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Per- and polyfluoroalkyl substances (PFASs) modify lung surfactant function and pro-inflammatory responses in human bronchial epithelial cells

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ABSTRACT

The toxicity of some per- and polyfluoroalkyl substances (PFASs), such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) has been studied thoroughly, showing that systemic PFASs targets the lungs. However, regulators lack data to assess the impact of other PFASs on the lungs and alternative methods to test substances for lung toxicity are needed. We combined two *in vitro* models to assess toxicity to the respiratory system; i) a lung surfactant (LS) function assay to assess the acute inhalation toxicity potential, and ii) a cell model with human bronchial epithelial cells to study pro-inflammatory potential and modulation of inflammatory responses. We tested salts of four PFASs: perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), PFOS, and PFOA as well as the fluorotelomer 8:2 FTOH. The results show that PFHxS, PFOA and PFOS can inhibit LS function. High PFOS concentrations induced a pro-inflammatory response, measured as increased IL-1α/β release. Moderate concentrations of PFOS suppressed release of the chemokines CXCL8 and CXCL10, whereas both PFOS and PFOA stimulated the release of the pro-inflammatory cytokine IL-1β in immune stimulated human bronchial epithelial cells. These findings support the concern that some PFASs may increase the risk of acute lung toxicity and of airway infections.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) have diverse technical applications due to their superior surface-active properties. Emerging evidence shows a link between exposure to indoor air pollutants, like PFASs, and lung diseases (Qin et al., 2017; Impinen et al., 2018). In addition, epidemiological studies indicate an association between prenatal PFAS exposure and increased number of infections in children (Granum et al., 2013; Dalsager et al., 2016;Impinen et al., 2018; Impinen et al., 2019) and lower vaccination response (Granum et al., 2013; Grandjean et al., 2017), suggesting that PFASs have an immunosuppressive effect. Although PFAS exposure and the risk of developing asthma has been studied, the relation is not clear (Humblet et al., 2014; Impinen et al., 2018; Impinen et al., 2019).

The respiratory system is a target for PFAS toxicity, both by inhalation and systemically from the circulating blood. Volatile PFASs, such as fluorotelomer alcohols (FTOHs), can be measured at relatively high levels in indoor air. In addition, consumer products containing PFASs such as fluorinated ski wax, cosmetic sprays or impregnation products may lead to inhalation exposure and add to the total PFAS

Abbreviations: CDS, constrained drop surfactometer; CMC, critical micelle concentration; DMSO, dimethylsulfoxide; DPPC, dipalmitoylphosphatidylcholine; EFSA, European Food Safety Authority; ELISA, enzyme-linked immunosorbent assay; LOAEC, lowest observed adverse effect concentration; LOQ, limit of quantification; LPS, lipopolysaccharides; LS, lung surfactant; NOAEC, no observed adverse effect concentration; PBS, phosphate buffered saline; PFAS, per- and polyfluoroalkyl substance; PFBS, perfluorobutane sulfonate; PFDA, perfluorodecanoic acid; PFHxS, perfluorohexane sulfonate; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; Poly I:C, polyinosinic:polycytidylic acid; POP, persistent organic pollutant; POSF, perfluorooctanesulfonyl fluoride; REACH, registration evaluation authorisation and restriction of chemicals; TLR3, toll-like receptor 3; 8:2 FTOH, 1H,1H,2H,2H-perfluoro-1-decanol.

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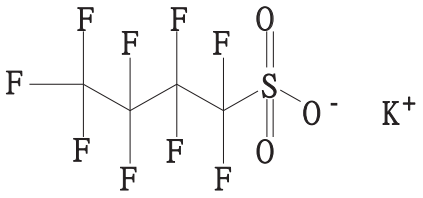
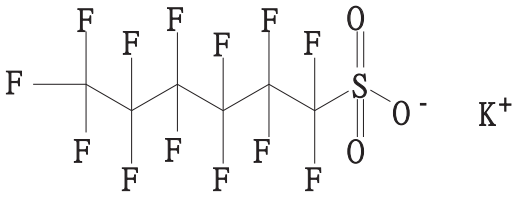
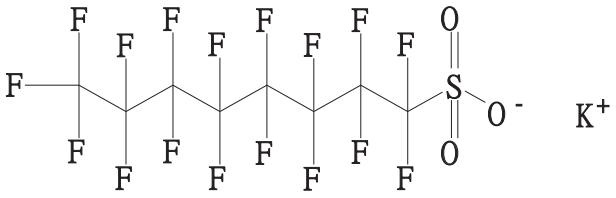
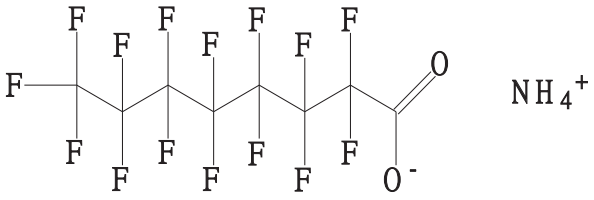
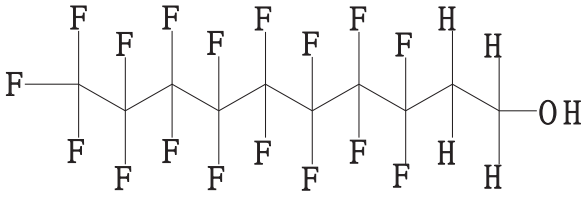
Table 1
Abbreviation, CAS number and structures of PFASs analysed.

