



Hyphae fragments from *A. fumigatus* sensitize lung cells to silica particles (Min-U-Sil): Increased release of IL-1 β

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ABSTRACT

Exposure to particulate matter (PM), such as mineral particles and biological particles/components may be linked to aggravation of respiratory diseases, including asthma. Here we report that exposure to *Aspergillus fumigatus* hyphae fragments (AFH) and lipopolysaccharide (LPS) induced both mRNA synthesis and release of pro-inflammatory interleukin-1 beta (IL-1 β) in both human THP-1 monocytes (THP-1 Mo) and phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 monocytes (THP-1 macrophages; THP-1 Ma); while Min-U-Sil alone enhanced the release of IL-1 β only in THP-1 Ma. Co-exposure to LPS or AFH with Min-U-Sil caused a synergistic release of IL-1 β when compared to single exposures. In contrast, Min-U-Sil did not markedly change LPS- and AFH-induced release of tumor necrosis factor alpha (TNF- α). The combined exposures did not increase the LPS- and AFH-induced expression of IL-1 β mRNA. Notably, the AFH- and LPS-induced IL-1 β responses with and without co-exposure to Min-U-Sil in THP-1 Mo were found to be caspase-dependent as shown by inhibition with zYVAD-fmk. Furthermore, co-exposure with AFH and Min-U-Sil resulted in similar synergistic releases of IL-1 β in primary human airway macrophages (AM; sputum), peripheral blood monocyte-derived macrophages (MDM) and in the human bronchial epithelial cell line (BEAS-2B). In conclusion, AFH induce both the synthesis and release of IL-1 β . However, Min-U-Sil further enhanced the cleavage of the induced pro-IL-1 β .

1. Introduction

Epidemiological studies link exposure to air pollution particulate matter (PM), including mineral particles (central component of PM₁₀) and biological particles/components arising from bacteria and fungi, to various respiratory diseases, including asthma (Denning et al., 2006; Denning and Chakrabarti, 2017; Munzel et al., 2017a; Munzel et al., 2017b; Orellano et al., 2017).

Organic particle components may not only be found on PM, but also in bio-aerosols. Bio-aerosols like airborne virus, bacteria, fungi and other biological fragments are found indoor and outdoor, and may

constitute up to 80% of ambient aerosols (Lee, 2011). Furthermore, some airborne bacteria are considered to be a natural part of the lungs microbiota and seem to play a crucial role in developing and maintaining the pulmonary immune system (Charlson et al., 2011; Ichinohe et al., 2011; Gollwitzer et al., 2014; Shima et al., 2016). Thus, lung cells are naturally exposed to and primed with various microbiota. Notably, microbiota like bacteria and fungi also play a central role in various lung diseases. Unbalanced exposure may cause infection and affect human health through allergic and non-allergic pro-inflammatory reactions, which are often linked to asthma and sinusitis (Shima et al., 2016; Denning and Chakrabarti, 2017).

Abbreviations: AFH, *Aspergillus fumigatus* hyphae; AM, Airway Macrophage; BEAS-2B, Human Bronchial Epithelial cell line; ELISA, Enzyme Linked Immunosorbent Assay; IL-1 β , Interleukin-1beta; LPS, Lipopolysaccharide; MDM, Monocyte-Derived Macrophages; Min-U-Sil, crystalline silica; NF- κ B, Nuclear Factor kappa light chain enhancer of activated B cells; NLR, NOD-like Receptor; PAMP, Pathogen Associated Molecular Pattern; PCR, Polymerase Chain Reaction; PI, Propidium Iodide; PM, Particulate Matter; PMA, Phorbol 12-myristate 13-acetate; RT-PCR, Reverse Transcriptase polymerase chain reaction; SiO₂, Silicon dioxide; TACE, TNF- α converting enzyme; THP-1, Human leukemia monocyte cell line; TLR, Toll-like Receptor; TNF- α , Tumor Necrosis Factor- alpha

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Lipopolysaccharide (LPS) is a glycolipid located in the outer membrane of most Gram-negative bacteria. It is a prime target for recognition by the immune system, can induce strong immune responses via TLR4 and is often used as a model for bacterial exposure/priming (Chandler and Ernst, 2017). With regard to fungi, human exposure to spores is more abundant than to hyphae. On the other hand, spores will form hyphae, which are suggested to have a stronger pro-inflammatory potential (Fung and Hughson, 2003; Gorny, 2004; Green et al., 2006; Øya et al., 2018). *Aspergillus fumigatus* is the most prevalent airborne fungal pathogen (Latge, 1999), and the spores and small sized hyphae fragments can reach small airways and alveolar spaces, resulting in inflammation and activation of different immune cells (Taramelli et al., 1996; Dagenais and Keller, 2009; Margalit and Kavanagh, 2015).

Crystalline silica (SiO₂) particles exist abundantly in nature, in soil, rocks and sand, and constitute part of the PM which the general population is exposed to through road abrasion and ongoing construction works. Airborne silica (quartz) particles are also encountered in numerous occupations, including mining, and about 2.3 million workers are known to be exposed to silica in US alone (WHO, 2007; OSHA, 2017). The mineral particles may damage the respiratory system through various genotoxic, cytotoxic and pro-inflammatory effects, including release of the strong pro-inflammatory cytokine interleukin beta (IL-1β) (Herseeth et al., 2008; Kawasaki, 2015; Øvrevik et al., 2015). These events may be of importance for explaining why long-term inhalation of crystalline silica may cause fibrosis (silicosis), lung cancer and autoimmune diseases (Cooper et al., 2002; Maciejewska, 2014).

Epithelial cells and resident airway macrophages in a healthy lung are the primary cellular targets for deposited inhaled particles and constitute the first line of defense against invading pathogens (Øvrevik et al., 2015). The macrophages derived from blood monocytes may be accumulated in the lungs during inflammation (Lambrecht, 2006; Shi and Pamer, 2011). Various lung cells recognize and respond to the different PM exposures through activation of distinct receptors, leading to increased synthesis and release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1α and -β. These cytokines will further regulate other cytokines such as IL-6 and CXCL8, involved in recruitment of several inflammatory cell types (Feghali and Wright, 1997; Arango and Descoteaux, 2014; Turner et al., 2014). Pattern recognition receptors (PRRs), mainly the Toll-like receptors (TLRs) are crucial in activation of immune responses. The TLRs are activated by ligation of pathogen-associated molecular pattern (PAMP) or danger associated molecular pattern (DAMP) molecules, triggering cellular signaling cascades. Notably, bacteria and fungi are in part recognized by different TLRs. While TLR4 is an essential receptor for recognition of LPS and Gram-negative bacteria (Netea et al., 2008), causing elevation of pro-IL-1β, both TLR2/4 are involved in host defense against Gram-positive bacteria and fungi (Akira et al., 2006).

IL-1 release is a two-step process involving inflammasome activation (He et al., 2016). Step 1 typically starts with an initial event, such as TLR-binding leading to activation of NF-κB, resulting in subsequent transcription and synthesis of pro-IL-1β. The further cleavage or maturation of pro-IL-1β to active IL-1β (step 2) is carefully controlled by inflammasomes in the cytosol, through a process involving cleavage of pro-caspase-1 to caspase-1 (Schroder and Tschopp, 2010; Takeuchi and Akira, 2010; Monteleone et al., 2015). This activation step differs for the respective inflammasome sensors such as NOD-like receptors (NLRs) and AIM2-like receptors (ALRs), and ultimately leads to the assembly of inflammasome complexes which are comprised of the NLR/ALR and adapter molecules. There are several NLRP (NLR family, pyrin domain-containing) proteins (Schroder and Tschopp, 2010). Interestingly, the activation of NLRP3 may be a result of particle phagocytosis, as well as the presence of PAMPs and DAMPs (Hornung et al., 2008). TNF-α also plays a crucial role in early stage of TLR-induced immune responses. Regulation of TNF-α involves the activation of NF-κB and transcription and production of a membrane bound pro-form. The

release of TNF-α requires proteolytic cleavage by the metalloprotease TNF-α converting enzyme (TACE) (Wajant et al., 2003; Arango and Descoteaux, 2014).

Several studies have documented the individual and combined effects of bacterial LPS and mineral particles (Morishige et al., 2010; Yazdi et al., 2010; Winter et al., 2011; Sandberg et al., 2012). However, co-exposures to other microbiota/organic components like fungal spores/hyphae and mineral particles has to our knowledge not been reported in the literature. We hypothesized that priming of cells with the *A. fumigatus* hyphae fragments (AFH) has a similar effect as LPS co-exposure to crystalline silica (Min-U-Sil), i.e. causing increased levels of intracellular pro-IL-1β that are cleaved to IL-1β upon crystalline silica exposure. Thus, we examined the pro-inflammatory potential of these particles both individually and upon co-exposure with AFH (and LPS) in several human in vitro cell models; THP-1 monocytes (THP-1 Mo), phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophages (THP-1 Ma), primary airway macrophages (AM)/sputum macrophages, primary peripheral blood monocyte-derived macrophages (MDM) and bronchial epithelial cells (BEAS-2B).

