

# Modulating the Barrier Function of Human Alveolar Epithelial (hAELVi) Cell Monolayers as a Model of Inflammation

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## Abstract

The incidence of inflammatory lung diseases such as acute respiratory distress syndrome (ARDS) remains an important problem, particularly in the present time with the Covid-19 pandemic. However, an adequate *in vitro* test system to monitor the barrier function of the alveolar epithelium during inflammation and for assessing anti-inflammatory drugs is urgently needed. Therefore, we treated human Alveolar Epithelial Lentivirus immortalised cells (hAELVi cells) with the pro-inflammatory cytokines TNF- $\alpha$  (25 ng/ml) and INF- $\gamma$  (30 ng/ml), in the presence or absence of hydrocortisone (HC). While TNF- $\alpha$  and INF- $\gamma$  are known to reduce epithelial barrier properties, HC could be expected to protect the barrier function and result in an anti-inflammatory effect. We investigated the impact of anti-inflammatory/inflammatory treatment on transepithelial electrical resistance (TEER) and the apparent permeability coefficient ( $P_{app}$ ) of the low permeability marker sodium fluorescein (NaFlu). After incubating hAELVi cells for 48 hours with a combination of TNF- $\alpha$  and INF- $\gamma$ , there was a significant decrease in TEER and a significant increase in the  $P_{app}$ . The presence of HC maintained the TEER values and barrier properties, so that no significant  $P_{app}$  change was observed. By using hAELVi cells to study anti-inflammatory drugs *in vitro*, the need for animal experiments could be reduced and pulmonary drug development accelerated.

## Key words

ARDS, alveolar epithelium, hydrocortisone, paracellular permeability, TEER, Three Rs

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## Introduction [L1]

The alveolar epithelium of the lung forms the 'air–blood barrier', and is essential for maintenance of lung function, including gas exchange, and immune responses after infection and lung injury.<sup>1</sup> Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are examples of conditions that occur when the alveolar epithelium is damaged. This damage results in hypoxaemia, limited ventilation perfusion in the lung, decreased respiratory supply and oedema formation, with a mortality of up to 50%.<sup>2–4</sup> With Covid-19 continuing to be a major health issue, the incidence of ARDS and ALI are

significant concerns.<sup>5</sup> ARDS and ALI are most commonly caused by viral or bacterial infections that trigger an inflammatory response by the immune system.<sup>6</sup> In addition to neutrophils and dendritic cells, alveolar macrophages play a major role in such an immune response to persistent infection in the respiratory region of the lung.<sup>7</sup> Alveolar pathogens interact with toll-like receptors (TLRs) of the resident macrophages<sup>8</sup> which then secrete pro-inflammatory cytokines (such as IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$ ) to activate the penetration of neutrophils into the alveolar space leading to a high-protein oedema.<sup>9,10</sup> This breakdown of the epithelial barrier has diverse consequences for cell communication, epithelial connection with the extracellular matrix (ECM), and the destruction of tight junctions (TJs).<sup>10</sup> TJs are essential for the formation and maintenance of the epithelial cell barrier, and control the permeability of dissolved substances of various sizes and charges.<sup>11</sup> This paracellular permeability is based on the interaction of transmembrane, peripheral and cytoskeletal proteins. Claudins represent the main family of transmembrane proteins that ensure a regulated exchange of substances between cells, and they are responsible for controlled paracellular diffusion. Other junctional plaque components that are associated with the transmembrane proteins — for example, the zonula occludens 1 (ZO-1) protein — interact with the junctional membrane proteins F-actin and microtubules to regulate the contractility of the actomyosin cytoskeleton.<sup>12–14</sup> The parameters of the resulting TJ permeability are defined by various complex cellular mechanisms. The main TJ regulators are phosphoinositide 3-kinase (PI3K)/serine and threonine kinase (Akt kinase), mitogen-activated protein kinase (MAPK), Rho-associated kinase (ROCK) and protein kinase C (PKC)-related signalling pathways, which can interact with each other in a network to change the phosphorylation of TJ proteins in response to infection in the microenvironment of the epithelial cells.<sup>15</sup> For example, as a result of an infection, the production of the pro-inflammatory cytokine TNF- $\alpha$  is essential for the expression of inflammatory genes, cell viability, and for the initiation of immune cell responses.<sup>16</sup> Through activation of the NF- $\kappa$ B signalling pathway, TNF- $\alpha$  opens the TJs by interacting with the Claudin-5 (Cldn-5) protein.<sup>11</sup> In combination with INF- $\gamma$ , TNF- $\alpha$  increases the permeability of the epithelial barriers over the MLC/MLCK pathway, and induces the rearrangement of Cldn-2 and Cldn-3 to Cldn-4.<sup>17,18</sup>

The complexity of these intracellular processes is one factor impeding the development of new therapies for ARDS/ALI. Experiments on animals are still the most common choice for testing drugs, as these animal models attempt to simulate the course of the diseases *in vivo*. The most widely used respiratory/inflammatory animal models in drug research are based on experiments that involve exposure to pro-inflammatory compounds, such as the aspiration of 0.1–0.5 M hydrochloric acid or treatment with lipopolysaccharide (LPS). These experiments are normally performed on mice.<sup>19</sup> In addition, ventilator-induced lung injury (VILI) has great clinical importance, as it simulates ARDS *in vivo* by the mechanical disruption of the animal's epithelial barrier.<sup>20</sup> Overall, these methods are used to induce different inflammatory processes in respiratory epithelia, leading to various symptoms of ARDS/ALI, such as the intrapulmonary release of cytokines and the breakdown of the blood–air barrier in the alveolar space.<sup>19,21</sup> However, besides the ethical concerns and economic limitations of performing animal experiments, the development of adequate *in vivo* models for testing new therapies is complicated by a range of species differences, including: TLR expression; the structure of the mononuclear phagocyte system; the chemokines and receptors involved; and the overall size of the lung.<sup>21</sup>

