The mycotoxin alternariol induces DNA damage and modify macrophage phenotype and inflammatory responses

A. Solhaug1*, C. Wisbech1, T.E. Christoffersen2,3, L.O. Hult3,4, T. Lea3, G.S. Eriksen1 and J.A. Holme5.

1Norwegian Veterinary Institute, 0454 Oslo, Norway, 2Faculty of Engineering, Ostfold University College, 1757 Halden, Norway, 3Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway, 4Ostfold Hospital Trust, 1603 Fredrikstad, Norway, 5Division of Environmental medicine, Norwegian Institute of public health, 0379 Oslo, Norway

*Corresponding author:
Anita Solhaug, PhD
Norwegian Veterinary Institute,
P.O.BOX 750 Sentrum, 0106 Oslo, Norway

Tel: +47 23216214; Fax: +47 23216201
E-mail: Anita.Solhaug@vetinst.no
Abstract
Alternariol (AOH), a mycotoxin produced by Alternaria fungi, is frequently found as a contaminant in fruit and grain products. Here we examined if AOH could modify macrophage phenotype and inflammatory responses. In RAW 264.7 mouse macrophages AOH changed the cell morphology from round to star-shaped cells, with increased levels of CD83, CD86, CD11b, MHCII and endocytic activity. TNFα and IL-6 were enhanced at mRNA-level, but only TNFα showed increased secretion. No changes were found in IL-10 or IL-12p40 expression. Primary human macrophages changed the cell morphology from round into elongated shapes with dendrite-like protrusions in response to AOH. The levels of CD83 and CD86 were increased, HLA-DR and CD68 were down-regulated and CD80, CD200R and CD163 remained unchanged. Increased secretion of TNFα and IL-6 were found after AOH exposure, while IL-8, IL-10 and IL-12p70 were not changed. Furthermore, AOH reduced macrophage endocytic activity and autophagosomes. AOH was also found to induce DNA damage, which is suggested to be linked to the morphological and phenotypical changes. Thus, AOH was found to change the morphology and phenotype of the two cell models, but either of them could be characterized as typical M1/M2 macrophages or as dendritic cells (DC).

Keywords:
Macrophages, differentiation, DNA damage, mycotoxins, alternariol
**Abbreviations:**

AOH, alternariol; AF, autofluorescence; Arg-1, arginase-1; BSA, bovine serum albumin; CBA, cytometric bead array; CD, cluster of differentiation; DC, dendritic cells; DSBs, DNA double stranded breaks; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte macrophage colony-stimulating factor; iNOS, Inducible nitric oxide synthase; IFN-γ, interferon-γ; IL-4, interleukin-4; IL-10, interleukin-10; IC, Isotype controls; SEM, scanning electron microscopy; SSBs, DNA single stranded breaks; MFI, median fluorescence intensity; NAC, N-acetyl-L-cysteine; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species
1. Introduction

Mycotoxins are secondary metabolites produced by fungi that may contaminate all stages of the food chain. Consumption of mycotoxins is considered an important risk factor for both human and animal health (Wu et al., 2014). The immune system is considered to be the most sensitive target for several mycotoxins, including the trichothecenes; with low doses of toxins having immune-stimulatory effects and higher doses causing immunosuppression (Pestka, 2010). The mycotoxin alternariol (AOH) is produced by the Alternaria fungi, which is ubiquitous in the environment. Alternaria seems to have a great ability to adapt to the environmental conditions and is therefore found in both humid and semi-dry regions (EFSA, 2011). Toxin production may also occur at lower temperatures, so food refrigerated during transportation and storage may also be contaminated (EFSA, 2011). Alternariol is often found in fruit and in processed fruit products such as juices and wine (Ackermann et al., 2011), as well as in vegetables and grain (Ostry, 2008; Uhlig et al., 2013). AOH has been found in 31% of samples of feed and agricultural commodities in Europe (n=300), with concentrations ranging from 6.3 – 1840 mg/kg (EFSA, 2011). The highest levels of AOH are found in legume, nuts and oilseed food (EFSA, 2011). At present, there are no regulations of AOH in food and feed (EFSA, 2011). Although the human dietary exposure is estimated to be low (1.9 - 39 ng/kg/bw/day), it exceeds the threshold of toxicological concern for potential genotoxic compounds (2.5 ng/kg/bw/day) (EFSA, 2011).