Substance	CAS#	Structure
Potassium perfluorobutanesulfonate (PFBS)	29420-49-3	
Potassium perfluorohexanesulfonate (PFHxS)	3871-99-6	
Potassium perfluorooctanesulfonate (PFOS)	2795-39-3	
Ammonium perfluorooctanoate (PFOA)	3825-26-1	
1H,1H,2H,2H-perfluoro-1-decanol (8:2 FTOH)	678-39-7	

body burden (Freberg et al., 2010; Haug et al., 2011b; Nilsson et al., 2013a, 2013b; Padilla-Sanchez et al., 2017). Inhaled PFASs can have both portal of entry effects as well as systemic effects. The pulmonary epithelium and the layer of lung surfactant (LS) covering the cells are physical barriers between the outside environment and blood circulation, and are the primary portal of entry targets of inhaled pollutants. PFASs are synthetic surfactant molecules and have therefore the potential to interfere with the integrity of the surface-active interfacial films formed by natural surfactant in the alveolar spaces. LS has several functions, but the most important is to maintain very low surface tension at the air–liquid interface in the lungs during respiration, particularly at the end of exhalation, to facilitate breathing mechanics (Zuo et al., 2008; Perez-Gil and Weaver, 2010). For this reason, this study focuses on minimum surface tension during cycling, however for a comprehensive review of the structure function relationship of LS and the study of LS hysteresis along compression-expansion cycling we suggest reading (Notter et al., 1982: Lopez-Rodriguez and Perez-Gil, 2014; Sosnowski, 2018; Autilio and Perez-Gil, 2019).

While inhalation adds to the local exposure in the lungs as well as to the total PFAS level in blood, the main contribution to the blood levels of the different PFASs in the general population is from food and drinking water (Emmett et al., 2006a; Haug et al., 2011a). Many PFASs have long terminal half-lives in humans and are persistent in the environment (Haug et al., 2009; Olsen et al., 2017). Consequently, high levels of PFASs can be measured in blood from heavily exposed populations, which leads to chronic systemic exposure of organs, including the lungs (Haug et al., 2009; Haug et al., 2011a). In a mouse study, the level of PFOS was shown to be higher in lung tissue than in blood in both perinatal and adult mice, suggesting the lung to be a target of PFOS toxicity (Borg et al., 2010).

The production and use of perfluorooctanoic acid (PFOA) and its salts and precursor substances are regulated in a REACH restriction that will apply from 2020 (ECHA, 2017b). In addition, perfluorooctane sulfonate (PFOS) and perfluorooctane sulfonyl fluoride (POSF) have been listed under the Stockholm Convention on Persistent Organic Pollutants (POP) for a global restriction since 2009 (Stockholm Convention, 2008) and PFOA as well as perfluorohexane sulfonate (PFHxS), and their precursors are currently being evaluated for listing (UN Environment, 2017; Stockholm Convention, 2018a, b). Recently, Denmark proposed a restriction on some PFAS-related compounds, in different types of spray products to prevent consumer exposure to these compounds via inhalation as they can inhibit LS function and cause acute inhalation toxicity (Nørgaard et al., 2014; ECHA, 2017a; Sørli et al., 2017). As a consequence of these restrictions, production and use have shifted towards shorter chain compounds (C4 and C6), and many novel PFASs have been developed to replace long-chain PFASs. According to the CAS Registry (OECD, 2018), > 4600 PFASs are potentially on the global market. The high number of novel PFASs

manufactured poses a challenge for regulators, and there is an obvious need for efficient screening for potential lung toxicity.

In the present study, we have used two *in vitro* screening strategies to measure effects of PFASs on central mechanisms of the respiratory system. Firstly, we used a LS function assay to screen PFASs for acute inhalation toxicity potential. Dynamic LS function was measured in the constrained drop surfactometer (CDS), where the effect of a chemical can be screened rapidly and at low cost (Da Silva and Sørli, 2018). The assay has previously been used to show a link between inhibition of LS function and induction of acute inhalation toxicity *in vivo* by impregnation products and inhaled pharmaceutical enhancers (Sørli et al., 2017; Sørli et al., 2018). Secondly, we studied the immunomodulatory effects of PFASs. Bronchial epithelial cells were exposed to PFASs with and without priming with the highly pro-inflammatory toll-like receptor 3 (TLR3) ligand polyinosinic:polycytidylic acid (Poly I:C), a synthetic double stranded RNA analogue often used to mimic virus infection. Chemical exposures may sensitize the cells to additional pro-inflammatory insults (Øvrevik et al., 2013; Bach et al., 2014; Øvrevik et al., 2014) or inhibits the pro-inflammatory effects normally induced by Poly I:C (Müller et al., 2013).

The substances examined for LS inhibition and immune modulatory effects were the salts of PFASs of differing chain lengths: potassium perfluorobutane sulfonate (PFBS), potassium PFHxS, potassium PFOS, ammonium PFOA, as well as the fluorotelomer alcohol 1H,1H,2H,2H-perfluoro-1-decanol (8:2 FTOH). Screening the compounds for their potential for affecting LS function, can be used to rank the PFASs in relation to their potency, whereas measurements of cytokine release might suggest which PFASs are likely to modulate immune responses from bronchial epithelial cells at human relevant concentrations. This information can support risk assessment of acute inhalation toxicity for data-poor substances and prioritization of substances for further testing.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharides (LPS; from *Escherichia coli*), Poly I:C, dimethyl sulfoxide (DMSO) and PFASs (Table 1) were purchased from Sigma-Aldrich (Oslo, Norway). Gibco LHC-9 medium was purchased from Thermo Fisher Scientific (Oslo, Norway).

