airway epithelial cell cultures were inoculated with 10000 $TCID_{50}$ of IDV and incubated at either 33°C or 37°C. The monitored viral RNA yield is given as genomic equivalents (GE) per 2 µL of isolated RNA (y-axis) at indicated hours post-inoculation (x-axis) for 33°C **(A)** and 37°C **(B)**. Whereas the viral titer is given as $TCID_{50}$/mL (y-axis) for 33°C **(C)** and 37°C **(D)** at indicated hours post-inoculation (x-axis). These results are displayed as means and SD from duplicates from three independent donors. Human airway epithelial cell cultures were formalin-fixed and immunostained with a custom generated antibody against the Nucleoprotein of the Influenza D virus to detect virus infected cells. A representative image from one of the three independent donors is shown for Influenza D virus infection at 33°C and 37°C **(E&G)** as well as their respective controls **(F&H)**. Magnification 60x, the scale bar represent 10 µM.

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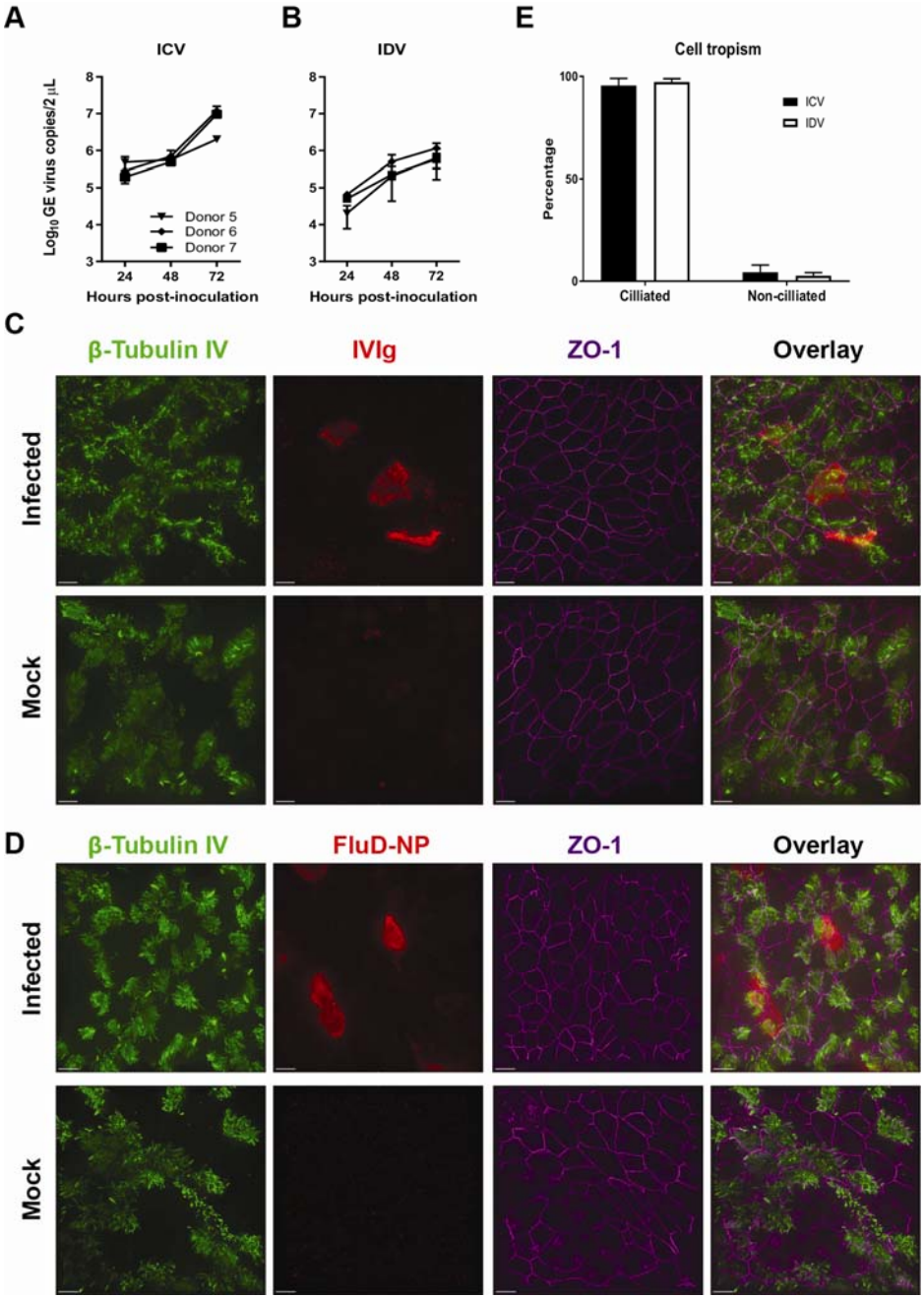