Several in vitro studies have reported that AOH shows genotoxic effects by the induction of DNA damage, including single-stranded DNA breaks (SSBs)- and double-stranded DNA breaks (DSBs) (Pfeiffer et al., 2007; Fehr et al., 2009). A recent in vivo study reported that AOH was negative in the bone marrow micronuclei test and comet assay using liver tissue (Schuchardt et al., 2014). The target organ is, however, most likely the gastrointestinal tract with associated immune cells and corresponding microbiota (Maresca and Fantini, 2010). Thus, although negative in the study of Schuchardt and co-workers, possible genotoxic effect of AOH in vivo can still not totally be excluded. The DNA damaging properties found in vitro has been suggested to be due the ability of AOH to act as a topoisomerase poison (Fehr et al., 2009). DNA topoisomerases are enzymes that regulate the DNA topology during transcription, replication, chromosome condensation and the maintenance of genome stability (Vos et al., 2011). Our recent studies using the RAW 264.7 macrophage cell line (Solhaug et al., 2012, 2013, 2014), showed that exposure to AOH increased the production of reactive oxygen species (ROS) and the level of...
DNA damage (SSBs, DSBs and oxidative DNA damage). Cells accumulated in G2-phase (4N), with diploid or abnormal partly divided nuclei. Furthermore, the AOH-induced cell cycle arrest was accompanied by increased autophagy and senescence, which were suggested to be a consequence of DSBs.

Macrophages play a key role in innate and adaptive immunity. Their main function is to perform phagocytic clearance of pathogens and dying cells and to modulate the adaptive immune response through antigen processing and presentation and by cytokine secretion. Both tissue-resident macrophages and monocyte-derived macrophages are recruited during inflammation (Hume et al., 2008; Mowat and Bain, 2010). Monocytes develop into various forms of macrophages according to the nature of environmental signals (Hume, 2008; Bain and Mowat, 2014). The main macrophage polarization states are referred to as classically activated macrophages (M1) and alternatively activated macrophages (M2), thus mirroring the Th1/Th2 differentiation paradigm (Biswas et al., 2012; Martinez and Gordon, 2014). Th1-related cytokines such as interferon-γ (INF-γ), as well as microbial stimuli such as lipopolysaccharide (LPS) polarize macrophages to an M1 phenotype. These cells produce pro-inflammatory cytokines such as TNFα, IL-12/23 and IL-8; and have inducible nitric oxide synthase (iNOS). They are able to ingest (endocytic activity) and kill pathogens rapidly; however, the use of ROS and NO will also result in tissue damage (Laskin et al., 2011; Biswas et al., 2012; Mills and Ley, 2014). M1 macrophages have increased antigen presenting capacity and display increased levels of major histocompatibility complex class II (MHCII) and B7 co-stimulatory molecules such as CD80 and CD86 (Ambarus et al., 2012; Biswas et al., 2012). The integrin CD11b is considered being a pan-macrophage marker, associated with adherence and phagocytosis. M2 polarization is broader and includes IL-4/IL-13 stimulated macrophages (M2a), IL-10 induced macrophages (M2c) and immune complex-triggered macrophages (M2b) (Mantovani et al., 2004). M2 macrophages produce anti-inflammatory cytokines such as IL-10, and are generally involved in tissue regeneration (Mantovani et al., 2004; Biswas et al., 2012). Typical surface receptors expressed by M2 cells are the membrane glycoprotein CD200r, which is expressed on M2a cells and the scavenger receptor CD163, which is expressed on M2c cells (Koning et al., 2010; Ambarus et al., 2012). Interestingly, proliferation of M2 macrophages rather than recruitment from the blood has recently been suggested to be important for Th2 responses (Jenkins et al., 2011). Other important
markers may include arginase-1 (Arg-1) (Mills and Ley, 2014). Monocytes may also differentiate into dendritic cells (DC), which are professional antigen-presenting cells (APCs) bridging the innate and adaptive immune system through activation and expansion of T cells (Coombes and Powrie, 2008). Immature DCs are located in peripheral tissues to continuously monitor the environment through the uptake of particulate and soluble products, thus have increased endocytic and phagocytic activity. Antigen-loaded DCs acquire a mature phenotype, associated with reduced endocytic and phagocytic capacities, and enhanced production of pro-inflammatory cytokines (IL-12p70, TNFα, IL-6 and IL-23). The mature DCs, then, migrate towards the lymphoid organs where they interact with, and activate, naive T cells. In addition to the cytokine profile, mature DCs are often characterized by increased expression of co-stimulatory molecules (e.g. CD80, CD86), MHCII, HLA-DR as well as CD83, a conserved marker for mature DC (Jensen and Gad, 2010, Zhou and Tedder, 1996).

Immune cells are potential targets for the adverse health effects of mycotoxins. An optimal immune response depends on the delicate balance between M1, M2 and DC. We have therefore investigated the potential effect of AOH on macrophage phenotypes and inflammatory responses. In our previous studies, the RAW 264.7 macrophage cell line has proven to be a good model for AOH toxicity (Solhaug et al., 2012, 2013, 2014). To further enhance the relevance for human risk evaluation, we have extended our studies to also include macrophages derived from human primary blood monocytes.