Therefore, suitable *in vitro* methods for the simulation of ALI/ARDS are still urgently needed. In compliance with the Three Rs principles (in particular, *replacement*), and to overcome the challenges resulting from species differences, the further development and optimisation of human cell-based *in vitro* systems are required. Gordon et al.<sup>22</sup> summarised the available *in vitro* models for simulating the lung, divided into those

representing the central airway (e.g. Calu-3, 16HBE14o-, NCI-H441, BEAS-2B and primary cells) and those representing the peripheral airway, such as A549 cells.<sup>22</sup> The latter model is commonly used for the investigation of pro-inflammatory effects after LPS stimulation<sup>23</sup> and hypoxia.<sup>21</sup> However, the main disadvantages of a A549 cell-based model in the simulation of ARDS/ALI is their inability to form TJs, leading to a high paracellular permeability without the ability to evaluate transepithelial electrical resistance (TEER).<sup>24</sup> Complex multicellular test systems consisting of epithelial cells (e.g. primary cells, A549, Calu-3, BEAS-2B or CFTE29o- cells), macrophages and dendritic cells are suitable for investigating pulmonary fibrosis or the efficacy of anti-inflammatory drugs *in vitro*.<sup>25,26</sup> These sophisticated cell culture models can be used as part of lung-on-a-chip systems,<sup>26</sup> with a more physiological reconstitution of the lung being feasible, but they still lack standardised end-user formats.<sup>27</sup>

Commercially available 3-D human airway models like EpiAirway™ (MatTek Life Sciences) and MucilAir™ (Epithelix Sàrl) are now being used for respiratory safety assessments.<sup>28,29</sup> These cell culture systems represent the airway epithelium of the bronchi and not the alveolar epithelium of the deep lung. Therefore, the EpiAlveolar™ model (MatTek Life Sciences) is suitable for the *in vitro* simulation of the alveolar region, which can be used, for example, to mimic pulmonary fibrosis development after nanoparticle exposure.<sup>30</sup> Epithelial monolayers are perhaps of limited use for modelling inflammatory processes, due to the lack of complex immune cell communication.<sup>31</sup> In the current study, this limitation was addressed by combining primary human alveolar macrophages with primary epithelial type I-like cells from the same donor.<sup>32</sup> However, while such a primary cell culture model probably most closely reflects human epithelial responses, it is not commercially available and it is not very practical to implement, due to limited access to human lung tissue.

For use as an alternative to primary human alveolar cells, human Alveolar Epithelial Lentivirus immortalised cells (hAELVi cells) have been recently developed and characterised. These cells were originally derived from primary human cells and have pneumocyte type I characteristics, with functional TJs characterised by a high TEER.<sup>33</sup> Based on this new cell line, the present study aims to establish an *in vitro* test system for pulmonary inflammatory studies. The inflammatory response is observed through changes in the barrier properties of the hAELVi cells after stimulation with the pro-inflammatory cytokines TNF- $\alpha$  and INF- $\gamma$ . By measuring TEER and  $P_{app}$  of the hAELVi barrier, we demonstrate how hAELVi cells react to pro-inflammatory cytokine stimulation (TNF- $\alpha$  and INF- $\gamma$ ) at different time points (0, 24 and 48 hours), in the presence or absence of fetal calf serum (FCS) and the anti-inflammatory glucocorticoid, hydrocortisone (HC). To provide a useful model for *in vivo* conditions, experimental conditions were optimised to clearly show any HC-mediated amelioration of TEER decreases and  $P_{app}$  increases that were occurring as a result of inflammatory stimulation.

## Materials and Methods [L1]

### Culture of the hAELVi cells [L2]

The human cell line, hAELVi, was obtained from InSCREENeX GmbH (Braunschweig, Germany) and cultured in Small Airway Epithelial Cell Growth Medium™ (SAGM™; Lonza, Basel, Switzerland) supplemented with 1% v/v fetal calf serum (FCS; South American origin, Superior; Biochrom, Berlin, Germany) and 1% v/v antibiotics (Gibco™ penicillin (10,000 U/ml)/streptomycin (10,000  $\mu$ g/ml); Fisher Scientific, Waltham, MA, USA). For the HC-free conditions, no HC from the supplement kit (Lonza) nor FCS were added to the

medium. Otherwise, the concentration of HC in SAGM™ was 400–600 ng/ml when prepared according to the manufacturer's instructions. For the routine cell culture, the cells were passaged once a week and grown in parallel in either HC-free or HC-containing medium (both without FCS). The cells were detached with 0.05% trypsin–EDTA (Gibco™; Fisher Scientific) for 5 minutes at 37°C. As hAELVi cells cultured in the presence of HC and FCS have a faster growth rate,  $1.5 \times 10^5$  cells and  $3 \times 10^5$  cells were seeded per T75 cm<sup>2</sup> flask in SAGM with HC/FCS and SAGM without HC/FCS, respectively. The effects of FCS as media supplement on a successful stimulation were primarily investigated, in order to omit FCS when possible, with the goal of generating a non-animal product-based model in line with Three Rs principles.

For the inflammation experiments,  $1 \times 10^5$  hAELVi cells per 1.12 cm<sup>2</sup> were seeded in the Transwell® filter devices (Cat. No. 3460; Corning, New York, NY, USA) without media supplementation (i.e. in SAGM only). The pore size of the Transwell filter devices used was 0.4 µm, which is optimised for transport and permeability studies.<sup>34</sup> Cell counting was performed with a LUNA™ Automated Cell Counter (Logo Biosystems, Anyang-si, South Korea). All plastic devices were pre-coated with a 1% v/v fibronectin–collagen solution (Corning and Sigma-Aldrich, Darmstadt, Germany, respectively) solution.<sup>33</sup> The hAELVi cells were maintained in a humidified atmosphere with 5% v/v CO<sub>2</sub> at 37°C, from passage number 29 to 41.