2. Materials and methods

2.1. Chemicals

Hoechst 33342, phorbol 12-myristate 13-acetate (PMA), and trypan blue were purchased from Sigma Aldrich (St. Louis, MO, USA). Granulocyte macrophage colony-stimulating factor (GM-CSF) was from ImmunoTools (Germany). Propidium Iodide (PI), cDNA Archive Kit and TaqMan gene expression assay were purchased from Life Technologies (CA, USA). LHC-9 cell culture medium was from Invitrogen (Carlsbad, CA, USA), and RPMI 1640 medium from Lonza (Verviers, Belgium). Cytokine ELISA assay for TNF-α (Human TNF-α Cytoset) was purchased from Life Technologies (Camarillo, CA, USA), and cytokine ELISA assay for IL-1β (Human IL-1β DuoSet) from R&D Systems, Inc. (Minneapolis, MN, USA). PerfectPure RNA cultured cell kit-50 was purchased from GmbH (D-22767 Hamburg, Germany). Caspase-1 inhibitor (zYVAD-fmk) was from EMD Chemicals Inc., Gibbstown, USA. All other chemicals used were purchased from commercial sources at the highest purity available.

2.2. Cell models

2.2.1. THP-1 monocytes and macrophages

The human monocyte cell line THP-1 was obtained from American Tissue Type Culture Collection (Rockville, MD, USA) and was maintained in RPMI 1640 medium with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 1 mM pyruvic acid and 0.1% gentamicin and were grown in humidified atmosphere at 37 °C with 5% CO₂. The cells were plated at a concentration of 5×10^5 cells/mL in 75 cm² cell culture flasks (Sarstedt AG & Co, Numbrecht, Germany) and were passaged every week and refreshment of medium was done every second day. For the exposures, THP-1 monocyte (THP-1 Mo) cells were plated into 35 mm 6-well culture dishes (8×10^5 cells/well), and rested for 3 h before exposure.

Phorbol 12-myristate 13-acetate (PMA) treatment is known to induce differentiation of THP-1 cells as reflected by increased adherence and expression of surface markers associated with macrophage differentiation (Takashiba et al., 1999; Park et al., 2007; Daigneault et al., 2010). Thus, THP-1 Mo (seeded at a concentration of 2×10^5 cells well in 10 mm 12-well culture dishes or 4.15×10^5 cells/well in 6-six well culture dishes) were also treated with 200 nM PMA for 72 h and differentiated to macrophage like cells (THP-1 Macrophages; THP-1 Ma). THP-1 Ma rested for 3 h after media exchange/before exposure.

2.2.2. Airway macrophages (AM)

Human airway macrophages (AM)/sputum were isolated from

central airways from consenting, healthy donors by induced sputum as described previously (Sikkeland et al., 2012). In short, hypertonic saline was inhaled from an ultrasonic nebuliser (DeVilbiss, Sunrise Medicals, Longmont, CO, USA) during three 7 min inhalation periods. Expecterates were collected after each inhalation period. The sputum samples were dissolved in PBS with DTT, filtered, centrifuged and resuspended in complete cell culture medium, consisting of RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL)/streptomycin (100 µg/mL), 2 mM L-glutamine and 0.5 mg/mL Gibco™ Amphotericin B.

Based on the viability (trypan blue exclusion) and differential cell counts, the cells were seeded in 12-well cell culture plates (3.2×10^5 macrophages/well) or 48-well cell culture plates (10^5 macrophages/well) (Falcon, Corning inc., NY, USA) for analysis of cytokine release. The cells were incubated for 1 h in an atmosphere of 37 °C and 5% CO₂ to allow macrophages to attach. Then, the wells were washed three times with complete cell culture medium, detached cells were discarded (supernatant), and fresh medium was added. The airway macrophages were rested overnight prior to exposure (Tomlinson et al., 2012).

2.2.3. Peripheral blood monocyte derived macrophages (MDM)

Elutriation-purified, cryopreserved human monocytes (> 90% purity) (Lund et al., 2000) from consenting, healthy donors (3) were thawed and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS and penicillin (100 U/mL)/streptomycin (100 µg/mL), at 37 °C under 5% CO₂, and plated at a density of 1.0×10^6 cells/cm². Monocytes were cultured for 24 h to allow adhering to the plastic and non-adherent PBMC were washed off. Monocyte-derived macrophages (MDM) were differentiated in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF, 50 ng/mL) for 7 days (Solhaug et al., 2015). The medium was replenished on day four and on the day of exposure. Macrophage purity and differentiation were verified for presence of CD68 by flow cytometric analysis. The cells were checked for viability and were approximately 70% confluent at the day of exposure.

2.2.4. Bronchial epithelial cells (BEAS-2B)

The BEAS-2B cell line, which is a SV-40 hybrid (Ad12SV40) transformed human bronchial epithelial cells, was purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in serum-free LHC-9 medium on collagen-coated flasks in a humidified atmosphere at 37 °C with 5% CO₂, with refreshment of medium every second day and passaged twice per week. Two days prior to exposure, cells were plated into collagen-coated 10 mm 12-well culture dishes (8×10^4 cells/well) and grown to near confluence. In all experiments medium was changed the day after seeding and before exposure.

2.3. Preparation of AFH and LPS stock-solution

A. fumigatus hypha (AFH) sample preparation and characterization have been previously described elsewhere (Øya et al., 2018). In short, AFH was grown on agar covered with cellophane membrane. The mycelial mass was harvested prior to sporulation by scraping the mass into a polypropylene centrifuge tube. The biomass was freeze-dried and then ground in a RetchMM301 mixer mill (Retsch GmbH, Haan, Germany). The resulting mycelial mass powder was vacuumed through a cyclone (GK2.69 Cyclone; BGI, Waltham, MA, USA) operated in the respirable mode at 4.2 L/min onto polycarbonate filters which were eluted in phosphate buffered saline. Following centrifugation, the fragment pellet was resuspended in LHC-9 cell culture medium and incubated at 37 °C for 20 h and then kept at −80 °C. The fungal fragments were inactivated on ice with x-ray (17.45 Gy/min, 225 kV, 13 mA, no filter, 5 cm distance from the source) from X-RAD 225 (Precision X-ray Inc., North Branford) receiving a total dose of 5 kGy. AFH stock solution (31.8 mg/mL) was prepared in LHC-9 culture medium and

characterized (Øya et al., 2018). In the AFH sample, fragments of 1–10 µm dominated by relative number (84%). Lipopolysaccharide (LPS) was purchased from Sigma Aldrich (St. Louis, MO, USA) and prepared at a stock concentration of 1 mg/mL in sterile water.

2.4. Particle samples and preparations

Min-U-Sil 5 quartz, a high purity natural crystalline silica particle, was obtained from U.S. Silica Company (Berkeley Springs, WV, USA). According to the manufacturer it has a diameter of ≤ 5 µm (median size 1.6 µm) and contains 99% SiO₂. Min-U-Sil was suspended in FBS free RPMI1640 medium at a concentration of 5 mg/mL, stirred overnight at room temperature (RT), sonicated for 30 min in an ultrasonic water bath (Sonorex RK 100, Transistor) and stored at 4 °C (maximum 3 weeks) as also described elsewhere (Herseeth et al., 2008). The particle suspension was vortexed for 30 s before added to the cells.

2.5. Cytokine release

Pro-inflammatory cytokines IL-1β and TNF-α were analyzed in the supernatants after exposures using quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit. The kits were used according to the manufacturer's standard protocols. The corresponding limits of detection were as follows: TNF-α, 1.7 pg/mL; IL-1β, 3.9 pg/mL. Absorbance was read using a plate reader (TECAN sunrise, Austria GmbH) employing Magellan V1.10 software.

2.6. Cytotoxicity

Cells were examined after exposures and their viability was determined using fluorescence microscopy after staining with Propidium Iodide (PI) and Hoechst 33342 (Øya et al., 2018). At least 400 cells were counted per sample.

2.7. Gene expression

Total RNA was isolated using NukleoSpin RNA Plus (Macherey-Nagel, Düre, Germany) and reverse transcribed to cDNA on a PCR System 2400 (PerkinElmer, Waltham, MA, USA), using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using pre-designed TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix and run on ABI 7500 fast (Applied Biosystems). Gene expressions of induced IL-1β (Hs01555410_m1) and TNF (Hs01113624_g1) were normalized against ACTB (Hs01060665_g1), and expressed as fold change compared to untreated control as calculated by the $\Delta\Delta C_t$ method ($\Delta C_t = C_t[\text{Gene of Interest}] - C_t[\text{ACTB}]$; $\Delta\Delta C_t = \Delta C_t[\text{Treated}] - \Delta C_t[\text{Control}]$; Fold change = $2^{[-\Delta\Delta C_t]}$).