2.2. Preparation of native lung surfactant

Native LS was prepared and the phospholipid content determined as described previously (Taeusch et al., 2005). LS was isolated from bronchoalveolar lavage of porcine lungs by multiple centrifugations, including a density gradient ultracentrifugation that cleans potential blood contaminants from the surfactant. The method consistently yields surfactant preparations with highly reproducible batch-to-batch biophysical performance. To perform the described LS function assay, lyophilized LS was diluted to 5 mg/ml in a buffer containing 0.9% NaCl, 1.5 mM $CaCl_2$, and 2.5 mM HEPES, adjusted to pH 7.0 (Valle et al., 2015).

2.3. Lung surfactant function analysis using the constrained drop surfactometer

2.3.1. LS inhibition analysis

Inhibition of LS function by PFASs *in vitro* was tested using the CDS (Sørli et al., 2015; Valle et al., 2015; Sørli et al., 2017) by mixing the LS with increasing concentrations of the different PFASs. The final concentration of LS was 2.5 mg/ml in all experiments. Each experimental series started with a control, where water was added to LS to reach a final concentration of 2.5 mg/ml. A small drop (10 µl) of LS was placed on the hollow based pedestal, and dynamically cycled at 20 cycles/min and < 25% compression rate (between 18 and 24% for all experiments), to mimic the frequency and percentage area change as in the breathing lungs. The same LS suspension was used in all the subsequent experiments. The pedestal and tubing were cleaned between each testing of the different PFASs. The individual PFASs were tested by dissolving in DMSO at a concentration of 200 mM, and further diluted in water before mixing with LS, with the exception of 8:2 FTOH that was diluted in PBS to avoid precipitation. The LS-PFAS mixture was tested as described for the control LS. The PFAS content was halved with each dilution, the concentration of DMSO in each sample depended on the amount of PFAS being tested. Prior to removing 10 µl of the mixture for testing, the vial was vortexed for 30 s and each concentration was measured at least 3 times. Images were continuously taken of the drop and analysed by axisymmetric drop shape analysis (ADSA) software and surface tension of the drop was recorded (Yu et al., 2016). The CDS was kept at 23 °C inside a heating box for all measurements (with the exception of the highest concentration tested for PFBS, in this case the CDS was heated to 32 °C). The surface tension was recorded for the first 10 cycles, and the minimum surface tension of the 10 first cycles was found.

2.3.2. Control for system interference from other factors than PFASs

To exclude that the observed inhibition of function was from the DMSO in which all PFASs were dissolved, the solvent effect on surface tension function was assessed. Inhibition caused by DMSO could be excluded as the concentration of DMSO that inhibited LS function was 60 times higher than the concentrations used in any of the inhibitory dilutions of PFASs, and for PFASs that did not cause inhibition of the function, the DMSO concentration approached the concentration where pure DMSO showed inhibition (data not shown). LS function is also affected by extreme pH, and the optimal pH range is between 4 and 7 (Amirkhanian and Merritt, 1995), therefore the dilutions of PFASs in water were tested using pH indicator strips (Alkalit, Merck). All solutions had a pH of 6, therefore pH was excluded as a cause of LS function inhibition.

2.3.3. Critical micelle concentration (CMC) determination

The aggregation state of the PFASs can affect the distribution of surfactants in the test solutions, therefore the CMC was determined for all PFASs in water. The CMC was measured in the absence of LS. The CMCs were determined by recording the surface tension of a drop of water with different concentrations of the specific PFAS in the CDS. The drop was cycled using water in the syringe, 3 min were recorded during which the surface area change was 30% and at 3 s cycles. The PFASs were dissolved in DMSO at 200 mM, and dilutions between 1.25 mM and 80 mM in water (except 8:2 FTOH that was diluted in PBS and for which the highest dilution was 40 mM, because of precipitation in water and at higher concentrations) were tested in triplicate. The average surface tension measured was plotted against the concentration of PFAS, and this plot was used to determine CMC (See supplementary fig. 1). CMC was determined by drawing a superimposed line on the points of the graphs where surface tension dropped with increasing PFAS concentration. A second line was drawn through the points on the graph where the surface tension did not decrease with increasing PFAS concentration. The PFAS concentration at the point where these two lines intersect is the CMC, and this has been summarized in Table 2 for each PFAS under “CMC”.

2.4. Cell culture

HBEC3-KT human bronchial epithelial cell line (ATCC CRL-4051) was grown in serum free LHC-9 medium in collagen-coated flasks (PureCol™) in a humidified atmosphere at 37 °C with 5% CO_2. The medium was changed every 2–3 days. Prior to exposure, cells were plated in 35 mm 6-well culture dishes (220,000 cells per well), grown to near confluence in serum free LHC-9 medium and exposed as described below.

Table 2
LOAEC and CMC values for five PFASs.