2. Materials and Methods

2.1. Reagents and chemicals

Dulbecco’s Modified Eagle Medium (DMEM), penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Lonza (Verviers, Belgium). FITC-dextran (42 kDa), Hoechst 33342, LPS and collagen were from Sigma-Aldrich (St. Louis, MO, USA). OneComp beads were from eBioscience. BD OptEIA mouse TNF ELISA kit, BD OptEIA mouse IL-6 ELISA kit and Human inflammatory bead assay CBA was from BD Biosciences (San Diego, CA, USA). Interleukin 4 (IL-4), Interleukin 10 (IL-10), interferon-γ (INFγ) and granulocyte macrophage colony-stimulating factor (GM-CSF) were from ImmunoTools (Germany). Millecell EZ slides and
Mowiol were from Millipore (Hayward, CA, USA) and Upcell plates were purchased from Nunc (Rochester, NY, USA). H$_2$DCFDA-CM, DHE, N-acetyl-L-cysteine (NAC), propidium iodide, Hoechst and RNase were from, Life technologies (Grand Island, NY, USA).

**Antibodies:** Anti-human CD163 Alexa Fluor 647, Alexa Fluor 488 Mouse IgG2b (κ Isotype Ctrl), anti-human CD83 PE, anti-human CD86 PerCP/Cy5.5, anti-human CD80 Alexa Fluor 488, anti-human CD200r PE, anti-human CD68 Alexa Fluor 488, anti-mouse CD83 PE, Rat IgG1 PE, human Trustain FcX (Fc Receptor Blocking Solution) and TruStain fcX anti-mouse CD16/32 (mouse Fc receptos blocker), were from BioLegend (San Diego, CA, USA). anti-human HLA-DR Alexa Fluor 674 were from Bioss (Woburn, MA, USA), anti-mouse CD80 FITC, Armenian Hamster IgG FITC isotype Ctrl, anti-mouse MHCII FITC, Rat IgG2b FITC isotype Ctrl, anti-mouse CD11b Alexa Fluor 488, Rat IgG2b Alexa Fluor 488 isotype Ctrl, anti-mouse CD86 APC and Rat IgG2ακ Iso Control APC were from eBioscience (San Diego, CA, USA). γH2AX and LC3B were from cell signaling (Beverly, MA, USA) and anti-rabbit Alexa Fluor 647 were from molecular probes (Life Technology, Grand Island, NY, USA).

### 2.2. Cell cultures

**RAW 264.7 macrophages:** The mouse macrophage cell line RAW 264.7 was obtained from European Collection of Cell Cultures (ECACC) and grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS; EU standard, Lonza), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were cultured at 37 °C with 5% CO$_2$ in a humidified atmosphere and kept in logarithmic growth phase at 1 x 10$^6$ - 10 x 10$^6$ cells/75 cm$^2$ through routine sub-culturing by scraping, according to standard ECACC protocol. Cells were plated (0.3 x 10$^6$ cells/cm$^2$) 24 h prior to the experiment, which resulted in approximately 70% confluence at the day of exposure. Medium were refreshed before exposures. For measurement of cell surface receptors, endocytosis and apoptosis/necrosis, the cells were grown on temperature-responsive UpCell plates (Nunc, Rochester, NY, USA) from which cells detach at temperatures below 32 °C. Otherwise the cells were harvested by scraping, as recommended by ECACC.

**Primary human macrophages:** Peripheral blood mononuclear cells (PBMCs) were isolated fromuffy coats obtained from healthy donors according to the international ethical guidelines (CIOMS) (Østfold Hospital Trust, Norway) by Ficoll density gradient centrifugation. About 10% of the PBMC isolated from human blood is monocytes (Auffray et al., 2009). The PBMCs were
cultivated 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 x 10⁶ cells/cm². The PBMCs were cultivated for 24 h to allow the monocytes to adhere to the plastic. The non-adherent PBMC were washed off with PBS and the monocytes were allowed to differentiate into macrophages in the presence of granulocyte macrophage colony-stimulating factor (GM-CS, 50 ng/ml) for 7 days. The cells were approximately 70% confluent at the day of exposure. The medium was replenished on day four and on the day of exposure. Cells from different donors were used for the biological replicates of the experiments. Macrophage purity and differentiation were verified by flow cytometric analysis of CD68 (Supplementary, Fig. S1). Positive controls for M1 and M2 differentiation were generated by treatment with GM-CSF (50 ng/ml) alone for the first 4 days, then INF-γ (50 ng/ml) and IL-10 (50 ng/ml) were added, respectively. For the generation of DCs, the cells were stimulated with GM-CSF (50 ng/ml) + IL-4 (25 ng/ml) for 6 days and LPS (100 ng/ml) were added and the cell incubated further for 24 h. The supplemented RPMI 1640 media was exchanged on day four and six. Cell differentiation was verified by flow cytometric analysis of specific markers associated with the cell type as M1 express CD80, M2 express CD163 and CD200r and DCs express CD83 and DC86 (data not shown). The cells were harvested by trypsination.