2.8. Statistical analysis

All statistical analyses were performed in GraphPad Prism 7 (GraphPad Software Inc., La Jolla, California, USA). One- or two-way analysis of variance (ANOVA) with Dunnett or Sidak as a post-test was used to analyze the data sets depending on the number of explanatory variables in the experimental design. As specified in the figure legends, some data were log transformed before performing ANOVA to fulfil the assumption of equal standard deviations of all sets of replicates. The co-exposure data in Figs. 6 and 7 were normalized using a two-step procedure to scale the maximum response level (Fig. 6) or controls (Fig. 7) within in each data set to the same level while still retaining variation, allowing for ANOVA analysis. This was done due to large variations in the basal response levels between different donors/experiments. The data from each experiment was first normalized to the mean of that experiment. Then, the data in each data set, consisting of 3–6 different experiments, were normalized to the mean of the controls in those

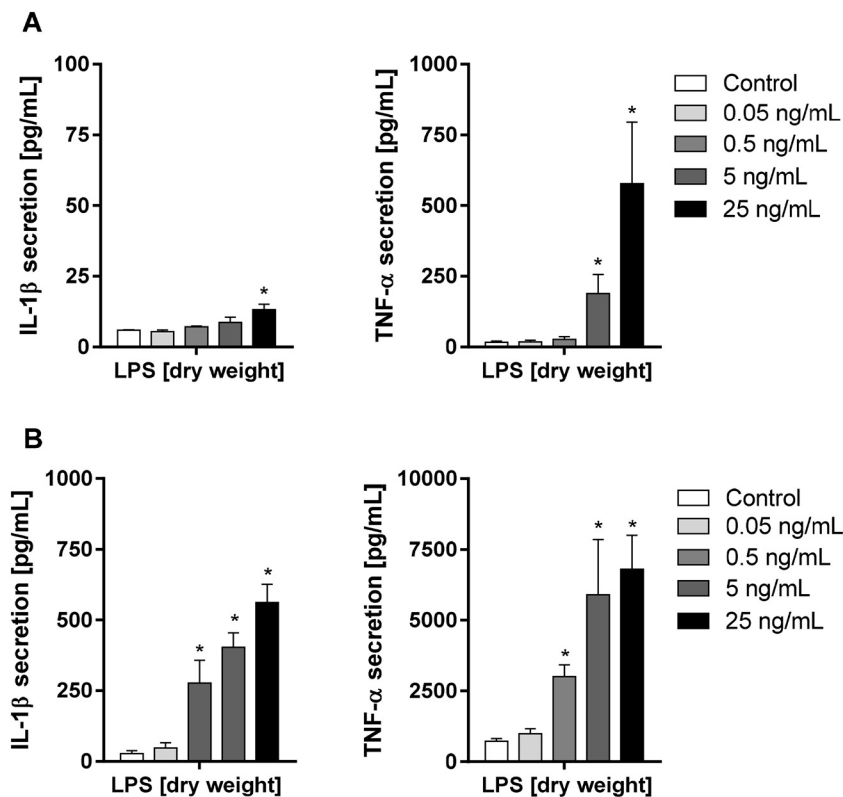


Fig. 1. IL-1 β and TNF- α release from THP-1 Mo/Ma after single exposures of LPS. (A) THP-1 Mo and (B) THP-1 Ma were exposed to four different concentrations of LPS (0.05, 0.5, 5, 25 μ g/mL) for 24 h and assessed for release of IL-1 β and TNF- α . Bars represent mean $\pm$ SEM concentrations of 3–4 independent experiments. Statistical analysis was based on log transformed data (one-way ANOVA, Dunnett post-test). Significant difference compared to the control denoted by * $P < 0.05$.

experiments. The resulting values were multiplied by 100 to achieve data reflecting % of control values. Results are expressed as mean $\pm$ standard error of mean (SEM). P values < 0.05 were considered as significant.

3. Results

3.1. Effect of single exposures on cytokine release in THP-1 Mo/Ma

In order to study the responsiveness of THP-1 Mo and THP-1 Ma towards LPS, AFH and Min-U-Sil, cells were exposed for 24 h to a range of concentrations individually and assessed for cytokine secretions (Figs. 1, 2 and 3). In THP-1 Mo exposed to four different concentrations of LPS in the non-cytotoxic range (Fig. 1A), the IL-1 β and TNF- α release increased moderately but statistically significant at the highest concentrations (5 and/or 25 ng/mL). In contrast, THP-1 Ma exposed to LPS showed a marked increase in both IL-1 β and TNF- α release from 0.5 ng/mL (Fig. 1B).

Exposure to AFH concentrations up to 100 μ g/mL did not induce apoptosis nor membrane damage in THP-1 Mo or THP-1 Ma as judged by microscopic examination (data not shown). THP-1 Mo exposed to 0.5–100 μ g/mL of AFH showed a marked release of IL-1 β and TNF- α (Fig. 2A), while THP-1 Ma responded to AFH at much lower concentrations (1–10 ng/mL). More specifically, a significant release of IL-1 β from 1 ng/mL and of TNF- α from 10 ng/mL was seen in THP-1 Ma (Fig. 2B), while AFH concentrations in the μ g/mL range were necessary to induce release in the THP-1 Mo.

THP-1 Mo exposed to Min-U-Sil showed a slight statistically increase in cell death/membrane damage starting at 20 μ g/cm² ending up to 16% at 160 μ g/cm² compared to 1–5% in control. Min-U-Sil did not increase the release of IL-1 β and TNF- α in THP-1 Mo (Fig. 3A). However, the release of IL-1 β , but not TNF- α (Fig. 3B) was enhanced following 24 h Min-U-Sil exposure in THP-1 Ma. The IL-1 β response was dose-dependent, and significantly increased at 80 μ g/cm² and 160 μ g/cm² Min-U-sil compared to the control. Min-U-Sil did not induce cell

death in THP-1 Ma (data not shown).

3.2. Effect of co-exposures on cytokine secretion in THP-1 Mo/Ma

For the combined exposures, low concentrations of Min-U-Sil were used (20 and 40 μ g/cm² exhibiting low cytokine release) in combination with inflammatory agent concentrations inducing moderate effects (25 and 500 ng/mL of LPS and AFH in THP-1 Mo and 0.5 and 50 ng/mL in THP-1 Ma). In both cell models, a priming exposure to LPS or AFH was done for 3 h, followed by a co-exposure to Min-U-Sil for another 18 h (Fig. 4). THP-1 Mo exposed to AFH and Min-U-Sil showed an enhanced release of IL-1 β when compared to the two single exposures, whereas the TNF- α release was not further increased (Fig. 4A). Similarly, in LPS primed THP-1 Mo, the IL-1 β release increased markedly after the Min-U-Sil co-exposure, while the TNF- α release was not affected (Fig. 4A).

In THP-1 Ma, AFH priming followed by exposure to the highest dose of Min-U-Sil increased the IL-1 β release significantly compared to AFH alone (Fig. 4B). Min-U-Sil also enhanced the release of IL-1 β following LPS exposure. On the contrary, TNF- α release from THP-1 Ma primed with LPS or AFH and co-exposed to Min-U-Sil was not significantly affected (Fig. 4B). None of the co-exposure increased cell death.

3.3. Effects of single and co-exposures on mRNA expression in THP-1 Mo/Ma

In THP-1 Mo, exposure to AFH (500 ng/mL) alone increased IL-1 β expression 75-fold and TNF- α expression 25-fold compared to the controls. Also LPS (25 ng/mL) enhanced IL-1 β and TNF- α expressions when compared to control, whereas no effects were seen after Min-U-Sil (20 and 40 μ g/cm²) exposure. Min-U-Sil had no effects on LPS induced expression of IL-1 β and TNF- α , whereas the expressions of both cytokines following AFH were rather reduced (Fig. 5A).

THP-1 Ma primed with LPS (0.5 ng/mL) or AFH (50 ng/mL) for 3 h and co-exposed with Min-U-Sil (40 μ g/cm²) for 3 h were also analyzed

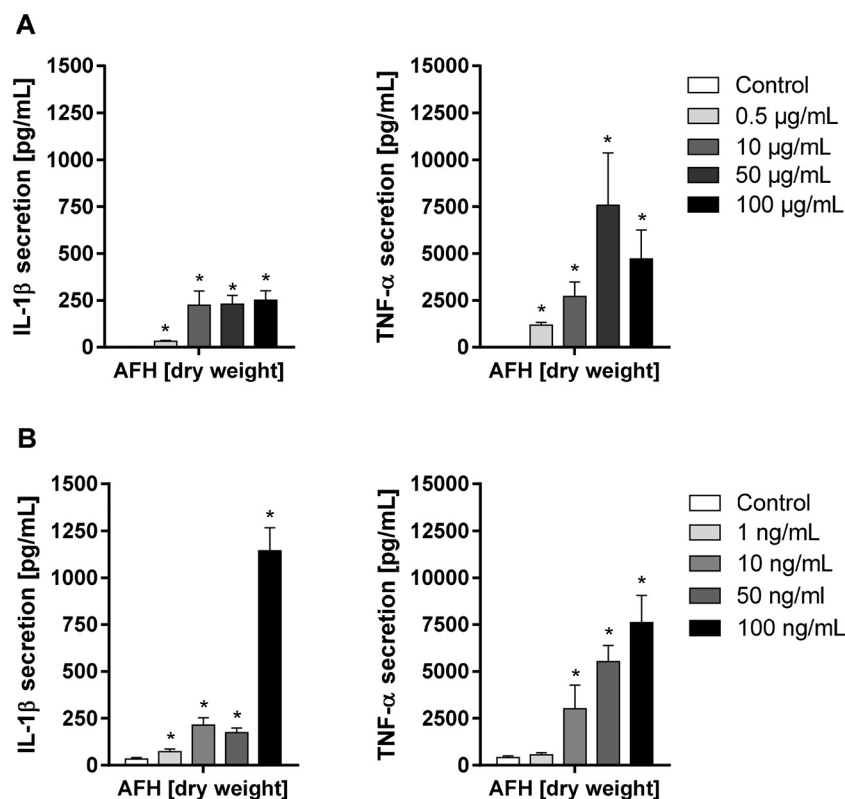


Fig. 2. IL-1 β and TNF- α release from THP-1 Mo/Ma after single exposures of AFH. (A) THP-1 Mo and (B) THP-1 Ma were exposed to four different concentrations of AFH (0.5, 10, 50, 100 $\mu\text{g/mL}$ and 1, 10, 50, 100 ng/mL respectively) for 24 h and evaluated for release of IL-1 β and TNF- α . Bars represent mean $\pm$ SEM concentrations of 3–4 independent experiments. Statistical analysis was based on log transformed data (one-way ANOVA, Dunnett post-test). Significant difference compared to the control denoted by * $P < 0.05$.

