Variability of interferon-λ induction and antiviral activity in Nipah virus infected differentiated human bronchial epithelial cells of two human donors

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Abstract
Highly pathogenic Nipah virus (NiV) generally causes severe encephalitis in humans. Respiratory symptoms are infrequently observed, likely reflecting variations in infection kinetics in human airways. Supporting this idea, we recently identified individual differences in NiV replication kinetics in cultured airway epithelia from different human donors. As type III interferons (IFN-λ) represent major players in the defence mechanism against viral infection of the respiratory mucosa, we studied IFN-λ induction and antiviral activity in NiV-infected primary differentiated human bronchial epithelial cells (HBEpCs) cultured under air–liquid interface conditions. Our studies revealed that IFN-λ was upregulated in airway epithelia upon NiV infection. We also show that IFN-λ pretreatment efficiently inhibited NiV replication. Interestingly, the antiviral activity of IFN-λ varied in HBEpCs from two different donors. Increased sensitivity to IFN-λ was associated with higher expression levels of IFN-λ receptors, enhanced phosphorylation of STAT1, as well as enhanced induction of interferon-stimulated gene expression. These findings suggest that individual variations in IFN-λ receptor expression affecting IFN responsiveness can play a functional role for NiV replication kinetics in human respiratory epithelial cells of different donors.

INTRODUCTION
Nipah virus (NiV) is a biosafety level (BSL) 4 classified zoonotic member of the Paramyxoviridae family that originates from fruit bats. NiV can cause symptomatic infections in livestock and humans, and was identified in 1998 during an outbreak of severe encephalitis among pig farmers in Malaysia [1]. In addition to the encephalitic disease that is mainly caused by an inflammatory infection of microvascular brain endothelial cells, 14–27% of NiV-infected patients develop respiratory symptoms [2]. This suggests that NiV productively replicates in human airways and induces inflammation that in some individuals causes clinical respiratory disease. Studies in cultured primary human airway epithelial cells supported the idea that the respiratory mucosa is highly susceptible to NiV infection and secretes pro-inflammatory cytokines in response to infection. Microarray analysis had furthermore revealed an efficient induction of interferon-stimulated genes (ISGs) in NiV-infected airway epithelia. While some transcriptional activation of interferon (IFN) β was observed in non-differentiated respiratory epithelial cells, type I IFN production was not detected in differentiated airway cultures [3, 4]. The role of type III IFNs (IFN-λ) is still completely unclear. In response to viral infections, almost any cell type is able to express mRNA for the three closely related major IFN-λ proteins: IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) (for a review, see [5]). While IFN-λ binds to distinct receptors, IL-28R1 and IL-10R2, it activates signal transduction pathways via STAT phosphorylation similar to the activation cascade induced by type I IFNs[6, 7]. IFN-λ thus possesses similar biological activities, including the intrinsic ability to induce the expression of antiviral ISGs. Expression of the IFN-λ receptor subunit IL-28R1 is restricted to epithelial cells in the lung, intestine and liver tissues. Thus, its antiviral activity is most evident against pathogens targeting mucosal barriers and the liver [8, 9]. As IFN-λ is known to be primarily responsible for the defence against influenza and other respiratory viruses [10–13], the aim of this study was to characterize its role during the infection of...
differentiated human airway epithelial cells with highly pathogenic NiV.