Primary mouse peritoneal macrophages: Female B6C3F1 mice (5 weeks old) weighing 16 to 18 g were obtained from Charles River Laboratories, Inc (Wilmington, MA, USA) or Harlan (Indianapolis, IN, USA). Housing, handling and sample collection procedures conformed to the policies of the Michigan State University All-University Committee on Animal Use and Care in accordance with NIH guidelines. Mice were injected ip with 1.5 ml of sterile 3% (w/v) thioglycollate broth. After 4 days, mice were euthanized and macrophages collected by peritoneal lavage with ice-cold Hank's BSS (Invitrogen Corporation, Carlsbad, CA, USA). Cells were pelleted by centrifugation at 1100 g for 5 min. Cells were washed with PBS once and re-suspended in RPMI-1640 containing 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml)/streptomycin (100 µg/ml) and cultivated cultured at 37 °C under 5% CO₂ in a humidified incubator (0.4 x 10⁶ cells/cm²). After 3 h incubation, non-adherent cells were removed. The cells were then cultivated further for 24 h before treatment. The cells were approximately 70% confluent at the day of exposure. The cells were harvested by trypsination.
Exposure: AOH was dissolved in DMSO and the final concentration of solvent in cell culture was 0.1%. Appropriate controls containing the same amount of solvent were included in each experiment. For experiments with the antioxidant N-acetyl-L-cysteine (NAC); NAC was dissolved in complete medium complemented with HEPES (25 mM) and pH adjusted to 7.2. The cells were pre-treated with NAC for 1 h before exposure to AOH.

2.3. Evaluation of cell morphology

Fluorescence microscopy:
The cells were seeded and cultivated on Millicell EZ slides. After exposure to AOH, the cells were fixed in 4% PFA for 10 min at ambient temperature followed by permeabilization and blocking in 3% BSA/PBS, 0.05% saponin for 1 h. The cells were then stained with Phalloidin Alexa Fluor 555, diluted in 3% BSA / PBS / 0.05% saponin for 1 h, and washed 3 times for 5 min with 3% BSA / PBS / 0.05% saponin. The nuclei were stained with Hoechst 33342 (1µg/µl) and coverslips mounted with mowiol. Pictures were captured with a fluorescence microscope (Nikon Eclipse 80i, equipped with a DS-Ri1 camera, Amsterdam, Netherlands).

Scanning electron microscopy (SEM):
SEM was done as previously described (Christoffersen et al., 2015). Samples were washed and fixed with 5% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 45 min; then replaced with 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 30 min. Samples were then washed, dehydrated in graded ethanol series and dried using a critical-point dryer (CDP 030, BAL-TEC GmBH, Germany). Dry samples were mounted on aluminum stubs using double-faced carbon tape (Agar Scientific, UK), and coated with approximately 500 Å platinum using a sputter coater (Polaron SC7640, Quorum Technologies, UK). Microscopic analyses were performed using an EVO-50 Zeiss microscope (Carl Zeiss AG, Germany).

Light microscopy:
Cell morphology was evaluated by light microscopy (Leica DMIL. Solms, Germany). Random pictures were taken by Moticam 1000 (Motic, Hong Kong, China).

2.4. Analysis of phenotypic cell markers by flow cytometry
RAW 264.7 macrophages: Following AOH exposure the cells were collected and incubated with Fc-blocker 30 min on ice. The samples were then stained with direct-conjugated antibodies toward CD86 APC, CD80 FITC, MHCII FITC, CD11b Alexa Fluor 488 or CD83 PE, or with their associated isotype controls (at respectively equal concentrations) for 30 min on ice in the dark. The cells were then washed with 0.5% BSA-PBS twice, re-suspended in PBS and analyzed by flow cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA). Alexa Fluor 488 or FITC were detected by using 488_ex:530/30_em, PE: 488_ex:585/42_em, APC: 647_ex:675/25_em. Live (non-fixated) AOH treated RAW 264.7 cells generated some autofluorescence (AF) compared to untreated cells. The AF was calculated by using isotype controls (IC) and median fluorescence intensity (MFI) measurements and subtracted from the MFI of AOH treated cells: AF = MFI (IC AOH treated cells) – MFI (IC Ctrl cells).