for IL-1 β and TNF- α gene expression. When compared to THP-1 Mo, the relative responses were less marked in THP-1 Ma. The maximum IL-1 β gene expressions observed were only about 5-fold in cells exposed to LPS alone and 4-fold with AFH alone. Furthermore, the gene expressions were not significantly influenced upon co-exposure to Min-U-Sil.

Similar effects were noted for TNF- α gene expression. All exposures except Min-U-Sil alone, were significantly increased compared to control. However, none of the co-exposures showed statistically significant differences in neither IL-1 β nor TNF- α expressions compared to their individual controls (LPS and AFH alone) (Fig. 5B).

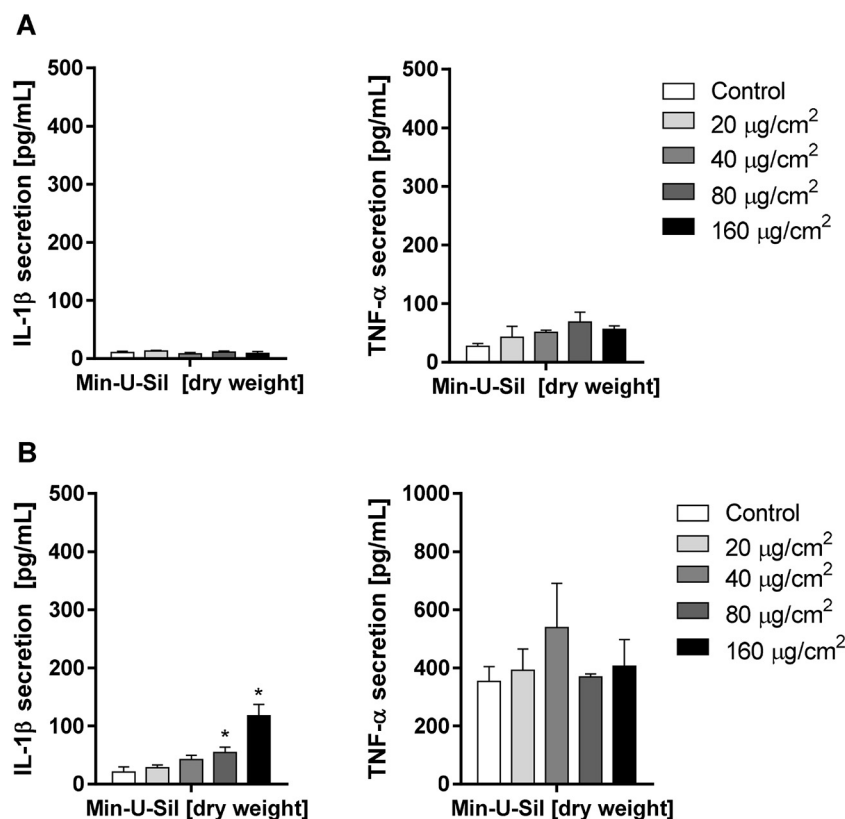


Fig. 3. IL-1 β and TNF- α release from THP-1 Mo/Ma after single exposures of Min-U-Sil. (A) THP-1 Mo and (B) THP-1 Ma were exposed to four different concentrations of Min-U-Sil (20, 40, 80, 160 $\mu\text{g/cm}^2$) for 24 h and evaluated for release of IL-1 β and TNF- α . Bars represent mean $\pm$ SEM concentrations of 3–4 independent experiments. Statistical analysis was based on log transformed data (one-way ANOVA, Dunnett post-test). Significant difference compared to the control denoted by * $P < 0.05$.

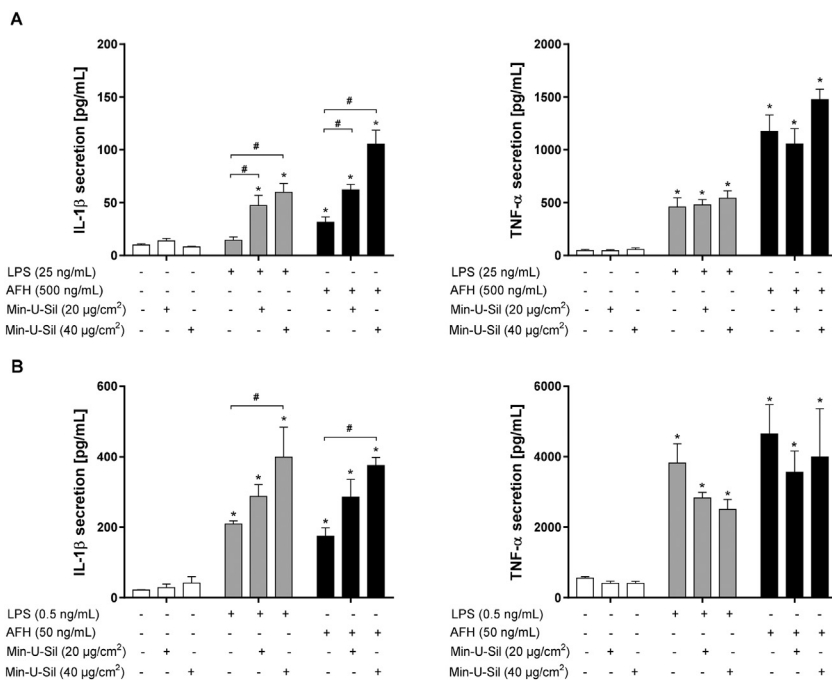


Fig. 4. Effects of co-exposure on IL-1 β and TNF- α release from THP-1 Mo/Ma. (A) THP-1 Mo and (B) THP-1 Ma primed with AFH (500 ng/mL and 50 ng/mL respectively) or LPS (25 ng/mL and 0.5 ng/mL respectively) for 3 h and co-exposed to Min-U-Sil (20, 40 μ g/cm²) for 18 h assessed for IL-1 β and TNF- α release. Bars represent mean $\pm$ SEM concentration of three independent experiments. Statistical analysis was based on log transformed data (two-way ANOVA Dunnett post-test). Significant difference is denoted by * $P < 0.05$ (medium control vs exposed) or by # $P < 0.05$ (co-exposed cells vs individual controls (medium, LPS, AFH)), + treated, - untreated.

3.4. Effect of caspase-inhibition on IL-1 β release from THP-1 Mo

To further elucidate the effects of AFH and LPS alone and in combination with Min-U-Sil THP-1 Mo were pretreated with the general caspase inhibitor zYVAD-fmk one hour before single or combined exposures. As seen in Fig. 6 pretreatment with zYVAD-fmk (also inhibiting caspase-1) markedly decreased the IL-1 β -release in both single and combined exposures regimes (Fig. 6). Min-U-Sil induced no effects alone (data not shown). In contrast, zYVAD-fmk had no effects on the release of TNF α (Supplementary Fig. S1).