**RESULTS AND DISCUSSION**

We first wanted to study type I and type III IFN induction in differentiated respiratory epithelial cells in response to NiV infection. We therefore cultured primary bronchial epithelial cells (HBEpCs) from a human donor (donor 73), as described in the Methods. After differentiation under air–liquid interface (ALI) conditions for 30 days, the cell cultures were infected at a multiplicity of infection of 2 with a recombinant wild-type NiV [14]. In contrast to NiV infection of non-differentiated bronchial epithelial cell cultures, in which pronounced syncytium formation and virus production was already observed at 24 h post infection (p.i.) [3, 15], virus replication in differentiated airway cultures progressed at a slower rate. Cytopathic effects were minimal, and substantial infection rates were only observed at 5–8 days after infection [4]. We therefore isolated the total RNA from cell lysates 6 days after infection to analyse type I and type III IFN induction by quantitative real-time PCR (qPCR). Consistent with a previous report [4], NiV infection poorly induced IFN-β. We however observed a clear upregulation of IFN-λ1 mRNA transcripts (Fig. 1a). To monitor IFN-λ induction, upregulation of ISGs and virus spread at early (day 1), middle (day 4) and at late (day 7) time points, we infected differentiated HBEpCs with a recombinant NiV encoding an eGFP gene [14]. This allowed us to visualize viral focus formation in live cells. Fig. 1(b) shows a representative
area in the differentiated cell layer illustrating that a single infected cell detected at day 1 (Fig. 1b, arrow) had developed within 3 days to an enlarged focus with numerous virus-infected cells. At 7 days p.i., extended virus-positive foci were formed, indicating a further spread of infection (Fig. 1b, day 7). At all three time points p.i., cells were harvested to isolate total RNA for qPCR analyses. Reflecting the increasing number of virus-infected cells and ongoing viral replication, the amount of cell-associated viral mRNA increased over time (Fig. 1c). IFN-α and ISG mRNA transcripts (represented by the immediate early response genes IP10 and ISG56) were not significantly induced at day 1 p.i. This was most likely due to the minimal amounts of viral RNA present at this time point. Increased upregulation of these genes was, however, observed at days 4 and 7 p.i. (Fig. 1d), indicating that the extent of IFN and ISG induction correlated with the number of NiV-infected cells in differentiated primary airway cultures.

The infrequent respiratory symptoms observed in NiV-infected humans during the first NiV outbreak in Malaysia [2] likely reflect individual variations in the in vivo lung infections. In agreement with this concept, we recently identified donor-dependent differences in NiV replication kinetics in human airway epithelial cultures [15]. The differences correlated with variations in ephrin-B2, known to be the main NiV entry receptor [16, 17]. Although ephrin-B2 expression levels can affect initial virus entry rates and early replication kinetics, there likely exist additional host factors that vary individually and can influence the final outcome of a NiV infection. To address this question, we searched for HBEpCs that did not differ in their endogenous NiV receptor levels compared with HBEpCs from donor 73. We were able to identify another human donor (designated donor 100). The flow cytometric analysis shown in Fig. 2(a) demonstrates that the surface expression of ephrin-B2 in differentiated cultures from donor 100 and donor 73 were comparable. Strengthening our idea of further individually varying host factors influencing the final outcome of an NiV infection, we found an enhanced NiV replication kinetic in HBEpC cultures from donor 100 compared with that in cell cultures from donor 73. As shown in Fig. 2(b), NiV infection of HBEpCs from donor 100 also started with single infected cells (day 1) but then spread more efficiently, forming larger foci at days 4 and 7 after infection compared with those formed in cell cultures from donor 73 (Fig. 1b). The higher total number of infected HBEpCs from donor 100 was reflected by a fourfold higher viral titre in the cell supernatants at 7 days p.i. ($8 \times 10^5$ 50% tissue culture infective dose [TCID50] ml$^{-1}$ versus $2 \times 10^5$ TCID50 ml$^{-1}$ for donor 73), as well as the increased amount of viral RNA in the cell lysates at days 4 and 7 p.i. (Fig. 2c). Consistent with the idea that IFN/ISG induction correlates with the number of infected cells, induction of IFN-α, IFN-β, ISG56, OAS, PKR and MxA expression at 7 days p.i. was also increased (Fig. 2d). In contrast to IFN-α, upregulation of IFN-β was minimal and did not significantly differ between HBEpCs from both donors (Fig. 2d). This finding suggests that type 1 IFNs do not play a major role in controlling NiV replication in HBEpCs.

As there is evidence for other primary cell types that responsiveness to IFNs can vary donor-dependently [18–20], we wanted to determine if the differences in NiV propagation in HBEpC from donors 73 and 100 correlated with differences in the antiviral activity of IFN-α against NiV. To compare the effect of IFN-α on HBEpC infection, differentiated cultures from the two donors were pretreated with 10 ng recombinant IFN-α2 (IL-28A). After 16 h, cells were infected with NiV, and viral RNA in the cell lysates was quantified at 48 h p.i. by qPCR. As shown in the left panel of Fig. 2(e), IFN-α2 inhibited virus replication in the primary cultures from donor 73 more than 1000-fold, while NiV infection of differentiated HBEpCs from donor 100 was only reduced about 100-fold. Such a difference in blocking virus replication was not observed in cells that were pre-treated with 10 ng ml$^{-1}$ IFN-β (Fig. 2e, right panel). IFN-β activates the same antiviral signalling pathways but uses a distinct receptor [6, 7]. This finding therefore indicates that HBEpCs from the two donors do not differ in their principle intrinsic ability to block NiV infection but rather specifically differ in the extent of antiviral activity induced by IFN-α.

