Characterization of a lung/liver organ-on-a-chip model

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Abstract

Until few years ago, in vitro models could only poorly mimic the processes occurring in the human body. In drug development, the inability of in vitro models to mimic complex physiological processes was an important reason for drug withdrawals. The combination of 3D in vitro models with an engineered microenvironment, resulting in the "organ-on-a-chip" technology can potentially minimize these limitations. This technology enables the study of complex organ interactions, better simulates processes occurring in vivo and therefore can lead to better prediction of drug-associated toxicity.

With the aim of creating a model able to assess the toxicity of aerosols accurately, Philip Morris International recently developed a new lung/liver-on-a-chip device combining a bronchial tissue at the air-liquid interface with HepaRG™ liver spheroids. A specifically designed peristaltic pump allows for a continuous medium circulation and thereby enables lung-liver crosstalk. Stability of both tissues in the chip over 28 days, alone and in combination, was first evaluated. At the end of the experimental period, key liver functionalities (albumin secretion, cytochrome P450 activity) and bronchial tissue characteristics (transepithelial electrical resistance (TEER), cilia beating frequency, morphology) were comparable to control tissues that were maintained in the incubator. Using this lung/liver-on-a-chip platform, we further demonstrated the role of the liver spheroids in metabolizing and inactivating a pulmonary toxicant, aflatoxin B1. Without the presence of the liver spheroids, bronchial tissues were exposed to this compound, a severe decrease of TEER values and adenosine triphosphate content was observed, along with an increased number of apoptotic cells. Conversely, in the presence of liver spheroids, bronchial tissues were unaffected by aflatoxin B1. In parallel, specific inhibitors of enzymes involved in the metabolism of this compound were used to demonstrate the toxicity of the parent compound and their metabolites.

Our results suggested that the lung/liver-on-a-chip platform is highly valuable and relevant for future toxicological analysis.

Methods

Bronchial organotypic cultures were prepared according to the protocol from STEMCELL. Briefly, normal human bronchial epithelial (NHBE) cells (Lonza, Basel, Switzerland) were thawed and cultured in T-flask using PneumaCult™-EX PLUS medium (STEMCELL, Vancouver, Canada) at 37°C with 5% C02 and 90% relative humidity. Once the cells were 80% confluent, they were detached from the flask using trypsin-EDTA (Lonza) and 50'000 cells were seeded on a collagen-1 coated Transwell® insert (Ref. 3470, Corning®, Corning, NY, USA). Both apical and basal sides of the cultures were filled with the PneumaCult™-EX PLUS medium and maintained for three days. Subsequently, the culture was air-lifted by removing the apical medium; the basal medium was replaced with the PneumaCult™-ALI medium (STEMCELL). Tissues were used for experiments starting from day 28 after they were air-lifted.

Liver spheroids - Cryopreserved and differentiated HepaRG™ cells (ThermoFisher, Waltham, MA, USA) were thawed and seeded at approximately 12,500 cells per ultra-low-adhesion well (Corning) using the William's E medium containing Thaw, Plate & General Purpose Supplement (ThermoFisher). 96-well plates were kept in the incubator at 37°C for four days before medium replacement. On the 4th day, cells formed dense aggregates with a visible extracellular matrix confirming the formation of a spheroid. Thereafter, the cell culture medium was renewed every two to three days. Spheroids were used once mature, approximately one week after thawing.

A 2-well chip connected to a peristaltic pump was used to expose lung and liver tissues to a medium flow. The chip was first loaded with complete coculture medium, ran without tissues to fill the tubing, and incubated at 37°C to equilibrate the medium in the CO_2 incubator. Organ cultures were placed in the chip, which was then returned to the incubator for 28 days. For lung-on-a-chip experiments, two bronchial tissues were placed in each circuit. For liver-on-a-chip experiments, 48 spheroids were pooled (total of 600,000 liver cells) together in one well of each circuit. For lung-liver-on-a-chip experiments, one bronchial tissues was cocultured with a total 48 spheroids (or 600,000 liver cells). Medium was changed every two or three days. In experiments using aflatoxin B1 (AFB1)(Ref. A6636, Sigma, Saint-Louis, MO, USA), the compounds was diluted in DMSO before being diluted in the medium circulating in the chip. The AFB1 working concentration was 100 µM.

Morphology of bronchial organotypic cultures was evaluated following fixation and paraffin embedding, sectioning and staining with hematoxylin and eosin (H&E) and Alcian blue.

