Environmental Toxicology

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ENVIRONMENTAL TOXICOLOGY

RESEARCH ARTICLE OPEN ACCESS

Establishment of a Human Cell-Based In Vitro Test System for Investigating and Evaluating Cytotoxic and Immunomodulatory Effects of Indoor Mold

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Received: 27 May 2025 | **Revised:** 6 July 2026 | **Accepted:** 14 July 2026

Keywords: cytotoxicity | immunomodulation | in vitro | indoor mold | mycotoxins

ABSTRACT

Mold growth in damp indoor environments is a widespread problem associated with respiratory health effects, though causal mechanisms remain incompletely understood. We established a human cell-based in vitro test system using bronchial epithelial cells (NuLi-1) and macrophages (THP-1) to assess inhalation toxicity and immunomodulatory effects of indoor molds, focusing on cellular responses and mycotoxin patterns from contaminated building materials. The system integrates endpoints and impedance-based real-time cell viability analysis, quantification of released cytokines, and targeted mycotoxin profiling for comprehensive toxicological insights. Methanolic extracts from plasterboard, woodchip wallpaper, and malt extract agar (sterile PBS-treated or contaminated with *Alternaria botrytis*, *Aspergillus versicolor*, *Penicillium chrysogenum*, and *Stachybotrys chartarum*) were prepared via direct extraction of the material or surface swabs of the contaminated material. The system detected fungus- and material-specific effects, with pronounced cytotoxicity and immunomodulation, especially in *Stachybotrys chartarum-contaminated* samples. This material increased cytotoxicity in both tested cell types, decreased GM-CSF release from NuLi-1 cells, as well as increased interleukin-1β release from THP-1 macrophages. Therefore, mycotoxin composition and concentration were analyzed and confirmed elevated levels of the mycotoxins Roridin L2, Verrucarin J, and Stachybotrylactam. These findings reveal a substantial inhalation toxicity potential from *Stachybotrys chartarum*-contaminated indoor materials, highlighting health risks in damp environments and necessitating further in vivo and epidemiological studies.

1 | Introduction

Mold in damp indoor environments is a widespread global problem and is often discussed in the context with respiratory diseases and allergic reactions, including asthma [1]. However, the causal relationship between mold exposure and specific health effects is difficult to clarify due to differences in individual susceptibility, exposure levels, and fungal species [2]. Mold exposure can lead to three primary health effects: allergic reactions including asthma exacerbation

Abbreviations: AECBM, airway epithelial cell basal medium; ATCC, American tissue culture collection; AUC, area under the curve; DO-Na-S-S, dioctyl sodium sulphosuccinate; ECIS, electric cell-substrate impedance sensing; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; HPLC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; IC_{50}, half-maximal inhibitory concentration; IL-1β, interleukin-1beta; IL-6, interleukin-6; IL-8, INTERLEUKIN-8; LLOQ, lower limit of quantification; MRM, multiple-reaction monitoring; Pam2CSK4, Pam2CysSerLys4; PBS, phosphate buffered saline; PVDF, polyvinylidenfluorid; TMB, tetramethylbenzidine; TNF-α, tumor-necrosis-factor alpha; WT, wild type.

and rhinitis, infectious diseases particularly in immunocompromised individuals, and toxic effects caused by secondary metabolites known as mycotoxins [3]. Mycotoxins are compounds produced by species of mold genera such as *Aspergillus, Penicillium*, and *Stachybotrys*. They can cause a range of health effects depending on the level and duration of exposure. It is well known that the production of mycotoxins depends on various environmental factors such as temperature, humidity, and nutrient sources [4]. Interestingly, strong mold growth does not always correlate with high toxin production, while even small amounts of mold can sometimes produce high concentrations of mycotoxins [5]. Thus, it is difficult to estimate whether mold infestation is associated with mycotoxin contamination.

Even at low levels, mycotoxins can pose acute and chronic health risks, affecting respiratory health (e.g., inflammation, lung damage), the immune system (e.g., immunosuppression), the nervous system (e.g., neurotoxicity) and genetic stability (e.g., mutagenic effects) [6, 7].

Mycotoxins can enter the human body in three ways through (i) ingestion via contaminated food, (ii) inhalation (through airborne spores and particles), and (iii) skin contact. Indoor environments with moisture damage are particularly concerning, as bioaerosols contaminated with mycotoxins can be inhaled, leading to chronic inhalation exposure at low doses which contributes to inflammation of airways and lung diseases [8, 9]. In healthy individuals, inhaled mold particles are usually removed from the respiratory tracts via the mucociliary clearance system. In addition, alveolar macrophages play a crucial role in the uptake and clearance of mold spores [10]. However, acute and repeated exposure to mold can impair these defense mechanisms, allowing the spores and mycotoxins to adhere to epithelial cells of the respiratory tract. If internalized, spores can germinate and cause further effects, and mycotoxins can exert their toxic potential [11].

Mycotoxins can induce cytotoxic effects which can be monitored by a variety of endpoint cell viability assays [12]. Real-time monitoring of cells also offers advantages for investigating cytotoxic effects. Electric cell-substrate impedance sensing (ECIS) is a label-free method for real-time monitoring of cellular processes by measuring impedance changes of adherent cells exposed to a substrate [13]. It continuously captures dynamic alterations in cell adhesion, proliferation, and morphology without relying on chemical dyes or endpoint analyses. ECIS overcomes limitations through direct physical measurement, providing detailed kinetic profiles from acute toxicity (rapid impedance drop) to delayed effects or recovery. It is therefore ideal for toxicological screening of multi-component mixtures, delivering comprehensive data from a single experiment for detection of the cell behavior especially cell adhesion not only after but also during mycotoxin exposure. In addition to cytotoxic effects, mycotoxins can modulate the immune system and can trigger significant inflammatory responses, primarily by stimulating the release of proinflammatory cytokines. It was shown that 24 h after exposure to fungal extracts from *A. versicolor* and *S. chartarum*, mRNA expression of interleukin-1β (IL-1β), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α) genes was increased in human THP-1 macrophages [14]. In lung epithelial cells the proinflammatory cytokines interleukin-6 (IL-6) and IL-8 were released after exposure to trichothecenes type B [15].

A further cytokine involved as response to mold contamination is granulocyte macrophage-colony stimulating factor (GM-CSF). It is a pivotal cytokine in lung immunity, regulates alveolar macrophage differentiation, maturation, and homeostasis, ensuring effective surfactant clearance and pathogen defense [16]. GM-CSF activates alveolar macrophages to combat pathogens like bacteria, viruses, and fungi through enhanced recruitment, activation, and phagocytosis. It is crucial for antifungal defense, as GM-CSF deficiencies impair control of fungi such as Aspergillus [17]. Mycotoxins from molds like *Aspergillus fumigatus* can damage lung tissue and promote fungal infection; GM-CSF counters this by amplifying antifungal responses via neutrophil and macrophage activation, oxidative burst, and fungal clearance. Disrupted GM-CSF signaling increases susceptibility to mycotoxin-linked lung infections, as shown by the Aspergillus studies. These examples show that cytokine profiling is essential for mapping the immune system's response to such stimuli, as these molecules act as mediators in inflammation, immune regulation, and cell signaling.

Currently, there is no standardized in vitro test system that enables a comprehensive assessment of the health effects caused by inhalation of mold. Previous studies have examined the impact of mold spores (both active or inactive), hyphal fragments, their produced metabolites or their combinations on human and animal cell models [18, 19]. Results from animal models often do not accurately reflect human responses [20]. Therefore, to improve the relevance and predictive value of such studies, future research should prioritize the use and development of human cell-based in vitro models that more closely mimic real-life exposure conditions; for example, in the human respiratory tract [21].

The aim of this study was therefore to establish a human cell-based in vitro test system to investigate the cytotoxic and immunomodulatory effects of common fungal species in indoor environments and to compare different fungal species, materials, and sample preparation methods with regard to their inhalational toxic potential. The combination of endpoint viability assays and cytokine measurement with real-time monitoring to determine cell viability as well as mycotoxin analysis of the prepared extracts discloses more information and completes the data a bit more. The introduction of such an approach should provide researchers a better insight into the health risks associated with mold and enables a standardized assessment of indoor air quality.