3.5. Cytokine secretion from AM, MDM and BEAS-2B cells after single and co-exposures

In order to further elucidate the relevance of the synergistic IL-1 β responses, we explored the effects of AFH and Min-U-Sil after single and

co-exposure in two primary macrophage models: human airway macrophages (AM)/sputum macrophages and monocyte-derived macrophages (MDM), as well as a bronchial epithelial cell line (BEAS-2B). Based on previous experiments (Øya et al., 2018, Øya et al. unpublished) non cytotoxic concentrations of AFH giving low or no effects on IL-1 β were chosen. With regard to Min-U-Sil we used the highest non-cytotoxic dose. As judged by light microscopy, neither of the test materials alone nor their combinations affected viability in any of the cell models at the concentrations used (data not shown). Following single exposures in AM, a slight increase in IL-1 β was induced by Min-U-Sil, while AFH did not enhance the release of IL-1 β . Priming and co-exposure with AFH and Min-U-Sil showed a clear synergistic increase in the IL-1 β release (Fig. 7A). In MDM, neither single exposures with AFH nor Min-U-Sil induced IL-1 β release. However, combined exposure again increased IL-1 β release markedly (Fig. 7B). Similarly, in the bronchial epithelial cells line BEAS-2B the single exposures did not

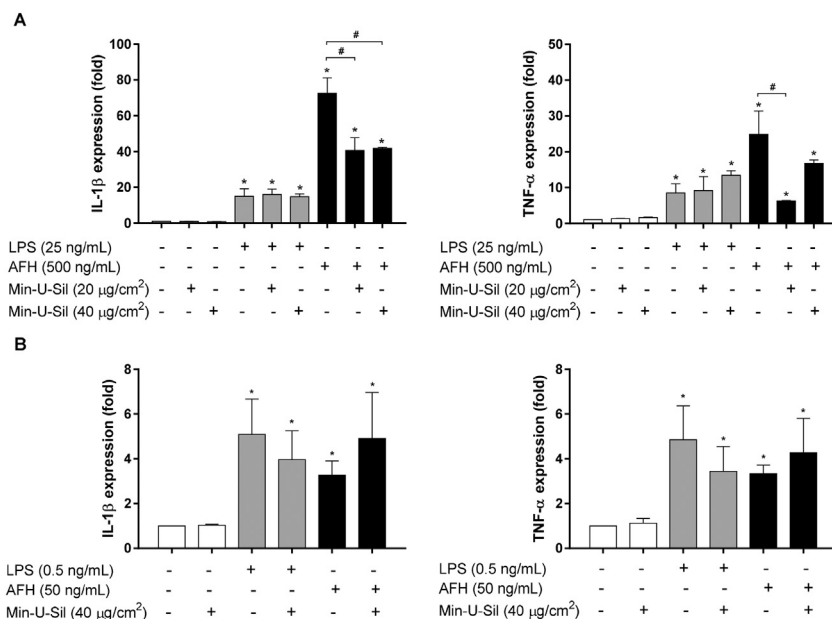


Fig. 5. Effects of co-exposure on IL-1 β and TNF- α gene expression in THP-1 Mo/Ma. (A) THP-1 Mo and (B) THP-1 Ma were primed with AFH (500 ng/mL and 50 ng/mL respectively) or LPS (25 ng/mL and 0.5 ng/mL respectively) for 3 h and co-exposed with Min-U-Sil (20, 40 μ g/cm²) for further 3 h. The mRNA level of IL-1 β and TNF- α were assessed by using real time RT-PCR. Bars represent means $\pm$ SEM of the fold increase relative to control of three independent experiments. Statistical analysis was based on log transformed data (two-way ANOVA Dunnett post-test). Significant difference is denoted by * $P < 0.05$ (medium control vs exposed) or by # $P < 0.05$ (co-exposed cells vs individual controls (medium, LPS, AFH)), + treated, - untreated.

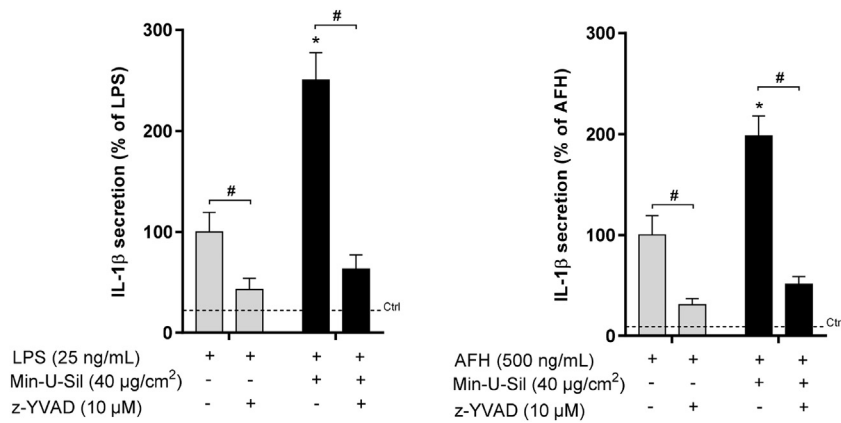


Fig. 6. Effects of caspase inhibitor on IL-1 β release after single and co-exposures from THP-1 Mo. THP-1 Mo were exposed to AFH (500 ng/mL), LPS (25 ng/mL) and Min-U-Sil (40 μ g/cm²) for 21 or 18 h, or primed with AFH or LPS for 3 h and co-exposed with Min-U-Sil for further 18 h. Both setups were with or without caspase inhibitor (z-YVAD-fmk, 10 μ M) pre-treatment for 1 h and assessed for IL-1 β release. All data were normalized by using a 2-step normalization procedure. Bars represent mean $\pm$ SEM concentrations of four independent experiments. Statistical analysis was based on log transformed data (two-way ANOVA with Sidak post test). Significant difference is denoted by * $P < 0.05$ (exposed cells vs co-exposed) or by # $P < 0.05$ (exposed cells vs z-YVAD), + treated, – untreated. Control without zYVAD 2.3 ± 0.9 pg/mL, and control with zYVAD 2.5 ± 1.0 pg/mL. Control (Ctrl) shown as dotted line.

induce significant cytokine release, while combined exposure to AFH and Min-U-Sil induced release of IL-1 β (Fig. 7C). The TNF- α release was not affected by AFH or Min-U-Sil exposures alone or in combination in any of the three cell models (data not shown).

4. Discussion

Production of pro-inflammatory cytokines plays a crucial role in regulating immune responses and inflammation. Here we investigated the effects of AFH, LPS and Min-U-Sil, alone and upon co-exposures, on the release of IL-1 β and TNF- α , using THP-1 Mo, THP-1 Ma, AM, MDM and BEAS-2B cells as experimental models. Our results show that exposures to LPS and AFH alone increase both the synthesis and release of IL-1 β and TNF- α in THP-1 Mo and THP-1 Ma. Min-U-Sil alone did not appear to increase synthesis, but enhanced the release of IL-1 β in THP-1 Ma and AM. Like LPS, co-exposure to AFH and Min-U-Sil synergistically enhanced the release of IL-1 β in both THP-1 Mo and THP-1 Ma. The apparent synergistic effects on the release of IL-1 β following co-exposure was apparently not reflected by a correspondingly increase in mRNA. The general caspase inhibitor zYVAD almost completely inhibited the induced IL-1 β release in THP-1 Mo. AFH and Min-U-Sil also synergistically enhanced the release of IL-1 β in AM, MDM and BEAS-2B cells; while no synergism was observed in any of the cell models with respect to TNF- α .

IL-1 β and TNF- α are known to have a critical role in regulation of a

large range of other cytokines (Turner et al., 2014). Both of these pro-inflammatory cytokines require a two-step activation (Lopez-Castejon and Brough, 2011; Monteleone et al., 2015). Partly depending on the experimental model, LPS has been reported to increase synthesis of pro-IL1 β via NF- κ B-activation (step 1) and activation of the inflammasome (NLRP3; step 2), leading to cleavage of pro-IL1 β and release of the active form (Netea et al., 2009). Accordingly, we found that LPS increased the synthesis of mRNA for pro-IL1 β and release of IL1- β in both THP-1 Mo and THP-1 Ma. Similarly, AFH alone enhanced both the expression and release of IL1- β in both the THP-1 models, illustrating a pro-inflammatory potential of fungal components as also previously reported (Bozza et al., 2002; Gersuk et al., 2006; Bellanger et al., 2009; Pei and Gunsch, 2013; Øya et al., 2018).

A critical question is at what concentrations of LPS and AFH the pro-inflammatory effects are exerted in the different cell models. As macrophages in a healthy lung are resident, while those recruited during invading of pathogens derive from monocytes, we decided to compare THP-1 monocytes with various macrophages models. THP-1 monocytic leukemia cells can be differentiated into macrophages using PMA, which has been suggested as a reliable in vitro model (Chanput et al., 2014) for studying immune cell processes. Notably, in the present study the synthesis and release of both IL1- β and TNF α were markedly facilitated by PMA pre-treatment of THP-1 Mo, as the stimulatory effects of LPS and AFH were exerted at lower concentrations in THP-1 Ma compared to THP-1 Mo. A possible explanation could be that the PMA-