Transepithelial electrical resistance (TEER) was measured using an EndOhm-6 chamber (WPI) connected to an EVOM™ Epithelial Voltohmmeter (WPI, Sarasota, FL, USA), according to the manufacturer's instruction. Cilia beat frequency (CBF) measurements were conducted on bronchial tissues. Samples were first placed in a 37°C chamber, under the objective of an inverted microscope connected to a high-speed camera. 512 frames were then captured and analyzed with the SAVA software.

Cilia beating frequency (CBF) was measured using an inverted microscope (Zeiss, Oberkochen, Germany) equipped with a 4x objective, a chamber warmed at 37°C and connected to a high-speed camera (Basler AG, Ahrensburg, Germany). Short movies composed of 512 frames recorded at 120 images per seconds were analyzed using Sisson-Ammons Video Analysis (SAVA) analysis software (Ammons Engineering, Clio, MI, USA).

The metabolic capability of both culture systems was further confirmed by examining **inducibility and activity of CYP1A1/B1** using P450-Glo™ Assays (Promega) according to the manufacturer's instruction. CYP activities were induced 48 hours prior measurement by treatment of TCDD at a final concentration of 10 nM (Sigma-Aldrich).

The ATP content was measured in tissues to evaluate their viability. The ATP content in tissues was measured using the CellTiter-Glo® 3D cell viability assay (Promega, Madison, WI, USA). For liver spheroids, the tissues were first transferred into a 96-well white polystyrene microplate before a mixture of one part of CellTiter reagent for one part of culture medium was added. For 3D bronchial cultures, 150 µl CellTiter-Glo® reagent was added to the apical surface. After 30 minutes, the luminescence signal was detected using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany).

Glucose, lactate, and albumin concentrations were measured in medium collected before each medium renewal. The concentration of albumin in the medium was measured using Human Albumin ELISA Kit (Abcam) and following the manufacturer's instructions. Glucose and lactate concentration in the culture media was measured using the Biosen kit (EKF, Cardiff, United Kingdom).

Experimental design

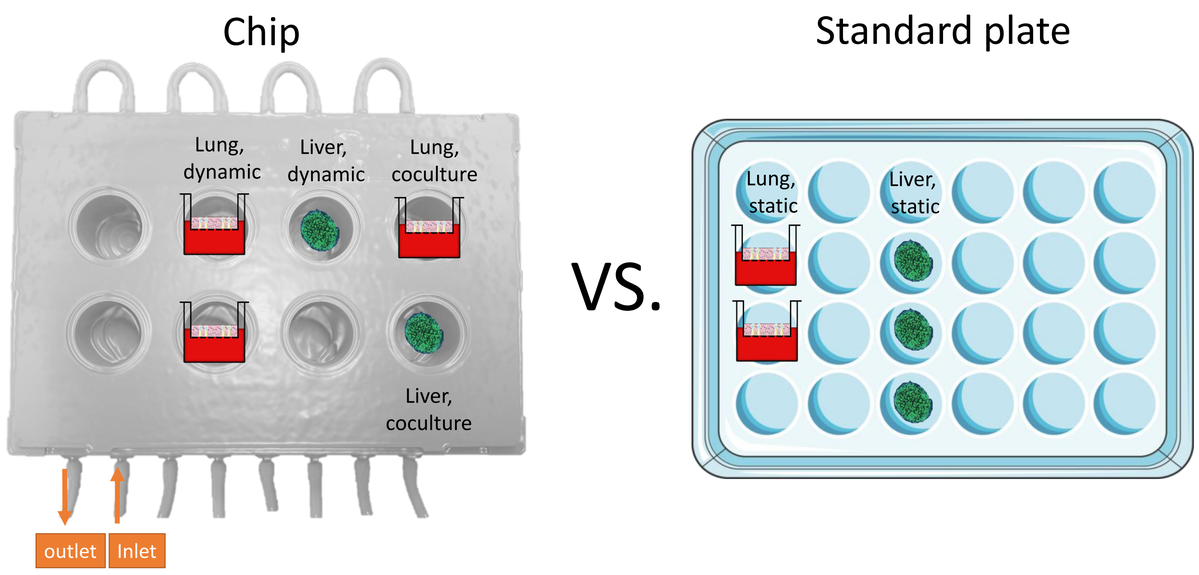

Figure 1: *Bronchial tissues and liver spheroids stability in the chip was evaluated by comparing tissues maintained in the chip and exposed to a medium flow **(dynamic)** for 28 days with tissues maintained in standard plates **(static)**. The effects of coculturing bronchial and liver spheroids tissues in the same circuit (as a **coculture**) were later evaluated comparing the results obtained when each culture type was maintained as monoculture.*