Substance	LOAEC, mM	CMC in water at 32 °C, mM
PFBS	> 80	22
PFHxS	1	12
PFOS	0.5	7
PFOA	0.5	14
8:2 FTOH[a]	> 40	20

[a] diluted in PBS; All substances were dissolved in DMSO (200 mM) and then diluted in water (or PBS) before mixing with LS to determine LOAEC. The PFAS in DMSO was diluted in water (or PBS) prior to analysis to determine CMC.

2.5. Cell culture exposures

Stock solutions of 100 mM PFAS in DMSO were diluted in culture medium prior to cell exposure. HBEC3-TK cells were exposed to concentrations ranging from 0.13 µM to 60 µM of PFOS, and 0.13 µM to 10 µM of the remaining PFASs. DMSO (0.02%) in culture medium was used as vehicle control. In a pilot experiment the release of CXCL8 after PFOS exposure was investigated after 24 and 48 h, and the 48 h exposure resulted in higher concentration-response pattern. Therefore, the remaining exposures were performed for 48 h. In parallel to the exposure to PFASs, the cells were incubated with 5 µg/ml Poly I:C or with Lipopolysaccharide (LPS) 3 h prior to exposure to PFASs.

2.6. Cell viability analysis

Cell viability was measured using AlamarBlue cell viability reagent (Thermo Fisher Scientific, Oslo, Norway) according to the manufacturer's protocol.

2.7. Cytokine analysis

Cell culture medium was centrifuged to remove cell debris, and stored at −70 °C prior to analysis of cytokines. Concentrations of CXCL8 (IL-8), IL-6 (both from Invitrogen, Thermo Fisher Scientific, Oslo, Norway), CXCL10 (IP-10), IL-10, IL-1α and IL-1β (all from R&D Systems, Minneapolis MN, USA) in cell culture medium were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Absorbance was measured using a plate reader (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA) equipped with Magellan (v1.10) software.

2.8. Statistical evaluation

The LS function data were analysed by calculating the statistical difference between the minimum surface tension at the 10th cycle of the control and the treated sample using ANOVA in the statistical platform R (version 3.4.1). If there was a significant difference between the control and the treated sample, and the minimum surface tension was higher, a *t*-test with Bonferroni correction was performed to find the group(s) different from control. The sample with the lowest concentration and statistical difference from the control was determined to be the lowest observed adverse effect concentration (LOAEC) of the PFAS.

For the cell culture study, statistical analysis of differences in cytokine secretion was performed by one-way ANOVA with Dunnett's post-test for multiple comparisons. Non-normally distributed data were log-transformed. All calculations were performed using GraphPad Prism 5.04 software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. LS function analysis

LS function was tested by mixing the individual PFASs with LS (in molar ratios between 0.04 and 24:1, PFAS:LS, using the molecular weight of DPPC), and measuring the reduction in surface tension during the first 10 rounds of compression-expansion cycling. The LOAEC was defined as the lowest concentration of a given PFAS producing a minimum surface tension at the 10th round of compression/expansion that was significantly different from the minimum surface tension of the control LS in the absence of the compound (Table 2). As a negative control, LS was diluted with water, except for 8:2 FTOH, where the control was diluted in PBS. For PFBS and 8:2 FTOH an inhibitory concentration could not be found. 8:2 FTOH could not be tested at higher concentrations than 40 mM, above this concentration the sample precipitated.

3.2. CMC determination

The CMC for each PFAS was determined in water at 32 °C for all PFAS using the CDS (Table 2) to be able to control for potential influence of micelle formation on the function of LS. The data shows that the LOAEC was below CMC for the compounds where a LOAEC could be found. For the PFBS and 8:2 FTOH a LOAEC could not be found, the tested concentration of PFAS was at least twice the CMC (Table 2).

3.3. Human bronchial epithelial cell analyses

The human bronchial epithelial HBEC3-KT cells were exposed to the different PFASs and cytokine release was measured. Furthermore, modulation of cytokine release was analysed after pre-treatment of the cells with the immune stimulants Poly I:C or LPS.

3.3.1. Cell viability

The effects of the PFASs on cell viability was measured using the AlamarBlue assay. In an initial experiment, cells were exposed to a wide concentration range of PFOS for 48 h to determine the effect on viability. The data showed no apparent reduction in cell viability at concentrations ≤ 30 µM (Fig. 1A). Furthermore, the other PFASs did not show any reduced cell viability at the highest tested concentration (10 µM) (Fig. 2, Fig. S2).

3.3.2. Cytokine release

Pro-inflammatory responses were analysed in the initial experiment that included high PFOS concentrations. As shown in Fig. 1, exposure to PFOS alone did not induce any biologically significant changes in CXCL8 secretion at the tested concentrations. However, at concentration ≥ 30 µM, IL-1α and IL-1β releases were increased.

Subsequently, all PFASs were tested at non-cytotoxic concentrations relevant to human exposures, ranging from 0.13 µM to 10 µM, with and without priming with an immune-stimulating agent (Fig. 2). Two pro-inflammatory agents were tested, Poly I:C and LPS, mimicking virus and bacterial infection, respectively. In response to LPS, only a modest increase of cytokine release was observed in the HBEC3-KT cells (data not shown), whereas exposure to Poly I:C resulted in a marked upregulation of cytokine release (Fig. 2). Poly I:C was thus used in the subsequent co-exposure experiments.

None of the PFASs tested induced significant changes in release of the cytokines measured at non-cytotoxic concentrations in unstimulated cells (Fig. 2 and Fig. S2).