Figure 2. **Comparison of Influenza C and Influenza D virus infection.**

Human airway epithelial cell cultures were inoculated with 32 Hemagglutination assay units of ICV or IDV and incubated at 33°C. The monitored viral RNA yield is given as genomic equivalents (GE) per 2 µL of isolated RNA (y-axis) at indicated hours post-inoculation (x-axis) for Influenza C virus **(A)** and Influenza D virus **(B)**. The results are displayed as means and SD from duplicates from three independent donors.

Formalin-fixed ICV and IDV infected hAEC cultures and their respective controls were immunostained with antibodies to visualize the cilia (β-tubulin IV, green), tight junction borders (ZO-1, purple).

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Whereas virus-infected cells (red) were visualized with either a custom generated IDV NP-antibody or intravenous immunoglobulins (IVIg) for ICV **(C&D)**. Magnification 60x, the scale bar represent 10 µM. The cell tropism of ICV (Black bars) and IDV (white bars) was quantified by calculating the percentage of viral antigen-positive signal co-localization with either ciliated or non-ciliated cells **(E)**. The mean percentage and SEM from ten random fields from three independent donors are displayed.

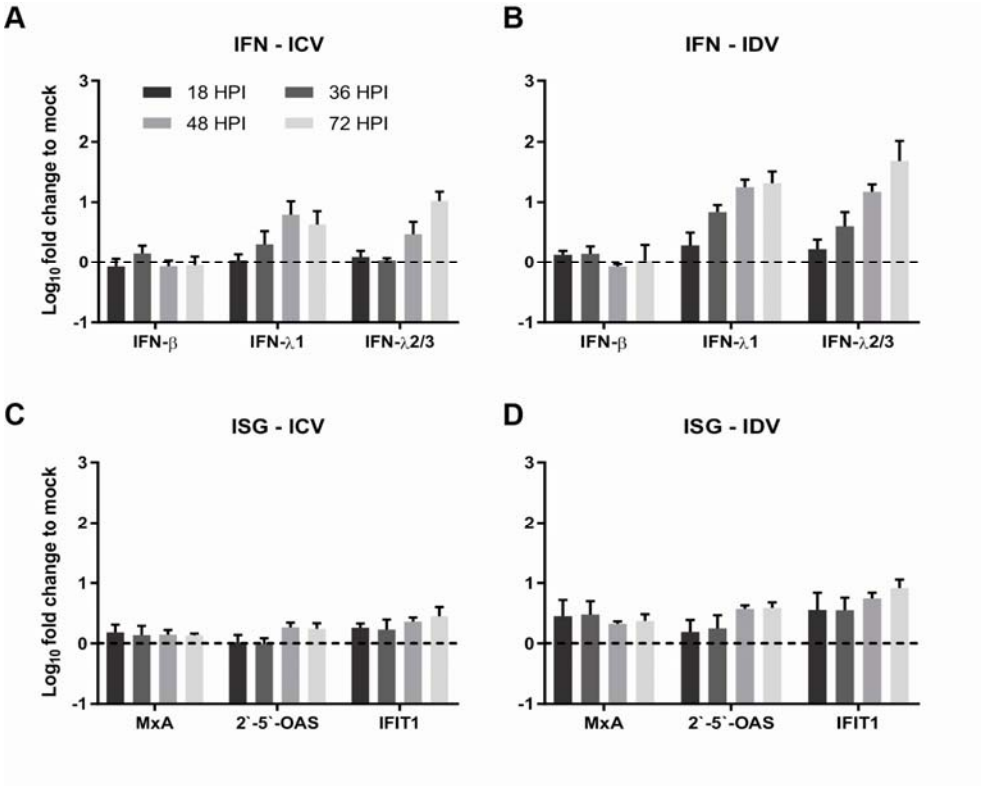

Figure 3. Transcriptional host response during ICV and IDV infection in hAECs.

Human airway epithelial cell cultures were inoculated with 32 HAU of ICV and IDV after which the transcriptional host response was quantified for Type I and III interferon (IFN) **(A,B)** and Interferon Stimulated Genes (ISG) mRNA-transcripts **(C, D)** at 18, 36, 48 and 72 hours post-inoculation using the ΔΔCt-method (44). The results are displayed as means and SD from three technical replicates from three independent donors.