### *TEER measurements [L2]*

The transepithelial electrical resistance (TEER) of the hAELVi cells was measured every 48 hours in the Transwell inserts, in order to evaluate barrier formation and integrity. During the measurements, the cells were placed on a heating plate at 37°C to avoid stress-related temperature variations that might affect the TEER. The TEER values were determined with a chopstick electrode connected to an EVOM<sup>2</sup> epithelial volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA). The chopstick electrode was cleaned and sterilised by soaking in 70% v/v isopropanol for 5 minutes, at the start of each session. The EVOM<sup>2</sup> was also calibrated with a reference resistance of 1000 Ω, prior to any measurements being taken. Between measurements of the different experimental groups, the electrode was cleaned with 1× Dulbecco's Phosphate-Buffered Saline (DPBS; no Calcium (CaCl<sub>2</sub>), no Magnesium (MgCl<sub>2</sub>); Fisher Scientific) to prevent any carry-over of the cytokines, etc. After the TEER measurement, a medium change was carried out. To avoid damage to the cells, the medium was first discarded from the basolateral compartment of the Transwell system, followed by removal from the apical side. Then, 0.5 ml of fresh medium was gently pipetted first into the apical compartment, and 1.5 ml of fresh medium were pipetted into the basolateral compartment. The hAELVi cells on the Transwell filters were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

### *Stimulation process [L2]*

Based on the correlation of intestinal TEER values and intact barrier properties that was previously reported by Srinivasan et al.,<sup>35</sup> the hAELVi cells were stimulated with cytokines when TEER values of  $> 800 \Omega \cdot \text{cm}^2$  were obtained (this being indicative of tight barrier formation). In preliminary experiments, hAELVi cells were stimulated apically and basolaterally with TNF-α (Fisher Scientific). A stock solution of 5 µg/ml TNF-α was initially prepared with 1× DPBS without CaCl<sub>2</sub> and MgCl<sub>2</sub> and stored at –20°C. The stimulation was carried out with TNF-α at concentrations of 50 ng/ml apically and 17 ng/ml basolateral, in SAGM without HC or FCS. For preparation of the apical working solution,

40  $\mu\text{l}$  of the 5  $\mu\text{g}/\text{ml}$  TNF- $\alpha$  stock were added to 4 ml of SAGM –HC/–FCS and 500  $\mu\text{l}$  of this solution transferred to the apical compartment for the treatment. For the basolateral working solution, 40  $\mu\text{l}$  of the 5  $\mu\text{g}/\text{ml}$  TNF- $\alpha$  stock were added to 12 ml of SAGM –HC/–FCS and 1.5 ml of this solution transferred to the basolateral compartment. After 24 and 48 hours, the TEER was determined again without an additional medium change. It should be noted that TNF- $\alpha$  was not present during the transport studies during the optimisation experiments.

After optimising the protocol, inflammation of the hAELVi cells was induced by exposing one group to 25 ng/ml TNF- $\alpha$ , another group to 30 ng/ml INF- $\gamma$  (Fisher Scientific), and a third one to both cytokines in a 'cocktail' (at these same concentrations) in SAGM with or without HC. The stock of INF- $\gamma$  was prepared in sterile and purified water at a concentration of 0.1 mg/ml and stored at  $-20^{\circ}\text{C}$ . A further dilution was performed to obtain 1  $\mu\text{g}/\text{ml}$  in Hanks' Balanced Salt Solution (HBSS; Gibco; Fisher Scientific), from which 210  $\mu\text{l}$  were diluted in 7 ml SAGM with or without HC and FCS to obtain the working concentration of 30 ng/ml. In all experiments, SAGM +/-HC, SAGM +/-FCS, all without any cytokine addition, were used as the control group.

The hAELVi cells on the Transwell filters were maintained in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The TEER was measured pre-treatment (0 hours), and after 24 and 48 hours; a transport study with sodium fluorescein was performed after 48 hours. It should be noted that, following optimisation of the protocol, the cytokine stimuli were present at the given concentrations in HBSS, during the transport studies.

### *Transport studies and calculation of the apparent permeability coefficient ( $P_{app}$ ) [L2]*

In addition to the TEER experiments, apparent permeability coefficients ( $P_{app}$ ) were determined to assess potential damage to the barrier after stimulation with the cytokines. After 48 hours of stimulation, the TEER values of the hAELVi cells were measured in the Transwell plate. Thereafter, the cells were incubated in HBSS for 30 minutes, 500  $\mu\text{l}$  apically and 1.5 ml basolaterally, in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . For the preliminary experiments, in which TNF- $\alpha$  was used alone for the stimulation experiments, the cytokine was not present during the transport studies. However, for the optimised experiments with 25 ng/ml TNF- $\alpha$  and 30 ng/ml INF- $\gamma$ , the cytokines were present during the transport studies. The TEER values were measured after 48 hours of stimulation (the TEER values are presented in Figures 1, 3 and 5) and after the transport studies (Figure 2, 4 and 6), in order to check the effectiveness of the barrier properties during the experiments. After assessing the TEER values of the cells in HBSS, 0.5 ml of fresh HBSS with 10  $\mu\text{g}/\text{ml}$  sodium fluorescein (NaFlu; Merck, Darmstadt, Germany) was added to the apical compartment, and 1.5 ml HBSS without NaFlu added to the basolateral compartment. To obtain time 0 readings, 20  $\mu\text{l}$  and 200  $\mu\text{l}$  aliquots were immediately taken from the apical and basolateral compartments, respectively, and transferred to a 96-well plate. After 20, 40, 60 and 90 minutes, 200  $\mu\text{l}$  were taken from only the basolateral compartments; after 120 minutes, 20  $\mu\text{l}$  were taken from the apical compartments and a further 200  $\mu\text{l}$  from each basolateral compartment. During the transport study, the Transwell plate was placed on a 35 rpm shaker (Heidolph, Germany) at  $37^{\circ}\text{C}$ , and protected from the light. For the fluorescence measurements, the 20  $\mu\text{l}$  apical compartment samples were diluted in 180  $\mu\text{l}$  HBSS in a 96-well plate, and the 200  $\mu\text{l}$  samples from the basolateral compartments were transferred to the plate without further dilution. To replace the medium lost and maintain the total volume throughout the study, 20  $\mu\text{l}$  and 200  $\mu\text{l}$  of HBSS were added after sampling, to the apical and basolateral compartments, respectively. The fluorescence of the samples was quantified with a plate

reader (Synergy2; Biotek, Winooski, VT, USA) at a wavelength of 485 nm (emission) and 528 nm (excitation). The TEER of the hAELVi cells was measured at the end of each experiment (the TEER values are presented in Figure 2, 4 and 6).

The  $P_{app}$  was determined by means of a calibration curve and calculated by using the formula shown:<sup>36</sup>

$$P_{app} = \left( \frac{1}{AC_0} \right) \times \left( \frac{dQ}{dt} \right)$$

Where,  $A$  = area,  $C_0$  = initial concentration of sodium fluorescein,  $Q$  = amount of sodium fluorescein that permeated across the Transwell membrane,  $t$  = time.