2 | Materials and Methods

2.1 | Mold Samples

2.1.1 | Mold

The five mold strains were provided from the German Environment Agency's strain collection (MR strains) or purchased from the Westerdijk Fungal Biodiversity Institute (CBS strains) for the study. These include *Alternaria botrytis* (CBS 197.67; *A. botrytis*), *Aspergillus versicolor* (MR-393-17;

A. versicolor), *Penicillium chrysogenum* (CBS 306.48; *P. chrysogenum*), and two different strains of *Stachybotrys chartarum*, firstly CBS (CBS 182.80; *S. chartarum*) and secondly *Stachybotrys chartarum* wild type (MR-386-17; *S. chartarum* WT).

2.1.2 | Preparation of Spore Suspensions

Spore suspensions of the fungal strains were prepared as follows: After 14 days of cultivation at 25°C, the spores of the fungal strains were harvested from each of 50 potato extract-glucose agar plates (Thermo Fisher Diagnostics GmbH) using a sterile 0.05% (w/v) dioctyl sodium sulphosuccinate (Do-Na-S-S) solution (Sigma-Aldrich Chemie GmbH). For this purpose, the colonized agar plates were first washed with 3 mL of the Do-Na-S-S solution and wiped over their surface with an inoculation loop. The resulting suspension was then filtered through a sterile syringe filled with glass wool to remove mycelial fragments and spore aggregates. This process was repeated twice with a further 3 and 4 mL of the Do-Na-S-S solution. The glass wool was then rinsed with 2 mL of the solution. In order to concentrate the spore suspension, it was centrifuged at 3438 × g for 5 min (centrifuge 5804R, Eppendorf SE). The spore pellets were pooled and their concentration was determined using a Neubauer counting chamber. The final spore suspensions obtained were aliquoted and stored at −20°C until further use.

2.1.3 | Used Materials and Mold Growth

For preparation of mold-contaminated indoor building materials, plasterboard (Saint-Gobain Rigips GmbH) and woodchip wallpaper (Rauhfaser Classico, Erfurt & Sohn KG) were used. Malt extract agar (Carl Roth GmbH) was used as reference material for excellent growth conditions. Plasterboard and wallpaper pieces of 2 cm x 7 cm were autoclaved in sterilization bags (stericlin, Carl Roth GmbH), moistened with sterile water and placed in square petri dishes (Sarstedt AG & Co. KG). Malt extract agar was prepared in square petri dishes, from which pieces of the same size (2 cm × 7 cm) were cut out with a scalpel and also placed in square petri dishes. Spore suspensions were thawed and diluted to a concentration of 10^6 spores/mL in phosphate buffered saline (PBS; w/o: Ca and Mg, PAN-Biotech GmbH). 100 µL of the spore suspension were dropped evenly onto the surface of each piece of material resulting in 10^5 spores/material piece. As negative control, pieces of plasterboard, wallpaper and malt extract agar were treated with sterile PBS in the same way. Inoculated material pieces were placed in petri dishes and transferred into boxes filled with glass beads and sterile water to ensure a humid environment and incubated for 28 days at 25°C in the darkness. Before extract preparation it was verified that these conditions led to an evenly contaminated material surface.

2.1.4 | Preparation of Extracts From Mold-Contaminated Materials and Material Surfaces

Extracts were prepared and analyzed from two types of samples, for each a separate set of material pieces was prepared. In the first experimental approach, extracts were generated from the whole piece of material to detect mycotoxins both on the surface and within the material. For the preparation of extracts from the whole piece of material contaminated or sterile material pieces were placed together with 7 mL methanol (100%, ROTISOLV HPLC Gradient Grade, Carl Roth GmbH) in 50 mL centrifuge tubes (Sarstedt AG & Co. KG) and rotated for 60 min at 45 rpm using an overhead shaker (Trayster digital, IKA-Werke GmbH & Co. KG). The material pieces were removed and discarded. The extracts were each centrifuged twice for 10 min at 15 000 × g (centrifuge 5804R, Eppendorf SE) to remove remaining pieces of material or fragments of spores and hyphae. The supernatants were transferred to 10 mL reaction vessels (Sarstedt AG & Co. KG) and completely dried using an evaporator (Martin Christ Gefriertrocknungsanlagen GmbH) at 1500 min^{-1} and 40°C. The extracts were dissolved in 5 mL of 10% methanol/Airway Epithelial Cell Basal Medium (AECBM; with supplements), sterile filtrated using a syringe filter (ROTILA-BO Mini-Tip polyvinylidene fluoride (PVDF), 0.2 µm, Carl Roth GmbH and Injekt Solo Luer-Lock 10 mL, B. Braun SE) and stored at 4°C until further analysis. In the second experimental approach, swabs were taken from the material surfaces for determination of spore concentration and for extraction of only mycotoxins from the surface that could potentially aerosolised. Here, a sterile moistened cotton swab (Heinz Herenz Medizinalbedarf GmbH) was used to wipe repeatedly over the entire surface of the material. The cotton swab was then rinsed in 0.5 mL of sterile water in an Eppendorf reaction vessel and the spore concentration in the suspension was determined microscopically using a Neubauer counting chamber. Subsequently, 1 mL methanol was added to this spore suspension and rotated for 60 min at 45 rpm. The extracts were centrifuged once for 5 min at 15 000 × g and further processed in the same way as the extracts from the whole pieces of material.

2.2 | Cell Culture and Assays

2.2.1 | Cell Culture

Human bronchial epithelial cell line NuLi-1 (American type culture collection [ATCC]) and the human monocyte cell line THP-1 (German collection of microorganisms and cell cultures GmbH [DSMZ]), were used. NuLi-1 cells were cultivated in airway epithelial cell based medium (AECBM) supplemented with bronchial epithelial cell growth kit (AECBM complete medium, ATCC) in cell culture flasks with CellBIND surface (Corning) at 37°C and 5% CO_2. The cells were used in passages 5 to 11. THP-1 cells were cultivated in Panserin 411S medium (PAN Biotech GmbH) in cell culture flasks (Techno Plastic Products AG) at 37°C and 5% CO_2. THP-1 macrophages were used in passages 10–15.

2.2.2 | PrestoBlue Assay

Cell viability was assessed by using the resazurin based reagent PrestoBlue (ThermoFischer GmbH) [22, 23]. 1×10^4 NuLi-1 cells were seeded in black 96 well plate with CellBIND surface (Corning) and cultivated at 37°C and 5% CO_2. 4×10^4

THP-1 cells were seeded in black tissue culture-treated 96 well plates (Corning). THP-1 macrophages were resuspended in Panserin medium supplemented with 61.7 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich GmbH) for the differentiation to macrophages. 48 h after seeding and cultivation at 37°C and 5% CO_2, the medium was replaced by 200 μL of fresh medium and 100 μL of prepared extracts from contaminated materials and surfaces (1:3 dilution). After 24 h, representing a commonly used exposure time for acute cytotoxicity studies, 200 μL of cell culture supernatants were collected and stored at −80°C until further ELISA analysis. Residual medium was removed from wells and 100 μL of PrestoBlue solution diluted 1:10 in the appropriate medium was added to cells and incubated for 2 h at 37°C and 5% CO_2. Fluorescence was measured at excitation of 530 nm and emission of 610 nm. Prestoblue solution without cells served as blank and was subtracted from all measured values. Cells exposed to AECBM complete medium and 10% methanol/AECBM complete medium (solvent control) served as controls. Fluorescence values of negative control (PBS treated materials) were set to 100% cell viability. The same procedure was used for viability measurement after a 48-h impedance measurement.

2.2.3 | Electric Cell-Substrate Impedance Sensing (ECIS)

Real-time cell viability assay by impedance measurements was performed using ECIS system [24–26] (ECIS-Z Theta; Applied Biophysics) equipped with a 96-well station as described by Klar et al. [27] with minor modifications. Briefly, 1×10^4 NuLi-1 cells/well were seeded in 96 well plate (96idf10e, Applied Biophysics) which was coated with 100 μL of 60 μg/mL human collagen IV solution (Sigma-Aldrich GmbH). 48 h after cell seeding, medium was removed completely and 200 μL AECBM complete medium was added to each well. Afterwards, 100 μL of prepared extracts from contaminated materials and surfaces were added to the cells (1:3 dilution) and monitored for a further 48 h. NuLi-1 cells exposed to AECBM complete medium, 10% methanol/AECBM complete medium (solvent control), and extracts obtained from the PBS treated materials served as controls. Data were recorded at three different frequencies of 4, 16, and 64 kHz. The 16 kHz data were used to calculate and evaluate the impedance, which represents a measure of real-time cell viability. Additionally, after impedance measurement, cell viability was determined using the PrestoBlue assay. For data analysis, the background signal of AECBM complete medium without cells was first subtracted from all measured values. Then, all values were normalized by dividing the values from each time point by the initial value directly after extract exposure ($t = 0$). The normalized values were plotted against time. The area under the curve (AUC) was determined for quantification using GraphPad Prism (version 9.3.1).