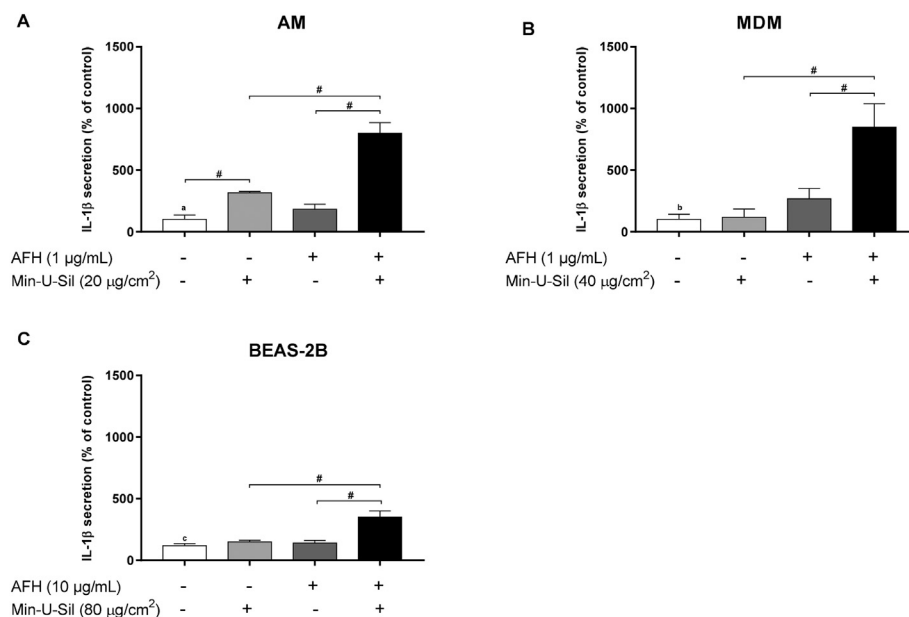


Fig. 7. Effects of co-exposure on IL-1 β release after single and co-exposures from AM, MDM and BEAS-2B cells. (A) AM (B) MDM (C) BEAS-2B cells were exposed to AFH (1, 1 and 10 μ g/mL respectively) and Min-U-Sil (20, 40 and 80 μ g/cm² respectively) for 24 h or 18 h, or primed with AFH for 3 h and co-exposed with Min-U-Sil for further 18 h and assessed for IL-1 β release. Bars represent mean $\pm$ SEM concentrations of 3–5 independent experiments. All data was normalized by using a 2-step normalization procedure. Statistical analysis was performed by two-way ANOVA with Sidak post-test. Significant difference compared to the respective control denoted by #, $P < 0.05$. Control ^a 25.7 ± 16.9 ^b 2.3 ± 1.1 ^c 2.2 ± 0.8 pg/mL.

treatment induced the expression of TLR's recognizing AFH and LPS. In line with this, the expression of TLR including TLR2 and TLR4 are reported to be enhanced in THP-1 Ma and other macrophages (Li et al., 2002; Zarembek and Godowski, 2002; Daigneault et al., 2010). Activation of signals from both these two receptors merge towards a common pathway involving activation of NF κ B (Mambula et al., 2002; Meier et al., 2003; Gersuk et al., 2006). As AFH may use both TLRs, it was expected that AFH had rather similar effects to that seen with LPS (Takeuchi et al., 1999; Wang et al., 2001). Thus, it is interesting to note that AFH seem to trigger a much higher response in THP-1 Ma versus THP-1 Mo when compared to LPS. Since TLR4 is considered to be important for LPS, while AFH recognises both TLR2 and TLR4, it is tempting to speculate that THP-1 Ma may have a higher TLR2/TLR4 ratio when compared to that seen in THP-1 Mo. However, further studies are needed in order to clarify this suggestion.

PMA is considered as an optimal differentiation agent of THP-1 Mo to macrophages, providing the desirable effects including phenotypes, loss of proliferation, adherence and expression of receptors (Daigneault et al., 2010). Compared to other myeloid cell lines, PMA-differentiated THP-1 Mo (THP-1 Ma) express many properties similar to primary human macrophages (Auwerx, 1991; Qin, 2012). However, it should not be excluded that PMA treatment also could interfere with essential mechanisms of the cytokine production (Lund et al., 2016), and that this may explain the somewhat high cytokine baseline observed in the THP-1 Ma. The mechanisms for inflammasome activation leading to processing and release of IL-1 β appear to differ in various types of monocyte and macrophage cellular models (Netea et al., 2008; Netea et al., 2009; Daigneault et al., 2010; Tedesco et al., 2018). Recently, two inflammasome receptors, AIM2 and NLRP3, were reported to recognize intracellular *A. fumigatus* and induce protective immune responses (Karki et al., 2015; Tomalka and Hise, 2015). AIM2 and NLRP3 activation initiates assembly of a single cytoplasmic inflammasome platform, composed of the adaptor protein ASC along with caspase-1 and caspase-8. It was reported that the combined actions of caspase-1 and caspase-8 lead to processing of the pro-form of IL-1 β . In another study, caspase-11 was found to be required for cleavage of caspase-1 and secretion of IL-1 β following *A. fumigatus* infection (Sun et al., 2018). In the present study, we observe that both LPS or AFH alone induced synthesis (IL-1 β mRNA) and release of IL-1 β in THP-1 Mo, as IL-1 β release was dramatically decreased by the caspase inhibitor z-VYAD in accordance with a role of caspases. However, following stimulation with LPS or AFH, Min-U-Sil gave a further enhanced release of IL-1 β .

Obviously, care should be taken when extrapolations from in vitro models to the in vivo situation, and also upon comparing primary cells and cell lines, macrophages with other cell types such as epithelial cells. THP-1 Ma was highly sensitive to AFH exposure, responding to much lower concentrations than in the other macrophage models (AM and MDM) as well as in epithelial BEAS-2B cells. It could however be that this model is too sensitive. As lung cells are naturally and constantly exposed to microbiota, IL-1 β release in the lung are carefully regulated (De Nardo et al., 2014). A sensitive response in lung cells would have resulted in deleterious inflammatory reactions if they responded promptly upon each danger signal to which they were naturally exposed. Thus, although THP-1 Ma is a very sensitive model with regard to AFH, further studies are needed to clarify the relevance of the various macrophage models for studying the effect of fungi on the synthesis and release of IL-1 β .

A lot of evidence has demonstrated that a range of agents like uric acid crystals and ATP, and also crystalline silica, may activate the inflammasome and caspase 1, leading to an increased cleavage of pro-IL-1 β to IL-1 β (Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008; Park and Park, 2009; Sandberg et al., 2012). In the present study, the effects of crystalline silica (Min-U-Sil) without and with LPS- and AFH-priming were explored in different cell models. Min-U-Sil did not increase the synthesis or the release of IL-1 β in THP-1 Mo when tested alone. In contrast, in THP-1 Ma, Min-U-Sil directly induced IL-1 β

release. No apparent increase in the corresponding level of mRNA was seen. This is suggesting that the increased release of IL-1 β seen in THP-1 Ma was due to an enhanced cleavage of pre-existing basal levels of pro-IL-1 β rather than an increased synthesis of the cytokine, and furthermore that the PMA-treatment resulted in an increased level of pro-IL-1 β as seen in the THP-1 Ma.

A central finding was that AFH similarly to LPS, seems to exert a synergistic effect in combination with Min-U-Sil on IL-1 β release in both the THP-1 Mo and THP-1 Ma cells. Furthermore, as Min-U-Sil rather reduced the AFH-induced IL-1 β -mRNA, Min-U-Sil seemed to stimulate release of pro-IL-1 β which were not released by AFH exposure. This assumption were supported by the reduced IL-1 β release as seen following addition of the general caspase inhibitor z-VYAD. Thus, both LPS as well as AFH seemed to form a pool of pro-IL-1 β which could be further activated by Min-U-Sil. Whether the Min-U-Sil-released IL-1 β represent some kind of a specific pro-IL-1 β pool different from that released after exposure to LPS or AFH alone remain to be elucidated.

Similar to IL-1 β , both LPS and AFH induce synthesis and release of TNF- α in THP-1 Mo and THP-1 Ma cells in line with what has been reported by others (Wang et al., 2001; Simitsopoulou et al., 2007). Interestingly, the release of TNF- α was independent of caspases, as judged by the lack of effects of the inhibitor zVYAD-fmk. As zVYAD-fmk strongly reduced the release of IL-1 β , this finding indicates that the TNF- α release occurs independently of IL-1 β . Furthermore, also in contrast to IL-1 β , co-exposure of cells to LPS or AFH in combination with Min-U-Sil did not induce synergistic effects on TNF- α release. This may seem somewhat surprising as we previously have demonstrated in BEAS-2B cells that non-crystalline silica nanoparticles induce the cleavage of pro-TGF- α to TGF- α via a TACE-dependent mechanism (Lag et al., 2018). Apparently, Min-U-Sil is not able to trigger a cleavage of the pro-form of TNF- α via a similar mechanism in THP-1 cells. It is interesting to note that Murray and co-workers have found that endosome delivery including TNF- α secretion facilitate phagocytosis in macrophages (Murray et al., 2005; Duque and Descoteaux, 2014). Thus, alternatively it could be that AFH (and LPS) directly cleaves all the pro-form of TNF- α in the THP-1 models directly into mature TNF- α .

There are both tissue-specific macrophages and macrophages derived from circulating monocytes during inflammation. Sputum macrophages from individuals without an ongoing inflammation are likely to be tissue-specific macrophages. It is still unclear whether tissue-derived and monocyte-derived macrophages have different functions in the lung (Chanput et al., 2014; Epelman et al., 2014). In the present study, we observe that Min-U-Sil enhanced IL-1 β release following exposure of LPS or AFH in monocytes, macrophages as well as lung epithelial cells, while no similar enhancement of the release of TNF- α were seen. Interestingly, a slight increase in release of IL-1 β following Min-U-Sil in AM was seen, suggesting that these cells may contain a pool of pro-IL-1 β . Possibly these cells represent macrophages that have been exposed to microbiota and are transferred up via the mucociliary escalator.

Lung macrophages and epithelial cells are naturally exposed to such microbiota, sensitized or primed cells may represent a relevant models to explore pro-inflammatory reactions to selected air pollution particles. Thus, the results support the hypothesis that inflammatory responses due to pulmonary particulate matter exposure are at least in part a result of a simultaneous exposure to various microbiota and particulate matter in the airways.