To test whether the observed differences in antiviral activities were due to differential responsiveness of the two donor cell cultures to IFN-α, we compared the phosphorylation of STAT1 and ISG expression upon IFN-α treatment. Flow cytometric analysis of pSTAT1 after treatment with 10 ng ml$^{-1}$ IFN-α2 for 30 min and 60 min revealed clear differences (Fig. 3a). In agreement with the delayed STAT1 phosphorylation in cells from donor 100, ISG expression, exemplified by the analysis of IP10, and the antiviral ISGs PKR and MxA, was significantly lower in these cultures (Fig. 3b). Thus, the more limited effect of IFN-α to block NiV replication in cells from donor 100 (Fig. 2e) clearly correlated with a decreased STAT1 phosphorylation and ISG expression in response to IFN-α. Our finding that HBEpCs from different individuals vary in their sensitivity to IFN-α are in good agreement with the donor-dependent variability in the responsiveness to IFNs observed in primary hepatocytes, blood mononuclear cells, T cells and dendritic cells [18–20].

We finally analysed the IFN-α receptor (IL-28R1) expression by flow cytometry. As shown in Fig. 3(c), we observed clear differences in the surface expression of IL-28R1. The number of IFN-α receptors in airway epithelial cultures from donor 100 was significantly lower, suggesting that the reduced responsiveness to IFN-α correlating with a delayed antiviral activity was the result of decreased IFN-α receptor cell surface expression in primary HBEpCs from donor 100. These results are in agreement with the findings that endogenous expression of the IFN-α receptors varied in primary human hepatocytes from different hepatitis C patients, and that the strength of IFN-α induced activation of signalling pathways and ISG expression in the liver is regulated to a large degree by the expression of the IFN-α receptor [18].

To our knowledge this is the first study showing (i) that NiV infection induces IFN-α in differentiated human...
airway epithelial cells, and (ii) that NiV replication can be blocked in these cells by IFN-α-mediated antiviral activity.

In addition, we provide the first evidence that IFN-α receptor expression and IFN-α sensitivity can vary in bronchial epithelial cells from different human donors. This finding might serve as a starting point for future in vivo studies evaluating individual differences in the response to IFN-α in human airways, which may influence the outcome of an infection with NiV.

METHODS

ALI cultures

For NiV infection in a well-differentiated mucociliary epithelium, ALI cultures were grown in line with methods previously described by Gray et al. [21]. HBEpCs (Provitro, Berlin, Germany) were cultured in T75 flasks (Corning, Costar Co.) and were passaged once. At 70% cell confluency, cells were detached with 0.05% trypsin-EDTA (Gibco)
and suspended in airway epithelial cell growth medium (AEGM; PromoCell, Heidelberg, Germany) supplemented with bovine pituitary extract (0.004 mg ml\(^{-1}\)), epidermal growth factor (10 ng ml\(^{-1}\)), insulin (5 µg ml\(^{-1}\)), hydrocortisone (0.5 µg ml\(^{-1}\)), epinephrine (0.5 µg ml\(^{-1}\)), triiodothyronine (6.7 ng ml\(^{-1}\)), transferrin (10 µg ml\(^{-1}\)) and retinoic acid (0.1 ng ml\(^{-1}\)). A total of 75 000 cells in 0.5 ml supplemented AEGM were seeded on 12 mm Transwell culture inserts (0.4 µm pore size; Costar Co., Cambridge, MA, USA). HBEpCs were cultured for 2 days until they had reached complete confluency. Then the apical medium was removed and the basal medium was replaced by a 1 : 1 mixture of supplemented AEGM and DMEM (Gibco) containing 50 ng ml\(^{-1}\) retinoic acid (Sigma-Aldrich), penicillin (50 U ml\(^{-1}\), Gibco) and streptomycin (50 µg ml\(^{-1}\), Gibco). The medium in the basal filter chamber was changed three times a week. Cultures were maintained under ALI conditions for 4–5 weeks to form well-differentiated, polarized cultures. Mucociliary differentiation was assessed by the presence of beating cilia and mucus production observed as a visible mucus layer on the apical surface of the cultures. To monitor cell polarization and barrier function, the transepithelial electrical resistance was regularly measured using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments). Development of a pseudostratified cell layer was furthermore controlled by histology, and the differentiation into different cell types (ciliated, basal, goblet and club cells) was monitored by immunostaining.