### Statistical analysis [L2]

The data are presented as the mean  $\pm$  standard deviation (SD), indicating the number of independent replicates ( $n$ ). The data were statistically analysed by a one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test (specified in the figure legends), and by a two-sampled  $t$ -test for comparisons of specific test groups. The  $p$  values are marked if there was a significant difference compared to the defined test group (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). The statistics were performed with Origin<sup>®</sup>Pro 2019.

## Results [L1]

### *Stimulation with TNF- $\alpha$ decreases the TEER of hAELVi cells, but does not increase paracellular permeability of sodium fluorescein (NaFlu) [L2]*

TNF- $\alpha$  can rearrange actin–myosin contractility through the activation of the NF- $\kappa$ B pathway, resulting in an impaired epithelial barrier formation.<sup>17</sup> Therefore, two experiments were performed, in order to investigate the effect of TNF- $\alpha$  on epithelial barrier formation in hAELVi cells. The parameters that were determined were: TEER values, to evaluate changes in epithelial barrier formation;<sup>35</sup> and  $P_{app}$  values, which represent a marker for paracellular permeability, which consequently increases with decreasing TEER values.<sup>37,38</sup>

Once TEER values above 1000  $\Omega \cdot \text{cm}^2$  were reached (see *Supplementary Figures S4 and S5*), hAELVi cells were stimulated in preliminary experiments with TNF- $\alpha$  (50 ng/ml and 17 ng/ml in the apical and basolateral compartments, respectively) for 24 and 48 hours. In the presence of HC and FCS in SAGM (SAGM +HC/+FCS), a decrease in the TEER was observed 24 hours after exposure to TNF- $\alpha$  ( $p < 0.05$ ), and this was even more marked after 48 hours ( $p < 0.001$ ; Fehler: Verweis nicht gefundena). In the absence of HC and FCS (SAGM –HC/–FCS), after TNF- $\alpha$  stimulation a significant reduction in the TEER, as compared to the control group, was observed after both 24 and 48 hours ( $p < 0.001$ ; Fehler: Verweis nicht gefundenb). The TEER values shown in Figure 1 were measured before stimulation (0 hours), and after 24 and 48 hours of stimulation in SAGM +HC/+FCS or SAGM –HC/–FCS, before the transport studies were performed.

After TNF- $\alpha$  stimulation for 48 hours, transport studies with the tracer molecule sodium fluorescein (NaFlu) were performed in order to assess paracellular permeability by calculating  $P_{app}$  values. The TEER values presented in Figure 2 were measured on completion of the transport studies. Upon stimulation by TNF- $\alpha$  for 48 hours, hAELVi cells previously cultured in SAGM +HC/+FCS (Figure 2a), showed a significant reduction ( $p <$

0.001) in TEER values, from  $1019 \pm 359 \Omega \cdot \text{cm}^2$  in the control group to  $553 \pm 236 \Omega \cdot \text{cm}^2$  in the stimulated group. However, the corresponding  $P_{app}$  values for the control and the stimulated group were not significantly different ( $2.23 \times 10^{-6} \pm 3.70 \times 10^{-6} \text{ cm/s}$  and  $1.02 \times 10^{-6} \pm 4.68 \times 10^{-7} \text{ cm/s}$ , respectively).

The same trend was observed in the absence of HC and FCS (SAGM –HC/–FCS), where stimulation of hAELVi cells with TNF- $\alpha$  significantly reduced ( $p < 0.001$ ) TEER values from  $1466 \pm 472 \Omega \cdot \text{cm}^2$  in the control group to  $846 \pm 153 \Omega \cdot \text{cm}^2$  in the stimulated group, whereas the  $P_{app}$  of NaFlu did not change significantly (Figure 2b). It is clear that TNF- $\alpha$  causes a moderate loss of barrier function, leading to a slight but significant drop in the TEER ( $p < 0.001$ ). This is more pronounced in the absence of HC and FCS in the medium. The permeability of the paracellular transport marker NaFlu was, however, not significantly affected by this moderate change in barrier function. A more pronounced disruption of the cellular barrier would have been required in order to instigate a more significant effect on barrier permeability.

### *Synergistic effects of TNF- $\alpha$ and INF- $\gamma$ reduced the TEER and increased the paracellular permeability of NaFlu [L2]*

As stimulation with TNF- $\alpha$  alone was not sufficient to mediate a significant increase in paracellular transport activity, as determined by the  $P_{app}$ , INF- $\gamma$  treatment — either alone or in combination with TNF- $\alpha$  — was included in subsequent experiments. This would also investigate whether these cytokines would act synergistically to bring about a reduction in epithelial barrier properties.<sup>17,39</sup>

Once a TEER above  $1000 \Omega \cdot \text{cm}^2$  was reached (see *Supplementary Figures S7 and S8*), the stimulation experiments with either 25 ng/ml TNF- $\alpha$ , 30 ng/ml INF- $\gamma$ , or TNF- $\alpha$  and INF- $\gamma$  combined in a cytokine ‘cocktail’, were performed. The TEER of hAELVi cells cultured in HC and FCS (SAGM +HC/+FCS) did not show a significant decrease in any group (Figure 3a). In contrast, when hAELVi cells were cultured in HC and FCS depleted SAGM (SAGM –HC/–FCS; Figure 3b), a significant decrease in TEER was achieved after a 48-hour stimulation with TNF- $\alpha$  alone ( $p < 0.05$ ), INF- $\gamma$  alone ( $p < 0.001$ ), and both in combination ( $p < 0.001$ ). The TEER values presented in Figure 3 were measured before stimulation (0 hours), and after 24 and 48 hours of stimulation in SAGM +HC/+FCS or –HC/–FCS, before the transport studies were performed.