Primary human macrophages: The cells were collected following exposure and washed twice with 0.5% BSA in PBS (500xg for 10 min). The cells were re-suspended in 50 µl 0.5% BSA in PBS, 2.5 µl fc-blocker was added and the cells incubated for 30 min on ice. The directly conjugated antibodies CD163 Alexa Fluor 647), CD83 PE, CD86 PerCP/Cy5.5), CD80 Alexa Fluor 488, CD200r PE, HLA-DR Alexa Fluor 647) were added directly to the cells in the blocking solution and incubated further on ice in the dark for 30 min. Cells were then washed twice with 0.5% BSA-PBS, re-suspended in PBS and analyzed by flow cytometry (Accuri C6). Alexa Fluor 488 was detected by using 488_ex:530/30_em, PE: 488_ex:585/42_em, PerCP/Cy5.5: 488_ex:LP670_em, Alexa Fluor 647: 647_ex:675/25_em. AOH treatment did not induce any AF in human macrophages. As CD68 is an intracellular marker, cells were permeabilized prior to staining according to manufacturer’s guidelines (BioLegend). Cells were stained with CD68 Alexa Fluor 488 or the corresponding isotype control (at equal concentration), as described above followed by flow cytometric analysis.

2.5. Endocytosis assay

In order to measure macrophage endocytosis (bulk-phase endocytic ability), FITC-dextran uptake assay was performed. Following AOH exposure the cells were collected and 5 x 10^5 cells were incubated at 37 °C for 30 min with FITC-Dextran, 42 kDa (1 mg/ml) and then washed three times with cold PBS. Cellular uptake of FITC-dextran was measured by flow cytometry (Accuri C6) using 488_ex:530/30_em. A negative control was performed in parallel by incubating cells with
FITC-dextran at 4°C instead of 37°C. Uptake of FITC-dextran was expressed as Δ median fluorescence intensity (MFI), i.e., MFI (uptake at 37°C) – MFI (uptake at 4°C). Background (negative control) was withdrawn and AOH-induced phagocytic activity calculated.

2.6. RT-PCR

Gene expression of TNFα, IL-6 and IL-12 in RAW 264.7 cells were quantified by RT-PCR using gene specific primer-probe technology (Life Technologies) as previously described in Solhaug et al., 2012. The following TaqMan® probes were used: Mm00443258_m1 for TNFα, Mm00446190_m1 for IL-6, Mm01288989_m1 for IL-12p40 and Mm00437762_m1 for B2M. Gene expression of iNOS and Arg-1 in RAW 264.7 cells were monitored using The TaqMan® Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) according to the manufacturer's recommendations and analyzed using a Rotor Gene 6000 Real-Time PCR Machine (Qiagen, Germantown, MD) as previously described (Christoffersen et al., 2015). The following TaqMan® probes were used: Mm00440502_m1 for Nos2, Mm00475988_m1 for Arg1 and Mm02528467_g1 for Rpl32.

2.7. Cytokine measurement:

ELISA: Following exposure of RAW 264.7 cells, conditioned media was centrifuged (500 g for 10 min) and supernatants collected to remove cell debris. TNFα and IL-6 were quantified by enzyme-linked immunosorbent (ELISA), according to the manufacturer’s guidelines (BD Biosciences) using a Viktor2 multilabel counter (Perkin Elmer, Boston, MA, USA) equipped with appropriate software (Magellan VI). Cytokine levels were normalized in regards to cell number as AOH is found to inhibit RAW 264.7 cell proliferation (Solhaug et al., 2012).

Cytokine bead assay (CBA): Following exposure of primary macrophages, conditioned media were centrifuged at 500 g for 10 min and supernatant collected to remove cell debris. Levels of IL-12p70, TNFα, IL-10, IL-6, IL-8 and IL-1β were measured simultaneously using CBA and flow cytometry (Accuri C6), according to the manufacturer’s guidelines.

2.8. Cytotoxicity

Microscopy: Changes in nuclear morphology and plasma membrane damages were evaluated after staining cells (~0.5 x 10⁶ cells) with propidium iodide (PI, 10 µg/ml) and Hoechst 33342 (5
µg/ml) for 30 min. Stained cells suspended in 10 µl FBS were applied to slides and air-dried.

Nuclear morphology associated with necrosis and apoptosis was determined using a Nikon Eclipse E400 fluorescence microscope. A minimum of 300 cells were counted per slide.

Flow cytometry: Necrotic and late apoptotic cells have impaired membrane integrity and cannot exclude PI like viable cells. Cells were harvested and stained with PI (5 µg/ml) for 10 min in the dark at ambient temperature and analyzed immediately by flow cytometry (Accuri C6) using 488<sub>ex</sub>:585/42<sub>em</sub>.

Alamar Blue: Viability of the primary mouse peritoneal macrophages was measured by Alamar blue as described in Solhaug et al., 2012.