2.2.4 | Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of released cytokines from NuLi-1 and THP-1 macrophages was quantified by ELISA [28, 29].

2.2.5 | Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) ELISA

The ELISA MAX Standard Set Human GM-CSF kit (Biolegend) was used to determine the concentration of GM-CSF after extract exposure and performed according to the manufacturer's instructions. As positive control, a 3.33 ng/mL (2.6 nM) synthetic diacylated lipopeptide Pam2CysSerLys4 (Pam2CSK4) solution (InvivoGen) was used.

2.2.6 | Interleukin-1β (IL-1β) ELISA

For determination of IL-1β concentration an in-house established ELISA protocol was used. At first, microtiter plates were coated with a human primary IL-1β antibody (MAB601, R&D Systems) overnight at 2°C–8°C and blocked with 2% bovine serum albumin (Sigma-Aldrich/Merck KGaA) for 2 h at room temperature. Subsequently, the standard concentration series (0 to 500 pg/mL IL-1β) was added to the wells in duplicates and the extract samples were added to the wells in triplicates and incubated for 45 min at 37°C. This was followed by incubation (45 min, 37°C) with a secondary biotinylated human IL-1β antibody (BAF201, R&D Systems), which binds to a site on the target protein. For the detection of the enzyme complex, a streptavidin-horseradish peroxidase-coupled antibody (streptavidin-HRPO; Jackson ImmunoResearch Laboratories), which binds to the biotin of the second antibody, was subsequently incubated for 30 min in the dark and room temperature. To detect the enzyme complex, the colorless substrate tetramethylbenzidine (TMB, SeramunBlau fast2, Seramun Diagnostica GmbH) was added for 10 min, which converted the substrate to a blue-colored solution in the presence of the peroxidase. The color change was stopped by adding sulfuric acid (2 N, Carl Roth GmbH) and the absorbance was measured at 450 and 630 nm. Between all incubation steps, the microtiter plates were washed several times with a wash solution consisting of 0.1% Tween20 (Carl Roth GmbH) and PBS. As positive control for IL-1β, a 1.67 μg/mL (2.2 μM) nigericin solution (InvivoGen) and a 3.33 μg/mL lipopolysaccharide (LPS) solution (Sigma-Aldrich GmbH) were used.

For data analysis, Gen5 software (version 3.14) was used. The standard curves of GM-CSF and IL-1β were fitted using a 4-parameter logistic regression enabling the concentrations of the cytokines in the extract samples to be calculated.

IL-6, IL-8, and TNF-α were quantified using the human ELISA MAX Standard Sets from Biolegend according to manufacturer's instructions.

2.3 | Mycotoxin Analysis

2.3.1 | Mycotoxins

Roridin L2 and Stachybotrylactam were purchased from Cayman Chemical, diluted in LC/MS grade methanol (Fisher Scientific GmbH) and stored at −20°C. Verrucarin J was

TABLE 1 | Substance-specific parameters for HPLC-MS/MS analysis of mycotoxins.

Mycotoxin	Precursor ion (m/z)	Product ions (m/z)	Declustering-Potential (V)	Colission energy (eV)
Roridin L2	531 [M + H] +	249.1	71	33
		231	71	33
Verrucarin J	502.1 [M + NH_4] +	343.2	51	25
		249.1	51	27
Stachybotrylactam	386.3 [M + H]+	178.2	131	51

purchased from MedChemExpress, diluted in DMSO (Sigma-Aldrich) and stored at −80°C.

2.3.2 | High-Performance Liquid Chromatography–Tandem Mass Spectrometry (HPLC-MS/MS) Analysis

At a later time point than the viability and ELISA measurements, mycotoxins were analyzed in extracts from *S. chartarum* contaminated materials by high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) using a Shimadzu Nexera XR system that was coupled to a Sciex QTRAP 5500 triple quadruple mass spectrometer. Therefore, a previously reported method was adapted [30]. Chromatographic separation was carried out on a Kinetex Biphenyl column (100 mm × 2.1 mm, 2.6 μm, 100 Å). Water (mobile phase A) and methanol (mobile phase B) both containing 1 mM ammonium acetate and 0.1% acetic acid were used as eluents with a flow rate of 0.45 mL/min. A solvent gradient was used as follows: 0–2 min, 2% B; 2–5 min, 2%–80% B; 5–5.2 min, 80%–98% B; 5.2–8 min, 98% B; 8–11 min, 2% B. The column oven was set to 40°C. The substance-specific parameters used for multiple-reaction monitoring (MRM) measurement are presented in Table 1. The following source parameters were applied: ion spray voltage (ESI+), 5500 V; temperature, 500°C; nebulizer gas, 50 psi; heating gas, 80 psi; curtain gas, 35 psi; and collision gas (nitrogen). Analyst (version 1.6.2) and MultiQuant (version 3.0.1), both provided by Sciex, were used for data acquisition and processing. For each sample series, a calibration curve was created. The concentration of the lowest calibration point was used as the lower limit of quantification (LLOQ). Samples were considered positive if the mycotoxin concentration was within the calibration range or test criteria (qualifier-ion-to-quantifier-ion ratio of ±20% and a signal-to-noise ratio of the qualifier ion of 10) were fulfilled. Extracts of the PBS treated materials were used as negative control.

2.4 | Half-Maximal Inhibitory Concentration (IC_{50}) Values

To analyze the dose-effect response, mycotoxin solutions of Roridin L2 (0 to 50 μM), Verrucarin J (0–100 nM) and Stachybotrylactam (0–125 μM) were exposed to NuLi-1 cells and THP-1 macrophages at different concentrations for 24 h and cell viability was analyzed by PrestoBlue assay. Mycotoxin solutions were prepared in 10% methanol/AECBM complete medium according to the solvent of extracts. Cells exposed to 10% methanol/AECBM complete medium served as control and were set to 100% cell viability. The relative IC_{50} values [31] were determined by fitting the curve with 4-parameter logistic regression (variable slope) using GraphPadPrism (version 9.3.1).

2.5 | Statistical Analyses

All analyses were performed in triplicates and three independent experiments were performed. Mycotoxin concentrations below LLOQ were assigned the value half of the LLOQ if the majority of the data set could be quantified. All data were tested for normal distribution. For statistical analysis, the groups were compared either with the PBS treated control group or the groups were compared with each other using the non-parametric Kruskall-Wallis test with Dunn's post hoc test. A $p < 0.05$ indicates statistical significance to extracts from PBS treated materials. Statistical analysis was performed using Graph Pad Prism (version 9.3.1).

Most data are presented in box and whisker plots (boxplots). The boxes represent the 25/75 percentile, the horizontal line in the middle of the box indicates the median (50 percentile) values, and the boundaries of the whiskers mark the minimum and maximum values. Dotted lines indicate the mean values of the control (exposure with PBS).

3 | Results

A human cell-based test system was developed in which extracts from mold-contaminated materials were prepared and tested on lung epithelial cells NuLi-1 and THP-1 macrophages. To detect cytotoxic effects, cell viability, particularly metabolic activity, was analyzed using PrestoBlue assay as well as impedance measurements as a real-time measure were carried out. To investigate immunomodulatory effects, the concentration of the released cytokines GM-CSF from NuLi-1 cells and IL-1β from THP-1 macrophages was determined. In preliminary investigations, it was shown that the baseline levels of further proinflammatory cytokines (IL-6 and IL-8) were very high in NuLi-1 cells. However, the cause is currently unknown, and there are no published references regarding this specific cell line. This makes it difficult to detect differences, which is why these cytokines were not included in the study and its role cannot be known yet. In THP-1 macrophages, the cytokines IL-6 and TNF-α were also analyzed after exposure to selected extracts but could not be detected in the supernatants (data not shown).