The significant reduction in TEER shown in Figure 3b raised the question as to whether stimulation with TNF- $\alpha$  alone, INF- $\gamma$  alone, or both in combination, would increase the permeability of NaFlu. Therefore, we performed transport studies to calculate the  $P_{app}$  of the stimulated hAELVi cells (Figure 4). When hAELVi cells were stimulated in SAGM +HC/+FCS, no significant changes in TEER values were observed (as shown in Figure 3a). This finding was also reflected in the permeability characteristics, with no significant changes apparent in the  $P_{app}$  (Fehler: Verweis nicht gefundena). However, this changed dramatically in the absence of HC and FCS (Fehler: Verweis nicht gefundenb). A significant change in the TEER values was noted between the groups stimulated with INF- $\gamma$  ( $541 \pm 313 \Omega \cdot \text{cm}^2$ ,  $p < 0.01$ ) or INF- $\gamma$  + TNF- $\alpha$  ( $195 \pm 74 \Omega \cdot \text{cm}^2$ ,  $p < 0.001$ ), as compared to the unstimulated control ( $1145 \pm 729 \Omega \cdot \text{cm}^2$ ). These results suggested that TNF- $\alpha$  and INF- $\gamma$  had a synergistic effect on the reduction of TEER, as stimulation with TNF- $\alpha$  alone did not have a significant impact ( $669 \pm 570 \Omega \cdot \text{cm}^2$ ), and INF- $\gamma$  stimulation alone mediated a less significant effect. The cytokine cocktail also had the greatest impact on permeability, with the  $P_{app}$  increasing significantly compared to the control group ( $1.14 \times 10^{-5} \pm 5.8 \times 10^{-6} \text{ cm/s}$  for the control group;  $1.27 \times 10^{-6} \pm 1.85 \times 10^{-6} \text{ cm/s}$  for the stimulated group). Stimulation with the cocktail also resulted in a significant increase in the  $P_{app}$ , as compared

to the groups stimulated with TNF- $\alpha$  and INF- $\gamma$  alone (Fehler: Verweis nicht gefundenb,  $p < 0.001$ ). The TEER values presented in Figure 4 were measured after the transport studies were performed.

### *Hydrocortisone maintains barrier properties independently of FCS supplementation [L2]*

From the experiments carried out to investigate the potential synergistic effects of the cytokine cocktail, it was clear that stimulation with combined TNF- $\alpha$  and INF- $\gamma$  significantly decreased the integrity of the epithelial barrier in the absence of HC and FCS (Figure 4b). It was also apparent that the presence of HC and FCS during the cytokine exposure ameliorated the detrimental effects on the barrier properties (Figure 4a). The contribution of FCS to the protective effect of this HC/FCS supplementation was investigated by omitting FCS completely from the medium during the cytokine exposure. This was with the overall aim of generating a non-animal product-based model in line with Three Rs principles.

The hAELVi cells were cultured in SAGM without FCS, in the presence or absence of HC (i.e. SAGM +HC/-FCS and -HC/-FCS), until a TEER value above  $1000 \Omega \cdot \text{cm}^2$  was reached (see *Supplementary Figure S8*). The cells were then stimulated with a cytokine cocktail of 25 ng/ml TNF- $\alpha$  and 30 ng/ml INF- $\gamma$  in SAGM +HC/-FCS or SAGM -HC/-FCS, and the TEER was measured after 24 and 48 hours, prior to performing the transport studies. These TEER values, as well as the values obtained before stimulation (0 hours), are presented in Figure 5.

Without HC added, in the absence of FCS, a significant decrease of TEER was observed after 48 hours to  $< 300 \Omega \cdot \text{cm}^2$ , as compared to the control with  $849 \pm 196 \Omega \cdot \text{cm}^2$  ( $p < 0.001$ ). Interestingly, the presence of HC in the unstimulated control groups resulted in increasing TEER values, from  $2927 \pm 295 \Omega \cdot \text{cm}^2$  at the 24-hour time-point, to  $4701 \pm 196 \Omega \cdot \text{cm}^2$  after 48 hours. Cytokine stimulation reduced the TEER significantly, from  $4701 \pm 196 \Omega \cdot \text{cm}^2$  to  $822 \pm 528 \Omega \cdot \text{cm}^2$  at this 48-hour time-point ( $p < 0.001$ ), even in the presence of HC. No FCS dependency was identified, but TEER is clearly enhanced by the addition of HC (Figure 5).

In the presence of HC, stimulation with the inflammatory cytokines did not lead to a significant increase in the  $P_{app}$  value (Figure 6a, right). In the absence of HC (Figure 6b), the TEER was significantly decreased ( $p < 0.001$ ) following the 48-hour exposure to inflammatory cytokines, when compared with the unstimulated control ( $175 \pm 56 \Omega \cdot \text{cm}^2$  versus  $1048 \pm 294 \Omega \cdot \text{cm}^2$ , respectively). The corresponding calculation of the  $P_{app}$  (Figure 6b, right) shows that the permeability of cytokine-stimulated hAELVi cells cultured without HC was significantly higher than that of the unstimulated control group ( $9.30 \times 10^{-6} \pm 3.89 \times 10^{-6} \text{ cm/s}$  versus  $1.08 \times 10^{-6} \pm 1.07 \times 10^{-6} \text{ cm/s}$ ;  $p < 0.001$ ). The TEER values presented in Figure 6 were measured after the transport studies were performed. Overall, these results suggest that hAELVi cells can mimic *in vitro* the reduction in epithelial barrier integrity induced by lung inflammation. TNF- $\alpha$  and INF- $\gamma$  in combination appeared to reduce barrier properties (TEER) and increase cell permeability ( $P_{app}$ ). Hydrocortisone acted as expected from clinical findings, in that it functioned as an anti-inflammatory drug and contributed to maintenance of the barrier function in hAELVi cell *in vitro* cultures. FCS did not seem to influence these findings, and therefore its use could be avoided when using this *in vitro* model.

### *The relationship between electrical resistance and increased permeability [L2]*

These studies demonstrated that stimulation with the pro-inflammatory cytokines, TNF- $\alpha$  and INF- $\gamma$ , reduced the barrier function of hAELVi cells. Increased paracellular permeability was observed at lower TEER values, in the absence of HC.

In principle, data points in the function of TEER and  $P_{app}$  follow a hyperbolic function ( $1/x$ ), and this can be seen in Figure 7a. All of the collected data points were plotted on this graph, regardless of the culture conditions or the stimulation parameters. From this graph, it was clear that, with decreasing TEER values the  $P_{app}$  increased.

An inverse function leads to a linear relation, and thus the reciprocal of the TEER values were calculated and plotted against the corresponding  $P_{app}$  values (see Figure 7b). The TEER is defined as the epithelial resistance ( $\Omega$ ) multiplied by the measurement area ( $\text{cm}^2$ ). The reciprocal of resistance is electrical conductivity — more specifically in this case, it is the transepithelial electrical conductance (TEEC; conductivity (S) per measurement area;  $\text{S}/\text{cm}^2$ ). The inverse plot follows a straight line up to a certain point (the 'critical TEEC'), after which the permeability increases abruptly. This is indicated by the vertical line in the graph in Figure 7b, with the vertical dashed lines indicating the standard error. A sharp increase in  $P_{app}$  was observed after this 'critical TEEC' point.