**NiV infections**

All experiments with live NiV were performed under BSL-4 conditions at the Institute of Virology, Philipps University Marburg. For infection studies, HBEpCs cultured under ALI conditions for 30 days were extensively washed to remove the mucus. Cells were then infected at an m.o.i of 2 from both the apical and the basal side with a GFP-encoding recombinant wild-type NiV based on the Malaysian NiV strain [14]. After virus adsorption at 37 °C for 60 min, cells were washed three times for 10 min with PBS, and then further cultured under ALI conditions. The complete cell culture was monitored microscopically for infected cells/foci on a daily basis. The total numbers and average sizes of the foci were determined in the living culture. For illustration, one representative field (growing foci) for each donor

![Fig. 3. Donor-dependent variations in IFN-α responsiveness and IL-28R1 expression.](image-url)
culture is shown. Virus titers in the apical supernatant were determined by the 50% tissue culture infective dose (TCID50) method on Vero76 cells (n=3).

At different time points after infection (1–7 days p.i.), the cells were harvested and lysed using RLT buffer (RNeasy Kit; Qiagen) containing 1% β-mercaptoethanol (ME) before the samples were brought out of the BSL-4 containment for RNA isolation and subsequent qPCR analysis.

To determine the antiviral activity of IFNs, differentiated HBEpCs maintained under ALI conditions for 34 days were stimulated from the basal side for 16 h with either 10 ng ml⁻¹ recombinant human IFN-α-2 (IL-28A; Peprotech) or IFN-β (ProSpec). Preincubated cells were washed and then infected with NiV as described above. At 48 h p.i., the cells were harvested for RNA isolation.

RNA extraction and qPCR
Total RNA was isolated from differentiated HBEpCs using the Qiagen RNeasy Kit according to the manufacturer’s protocol. A volume of 50 ng of the extracted RNA was reverse transcribed using oligo dT primers and the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher). Then 50 ng cDNA was mixed in a total volume of 25 μl with 2× Quantifast SYBR green PCR Master Mix (Applied Biosystems), and 100 nM of forward and reverse primers (primer sequences are described in [22], or are available on request). qPCR was performed using a StepOne Real-Time PCR System (Applied Biosystems). α-Tubulin served as housekeeping gene for normalizations. Viral nucleoprotein N mRNA and changes in IFN and ISG RNA levels (fold change over mock) were determined and calculated as described earlier [15]. Data were expressed as mean ± standard deviation (SD) from at least three replicates. The statistical significance of differences was analysed by unpaired t-test using GraphPad Prism Software.

Flow cytometry
Surface expression of ephrin-B2 was determined as previously described [15]. Briefly, HBEpC single-cell suspensions were fixed with 0.5% paraformaldehyde (PFA) for 15 min, followed by an incubation overnight with recombinant mouse EphB4/Fc, a soluble receptor for ephrin-B2 fused to the Fc region of human IgG (R&D Systems). EphB4/Fc was then detected using FITC-conjugated goat-anti-human IgG (Dianova).

To quantitate phosphorylated STAT1 protein after IFN-α stimulation, differentiated HBEpCs were treated with 10 ng ml⁻¹ IL-28A from the basal side for 30 and 60 min, respectively. After detachment of the cells using accutase (Life Technologies), cells were immunostained basically as described by Krutzik and Nolan [23]. After fixation with 1.5% PFA for 10 min, cells were washed with FACS buffer (PBS containing 1% BSA). The fixed cells were then permeabilized for 15 min with methanol, washed, and subsequently incubated for 16 h at 4°C with Phosflow anti-pSTAT1 antibodies (pY701, BD) conjugated with phycoerythrin (PE).

To control surface expression of the IFN-λ receptor (IL-28R1) on differentiated HBEpCs, the cells were detached as described above and then fixed with 0.5% PFA for 15 min. Cell suspensions were incubated overnight at 4°C with anti-IL28R (R&D) followed by treatment with FITC-labelled anti-mouse IgG antibodies (Dianova) for 30 min. All FACS analyses were carried out using the Guava easyCyte Flow Cytometer (Merck Millipore). Data were evaluated using software Cytosoft 4.2 (Guava Technologies, Merck Millipore).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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