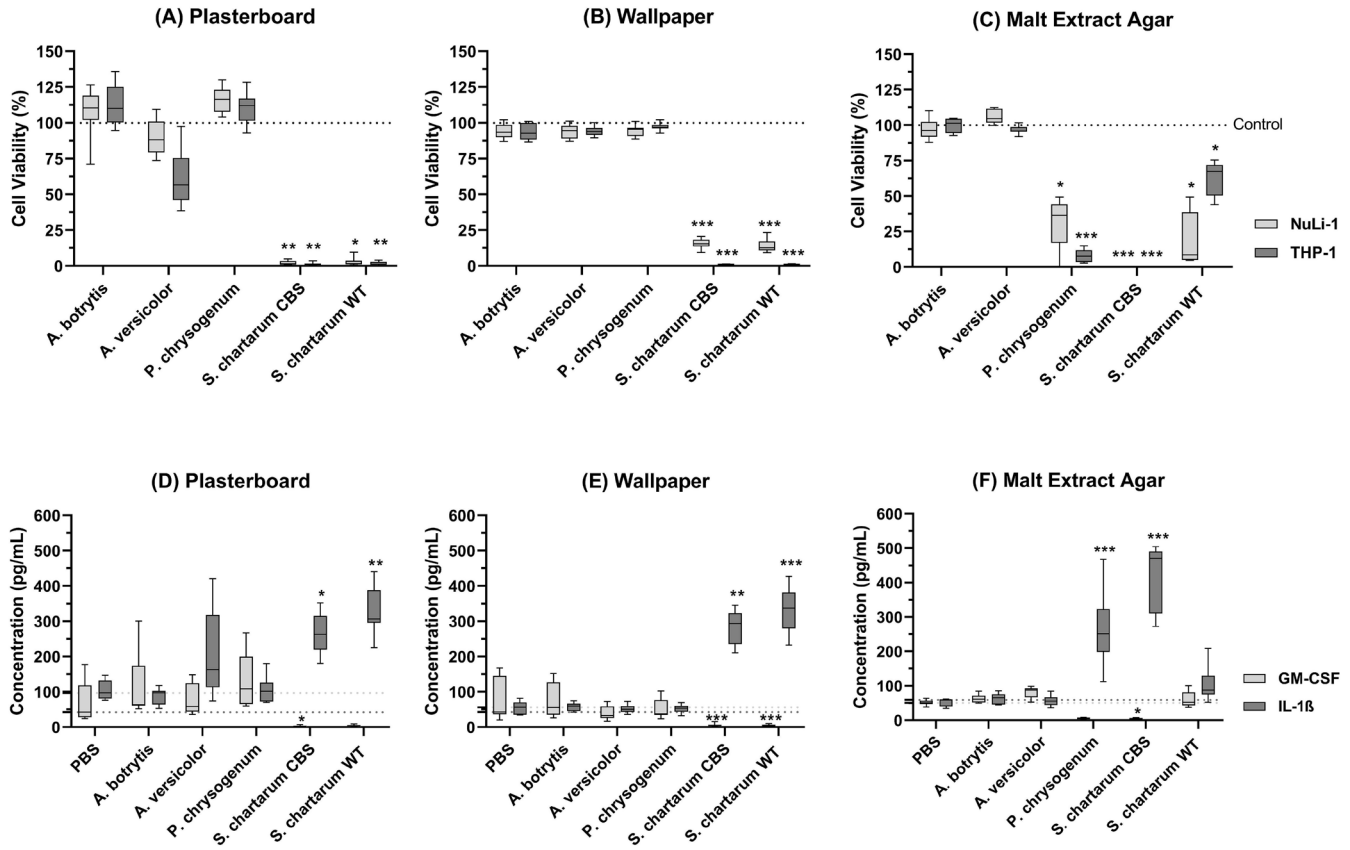

FIGURE 1 | Cytotoxic and immunomodulatory effects of extracts from contaminated materials depending on the fungal species. Cell viability of NuLi-1 cells (light gray) and THP-1 macrophages (dark gray) after exposure to extracts from contaminated (A) plasterboard, (B) wallpaper, (C) malt extract agar; Cytokine concentrations (GM-CSF, IL-1β) in cell culture medium after exposure to extracts from contaminated (D) plasterboard, (E) wallpaper, (F) malt extract agar; dashed line indicate either the cell viability (A–C) or the cytokine concentration (D–F) of extracts from PBS-treated materials (control); $n = 9$; data are presented in boxplots *$p < 0.05$ indicates statistically significance to extracts from PBS-treated materials, **$p < 0.01$, ***$p < 0.001$.

3.1 | Analysis of Extracts From Contaminated Materials

3.1.1 | Cell Viability and Cytokine Release

To assess the cytotoxic and immunomodulatory effects of different fungal species, cell viability and cytokine release (GM-CSF; IL-1β) were analyzed in human lung epithelial cells (NuLi-1) and macrophages (THP-1). Depending on investigated fungal species and contaminated material, in vitro experiments showed that methanolic extracts resulted in different effects on cell viability. Exposure to *S. chartarum* (CBS and WT) contaminated material extracts led to a significant reduction in cell viability after 24 h exposure, particularly in THP-1 macrophages, across all substrate types (Figure 1A–C). The strongest cytotoxic effects were observed with plasterboard-derived extracts. NuLi-1 cells also showed decreased viability in response to these extracts, although the effect was less pronounced than in THP-1 macrophages. A cytotoxic effect caused by the extracts from the PBS-treated plasterboard can be excluded because the cell viability of these extracts and the cell culture medium used were similar. In contrast, *A. botrytis* and *A. versicolor* contaminated material extracts did not significantly affect cell viability in either cell line. The extracts from the *P. chrysogenum*-overgrown plasterboard and wallpaper also showed no reduction of cell viability on the cells. However, extracts prepared from overgrown malt extract agar led to a statistically significant reduction in cell viability for NuLi-1 cells and THP-1 macrophages.

The analysis of cytokine release also showed fungal- and material-dependent differences. Compared to the extracts from the control materials, extracts from the materials contaminated with *A. botrytis* and *A. versicolor* showed no significant change in the cytokine concentration (Figure 1D–F). The same was observed for the extracts from the *P. chrysogenum*-contaminated plasterboard and wallpaper. However, exposure to extracts from the *P. chrysogenum*-contaminated malt extract agar resulted in a statistically significant inhibition of GM-CSF release from the NuLi-1 cells and a statistically significant higher IL-1β release from the THP-1 macrophages compared to the control. Exposure to the extracts from materials contaminated with both *S. chartarum* strains led to a statistically significant inhibition of GM-CSF release and a statistically significantly higher IL-1β release. However, the malt extract agar extracts from *S. chartarum* WT did not result in reduced GM-CSF release from NuLi-1 cells although cell viability was decreased. Analogous to the lower viability reduction, the extracts from malt extract agar contaminated with *S. chartarum* WT showed a lower IL-1β release than the extracts

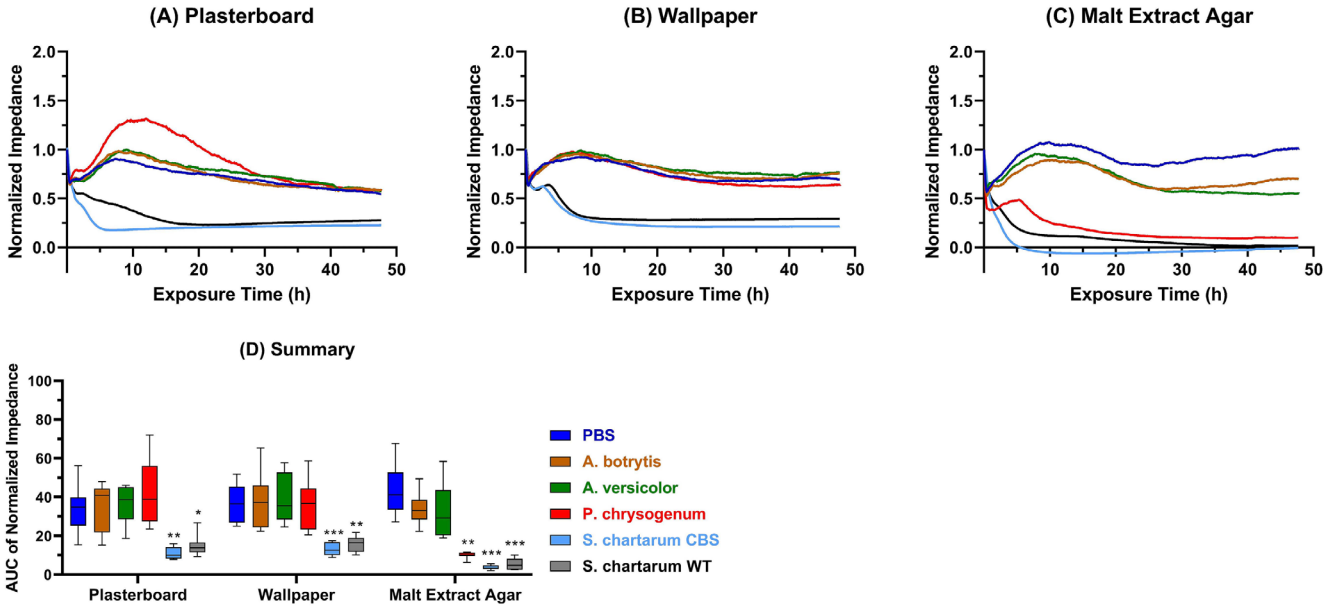

FIGURE 2 | Impedance measurements of NuLi-1 cells after exposure (48 h) to extracts from contaminated materials depending on fungal species. Normalized impedance courses of extracts from contaminated (A) plasterboard, (B) wallpaper, (C) malt extract agar (*Alternaria botrytis* [brown], *Aspergillus versicolor* [green], *Penicillium chrysogenum* [red], *Stachybotrys chartarum* CBS [light blue], *S. chartarum* WT [gray], control [dark blue]); (D) area under the normalized impedance curve; $n = 9$; data are presented in boxplots $^{*}p < 0.05$ indicates statistically significance to extracts from PBS-treated materials (control), $^{**}p < 0.01$, $^{***}p < 0.001$.