To calculate the exact value of the critical TEEC, a piece-wise linear fitting (PWLFF) of the curve was performed. By using this fitting procedure, the critical TEEC was calculated to be  $0.0041 \pm 3.63 \times 10^{-4} \text{ S}/\text{cm}^2$  and the  $P_{app}$  to be  $3 \times 10^{-6} \text{ cm}/\text{s}$ . By taking the reciprocal value of the 'critical TEEC', it was then possible to calculate a TEER value of  $245 \pm 20 \Omega \cdot \text{cm}^2$ . Thus, for hAELVi cells, a reduction in TEER to a range lower than  $245 \pm 20 \Omega \cdot \text{cm}^2$  will lead to a significantly increased permeability to NaFlu ( $p < 0.001$ , Figures 4b and 6b).

## Discussion [L2]

### *hAELVi cells represent a simple in vitro model for the simulation of inflammation [L2]*

Major interstitial lung diseases (ILDs), such as idiopathic pulmonary fibrosis (IPF), acute intestinal pneumonia (AIP), or the mostly aetiologically unclear ARDS, illustrate the heterogeneity of the life-threatening consequences initiated by inflammatory irregularities of the lung.<sup>40,41</sup> ARDS results from bilateral pulmonary infiltrates, hypoxaemia and diffuse alveolar damage.<sup>10</sup> The alveolar epithelium is mainly responsible for the normal lung fluid balance and, thus, for the avoidance of oedema.<sup>42</sup> As soon as the cells lose their barrier function, this fluid balance is disturbed and liquids can accumulate in the interstitial tissue or directly in the alveoli themselves.<sup>10,43</sup> To avoid lung oedema, it is essential that the physiological integrity of the epithelial barrier is maintained, and this is mostly mediated by the TJs and interacting proteins.<sup>44</sup>

To develop therapeutic options for the recovery of impaired TJs and barrier function, an appropriate test model is essential. Therefore, in this study, we tried to simulate a state of cell barrier property impairment by using simple *in vitro* methods. The hAELVi cells used here are particularly suitable for this purpose, since this immortalised cell line demonstrates strong barrier properties expressed by type I pneumocytes with associated stable TEER values, both in liquid–liquid and air–liquid culture conditions. Despite the great potential of hAELVi cells for the development of a stable barrier, the barriers formed by these cells have an increased range of TEER values (from 1000–7000  $\Omega \cdot \text{cm}^2$ , see *Supplementary Figures S4–S8*). EDTA was used as control, as a TJ opener, to decrease TEER values to  $< 500 \Omega \cdot \text{cm}^2$ , leading to  $P_{app}$  values of  $1 \times 10^{-5} \text{ cm}/\text{s}$ .<sup>33</sup> Overall, hAELVi cells can form a tight barrier with corresponding high TEER values and thus they

can provide an additional read-out in inflammation studies, as compared to non-barrier forming cell lines, such as A549.<sup>35</sup>

### *TNF- $\alpha$ decreases the TEER of hAELVi cells, but does not increase the paracellular permeability of NaFlu [L2]*

The stimulation of hAELVi cells with the pro-inflammatory cytokine TNF- $\alpha$  was tested first with 50 ng/ml TNF- $\alpha$  in the apical compartment (modelling the cytokine release from tissue macrophages)<sup>45</sup> and with 17 ng/ml in the basolateral compartment (simulating the systemic inflammation by activated neutrophils and monocytes).<sup>46</sup> Here, a significant reduction in the TEER was observed, but an effective opening of the TJs, demonstrated by a constant  $P_{app}$  value, was not observed. TNF- $\alpha$  alone significantly decreased the TEER by up to 500  $\Omega \cdot \text{cm}^2$ , as seen in Figures 1a and 3b. Whether the barrier function was subsequently reduced depended heavily on the initial TEER value of the hAELVi cells (Figure 1b *versus* Figure 3b). The higher the initial TEER of the cell barrier before stimulation, the less likely it was to exhibit increased permeability. In this preliminary experiment, we were able to show a significant decrease in TEER after stimulation with TNF- $\alpha$ , but not a significant increase in paracellular permeability. In future experiments, further tracer molecules of different molecular weights could be tested, in addition to NaFlu.<sup>47,48</sup>

The most appropriate concentration of TNF- $\alpha$  to use in *in vitro* assays is often debated. TNF- $\alpha$  is released from stimulated leukocytes and is a key mediator of inflammation.<sup>49</sup> Among other pro-inflammatory cytokines (e.g. INF- $\gamma$ , IL-1 $\beta$ , IL-6, IL-8), TNF- $\alpha$  leads to increased epithelial permeability.<sup>50,51</sup> This effect has been extensively investigated *in vitro* by using the intestinal epithelial cell line Caco-2. A time and concentration-dependent significant decrease in the TEER of Caco-2 cells arose from TNF- $\alpha$  stimulation from 1–100 ng/ml and remained stable within this concentration range.<sup>50,52</sup> Comparing this experimental concentration range to that found in ARDS patients, TNF- $\alpha$  increase has been detected at 932.6 pg/ml in bronchoalveolar lavage (BAL) samples, seven days after diagnosis.<sup>53</sup> Such an increase in cytokine level leads to a reduced barrier function, by downregulating aPKC (atypical protein kinase C), which is mainly responsible for the maintenance of cell polarity through the phosphorylation of the myosin light chains (MLCs).<sup>54</sup> Consequently, TNF- $\alpha$  induces NF- $\kappa$ B activation, resulting in the upregulation of myosin light chain kinase (MLCK) expression, causing cytoskeletal contraction and permeable TJs.<sup>55,56</sup>

While TNF- $\alpha$  stimulation has been reported to increase the permeability of the intestinal epithelium, we did not observe this effect in hAELVi cells (see Fehler: Verweis nicht gefunden and Fehler: Verweis nicht gefunden). The barrier function of human lung epithelial cells (HLECs) in comparison to that of Caco-2 cells is more robust against stimulation with cytokines (such as TNF- $\alpha$  and INF- $\gamma$ ), with the difference attributed to the mitochondrial energy state during acute inflammation.<sup>57</sup> This might explain the increased robustness of barrier properties exhibited by hAELVi cells after TNF- $\alpha$  stimulation and explain why an additional inflammatory mediator (INF- $\gamma$ ) was necessary.