As can be seen from Fig. 2, Poly I:C induced a marked induction of all tested cytokines except IL-10 that remained below the limit of quantification for all treated samples (data not shown). PFOS exposure resulted in a statistical significant concentration-dependent reduction of Poly I:C-induced CXCL8 and CXCL10 secretion. Furthermore, the IL-6

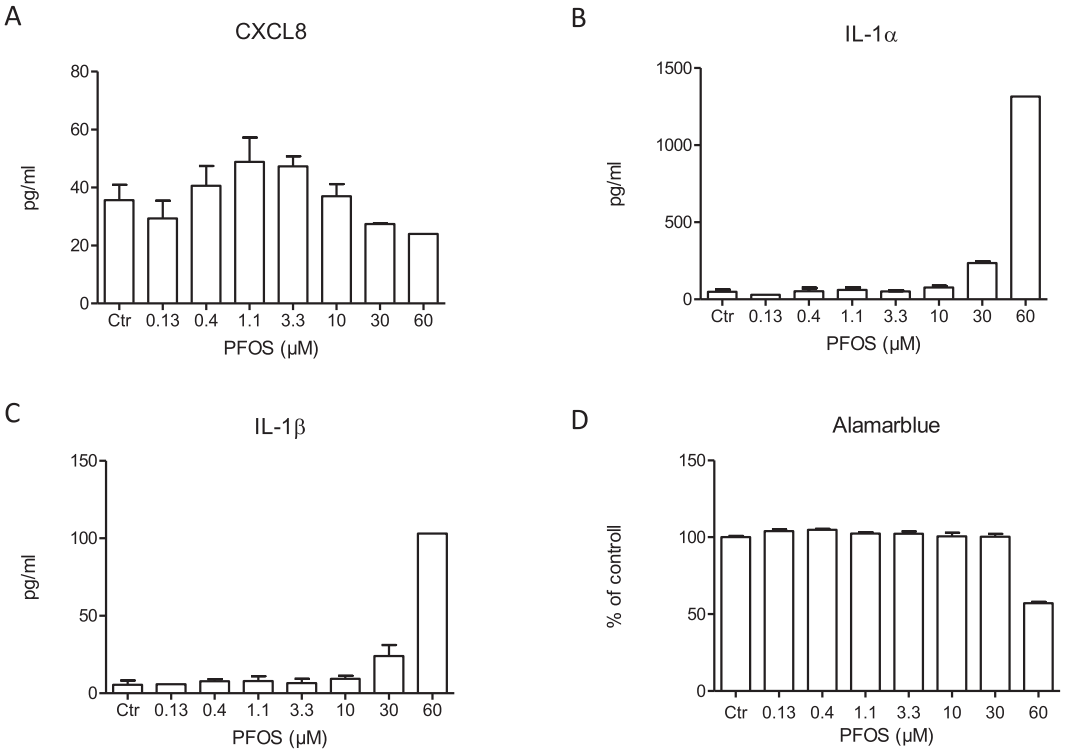


Fig. 1. Cell viability and cytokine release induced by PFOS in an initial concentration-response experiment. Human bronchial epithelial cells, HBEC3-KT, were exposed for 48 h to PFOS. A-C) concentration in medium of the cytokines CXCL8, IL-1α and IL-1β. D) Cell viability as measured by AlamarBlue staining. n = 1–5 within each treatment group.

release seemed to have a concentration-dependent reduction, but without reaching statistical significant difference from Poly I:C stimulated control. On the other hand, PFOS significantly enhanced the increase of IL-1α release, and both PFOS and PFOA enhanced the IL-1β release in response to stimulation with Poly I:C. The remaining PFASs (PFBS, PFHxS and 8:2 FTOH) did not induce any statistically significant changes in cytokine release at the concentrations tested (Fig. S2).

4. Discussion

PFASs represent a versatile group of ubiquitously occurring chemicals of increasing regulatory concern. For many of the PFASs, public information on their hazardous properties, environmental fate and transport, exposure, and toxic effects is still very limited, and in the public domain they remain largely unassessed and unregulated (Wang et al., 2015; Wang et al., 2016; Wang et al., 2017). PFASs can easily be synthesized in multiple forms, and screening the toxic potential of all novel PFASs is a daunting task. However, as these substances emerge on the market, regulators, producers and importers of the products containing them, still have to do a hazard assessment. Traditional hazard assessment is performed on experimental animals, however, this is time-consuming, costly and ethically questionable. We have tested two mechanism based *in vitro* screening procedures that assess the effects of PFASs on the respiratory system. The relative potencies of substances may be used in an integrated alternative approach to support hazard assessment and testing prioritization. We studied the effects of PFASs on LS function of which inhibition can cause acute inhalation toxicity. We also studied the immunomodulatory effects of PFASs on bronchial epithelial cells, in the presence or absence of pro-inflammatory priming with Poly I:C.