2.9. ROS measurements

ROS production was detected using oxidation-sensitive fluorescent probes. H<sub>2</sub>DCFDA-CM (1 µM) was used to detect H<sub>2</sub>O<sub>2</sub> and DHE (5 µM) to detect O<sub>2</sub>-. The cells were harvested and loaded with H<sub>2</sub>DCFDA-CM or DHE for 20 min at 37 °C under 5% CO<sub>2</sub>. The cells were washed twice with ice cold PBS and analyzed by flow cytometry (Accuri C6). H<sub>2</sub>DCFDA-CM was measured by using 488<sub>ex</sub>:530/30<sub>em</sub> and DHE using 488<sub>ex</sub>:585/42<sub>em</sub>. Relative expression was expressed as MFI. The pro-oxidant H<sub>2</sub>O<sub>2</sub> (1 mM, 10 min) was used as a positive control.

2.10. Measurement of γH2AX and LC3II by flow cytometry

Following treatments, cells were harvested, washed once in PBS, fixed in 2% paraformaldehyde (PFA) in PBS for 10 min on ice, and post-fixed/permeabilized in 90% ice-cold methanol for 24 h or more at -20 °C. For staining with antibody, 5 x 10<sup>5</sup> cells were washed twice with 0.5% BSA in PBS and then incubated with γH2AX or LC3B antibody in 0.5% BSA/PBS overnight at 4 °C. Cells were then rinsed twice in 0.5% BSA/PBS and incubated with secondary antibody conjugated to Alexa Fluor 647 for 2 h at room temperature in the dark. Finally cells were rinsed twice and analyzed by flow cytometry (Accuri C6) using 633<sub>ex</sub>:675/25<sub>em</sub>. For analysis of cell cycle distribution in addition to protein expression, the cells were washed with PBS, incubated with propidium iodide (PI; 10 µg/ml) / RNase A (100 µg/ml) in PBS for 30 min at 37 °C before analyses on a flow cytometer (Accuri C6). Single cells were gated and a minimum of 10,000 cells acquired and analyzed using 488<sub>ex</sub>:585/40<sub>em</sub> vs 647<sub>ex</sub>:675/25<sub>em</sub>.
2.11. Statistical analysis
Data analyses were performed using Sigma Plot version 13.0. Statistical significance (p<0.05) was assessed using 1-way ANOVA, followed by Dunnett’s post-test or paired t-test as indicated.

3. Results

3.1. AOH induces morphological and phenotypic changes in RAW 264.7 macrophages.
In order to evaluate the effect of AOH on macrophage morphology, RAW 264.7 cells were treated with AOH (15 and 30 µM) for 24 h followed by staining of the cytoskeleton and nuclei. Exposure to 15 µM AOH did not induce any obvious morphological changes (data not shown), but after exposure to AOH (30 µM) the morphology changed from small and round into more flattened cells, others were star-shaped or with elongated spindle shapes (Fig. 1). The morphological changes were found to be sustained also after 48 h exposure (data not shown). In agreement with our previous reports (Solhaug et al., 2013), AOH were also found to induce abnormal nuclei, such as micronuclei and polyplody (Fig. 1). Little or no cell death was observed after AOH exposure (Supplementary, Fig. S2), which is in agreement with that AOH reduce the proliferation rate by the induction of cell cycle arrest rather than cell death (Solhaug et al., 2012, 2013). We have previously shown that AOH increases the cellular level of ROS in RAW 264.7 cells, and that this ROS was without effects on the AOH-induced cell cycle arrest (Solhaug et al., 2012). Here, we added the antioxidant N-acetyl-L-cysteine (NAC, 5mM) to examine if ROS production was linked to the morphological changes. Even though antioxidants reduce AOH induced ROS production (Solhaug et al., 2012), the addition had no obvious effect on the AOH-induced changes in cell morphology (Supplementary, Fig S3).