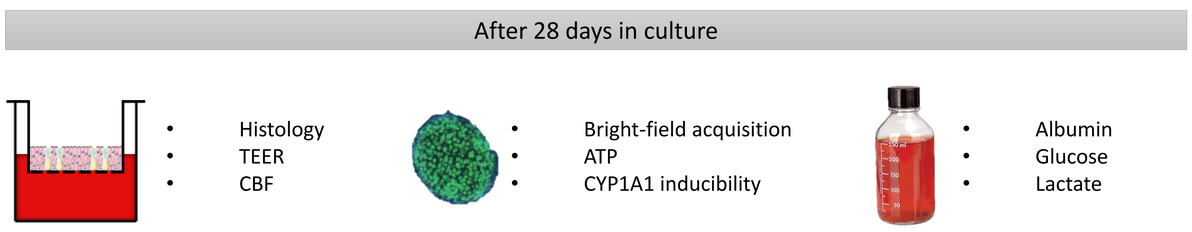

Figure 2: *Tissue characteristics were assessed after 28 days in static or dynamic conditions (both as mono- and cocultures). Bronchial tissues were evaluated based on their morphology (Histology) and functionality (TEER & CBF). Liver spheroids were evaluated based on their morphology (Bright-field acquisition), viability (ATP content) and metabolic capacity (CYP1A1 inducibility). Before every medium change, the medium was collected and analyzed for its albumin, glucose and lactate concentrations.*

Results

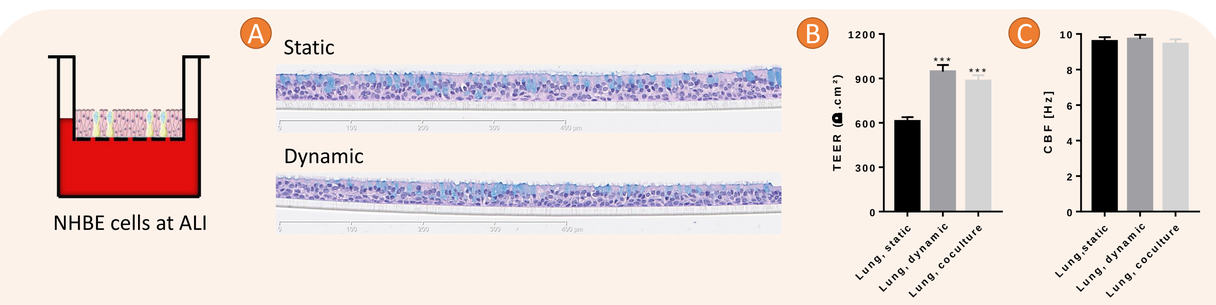

Figure 3: *Impact of medium flow on bronchial tissues as monoculture and coculture with HepaRG™ spheroids was assessed after 28 days in the chip. (A) Lung tissue morphology was evaluated at the end of the co-culture period following hematoxylin & eosin/Alcian blue staining (Objective 20x). (B) CBF and (C) TEER were measured in static, dynamic monocultured and dynamic cocultured bronchial tissues. Graphs show mean ± SEM value for a minimum of seven independent measurements. Student's t-test vs lung static, *** (p<0.0005).*