The LS lowers surface tension at the air-liquid interface of the alveoli, in a particular way that minimizes tension to values < 2 mN/m at the end of compression (upon exhalation), when the risk of lung collapse is maximum. This is possible due to extensive structural reorganizations occurring at the interfacial surfactant films during the reduction of respiratory area that occurs upon expiration. Surfactant structure supports these recurrent compression-driven structural transitions thanks to very defined lipid and protein composition that include coexistence of condensed and expanded lipid phases and the particular action of specialized surfactant proteins to impart proper dynamics (Lopez-Rodriguez and Perez-Gil, 2014; Autilio and Perez-Gil, 2019). Surfactant also provides some protection against hazardous inhaled substances (Zuo et al., 2008). However, surfactant structure and performance is sensitive to accumulation of deleterious components with potential to integrate into the hydrophobic environment provided by lipid/protein complexes, or with intrinsic surface activity that results in accumulation at the air-liquid interface. Damage to LS function causes the alveoli to collapse and the respiratory bronchioles to fill with liquid, making breathing laboured. PFASs can potentially disrupt the structure of the LS film and thereby inhibit its function. We tested five different PFASs to determine the lowest concentration where inhibition of LS function was observed, defined as the LOAEC value. PFHxS, PFOS and PFOA inhibited the LS function at comparable concentrations (1, 0.5 and 0.5 mM, respectively). For PFBS and 8:2 FTOH, a LOAEC could not be found. PFAS concentrations above CMC might affect the formation and stability of the LS film at the air-liquid interface in the CDS. Determination of the CMC is not trivial and values from literature could only be found for PFOS: 1.13 mM (Roberts et al., 2016) and 8 mM (Harada et al., 2005) and PFOA: 16 mM (Roberts et al., 2016), 30 mM (Lopez-Fontan et al., 2005) and 25 mM (Harada et al., 2005). We determined the CMC for PFOS and PFOA to be 7 and 14 mM, respectively. The LOAEC was lower than the CMC for the three PFAS that inhibited LS function, discarding that their deleterious action could have to do with a “detergent-like” effect of PFAS micelles across the surface film, removing surface-active molecules from the interface. The two remaining PFASs (PFBS and 8:2 FTOH), with no evident inhibitory action, were tested at concentrations at least twice the CMC. Thus, the CMC of these PFASs did not appear to directly determine the effect of the PFASs