### *TNF- $\alpha$ and INF- $\gamma$ synergistically increase the paracellular permeability of NaFlu [L2]*

It was not possible to consistently increase the permeability of the hAELVi barrier by using TNF- $\alpha$  alone as a pro-inflammatory mediator. Therefore, further experiments were carried out to assess the synergistic effects of co-stimulation with TNF- $\alpha$  and INF- $\gamma$  to decrease the barrier properties of hAELVi cells.

INF- $\gamma$  is mainly secreted by lymphocytes and dendritic cells, activating macrophages and triggering increased TJ permeability.<sup>56</sup> A time- and concentration-dependent decrease in TEER of up to 50% was observed with the intestinal epithelial cell line T84 after stimulation with 1–100 ng/ml INF- $\gamma$ ,<sup>58</sup> which was in line with a similar study involving TNF- $\alpha$  stimulation.<sup>50</sup> Mice infected with H1N1 have shown an increase in BAL fluid INF- $\gamma$  levels of up to 200 pg/ml.<sup>59</sup> Analysis of serum from severe acute respiratory syndrome (SARS) patients does not indicate an increase in INF- $\gamma$  concentration, which suggests that symptoms are not triggered by circulating INF- $\gamma$ .<sup>60</sup> Nevertheless, in *in vitro* experiments with T84 cells, Watson et al. observed impaired barrier function (TEER < 300  $\Omega$ ·cm<sup>2</sup>) after stimulation with 10 ng/ml INF- $\gamma$  for 48 hours, but an increased  $P_{app}$  value was only evident at concentrations of 10–100 ng/ml (i.e. 10 ng/ml was at the extreme lower end of the concentration range needed to successfully increase the  $P_{app}$ ). Therefore, in the present work, a stimulatory concentration of 30 ng/ml INF- $\gamma$  was used.<sup>61</sup> INF- $\gamma$  inhibits the Wnt signalling pathway to stop proliferation, and can trigger apoptosis, MLC and MLCK phosphorylation, as well as the release of intercellular adhesion molecule-1 (ICAM-1), to instigate rearrangement of actin components and the opening of TJs.<sup>62</sup> INF- $\gamma$  can open TJs by activating the NF- $\kappa$ B pathway, which is related to the hypoxia-inducible factor-1 (HIF-1) in T84 intestinal epithelial cells.<sup>63</sup>

Representing the bronchial epithelium, Calu-3 cells rearrange occludin and ZO-1 proteins in TJs through the epidermal growth factor receptor (EGFR) dependent MAPK/ERK 1/2 pathway, to increase permeability after INF- $\gamma$  stimulation. For hAELVi cells, this effect was observed in the reduction of TEER after 48 hours of INF- $\gamma$  stimulation (Fehler: Verweis nicht gefunden), but without a significant increase in permeability (as shown for the Calu-3 cells). As co-stimuli, TNF- $\alpha$  and INF- $\gamma$  were shown to induce a significant TEER reduction after a 48-hour stimulation of Calu-3 cells,<sup>64</sup> similar to the results obtained with hAELVi cell stimulation in the present study (see Fehler: Verweis nicht gefunden and Fehler: Verweis nicht gefunden). The hAELVi cells were co-stimulated with TNF- $\alpha$  and INF- $\gamma$ , in order to determine whether these two cytokines would exert any synergistic effects on TJ the opening (explained in more detail in the literature).<sup>17,56,65</sup> The co-stimulation of hAELVi cells in the current study showed a significant increase of permeability after 48 hours (see Fehler: Verweis nicht gefunden), demonstrating increased susceptibility to the cytokine cocktail than primary bronchial cells.<sup>66</sup> In addition, Figure 7 demonstrates that hAELVi cells behave as a permeability barrier to NaFlu in a similar manner to the bronchial epithelial cell line 16HBE14o analysed in Ehrhardt et al.,<sup>67</sup> particularly with regard to their paracellular activity and the culture method used. The observation that low TEER correlates with high  $P_{app}$  values enables the evaluation of the different transport rates of potential new drugs, depending on their molecular weight.<sup>68</sup>

### *The balance between inflammatory reactions and apoptosis [L2]*

The activation of NF- $\kappa$ B signalling protects cells against the initiation of apoptosis after exposure to inflammatory stimuli.<sup>69</sup> Pro-inflammatory cytokines, such as TNF- $\alpha$ , INF- $\gamma$  and IL-1 $\beta$ , can increase Fas-mediated apoptosis in ARDS patients, and this has been simulated *in vitro* in NHBE cells, resulting in the loss of barrier properties in the alveolar space.<sup>70,71</sup> In HT29 epithelial cells, pro-inflammatory cytokines cannot individually induce apoptosis, but INF- $\gamma$  can increase the susceptibility of TNF- $\alpha$  triggered apoptotic gene expression.<sup>72,73</sup> Additionally, TNF- $\alpha$  and INF- $\gamma$  can upregulate the expression of the TNF-related apoptosis inducing ligand (TRAIL), which induces cell-specific apoptotic pathways by caspase-8 activation. Alternatively, and directly dependent on the incoming stimuli, the

NF- $\kappa$ B pathway can initiate proliferation and survival of intestinal as well as airway epithelial cells.<sup>74,75</sup>

A change in morphology was observed in hAELVi cells cultured without HC and FCS, as stimulation led to the cells becoming more rounded (see *Supplementary Fehler: Verweis nicht gefunden–S3*). To clarify, if these are signs of apoptosis, the consequences of stimulating hAELVi cells with the pro-inflammatory TNF- $\alpha$  and INF- $\gamma$  must be examined more closely in further studies, to evaluate the balance between initiating events of the NF- $\kappa$ B pathway and the activation of potential apoptotic processes. Such studies would involve cell viability measurements (for example, the MTT assay), dUTP nick-end labelling (TUNEL) assay or caspase activity detection.