from malt extract agar contaminated with *S. chartarum* CBS. The results demonstrate that a reduction in cell viability is associated with a reduced GM-CSF release in NuLi-1 cells and with an increased IL-1β release in THP-1 macrophages. The results suggest that a reduction in cell viability may be associated with a reduced GM-CSF release in NuLi-1 cells and with an increased IL-1β release in THP-1 macrophages for extracts from malt extract agar contaminated with *S. chartarum* and *P. chrysogenum*. However, the absence of significant changes for *P. chrysogenum*-contaminated plasterboard and wallpaper indicates that this association is material- and extract-dependent and not a general rule.

Impedance-based measurements were also carried out with the extracts from contaminated materials to investigate the development of the cell behavior in real time. The impedance curves and the AUCs determined are shown in Figure 2 and reflect the results of the PrestoBlue assay (Figures 1A–F and S2). After exposure with extracts obtained from *P. chrysogenum* grown on plasterboard, the impedance first increased for up to 10 h and then slowly decreased with up to 30 h on a signal level similar to the control, reflecting morphological changes. Following exposure to the extracts from *P. chrysogenum* grown on malt extract agar, the impedance firstly decreased compared to the control. Afterwards, an increase in impedance was recorded up to around 6 h representing a little recovery process, followed by a continuous decrease over a period of 30 h resulting in decreased cell viability similar to *S. chartarum*. The extracts of all materials contaminated with both *S. chartarum* strains led to a decrease in impedance even within the first 10 h. In addition, in experiments with extracts obtained from contaminated plasterboard and malt extract agar, the impedance and thus the cell viability decreased faster for the extracts of *S. chartarum* CBS than for the extracts of *S. chartarum* WT contaminated material.

3.1.2 | Mycotoxin Composition

To explain the cytotoxic and immunomodulatory effects, the extracts from the materials contaminated with *S. chartarum* and *P. chrysogenum* were analyzed for mycotoxin composition and concentration in comparison to extracts from PBS-treated materials as control. For *P. chrysogenum*, no relevant mycotoxins could be detected. For *S. chartarum*, three mycotoxins were detected and quantified: Roridin L2, Verrucarin J, and Stachybotrylactam. No mycotoxins were detected in the extracts of the PBS-treated materials (negative control; data not included in the graph). Differences in mycotoxin concentrations were observed between the extracts of both *S. chartarum* strains from contaminated malt extract agar (Figure 3). The extracts of *S. chartarum* CBS showed more positive samples for Roridin L2 and higher concentrations for Verrucarin J compared to the extracts of *S. chartarum* WT contaminated material, in which no Roridin L2 and Verrucarin J could be quantified. For Stachybotrylactam, the number of positive samples was lower in the extracts of the CBS strain than in the WT strain. These results indicate a main responsibility of Roridin L2 and Verrucarin J for the cytotoxic and immunomodulatory effects observed in extracts of *S. chartarum* contaminated materials. In contrast, Stachybotrylactam appears to play a minor role.

3.2 | Analysis of Extracts From Surface Swabs of Contaminated Materials

3.2.1 | Cell Viability and Cytokine Release

Extracts from surface swabs of materials contaminated with *A. versicolor*, *P. chrysogenum*, and *S. chartarum* CBS showed a cytotoxic and immunomodulatory potential similar to that of

the extracts from the contaminated material pieces. A statistically significant reduction in cell viability was demonstrated for extracts from surface swabs of *P. chrysogenum*-contaminated malt extract agar and for all extracts from *S. chartarum* CBS-contaminated materials (Figure 4A–C). However, the extracts from surface swabs of *S. chartarum CBS*-contaminated malt extract agar showed a weaker loss in viability compared to the extracts from plasterboard and wallpaper, which is also reflected in the lower immunomodulatory effects (Figure 4D–F). Furthermore, for the surface swab extracts, reduced cell viability is associated with lower GM-CSF release from NuLi-1 cells and higher IL-1β release from THP-1 macrophages.

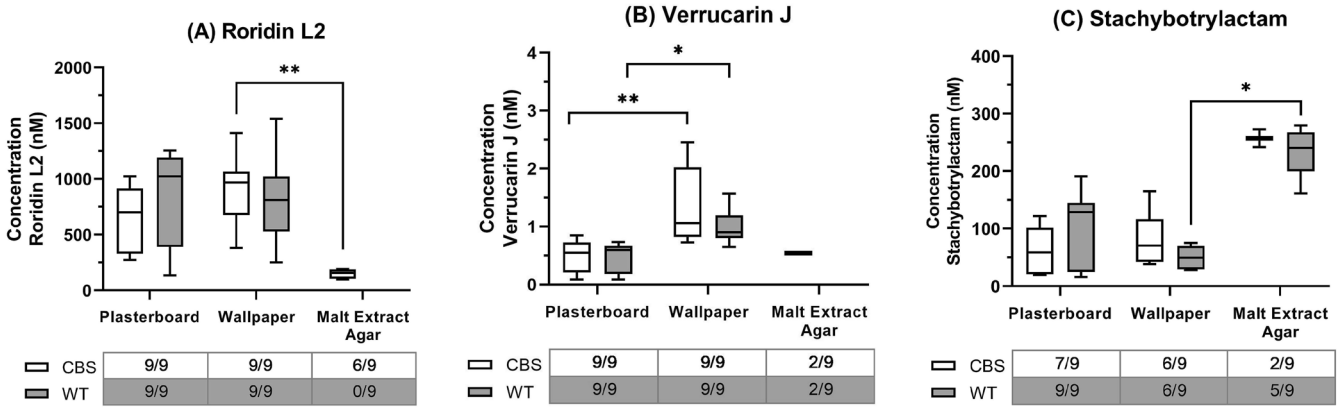

FIGURE 3 | Mycotoxins detected in extracts of *Stachybotrys chartarum* CBS and *S. chartarum* WT contaminated materials. Concentration and number of mycotoxin-positive samples of (A) Roridin L2, (B) Verrucarin J and (C) Stachybotrylactam; extracts of the PBS-treated materials were used as negative control containing no mycotoxins; $n = 9$; data are represented in boxplots. *$p < 0.05$ indicates statistically significance between the material-groups, **$p < 0.01$.