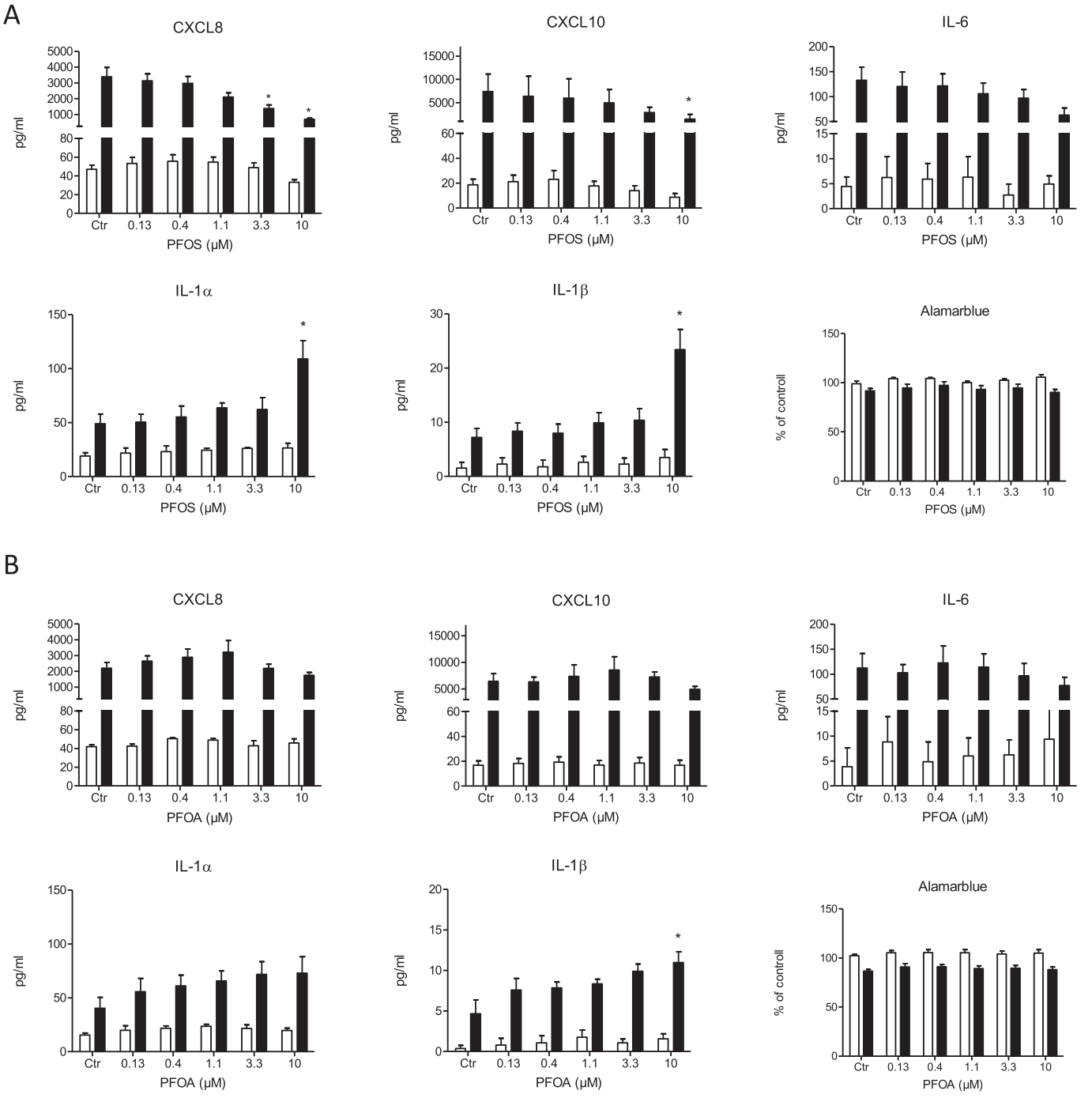


Fig. 2. Cytokine release. A) PFOS, B) PFOA. White bars are without and black bars with immune stimulation by the toll-like receptor 3 (TLR3) ligand Poly I:C, n = 5–6 (PFOS) and 4 (PFOA). *P < .05 significantly different from Poly I:C stimulated control, one-way ANOVA followed by Dunnett's *post hoc* test.

on LS function, because if that would be the case, one would expect qualitative differences on surfactant inhibition at concentrations above and below the respective CMC values.

Preliminary results have been published in an abstract where PFBS, PFHxS, PFOA and PFOS were tested for their ability to inhibit LS function in the captive bubble surfactometer (CBS) (Gordon et al., 2007). The CBS setup is different from the CDS, therefore direct comparison of concentrations is impossible. However, the authors found that all the PFASs inhibited LS function. At the highest concentrations of PFASs, a low minimum surface tension could not be reached, while at the lowest concentration tested, a low minimum surface could only be obtained with very large compression of the surface area (Gordon et al., 2007). A main difference is that the captive air bubble of the CBS has a limited air phase, while the sessile drop of the CDS is exposed to an open air compartment that mimics better the situation in the alveoli. How PFOA and PFOS partitions into a model of LS (dipalmitoylphosphatidylcholine, DPPC) has been studied in detail (Xie et al., 2010a; Xie et al., 2010b). PFOS and PFOA incorporate into the lipid layers more readily than their corresponding hydrocarbon surfactants (Xie et al., 2007).

Inhalation of impregnation spray products containing PFASs is a known cause of acute toxicity in humans (Duch et al., 2014; Scheepers et al., 2017; Sørli et al., 2017). These products cause acute symptoms such as coughing, shortness of breath, chest pain and reduced

oxygenation of the blood. The acute symptoms evolve into lung inflammation. In most cases, the symptoms resolve within days of the exposure, but in some cases, the problems continue for months after the exposure (Schicht et al., 1982; Burkhart et al., 1996; Ota et al., 2000; Wallace and Brown, 2005; Khalid et al., 2009; Fukui et al., 2011; Kikuchi et al., 2015; Sørli et al., 2017). Impregnation product toxicity was recently studied using the CDS approach. The study compared the *in vitro* and *in vivo* toxicity of impregnation products and found a strong correlation between inhibition of LS function *in vitro* and observed toxicity by inhalation in humans or mice (Sørli et al., 2017). We show here that some PFASs inhibit LS function in a similar way as the impregnation products. It is likely that inhaling contaminated air (*e.g.* during ski waxing or spraying of products containing PFASs) can cause acute inhalation toxicity. There can be local build-up of PFASs, but the extent is not known since the clearing rate in the lung tissue has not been reported. This may lead to a significant alveolar collapse and reopening of the alveoli. The long term consequences of repeated acute inhalation toxicity are not known, but there are indications of adverse effects on the lungs, such as development of asthma, fibrosis, COPD or other lung pathologies (Devendra and Spragg, 2002).

The European Food Safety Authority (EFSA) report on PFOS and PFOA recognizes immune suppression as a critical effect (EFSA, 2018). Thus, a screening approach to determine the potential for immunomodulation of the currently used PFASs and their potential substitutions is needed. In the present study, non-cytotoxic PFAS concentrations, up to 10 μM, caused no significant effects on cytokine release (Fig. 2, Fig. S2). However, in immune stimulated cells (with Poly I:C) exposed to PFOS, a dose-related suppression of CXCL8 and CXCL10 release was observed. A similar tendency was observed for IL-6, but this was not statistically significant. Cytokine suppressive effects has also been reported by Corsini and co-workers (Corsini et al., 2011; Corsini et al., 2012) that investigated the effect of PFAS exposures on cytokine release by human immune cells stimulated with LPS or phytohemagglutinin. The authors demonstrated reduced release of both pro- and anti-inflammatory cytokines, including CXCL8 and IL-6, depending on substance and cell type. PFOS was generally more potent than PFOA (Corsini et al., 2011). All PFASs tested were shown to decrease LPS-induced NF-κB activation (Corsini et al., 2014). Furthermore, IL-2 secretion from stimulated human T-cells or the Jurkat T-cell line was decreased in response to PFOS, but not PFOA exposure (Midgett et al., 2015).

Since the HBEC3-TK bronchial epithelial cell culture responded weakly to LPS stimulation, we used Poly I:C for priming prior to PFAS exposure. In contrast to the impaired release of CXCL8 and CXCL10, in the present study PFOS exposure led to enhanced Poly I:C stimulated secretion of IL-1α and IL-1β and PFOA induced an increase in IL-1β release (Fig. 2). Increased transcription and release of several pro-inflammatory cytokines, including IL-6, CXCL8 and IL-1β has also been observed in a study with mast cells exposed to PFOA (Singh et al., 2012). Furthermore, exposure of a gastric cell line to perfluorodecanoic acid (PFDA) induced inflammasome assembly and secretion of IL-1β and IL-18 (Zhou et al., 2017). Finally, assays analyzing immunomodulatory activity of several PFASs and their salts are reported in the ToxCast database (EPA's National Center for Computational Toxicology, 2018) where the salts of PFOS and PFOA were active in eight and three of a total of 82 cytokine assays, respectively. PFOS, PFOA and PFHxS were reported to induce a slight enhancement of CXCL8 and a downregulation of CXCL10 release in immune stimulated cells. PFOS was also reported to reduce the release of IL-1α.