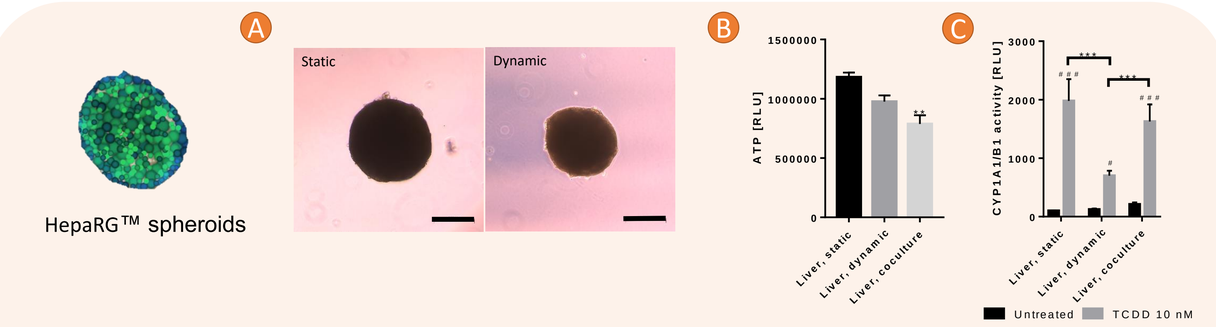

Figure 4*: Impact of medium flow on liver spheroids as monoculture and coculture with bronchial tissues was assessed after 28 days in the chip. (A) Bright-field acquisition of liver spheroids maintained in static or dynamic condition. Objective 5x. Scale bar: 200 µm. (B) ATP content and (C) CYP 1A1/1B1 basal and TCDD induced activity levels were measured in 5 replicates in static, dynamic monocultured and dynamic cocultured conditions on day 28. Data are mean ± SEM for five independent measurements. RLU: Relative light unit; TCDD: 2,3,7,8-Tetrachlorodibenzodioxin. Student's t-test ((B) vs. liver, static), * (p<0.05), ** (p<0.005) and *** (p<0.0005). Student's t-test vs untreated, # (p<0.05) and # # # (p<0.0005).*

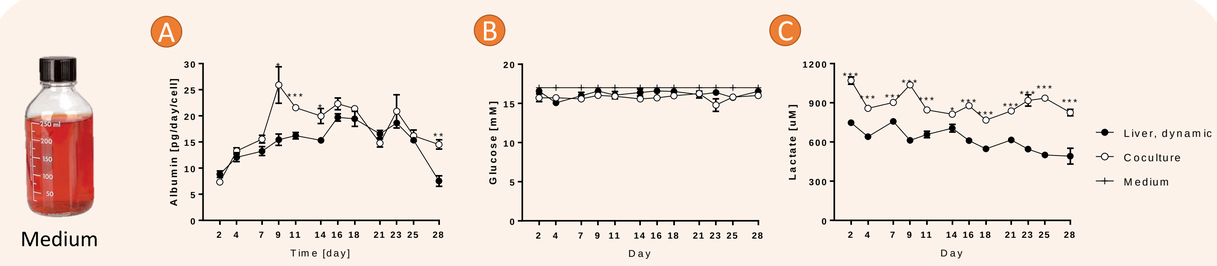

Figure 5: *Levels of a) albumin, b) glucose, and c) lactate in the media during a 28-day of culture. Measurements were performed every two or three days and compared between liver dynamic, liver coculture and medium content (glucose) . Data are mean ± SEM for four independent measurements. Student's t-test vs Liver dynamic, * (p<0.05), ** (p<0.005) and *** (p<0.0005).*