In conclusion, our data suggest that AFH similar to LPS induce the release of IL-1 β by increasing the synthesis of pro-IL-1 β , and in addition caused a marked cleavage of the pro-form to IL-1 β . The cleavage of the AFH-induced pro-form was, further enhanced in the presence of Min-U-Sil. The LPS, AFH- and the Min-U-Sil-induced IL-1 β were caspase-dependent as shown by inhibition by zVYAD-fmk. In contrast, the AFH- and LPS-induced TNF- α responses were not modified by Min-U-Sil.

Transparency document

The [Transparency document](#) associated to this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2018.11.002>.

References

- Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. *Cell* 124 (4), 783–801.
- Arango, Duque G., Descoteaux, A., 2014. Macrophage cytokines: involvement in immunity and infectious diseases. *Front. Immunol.* 5, 491.
- Auwerx, J., 1991. The human leukemia cell line, THP-1: a multifaceted model for the study of monocyte-macrophage differentiation. *Experientia* 47 (1), 22–31.
- Bellanger, A.P., Millon, L., Khouf, K., Rivollet, D., Bieche, I., et al., 2009. *Aspergillus fumigatus* germ tube growth and not conidia ingestion induces expression of inflammatory mediator genes in the human lung epithelial cell line A549. *J. Med. Microbiol.* 58 (Pt 2), 174–179.
- Bozza, S., Gaziano, R., Spreca, A., Bacci, A., Montagnoli, C., et al., 2002. Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J. Immunol.* 168 (3), 1362–1371.
- Cassel, S.L., Eisenbarth, S.C., Iyer, S.S., Sadler, J.J., Colegio, O.R., et al., 2008. The Nalp3 inflammasome is essential for the development of silicosis. *Proc. Natl. Acad. Sci. U. S. A.* 105 (26), 9035–9040.
- Chandler, C.E., Ernst, R.K., 2017. Bacterial lipids: powerful modifiers of the innate immune response. *(F1000Res)* 6.
- Chanput, W., Mes, J.J., Wichers, H.J., 2014. THP-1 cell line: an in vitro cell model for immune modulation approach. *Int. Immunopharmacol.* 23 (1), 37–45.
- Charlson, E.S., Bittner, K., Haas, A.R., Fitzgerald, A.S., Frank, I., et al., 2011. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am. J. Respir. Crit. Care Med.* 184 (8), 957–963.
- Cooper, G.S., Miller, F.W., Germolec, D.R., 2002. Occupational exposures and autoimmune diseases. *Int. Immunopharmacol.* 2 (2–3), 303–313.
- Dagenais, T.R., Keller, N.P., 2009. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *Clin. Microbiol. Rev.* 22 (3), 447–465.
- Daigneault, M., Preston, J.A., Marriott, H.M., Whyte, M.K., Dockrell, D.H., 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS ONE* 5 (1), e8668.
- De Nardo, D., De Nardo, C.M., Latz, E., 2014. New insights into mechanisms controlling the NLRP3 inflammasome and its role in lung disease. *Am. J. Pathol.* 184 (1), 42–54.
- Denning, D.W., Chakrabarti, A., 2017. Pulmonary and sinus fungal diseases in non-immunocompromised patients. *Lancet Infect. Dis.* 17 (11), e357–e366.
- Denning, D.W., O'Driscoll, B.R., Hogaboam, C.M., Bowyer, P., Niven, R.M., 2006. The link between fungi and severe asthma: a summary of the evidence. *Eur. Respir. J.* 27 (3), 615–626.
- Dostert, C., Petril, V., Van Bruggen, R., Steele, C., Mossman, B.T., et al., 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320 (5876), 674–677.
- Duque, G.A., Descoteaux, A., 2014. Macrophage cytokines: involvement in immunity and infectious diseases. *Front. Immunol.* 5.
- Epelman, S., Lavine, K.J., Randolph, G.J., 2014. Origin and functions of tissue macrophages. *Immunity* 41 (1), 21–35.
- Feghali, C.A., Wright, T.M., 1997. Cytokines in acute and chronic inflammation. *Front. Biosci.* 2, 12–26.
- Fung, F., Hughson, W.G., 2003. Health effects of indoor fungal bioaerosol exposure. *Appl. Occup. Environ. Hyg.* 18 (7), 535–544.
- Gersuk, G.M., Underhill, D.M., Zhu, L., Marr, K.A., 2006. Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. *J. Immunol.* 176 (6), 3717–3724.
- Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., Sherburn, R., et al., 2014. Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat. Med.* 20 (6), 642–647.
- Gorny, R.L., 2004. Filamentous microorganisms and their fragments in indoor air - a review. *Ann. Agric. Environ. Med.* 11 (2), 185–197.
- Green, B.J., Tovey, E.R., Sercombe, J.K., Blachere, F.M., Beezhold, D.H., et al., 2006. Airborne fungal fragments and allergenicity. *Med. Mycol.* 44 (Suppl. 1), S245–S255.
- He, Y., Hara, H., Nunez, G., 2016. Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends Biochem. Sci.* 41 (12), 1012–1021.
- Herseth, J., Refsnes, M., Lag, M., Hetland, G., Schwarze, P., 2008. IL-1 β as a determinant in silica-induced cytokine responses in monocyte-endothelial cell co-cultures. *Hum. Exp. Toxicol.* 27 (5), 387–399.
- Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., et al., 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat. Immunol.* 9 (8), 847–856.
- Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., et al., 2011. Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc. Natl. Acad. Sci. U. S. A.* 108 (13), 5354–5359.
- Karki, R., Man, S.M., Malireddi, R.K.S., Gurung, P., Vogel, P., et al., 2015. Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against *Aspergillus* infection. *Cell Host Microbe* 17 (3), 357–368.
- Kawasaki, H., 2015. A mechanistic review of silica-induced inhalation toxicity. *Inhal. Toxicol.* 27 (8), 363–377.
- Lag, M., Skuland, T., Godymchuk, A., Nguyen, T.H.T., Pham, H.L.T., et al., 2018. Silica Nanoparticle-induced Cytokine responses in BEAS-2B and HBEC3-KT Cells: significance of Particle size and Signalling Pathways in Different Lung Cell Cultures. *Basic Clin. Pharmacol. Toxicol.* 122 (6), 620–632.
- Lambrecht, B.N., 2006. Alveolar macrophage in the driver's seat. *Immunity* 24 (4), 366–368.
- Latge, J.P., 1999. *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12 (2), 310–350.
- Lee, B.U., 2011. Life comes from the air: a short review on bioaerosol control. *Aerosol Air Qual. Res.* 11, 921–927.
- Li, C., Wang, Y., Gao, L., Zhang, J., Shao, J., et al., 2002. Expression of toll-like receptors 2 and 4 and CD14 during differentiation of HL-60 cells induced by phorbol 12-myristate 13-acetate and 1 α , 25-dihydroxy-vitamin D(3). *Cell Growth Differ.* 13 (1), 27–38.
- Lopez-Castejon, G., Brough, D., 2011. Understanding the mechanism of IL-1 β secretion. *Cytokine Growth Factor Rev.* 22 (4), 189–195.
- Lund, P.K., Joo, G.B., Westvik, A.B., Ovstebo, R., Kierulf, P., 2000. Isolation of monocytes from whole blood by density gradient centrifugation and counter-current elutriation followed by cryopreservation: six years' experience. *Scand. J. Clin. Lab. Invest.* 60 (5), 357–365.
- Lund, M.E., To, J., O'Brien, B.A., Donnelly, S., 2016. The choice of phorbol 12-myristate 13-acetate differentiation protocol influences the response of THP-1 macrophages to a pro-inflammatory stimulus. *J. Immunol. Methods* 430, 64–70.
- Maciejewska, A., 2014. Health effects of occupational exposure to crystalline silica in the light of current research results. *Med. Pr.* 65 (6), 799–818.
- Mambula, S.S., Sau, K., Henneke, P., Golenbock, D.T., Levitz, S.M., 2002. Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. *J. Biol. Chem.* 277 (42), 39320–39326.
- Margalit, A., Kavanagh, K., 2015. The innate immune response to *Aspergillus fumigatus* at the alveolar surface. *FEMS Microbiol. Rev.* 39 (5), 670–687.
- Meier, A., Kirschning, C.J., Nikolaus, T., Wagner, H., Heesemann, J., et al., 2003. Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. *Cell. Microbiol.* 5 (8), 561–570.
- Monteleone, M., Stow, J.L., Schroder, K., 2015. Mechanisms of unconventional secretion of IL-1 family cytokines. *Cytokine* 74 (2), 213–218.
- Morishige, T., Yoshioka, Y., Inakura, H., Tanabe, A., Yao, X., et al., 2010. The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1 β production, ROS production and endosomal rupture. *Biomaterials* 31 (26), 6833–6842.
- Munzel, T., Sorensen, M., Gori, T., Schmidt, F.P., Rao, X., et al., 2017a. Environmental stressors and cardio-metabolic disease: part II-mechanistic insights. *Eur. Heart J.* 38 (8), 557–564.
- Munzel, T., Sorensen, M., Gori, T., Schmidt, F.P., Rao, X., et al., 2017b. Environmental stressors and cardio-metabolic disease: part I-epidemiologic evidence supporting a role for noise and air pollution and effects of mitigation strategies. *Eur. Heart J.* 38 (8), 550–556.
- Murray, R.Z., Kay, J.G., Sangermani, D.G., Stow, J.L., 2005. A role for the phagosome in cytokine secretion. *Science* 310 (5753), 1492–1495.
- Netea, M.G., van de Veerdonk, F.L., Kullberg, B.J., Van der Meer, J.W., Joosten, L.A., 2008. The role of NLRs and TLRs in the activation of the inflammasome. *Expert. Opin. Biol. Ther.* 8 (12), 1867–1872.
- Netea, M.G., Nold-Petry, C.A., Nold, M.F., Joosten, L.A., Opitz, B., et al., 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1 β in monocytes and macrophages. *Blood* 113 (10), 2324–2335.
- Orellano, P., Quaranta, N., Reynoso, J., Balbi, B., Vasquez, J., 2017. Effect of outdoor air pollution on asthma exacerbations in children and adults: Systematic review and multilevel meta-analysis. *PLoS ONE* 12 (3), e0174050.
- OSHA, 2017. Occupational Safety and Health Administration; Silica. <https://www.osha.gov/dsg/topics/silicacrystalline/>.
- Øvrevik, J., Refsnes, M., Låg, M., Holme, J.A., Schwarze, P.E., 2015. Activation of proinflammatory responses in cells of the airway mucosa by particulate matter: oxidant- and non-oxidant-mediated triggering mechanisms. *Biomol. Ther.* 5 (3), 1399–1440.
- Øya, E., Afanou, A.K.J., Malla, N., Uhlig, S., Rølen, E., et al., 2018. Characterization and pro-inflammatory responses of spore and hyphae samples from various mold species. *Indoor Air* 28 (1), 28–39.
- Park, E.J., Park, K., 2009. Oxidative stress and pro-inflammatory responses induced by silica nanoparticles in vivo and in vitro. *Toxicol. Lett.* 184 (1), 18–25.