The differential regulation of cytokines reported in literature, as well as in the present study, points to a likely cell type dependent regulation of cytokines in response to different PFASs. The mechanism behind such a complex regulation is not clear, but may be related to differences in regulation of cytokine release. Inhibition of NF-κB activation was shown to be related to the suppressive effects of PFASs on cytokine transcription in human leucocytes (Corsini et al., 2012Corsini et al., 2014). Notably, the IL-1β is synthesized as a precursor protein, stored in the cytoplasm, and is released in response to proteolytic activity (Ozaki et al., 2015). This suggests that various cell types relevant for lung tissue respond differently to the PFASs, but also that the cytokines are induced and/or released *via* different mechanisms in the same cell type.

Lung cells are affected by inhaled PFASs as well as by circulating PFAS, so the local concentrations in the respiratory system may be higher for some substances than the systemic concentrations. According to the recent EFSA report on PFOS and PFOA (EFSA, 2018) the range of blood concentrations of PFOS in adults is from 0.06 to 392 ng/ml, whereas for PFOA the range is 0.03 to 81 ng/ml. We have exposed bronchial epithelial cells to concentrations between 0.13 and 10 μM, corresponding to 65 ng/ml and 5000 ng/ml, in the case of potassium PFOS. This means that the lowest exposure concentration of PFOS in the culture medium is within the range of general human serum concentrations. The higher concentrations used are within the range of exposures reported among high exposed populations through drinking water and in production workers (Emmett et al., 2006b; Olsen, G. W., 2015; Li et al., 2018). The data thus supports the concern that modulation of immune responses by some PFASs may occur at human relevant exposures. The comparison of PFAS concentrations in culture media and serum are not trivial, since protein binding of PFASs in the culture medium is not characterized and protein binding may have an impact on their potential effect on immune responses.

Oral exposure of mice to radioactively labelled PFOS and PFBS has shown that these PFASs readily distribute to the lungs (Borg et al., 2010; Bogdanska et al., 2011; Bogdanska et al., 2014), although to a lesser extent for PFBS (Bogdanska et al., 2014). When pregnant mice and rats were exposed to PFOS or PFOA, the pups died shortly after birth (Lau et al., 2003; Lau et al., 2006). Impaired lung function has been proposed to contribute to the neonatal death (Borg et al., 2010). In line with this hypothesis, it has been shown that the alveolar walls in the lungs of rat pups exposed to PFOS during gestation were thicker than in the controls, but neither the LS composition nor the amount were changed (Grasty et al., 2005). Our results show that several PFASs can disrupt LS function, which may in turn contribute to impaired neonatal lung function.

There are some limitations to the methods employed in the present study. The acellular *in vitro* assay does not completely reflect the conditions in the lungs. Firstly, using air concentrations from literature, the deposited concentrations in the alveoli by a person inhaling contaminated air will be much lower than the concentrations tested in the CDS. In addition, the LS film would encounter aerosolized PFASs rather than being mixed in the liquid sub-phase. Mimicking such air-driven PFASs exposure of surfactant films is challenging, as the compound are very hazardous. Secondly, we do not know the retention time of the PFASs in the LS layer in the lungs, thus accumulation after exposure may occur. This combined with LS turnover in the lungs makes estimating local PFAS doses difficult. Even with these limitations the method has proven to be able to predict toxicity and correctly rank chemicals in earlier experiments with aerosolized impregnation products (Sørli et al., 2017), or bile salts (Sørli et al., 2018). The method has also been used to correlate *in vitro* and *in vivo* effects of nanomaterials (Yang et al., 2018). We therefore think that the LS test provides valuable information on the relative acute lung toxicity potencies of the tested PFASs. Concerning the use of human cells for determining immunomodulatory potential *in vitro*, available data strongly suggest the need to include both immune cells and epithelial cells relevant for lung tissue responses as the responses appears to be cell type dependent.

5. Conclusion

In this project, we have used two *in vitro* test systems: i) an *in vitro* surfactant assay that is being developed as an acellular test for acute lung toxicity, and ii) an *in vitro* human bronchial epithelial cell model of

inflammatory responses. These two assays are promising tools for the development of an *in vitro* integrated screening approach for acute inhalation toxicity and immunotoxicity of PFASs, respectively. However, additional cell types of the respiratory tract should be included in a screening strategy since they may differ in their responses to PFAS exposures. Both test systems support that PFASs may be toxic to the respiratory system and points to PFOS and PFOA as the two most potent substances.

Author contributions

JBS, ML, BL, KBG conceived and designed the experiments. MNM, LE supported the organization of the project and performed the experiments. JBS, LE, ML, BL, KBG analysed the data and interpreted the results. JBS, KBG, BL, ML drafted the manuscript, JPG, EDS, LSH critically reviewed the paper. All contributing authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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