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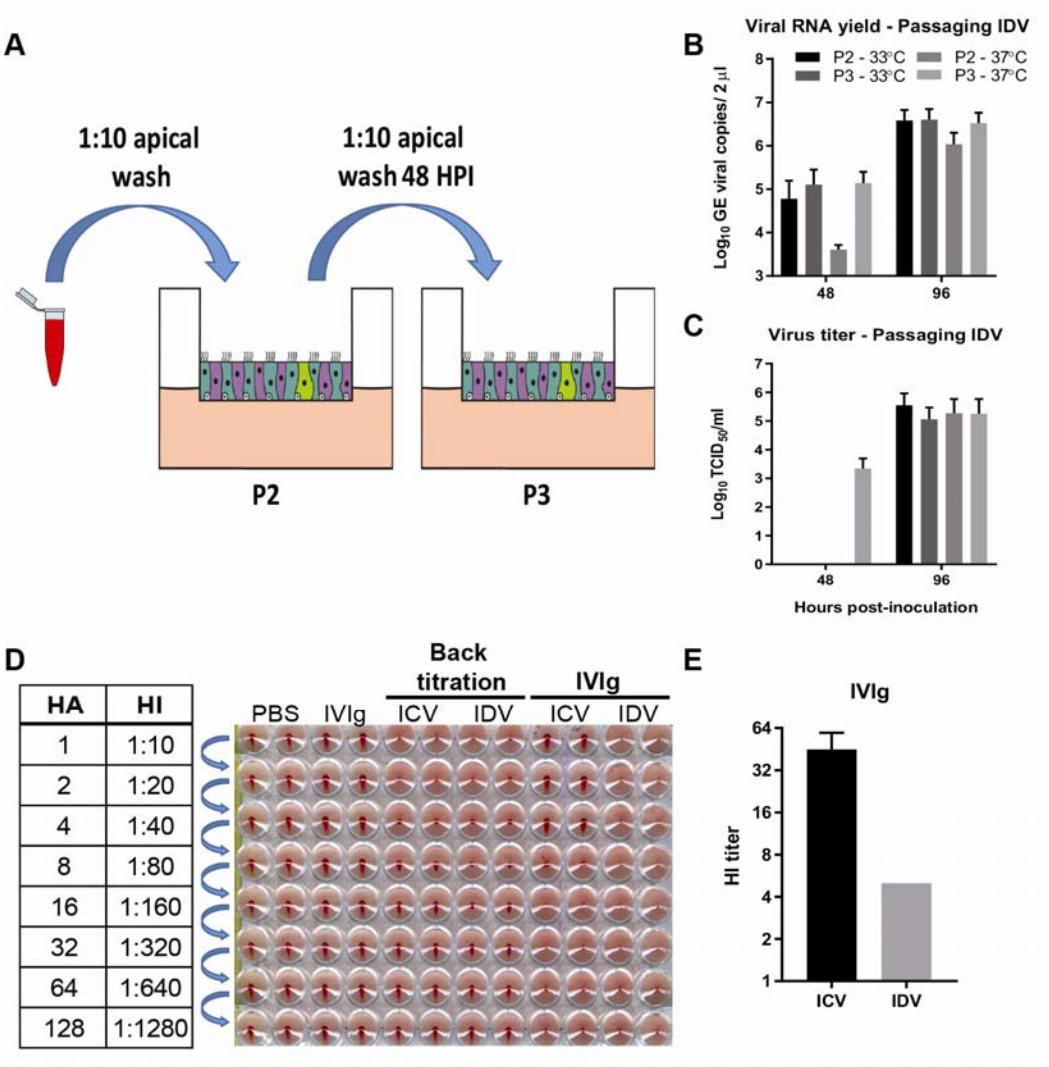

Figure 4. Sequential propagation and seroprevalence of IDV.

Human airway epithelial cell cultures were inoculated with tenfold-diluted apical wash and sequentially propagated upon new hAEC cultures to assess whether IDV viral progeny is replication competent and can be sub-passaged **(A)**. The monitored viral RNA yield is given as genomic equivalents (GE) per 2 µL of isolated RNA (y-axis) at indicated hours post-inoculation (x-axis) for each of the conditions **(B)**. Whereas the viral titer is given as $TCID_{50}$/mL (y-axis) for each condition, at indicated hours post-inoculation (x-axis) **(C)**. The results are displayed as means and SD from duplicates from three independent donors. A cross-sectional survey was performed using 8 hemagglutination units of virus antigen in combination with intravenous immunoglobulins (IVIg) in a hemagglutination inhibition (HI) assay to detect ICV- and IDV-directed antibodies among the general population **(D)**. The HI-titer was calculated and displayed as mean HI-titer **(E)**. Results are displayed as means and SD from duplicates from four independent experiments.