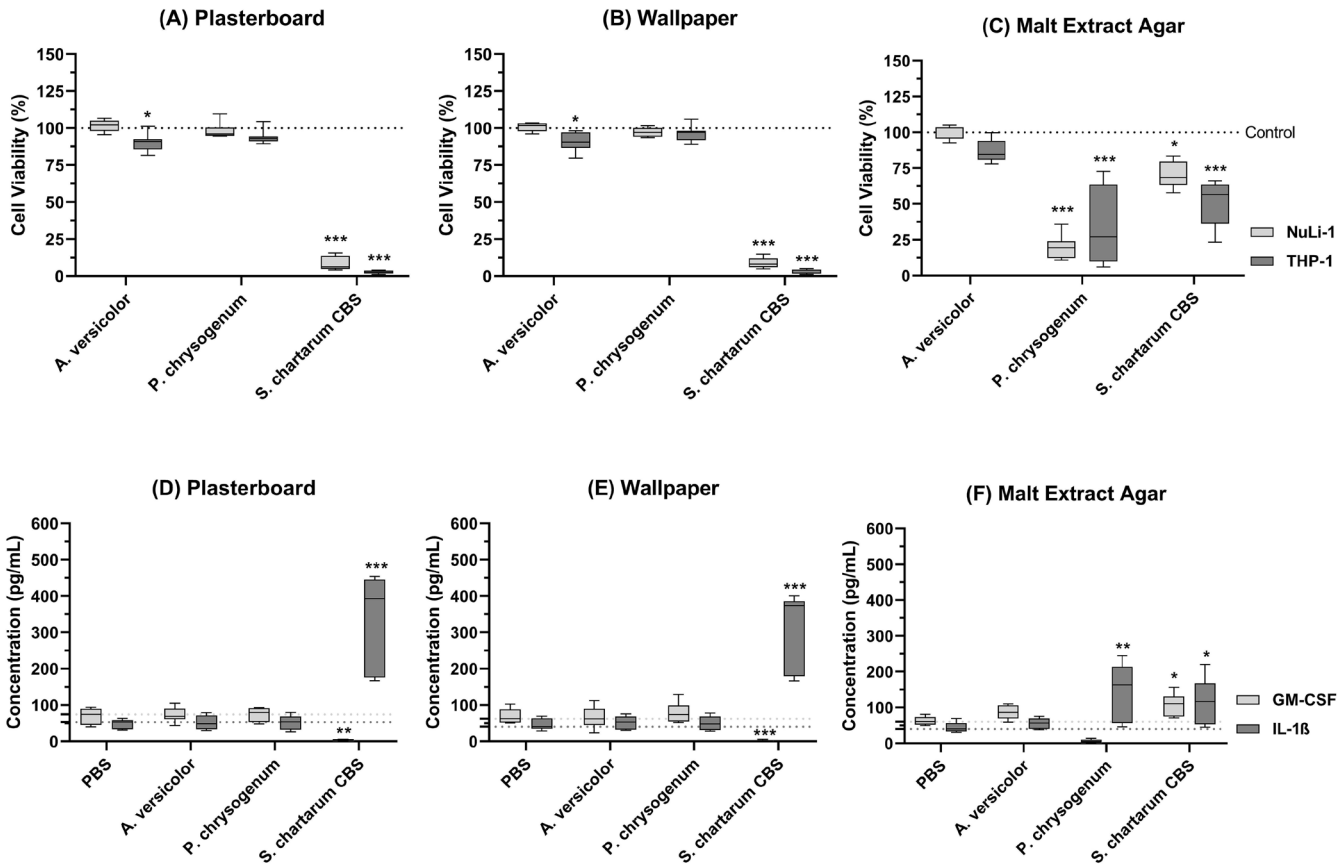

FIGURE 4 | Cytotoxic and immunomodulatory effects of extracts from surface swabs of contaminated materials depending on fungal species. Cell viability of NuLi-1 cells and THP-1 macrophages after exposure to extracts from contaminated (A) plasterboard, (B) wallpaper, (C) malt extract agar; Cytokine concentration (GM-CSF, IL-1β) in cell culture medium after exposure to extracts from contaminated (D) plasterboard, (E) wallpaper, (F) malt extract agar; $n = 9$; data are presented in boxplots *$p < 0.05$ indicates statistical significance to extracts from PBS-treated materials (control), **$p < 0.01$, ***$p < 0.001$.

The spore concentration from the surface swabs was determined and showed significant increased sporulation of *P. chrysogenum* and decreased sporulation of *S. chartarum* CBS on malt extract agar compared to the respective plasterboard and wallpaper samples (see Figure S1).

Material-dependent differences between the extracts from surface swabs of *S. chartarum*-contaminated malt extract agar and plasterboard or wallpaper could also be visualized in impedance measurements. In comparison to the decreasing curve progression for the extracts from the plasterboard and wallpaper, the curve for the extracts of the malt extract agar initially increases and then decreases again, indicating a lower cytotoxic potential for these extracts (Figures 5 and S3).

3.2.2 | Mycotoxin Composition

The mycotoxins Roridin L2, Verrucarin J, and Stachybotrylactam were also detected in the extracts from the surface swabs of *S. chartarum*-contaminated materials. Differences in mycotoxin concentrations were observed in the extracts from different materials (Figure 6). No mycotoxins were detected in the extracts of the PBS-treated materials

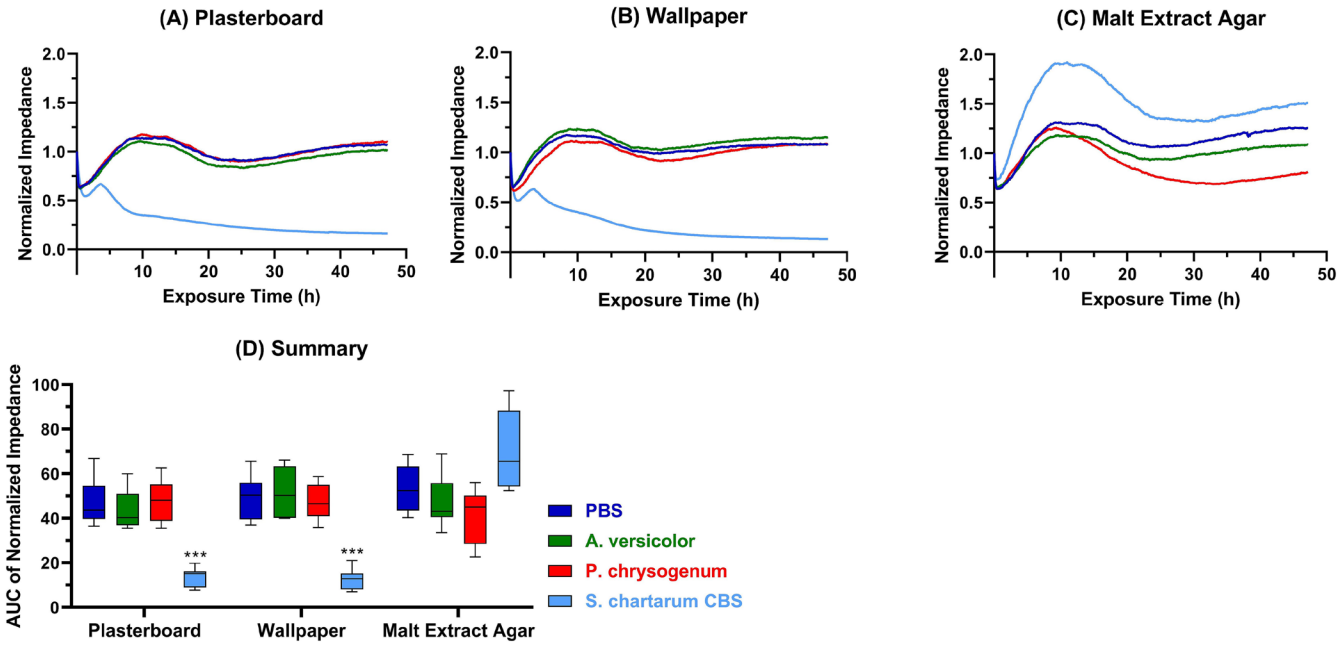

FIGURE 5 | Impedance measurements of NuLi-1 cells after exposure (48 h) to extracts from surface swabs of contaminated materials depending on the fungal species. Normalized impedance courses of extracts of contaminated (A) plasterboard, (B) wallpaper, (C) malt extract agar (*Aspergillus versicolor* [green], *Penicillium chrysogenum* [red], *Stachybotrys chartarum* CBS [light blue], control [dark blue]); (D) area under the normalized impedance curve; $n = 9$; data are presented in boxplots ***$p < 0.001$ indicates statistically significance to extracts from PBS-treated materials (control).