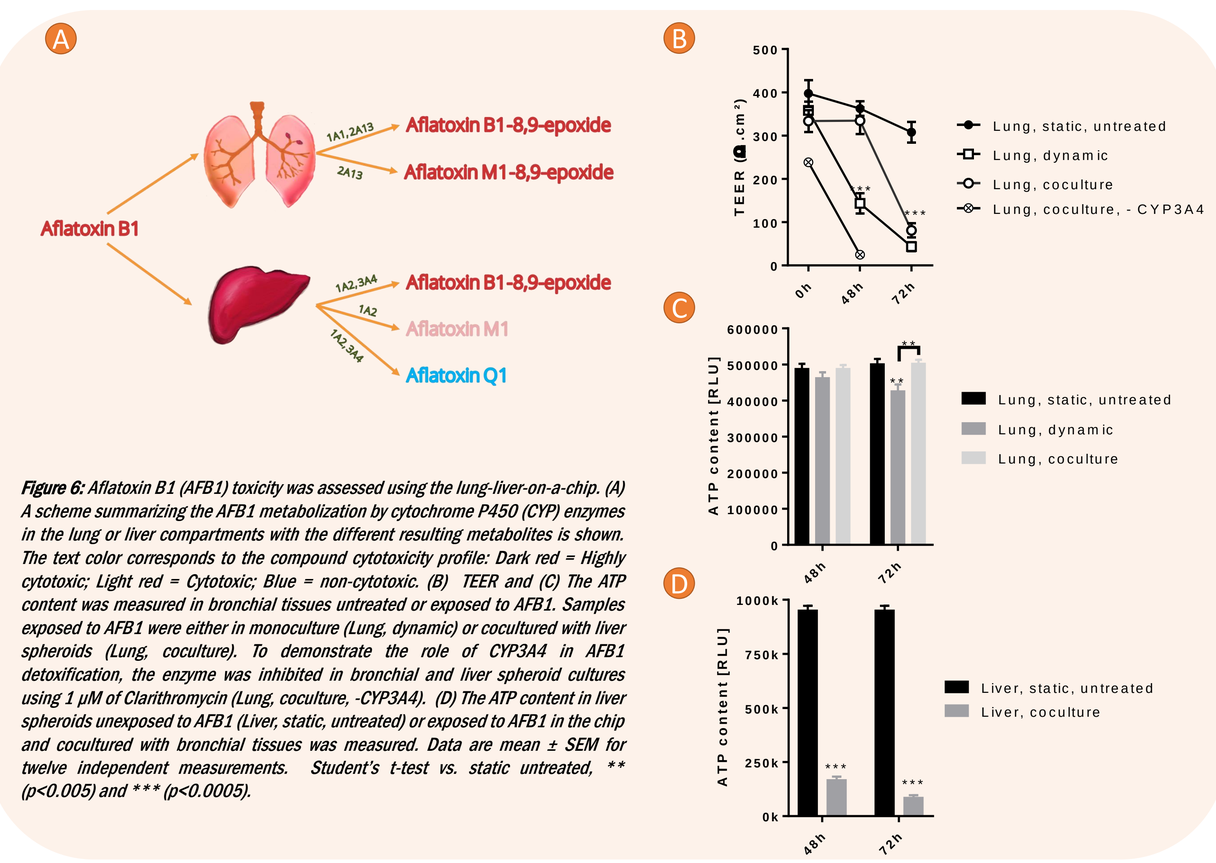

Figure 6: *Aflatoxin B1 (AFB1) toxicity was assessed using the lung-liver-on-a-chip. (A) A scheme summarizing the AFB1 metabolization by cytochrome P450 (CYP) enzymes in the lung or liver compartments with the different resulting metabolites is shown. The text color corresponds to the compound cytotoxicity profile: Dark red = Highly cytotoxic; Light red = Cytotoxic; Blue = non-cytotoxic. (B) TEER and (C) The ATP content was measured in bronchial tissues untreated or exposed to AFB1. Samples exposed to AFB1 were either in monoculture (Lung, dynamic) or cocultured with liver spheroids (Lung, coculture). To demonstrate the role of CYP3A4 in AFB1 detoxification, the enzyme was inhibited in bronchial and liver spheroid cultures using 1 µM of Clarithromycin (Lung, coculture, -CYP3A4). (D) The ATP content in liver spheroids unexposed to AFB1 (Liver, static, untreated) or exposed to AFB1 in the chip and cocultured with bronchial tissues was measured. Data are mean ± SEM for twelve independent measurements. Student's t-test vs. static untreated, ** (p<0.005) and *** (p<0.0005).*

Summary & Conclusion

To assess the potential toxicity of aerosols in the human lung tissue using in vitro approaches requires the development of a platform that simulate the crosstalk between the lung and liver tissues. Indeed, the liver is the primary organ involved in compounds metabolization forming metabolites with unknown toxicity. Here, we describe the development and characterization of a lung-liver-on-a-chip platform, which allowed long-term viability of both tissues with functionality to metabolize aflatoxin B1. In summary:

- Bronchial tissues under medium-flow condition and static condition were similar in terms of the epithelial thickness, pseudostratified organization, presence of the three differentiated cell types and cilia beat frequency (Figure 3). The TEER values under medium-flow were increased by 1.5- to 2-fold compared with those under static condition. Without any treatment, the characteristics of the bronchial tissues as monoculture and coculture were comparable.
- Liver spheroids under medium-flow condition in the chip were slightly smaller, had a 35% decreased ATP content and a 60% decreased CYP1A1 inducibility compared to spheroids in static condition (Figure 4). Coculturing with bronchial tissues partially restored their metabolic inducibility, suggesting that the bronchial tissues could have metabolized compounds present in the medium and affecting the liver spheroids.
- Analysis of the medium revealed that the flow increased significantly the production of albumin by liver spheroids (Figure 5). Glucose concentration remained high and stable over the 28 day-period. Lactate concentration in the medium was significantly higher in circuits with the cocultured tissues demonstrating the bronchial cells metabolism.
- Aflatoxin B1 was used as a test compound to demonstrate the benefits of coculturing bronchial and liver spheroid tissues to assess compound toxicity (Figure 6). Indeed, AFB1 cytotoxicity on the bronchial tissues decreased when the compound was metabolized by the liver spheroids present in the same circuit.

Together, these results demonstrate that long-term (28 days) coculturing of bronchial and liver spheroid cultures is feasible, with both tissues maintaining their key characteristics. The advantage of the chip compared with a single culture model to assess compound toxicity was demonstrated. Future experiments will test the impact of inhaled aerosols on the lung tissue with and without the presence of liver spheroids.

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Competing Financial Interest
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