Next we examined if the observed morphological changes corresponded to potential changes in cell surface markers expression relevant to macrophage differentiation. In contrast to the morphological changes, preliminary results showed that phenotypic markers, such as expression of surface receptors were more clearly up-regulated after 48 h compare to 24 h treatment with AOH (30 µM; data not shown). Further studies in order to characterize the phenotypic changes were therefore performed mainly with 48 h of AOH (30 µM). As can be seen from the data presented in Fig. 2, CD86, CD80 and MHCII, important co-stimulatory molecules for T cell
activation, were all up-regulated in RAW 264.7 cells after exposure to AOH (30 µM). Similarly, also the expression of the integrin CD11b was found to be up-regulated by AOH. In contrast, CD83 which is associated with DC maturation was not affected. To investigate if AOH induced changes of pro-inflammatory cytokines, we measured the expression of TNFα, IL-6 and IL-12p40 by RT-PCR. Both TNFα and IL-6 mRNA expression were found to be up-regulated in RAW 264.7 cells (Fig. 3A). IL-12 is composed of two heterologous chains; p40 and p35, which together form IL-12p70 and is a key cytokine released from immature and mature DCs (Steinman et al., 2007; Shortman et al., 2007). AOH was not found to induce expression of IL-12p40 in RAW 264.7 cells (Fig 3A). At the protein level, ELISA confirmed an AOH-induced up-regulation of TNFα release (Fig. 3B). In contrast, IL-6 could not be detected (data not shown), which might be caused by effects linked to post-transcriptional changes/ processes induced by AOH. To further characterize the AOH induced differentiation of the RAW 264.7 cells, we employed qPCR to measure expression of iNOS and Arg-1 as their up-regulation is commonly used as indicators for M1 or M2 macrophage polarization in mice, respectively (Stout et al., 2005; Kigerl et al., 2009). However, AOH (30 µM, 12-72 h) did not increase the expression of either iNOS or Arg-1 (data not shown). Endocytosis is required for the intracellular processing and presentation of exogenous antigenic fragments, and is a crucial function of macrophages as well as immature DCs. Endocytic capacity of the AOH treated cells was investigated by the uptake of FITC-dextran. A significantly higher amount of FITC-dextran was taken up by macrophages treated with AOH (30 µM) for 48 h (Fig. 4) compared to untreated cells, indicating a higher endocytic activity. This was not the case when cells were stimulated with lower concentrations of AOH (15 µM), which corresponds well with the morphological changes described above.

3.2. AOH induces morphological and phenotypically changes in human primary macrophages

To further study the effects of AOH on macrophage morphology, we next investigated if AOH induced similar morphological changes in primary human macrophages. Human blood monocytes were differentiated into macrophages by GM-CSF for 7 days, followed by exposure to AOH (30 µM) for 24 h. A dramatic change in morphology was observed in AOH-treated cells compared to the untreated control after 24 h (Fig. 5A) and 48 h exposure (Fig. 5B). Most cells appeared elongated with dendrite-like protrusions after AOH-exposure, while untreated cells
remained round. The morphological changes were similar after 24 and 48 h AOH (30 µM) exposure. No cell death was observed (Supplementary, Fig. S4). To further characterize the prominent changes in morphology induced by AOH (30 µM, 24 h), we measured the expression of several surface receptors, commonly associated with M1 (CD80, CD86), M2 (CD200r, CD163) or DCs (CD83, CD86, HLA-DR) in addition to the more common macrophage marker CD68. Interestingly, both CD83 and CD86 were found to be up-regulated in response to AOH treatment while HLA-DR and CD68 were down-regulated (Fig. 6). No significant changes were detected for CD80, CD200r and CD163 (Fig. 6). To assess the effects of AOH on pro-inflammatory cytokine production in primary macrophages, the secretion of TNFα, IL-6, IL-8, IL-1β, IL-10 and IL-12p70 was measured using cytokine bead assay (CBA). AOH induced increased secretion of TNFα and IL-6, but had no effects on IL-8, IL-1β, IL-10 or IL-12p70 levels (Fig. 7). In RAW 264.7 macrophages AOH were found to increase the endocytic capacity (Fig. 4). In contrast, the primary macrophages had lower uptake of FITC-dextran when treated with AOH compared to control (Fig. 8). Interestingly, similar effects of AOH on morphology and uptake of FITC-dextran, with no effects on cell viability, were observed in AOH-treated primary peritoneal macrophages isolated from mouse (Supplementary, Fig. S5).

Recently, we found that AOH induced autophagy in RAW 264.7 cells (Solhaug et al., 2014). Thus, as autophagy has been found to be essential during macrophage differentiation (Jacquel et al., 2012), we next examined the level of the autophagosome marker LC3II. In the primary macrophages exposure to AOH (30 µM, 24 h; Fig. 9) resulted in a down-regulation of LC3II, suggesting reduced autophagy.

### 3.3 AOH induced ROS, DNA damage and cell cycle arrest in human primary macrophages

AOH-induced ROS was found to be associated with SSBs and oxidative DNA damage (Solhaug et al., 2012). Thus, in order to further elucidate possible mechanisms involved in AOH-induced morphological changes, we next analyzed intracellular ROS levels using H₂DCFDA-CM and DHE probes detecting H₂O₂ and O₂⁻, respectively. In contrast to the RAW 264.7 macrophages (Solhaug et al., 2012), AOH (30 - 60 µM) showed only a slight up-regulation (not significant) of H₂O₂ production after 2 h, 6 h and 24 h exposure (Fig. 10AB). No O₂⁻ production was detected after 24 h of AOH exposure (30 – 60 µM, data not shown). Furthermore, in accordance with
observations in RAW 264.7 cells, addition of the ROS scavenger NAC (5 mM; 24 h) did not change the AOH-induced morphological changes as evaluated by light microscopy (Supplementary, Fig. S6).