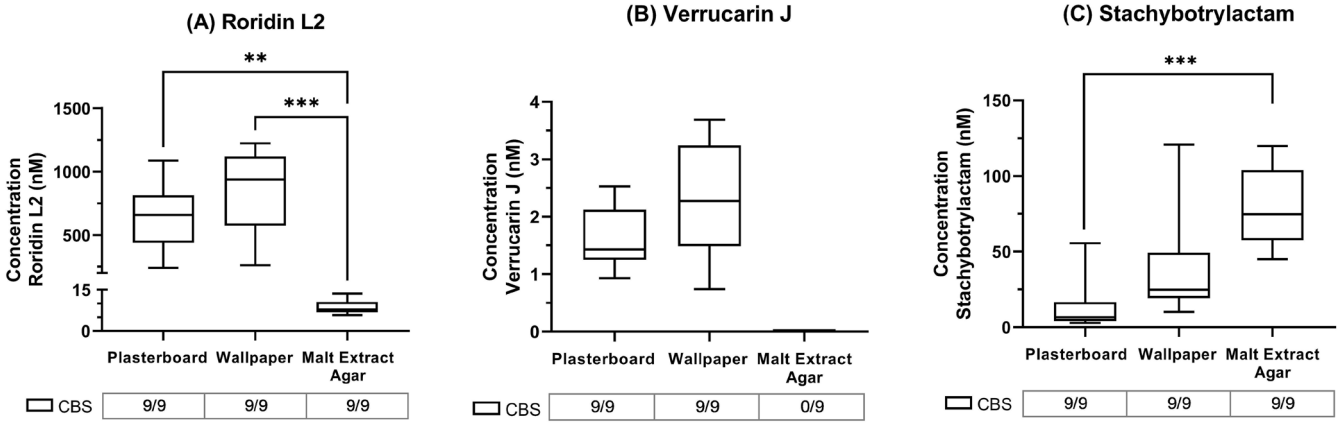

FIGURE 6 | Mycotoxins detected in the extracts from surface swabs of *Stachybotrys chartarum* CBS contaminated materials. Concentration and number of mycotoxin-positive samples of (A) Roridin L2, (B) Verrucarin J and (C) Stachybotrylactam; extracts of the PBS-treated materials were used as negative control containing no mycotoxins; $n = 9$; data are presented in boxplots indicates statistical significance between the material groups, **$p < 0.01$, ***$p < 0.001$.

(negative control; data not included in the graph). The concentrations of Roridin L2 and Verrucarin J were lower in extracts from *S. chartarum-contaminated* malt extract agar than in extracts from plasterboard and wallpaper, which could explain the lower viability loss observed after exposure to these extracts. In contrast, Stachybotrylactam was more present in the extracts from malt extract agar. These results support the assumption that Roridin L2 and Verrucarin J are primarily responsible for the cytotoxic and immunomodulatory effects of extracts obtained from *S. chartarum* extracts and that Stachybotrylactam plays only a minor role.

3.3 | Dose–Response Effects

To compare the cytotoxic potential of the three detected mycotoxins for cells, dose–response curves were generated and the corresponding IC_{50} values were calculated (Figure 7A–C). Verrucarin J has the lowest IC_{50} value in the nM range reflecting high cytotoxic potential. The IC_{50} value of Roridin L2 is in the lower μM range. In contrast, Stachybotrylactam shows a relatively high IC_{50} value in the two-digit μM range. The results demonstrate the different potential of mycotoxins and that the type and concentration are crucial for the evaluation of their cytotoxic and immunomodulatory effect.

4 | Discussion

This study aimed to establish a human cell-based test system to investigate and evaluate cytotoxic and immunomodulatory effects of common indoor fungal species. The test system was able to detect differences between different fungal species and materials. Reduced cell viability and altered cytokine release were observed for all extracts from the contaminated materials with *S. chartarum* CBS and *S. chartarum* WT. Mold can cause cytotoxic effects through the production of mycotoxins, proteases, and other by-products, with mycotoxins of high relevance concerning human health [32]. Depending on its chemotypes, *S. chartarum* is known to produce mycotoxins or metabolites of three main groups: macrocyclic trichothecenes, phenylspirodrimanes, and atranones [33–35]. *S. chartarum* chemotype S produces macrocyclic trichothecenes and phenylspirodrimanes, chemotype A mainly produces atranones and phenylspirodrimanes, and chemotype H produces phenylspirodrimanes but no macrocyclic trichothecenes [34–36]. In the extracts analyzed, both Roridin L2 and Verrucarin J, which belong to the macrocyclic trichothecenes, and the phenylspirodrimane Stachybotrylactam were detected. This demonstrates that the analyzed *S. chartarum* strains belong to the chemotype S.

Macrocyclic trichothecenes are highly toxic against eukaryotes, as they inhibit protein biosynthesis by irreversible binding to the peptidyl transferase centre of the 60S ribosomal subunit [37]. For human macrophages it was shown that exposure to Satratoxin positive *S. chartarum* spores as well as individual trichothecenes leads to the activation of caspase-3, which is involved in apoptosis, and represents a possible mechanism of action for the cytotoxic effects in THP-1 macrophages [38]. However, phenylspirodrimanes are less toxic than macrocyclic trichothecenes and their IC_{50} values are in the two-digit micromolar range [33, 39, 40]. This is consistent with our investigations of Stachybotrylactam and confirms the assumption that the cytotoxicity is primarily due to the mycotoxins Roridin L2 and Verrucarin J. However, combinatorial effects between the different mycotoxin groups cannot be excluded.

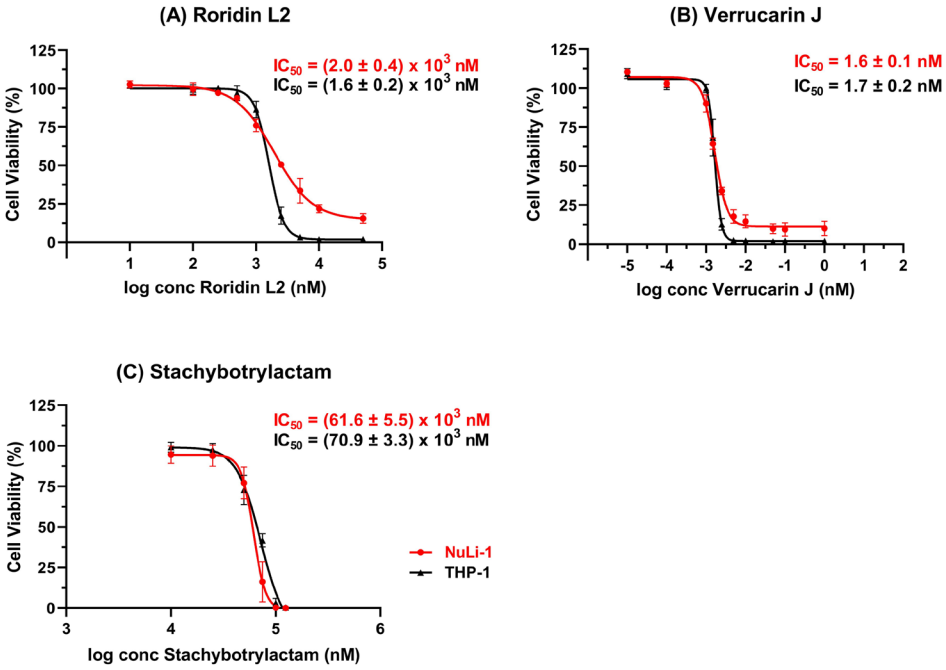

FIGURE 7 | Dose–response curves of Roridin L2, Verrucarin J and Stachybotrylactam. Cell viability in NuLi-1 cells (red) and THP-1 macrophages (black) after exposure to different concentrations of (A) Roridin L2, (B) Verrucarin J and (C) Stachybotrylactam with calculated relative IC_{50} values; $n = 3$; data are presented as mean ± standard deviation.

Furthermore, differences in cytotoxicity between the two *S. chartarum* strains were also detected for extracts from contaminated materials. The extracts from *S. chartarum* CBS contaminated malt extract agar caused a higher and faster reduction in cell viability than the extracts from *S. chartarum* WT. This was observed in the PrestoBlue assay as well as in the impedance measurements. Differences in the cytotoxicity of various fungal strains are already known [18]. Kováčiková et al. [41] investigated the cytotoxic effects of four different *S. chartarum* isolates and showed that all strains caused cytotoxic effects, but to a different extent. The different cytotoxic effects between the strains could be caused by different mycotoxin production [42]. Consequently, the higher number of positive samples for Roridin L2 and the higher Verrucarin J concentrations reflect the more rapid decrease and lower final level of cell viability caused by extracts of *S. chartarum* CBS contaminated malt extract agar compared to the extracts of *S. chartarum* WT.

In addition to the differences observed between the various fungal species and strains, differences in cytotoxicity were also found depending on the materials. This was particularly obvious for the extracts from surface swabs of contaminated material inoculated with *S. chartarum* CBS. The extracts prepared from contaminated malt extract agar showed lower cytotoxic effects than the extracts from contaminated plasterboard and wallpaper. It has already been shown that the type and quantity of mycotoxin production depends on the substrate [42–44]. Aleksic et al. [45] investigated the formation of four *S. chartarum* mycotoxins on different building material (glass fiber, painted glass fiber wallpaper, wallpaper, vinyl wallpaper, fir). They showed that mycotoxin formation was clearly the highest on wallpaper, while there was no mycotoxin formation on vinyl wallpaper due to a lack of fungal growth. *S. chartarum* grows preferentially on organic, mainly cellulose-containing materials, such as plasterboard, wallpaper or straw [36, 46, 47]. Therefore, more spores were formed on these contaminated materials than on the contaminated malt extract agar. The chemical composition of different materials or substrates offers a broad spectrum of nutrients and thus different energy states for the growth of microorganisms, which also influences mycotoxin production [9].