*The cytokine-dependent increase in paracellular permeability of NaFlu in the absence of hydrocortisone is independent of fetal calf serum supplementation [L2]*

In addition to the effects of pro-inflammatory cytokines discussed so far, the influence of HC and FCS in the stimulation studies was determined. The aim was to determine the optimal culture conditions that would cause an effective reduction in the barrier function of hAELVi cells, in order to simulate inflammation *in vitro*. hAELVi cells are normally cultured with 1% FCS<sup>33</sup> to support proliferation and cell attachment, and to promote strong growth conditions.<sup>76</sup> By omitting FCS from the culture medium, the new culture conditions contributed to an indirect reduction in animal use, according to the Three Rs principles.<sup>77</sup> FCS supplementation provided no benefit in terms of increased permeability of the stimulated model (see Figures 4 and 6), and thus the new culture conditions eliminated the need for animal-derived products.

The addition of HC to various cell cultures has been shown to increase cell proliferation and extend the utility of cell lines, especially young cultures.<sup>78</sup> This is also the case with the routine culture of hAELVi cells. HC is usually used as supplement of Lonza's SAGM. It was shown that HC absence is an immensely important factor in the successful induction of a significant increase in permeability after stimulation, as observed in Figure 5. hAELVi cells are usually kept under cortisone-supplemented conditions during routine cell culture, which is the same as for primary isolated lung cells.<sup>32</sup> The cells keep their barrier function for some days in culture, even in absence of HC, but this absence is necessary for their use as an inflammatory model. The TEER was significantly decreased after TNF- $\alpha$  and INF- $\gamma$  stimulation, regardless of the presence or absence of HC, but a significant increase in the  $P_{app}$  of stimulated hAELVi cells could only be achieved in the absence of HC (Figure 6b). This effect has also been observed in relation to the TJ properties of endothelial cells, especially in the blood–brain barrier (BBB).<sup>79</sup> TNF- $\alpha$  stimulation of hCMEC/D3 cells significantly decreased the TEER, which could be limited by the addition of HC.<sup>79</sup> Permeability also decreased after HC supplementation.<sup>79</sup> Glucocorticoids, including HC and dexamethasone, upregulate the expression of TJ proteins of endothelial cells, increasing the stability of the basal lamina as a result of increased levels of integrin  $\alpha$ 1, occludin, ZO-1<sup>80</sup> as well as VE-cadherin.<sup>81</sup> Drasler *et al.* investigated the anti-inflammatory effect of a reduced secretion of IL-8, after the methylprednisolone treatment of a multicellular human alveolar model consisting of macrophage, dendritic and epithelial cells.<sup>25</sup> In June 2020, dexamethasone was reported to reduce by one-third the 28-day mortality of Covid-19 patients with severe disease progression that were undergoing intensive mechanical ventilation.<sup>82</sup> Future studies should determine whether improved barrier properties in our inflammation model, as characterised by a higher TEER (up to 5000  $\Omega \cdot \text{cm}^2$ ; see Figure 5a), could be mediated and stabilised by treatment with corticosteroids such as dexamethasone or methylprednisolone. In our experiments, hAELVi cells that were cultured with HC clearly showed a more stable and robust barrier

formation over time than the cells cultured without HC (see Fehler: Verweis nicht gefunden and 5, and *Supplementary Figures S4–S8*).

### *Evaluation of the test system [L2]*

The TEER can serve as a simple starting point to draw conclusions about the inflammatory response of hAELVi cells. However, the TEER depends on many factors, such as the number of cell layers (e.g. one cell layer or multilayer), cytotoxic effects and media composition (e.g. the presence of HC).<sup>83–85</sup> The effects of these external influences on TEER values must therefore be evaluated in further studies. By examining the reciprocal relationship between TEER and TEEC (transepithelial electrical conductance), we were able to calculate a ‘critical’ TEER range of  $245 \pm 20 \Omega \cdot \text{cm}^2$ . Below this range, the cellular barrier of hAELVi cells exhibits increased permeability to NaFlu (see Figure 7). Consequently, assays with hAELVi cells could detect the anti-inflammatory potential of a compound by using the TEER or cell permeability parameters. Particular caution is recommended for compounds that interact with ions (e.g. EDTA) or are cytotoxic, as both cases will lead to a reduced TEER and increased barrier permeability.<sup>47</sup>

The nature of the cell barrier during inflammation should be characterised by using histology, immunofluorescence (ZO-1, occludin) and transmission electron microscopy (TEM), in addition to TEER and  $P_{app}$  measurements. Further studies of inflamed hAELVi cells should evaluate whether treatment with different anti-inflammatory drugs, such as other glucocorticoids or anti-inflammatory substances (such as curcumin),<sup>86</sup> can lead to the regeneration of the inflamed alveolar epithelium. The precise signalling pathways involved in these pro-inflammatory stimuli (NF- $\kappa$ B *versus* apoptosis) have not yet been investigated in hAELVi cells. To further reproduce lung physiology *in vitro*, coculture with macrophages (e.g. THP-1M or monocyte-derived macrophages) should be considered. Kletting et al. established a coculture system of hAELVi and THP-1M cells, which can be used as a functional barrier model<sup>87</sup> and should be tested for its potential to study lung inflammation.

Future testing may include a combination of hAELVi cells with macrophage-based assays. Rather than measuring cytokine release from immune cells by ELISA (or indirectly by PCR), supernatants could be added to hAELVi cells and the presence of inflammatory cytokines evaluated by TEER and/or  $P_{app}$  measurements. This approach would reduce the complexity of the methods, as compared to coculture systems, and permit faster screening by simple *in vitro* readouts.

### **Conclusions [L2]**

The present study established a simple *in vitro* test system that simulates the impaired barrier function of the alveolar epithelium that occurs during inflammation. These cells produced a stable barrier, which is essential for *in vitro* inflammation studies and anti-inflammatory drug testing strategies. Co-stimulation with INF- $\gamma$  and TNF- $\alpha$  in the absence of HC induced a significant increase in the paracellular permeability of NaFlu. In order to optimise and standardise this *in vitro* system, further studies should investigate potential cytotoxic effects, and the minimum effective cytokine and HC concentrations.

Overall, the generation of inflamed hAELVi cells provides a first step in the development of an *in vitro* system to test new therapeutic options for the treatment of ARDS, ALI and other causes of pulmonary inflammation, thus accelerating the discovery of new therapeutics for such conditions while reducing the need for animal experiments.

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## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. However, TMR is employed by Ferring Pharmaceuticals A/S; GC was sponsored by Ferring Pharmaceuticals A/S.

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## Ethical approval

Ethics approval was not required for this research article.

## Informed Consent

Informed consent was not required for this research article.

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