- Park, E.K., Jung, H.S., Yang, H.I., Yoo, M.C., Kim, C., et al., 2007. Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm. Res.* 56 (1), 45–50.
- Pei, R., Gunsch, C.K., 2013. Inflammatory cytokine gene expression in THP-1 cells exposed to *Stachybotrys chartarum* and *Aspergillus versicolor*. *Environ. Toxicol.* 28 (1), 51–60.
- Qin, Z., 2012. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis* 221 (1), 2–11.
- Sandberg, W.J., Lag, M., Holme, J.A., Friede, B., Gualtieri, M., et al., 2012. Comparison of non-crystalline silica nanoparticles in IL-1 β release from macrophages. *Part Fibre Toxicol.* 9, 32.
- Schroder, K., Tschopp, J., 2010. The inflammasomes. *Cell* 140 (6), 821–832.
- Shi, C., Pamer, E.G., 2011. Monocyte recruitment during infection and inflammation. *Nat. Rev. Immunol.* 11 (11), 762–774.
- Shima, K., Coopmeiners, J., Graspeuntner, S., Dalhoff, K., Rupp, J., 2016. Impact of micro-environmental changes on respiratory tract infections with intracellular bacteria. *FEBS Lett.* 590 (21), 3887–3904.
- Sikkeland, L.I., Dahl, C.P., Ueland, T., Andreassen, A.K., Gude, E., et al., 2012. Increased levels of inflammatory cytokines and endothelin-1 in alveolar macrophages from patients with chronic heart failure. *PLoS ONE* 7 (5), e36815.
- Simitsopoulou, M., Roilides, E., Likartsis, C., Ioannidis, J., Orfanou, A., et al., 2007. Expression of immunomodulatory genes in human monocytes induced by voriconazole in the presence of *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 51 (3), 1048–1054.
- Solhaug, A., Wisbech, C., Christoffersen, T.E., Hult, L.O., Lea, T., et al., 2015. The mycotoxin alternariol induces DNA damage and modify macrophage phenotype and inflammatory responses. *Toxicol. Lett.* 239 (1), 9–21.
- Sun, Y., Abbondante, S., Karmakar, M., de Jesus Carrion, S., Che, C., et al., 2018. Neutrophil Caspase-11 is Required for Cleavage of Caspase-1 and Secretion of IL-1 β in *Aspergillus fumigatus* Infection. *J. Immunol.* <https://doi.org/10.4049/jimmunol.1701195>.
- Takashiba, S., Van Dyke, T.E., Amar, S., Murayama, Y., Soskolne, A.W., et al., 1999. Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor kappaB. *Infect. Immun.* 67 (11), 5573–5578.
- Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. *Cell* 140 (6), 805–820.
- Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., et al., 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11 (4), 443–451.
- Taramelli, D., Malabarba, M.G., Sala, G., Basilio, N., Cocuzza, G., 1996. Production of cytokines by alveolar and peritoneal macrophages stimulated by *Aspergillus fumigatus* conidia or hyphae. *J. Med. Vet. Mycol.* 34 (1), 49–56.
- Tedesco, S., De Majo, F., Kim, J., Trenti, A., Trevisi, L., et al., 2018. Convenience versus biological significance: are PMA-differentiated THP-1 Cells a reliable substitute for blood-derived macrophages when studying in vitro polarization? *Front. Pharmacol.* 9, 71.
- Tomalka, J., Hise, A.G., 2015. Inflammasomes in aspergillosis—it takes two to tango. *Cell Host Microbe* 17 (3), 290–292.
- Tomlinson, G.S., Booth, H., Petit, S.J., Potton, E., Towers, G.J., et al., 2012. Adherent human alveolar macrophages exhibit a transient pro-inflammatory profile that confounds responses to innate immune stimulation. *PLoS ONE* 7 (6), e40348.
- Turner, M.D., Nedjai, B., Hurst, T., Pennington, D.J., 2014. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim. Biophys. Acta* 1843 (11), 2563–2582.
- Wajant, H., Pfizenmaier, K., Scheurich, P., 2003. Tumor necrosis factor signaling. *Cell Death Differ.* 10 (1), 45–65.
- Wang, J.E., Warris, A., Ellingsen, E.A., Jorgensen, P.F., Flo, T.H., et al., 2001. Involvement of CD14 and toll-like receptors in activation of human monocytes by *Aspergillus fumigatus* hyphae. *Infect. Immun.* 69 (4), 2402–2406.
- WHO, 2007. World Health Organization; The Global Occupational Health Network. http://www.who.int/occupational_health/publications/newsletter/gohnet12e.pdf?ua=1/.
- Winter, M., Beer, H.D., Hornung, V., Kramer, U., Schins, R.P., et al., 2011. Activation of the inflammasome by amorphous silica and TiO₂ nanoparticles in murine dendritic cells. *Nanotoxicology* 5 (3), 326–340.
- Yazdi, A.S., Guarda, G., Riteau, N., Drexler, S.K., Tardivel, A., et al., 2010. Nanoparticles activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and cause pulmonary inflammation through release of IL-1 α and IL-1 β . *Proc. Natl. Acad. Sci. U. S. A.* 107 (45), 19449–19454.
- Zarembek, K.A., Godowski, P.J., 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J. Immunol.* 168 (2), 554–561.

Supplementary

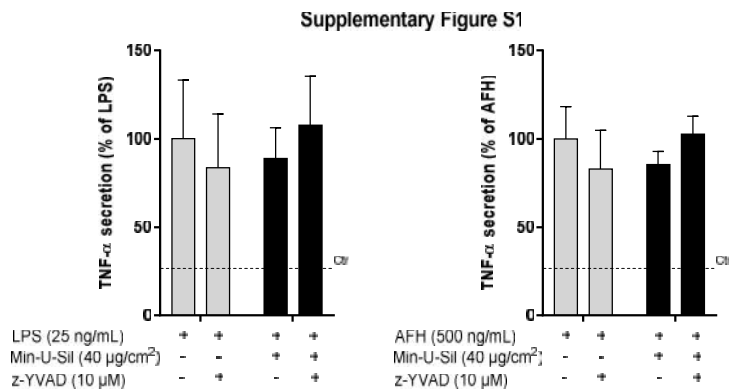


Figure S1. Effects of caspase inhibitor on TNF- α release after single and co-exposures from THP-1 Mo. THP-1 Mo were exposed to AFH (500 ng/mL), LPS (25 ng/mL) and Min-U-Sil (40 μ g/cm²) for 21 or 18 h, or primed with AFH or LPS for 3 h and co-exposed with Min-U-Sil for further 18 h. Both setups were with or without caspase inhibitor (z-YVAD-fmk, 10 μ M) pretreatment for 1 h and assessed for TNF- α release. All data were normalized by using a 2-step normalization procedure. Bars represent mean $\pm$ SEM concentrations of four independent experiments. Statistical analysis was based on log transformed data (two-way ANOVA with Sidak post test). Control without zYVAD 28.1 ± 11.1 pg/mL, and control with zYVAD 27.4 ± 9.9 pg/mL. Control (Ctrl) shown as dotted line.