Extracts that caused reduced cell viability were associated with reduced or absent release of GM-CSF concentrations from NuLi-1 cells and increased release of IL-1β concentrations from THP-1 macrophages. In addition to their cytotoxic effects, macrocyclic trichothecenes also have immunomodulatory effects. The reduced GM-CSF release from NuLi-1 cells could be due to the inhibition of protein biosynthesis by macrocyclic trichothecenes [8]. Thus, proteinogenic cytokines, such as GM-CSF, could no longer be expressed. Such protein inhibition can result in alveolar macrophages no longer being able to mature and function correctly, as GM-CSF is responsible for their differentiation and maintenance [16]. One consequence of this can be reduced clearance capacity in the lungs and an attenuated pro-inflammatory immune response. Over time, impaired clearance may, however, promote chronic, dysregulated inflammation in the lung tissue. Another process could be possible for IL-1β. The increased IL-1β release in THP-1 macrophages may have been caused by activation of inflammasome-associated caspase-1 via the $P2X_7$ receptor in human macrophages, as described by Kankkunen et al. [38, 48]. The inflammasome can be activated by various factors including bacterial factors, endogenous factors and exogenous factors such as asbestos, silica [49] and potentially also by macrocyclic trichothecenes [38]. Since the release of IL-18 is also induced by the inflammasome-associated caspase-1, it can be assumed that IL-18, analogous to IL-1β, is elevated in the extracts with toxic effects [38]. In our study, THP-1 macrophages did not show increased IL-6 and TNF-α release at the protein level after exposure to the extracts. On the one hand, this may be because the mycotoxins contained in the extracts did not induce their release. On the other hand, mRNA expression for these cytokines was not investigated and could be increased. Pei et al. [14] demonstrated increased mRNA expression of both cytokines, but did not investigate their protein level.

In addition, impedance measurements showed that NuLi-1 cells exhibited a strong morphological change during exposure to *P. chrysogenum* plasterboard material extracts. Impedance initially increased but then returned to the signal level of the control within 30 h. This biphasic pattern indicates a temporary change of the barrier function. *P. chrysogenum* is known to produce various secondary metabolites such as allergens, toxins, and proteases that can affect cell viability, membranes, or cell–cell contacts and can trigger morphological changes. Tai et al. [50], for example, described how the serine protease Pen ch 13 (oryzin) damaged tight junctions in epithelial lung cells, cleaved occludins, and released inflammatory mediators such as IL-8 or PEG-2. These previously described effects are tended to be consistent with our observed impedance profile and morphological changes, but our data not directly demonstrate a toxic mechanism. Our initial increase may reflect an acute stress response; however, underlying mechanisms (e.g., cytoskeletal reorganization, actin-myosin contractions, temporary cell tightening, or increased adhesion) remain to be investigated. The subsequent decline could be associated with proteolytic degradation of tight junctions and ROS-induced degeneration, but this interpretation is hypothetical and not directly supported by our current data. The fact that cells returned to control levels suggest that the concentrations of the unknown toxic component were insufficient to cause cell death under our conditions, allowing the cells to recover. The extracts obtained from *P. chrysogenum* grown on malt extract agar showed early cytotoxic effects with a small kind of recovery at 6 h and ongoing decrease and cell viability loss. This suggests that the concentration of cell-destroying metabolites produced by *P. chrysogenum* is considerably higher in malt extract agar than in plasterboard under our experimental conditions.

The established test system is sensitive to detect cytotoxic, including real time, and immunomodulatory effects of prepared extracts from contaminated materials between different fungal species, strains and materials. Additionally, the integrated mycotoxin analysis is an essential part to better understand the results because the strength of cytotoxicity is dependent on mycotoxin types and their concentration. The test system identified *S. chartarum* as a potentially harmful mold due to its highly toxic produced mycotoxins, which is consistent with published results [42, 51].

Adverse health effects of inhalative exposure to mycotoxins indoors have not yet been comprehensively investigated [9]. Some studies suggest that the concentrations of mycotoxins found

indoors are unlikely to cause adverse health effects in occupants through inhalation [52–54]. In view of methodological developments and climate change, further exposure and toxicological data should be collected. The collection and analysis of real samples from damp-associated indoor environments—including material pieces, surface swabs, water condensates, and bioaerosol samples using in vitro test systems is an established approach, as demonstrated in multiple previous studies [55–60]. This established strategy can now be applied to our test system.

Furthermore, the test system was established for a short-time exposure of the extracts (24–48 h) to assess acute effects. To detect also potential effects of extracts or mycotoxins with lower cytotoxicity, such as Stachybotrylactam, subacute or chronic effects by using repeated and long-term exposure scenarios should be considered in further investigations. For this air-liquid-interface cell cultures represent a suitable platform. Consequently, the existing test systems can be extended to more complex tissue cultures of the bronchus in order to investigate tissue-like structures, such as cilia beat analyses or mucus production as well as individual cell signaling pathways after single or repeated exposure to the extracts to take a closer look at the health effects on the respiratory tract that are relevant to humans [61, 62]. In addition, disease models can be used to investigate the health effects of indoor mold on pre-damaged and immunocompromised residents. Since mycotoxins have a harmful effect on lung epithelial cells and may therefore also have a negative effect on the lungs, it is important to investigate the release of mycotoxins into the air and determine their concentrations in the air in more detail in order to collect more data for risk assessment.

5 | Conclusion

In this study, a human cell-based in vitro test system was established to evaluate the cytotoxic and immunomodulatory effects of common indoor fungal species in the context of inhalation exposure. The developed test system is based on human lung epithelial cells and macrophages, which are sensitive enough to detect cytotoxic and immunomodulatory effects of moldy indoor materials. This was tested with selected fungal species that were found specifically in indoor environments. The test system was able to identify *S. chartarum* as a toxic mold among the tested molds because it produces highly toxic mycotoxins. The proposed test system thus offers a promising approach for detecting and differentiating cytotoxic and immunomodulatory effects of extracts from mold-contaminated materials. In combination with mycotoxin analysis, the applied system enables a comprehensive understanding of the inhalation-induced cytotoxic and immunomodulatory effects, which depend on the present types of mycotoxins and their concentrations. Standardization of such a test system could promote a standardized indoor air quality assessment.

Author Contributions

Stefanie Klar, Udo Jäckel: conceptualization. Stefanie Klar, Anja Wolff, Jessica Reinert, Marion Berger, Lennart Marske, Judith Neuhoff, Silke Steinborn: data acquisition. Stefanie Klar, Anja Wolff, Marion Berger: data analysis. Anja Wolff, Stefanie Klar: writing – original draft. Anja Wolff, Stefanie Klar, Udo Jäckel, Kerttu Valtanen: writing – review and editing.

Acknowledgments

We would like to thank Anne-Katrin Liebschner and Jeanette Farshadi-Nejad for preparing the spore solutions. Furthermore, we would like to thank Dierk-Christoph Pöther and Andy Schmied for critically reading the manuscript. Open Access funding enabled and organized by Projekt DEAL.

Funding

This work was supported by Federal Environment Agency.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Spore concentrations after surface swabbing of contaminated material depending on the mold. $n = 9$; data are presented in boxplots $^{*}p < 0.05$ indicates statistically significance between the material groups, $^{**}p < 0.01$, $^{***}p < 0.001$. **Figure S2:** Cytotoxic effects of extracts from contaminated materials depending on fungal species after 48 h exposure. Cell viability of NuLi-1 cells after 48 h exposure to extracts from contaminated (A) plasterboard, (B) wallpaper, (C) malt extract agar; $n = 9$; data are presented in boxplots $^{*}p < 0.05$ indicates statistically significance to extracts from PBS-treated materials (control), $^{**}p < 0.01$, $^{***}p < 0.001$. **Figure S3:** Cytotoxic effects of extracts from surface swabs of contaminated materials depending on fungal species after 48 h exposure. Cell viability of NuLi-1 cells after 48 h exposure to extracts from surface swabs of contaminated (A) plasterboard, (B) wallpaper, (C) malt extract agar; $n = 9$; data are presented in boxplots $^{*}p < 0.05$ indicates statistically significance to extracts from PBS-treated materials (control), $^{**}p < 0.01$, $^{***}p < 0.001$.