AOH’s effects on topoisomerase (Fehr et al., 2009) have been suggested to be linked to an increased level of DSBs (Solhaug et al., 2012). As increased phosphorylation of H2AX (γH2AX) is indicative of DSBs, we next examined γH2AX expression vs. cell cycle by flow cytometry (Sordet et al., 2009). As expected, the primary macrophages had only a limited proliferation rate as only approximately 2% of the cells were found to be in S phase (Fig. 11AD). Despite the low level of proliferation in these cells, AOH exposure markedly enhanced cell numbers in G2 (Fig. 11ACD). Most importantly, increased levels of γH2AX were seen following exposure to AOH (Fig. 11AB), suggesting an enhanced level of DSBs possibly via its known effect on topoisomerase. Interestingly, γH2AX was enhanced in all phases of the cell cycle, suggesting that inhibition of topoisomerase during replication as well as transcription could cause DSBs (Fig. 11A).

4. Discussion

Our previous studies show that AOH induces ROS and DNA damage followed by G2 arrest in RAW 264.7 macrophages (Solhaug et al., 2012, 2013). We have also identified AOH as an inducer of autophagy as well as senescence in these cells (Solhaug et al., 2014). These cellular effects were suggested to be linked to an AOH-initiating effect on topoisomerase thereby causing DSBs, rather than enhanced ROS (Solhaug et al., 2012, 2014). In the present study, we find that AOH modified the phenotype of proliferating RAW 264.7 and the slowly proliferating primary human macrophages. The AOH-induced changes on morphology and inflammatory cytokine responses were rather similar in the two models. In contrast, the effects of AOH with regard to endocytosis, autophagy as well as expression of CD-markers were markedly different (Table 1). The phenotypes did neither match with typical M1/ M2 macrophages nor with DC.

Plasticity and functional polarization are hallmarks of macrophages. Here we find that AOH induced star-shaped morphology of the RAW 264.7 cells. Similar morphological changes have been previously reported and associated with macrophage differentiation into DCs (Saxena et al.,
In this study, the levels of CD80, CD86, MHCII and CD11b were increased, as here also seen in AOH-exposed cells. In contrast, the expression of CD83, a marker of DC differentiation and maturation, was found to be unchanged after AOH exposure. Furthermore, AOH did not induce expression of IL-12p40, which is a key cytokine released from immature and mature DCs (Steinman et al., 2007; Shortman et al., 2007). Although, the changes in cell surface marker expression and enhanced endocytic activity shows that AOH exposure induced marked phenotypical changes in the macrophages. The AOH treated RAW 264.7 macrophages displayed several of the M1 characteristics, such as increased expression of MHCII, CD80, CD86 and increased secretion of TNFα. On the other hand, AOH did not enhance the expression of iNOS or release of IL-12p70, and the differentiated macrophages can therefore not be characterized as classical M1 macrophages. Similarly, the typical characteristics of M2 macrophages, such as elongated morphology (Bolling et al., 2012) and expression of IL-10 and Arg-1, were not fulfilled. Thus, the RAW 264.7 cells obtained after AOH exposure cannot be categorized as classical DC or as M1/ M2 macrophages.

In the primary human macrophage model, AOH induced even more DC-like morphology with elongated cells showing dendrite-like protrusions. Similar to what was seen in the RAW 264.7 cells, AOH also modifies the phenotype of the primary macrophages. More specifically, the AOH-exposed primary human macrophages show increased expression of CD83 and CD86 together with decreased CD68 levels, increased secretion of TNFα and IL-6 and decreased endocytosis, which may represent modifications into a more DC-like phenotype. On the other hand, rather a down regulation of HLA-DR and no increased secretion of IL-12p70 were found. As seen with the RAW 264.7 model, the phenotypically characteristic of the AOH treated primary macrophages did not fit with DC, M1 nor M2 polarized macrophages.

It is important to note that the DC and M1/M2 classification scheme of macrophages is generally considered to be an oversimplification of the true spectrum of macrophage phenotypes (Wermuth and Jimenez, 2015). Thus, it is not surprizing that the changes seen following exposure to a cellular stressor like the mycotoxin AOH could not be clearly categorized. The interesting and important part is that AOH did change the morphology and the macrophage phenotype in the experimental models tested, RAW 264.7 mouse macrophages, primary human macrophages and
primary mouse peritoneal macrophages (Supplementary, Fig. S5). Although the macrophages exposed were in different stage of differentiation due to their diverse origin. The macrophages response to AOH could have potentially important implications as the proper function of tissue macrophages and DCs are essential for the health of specialized parenchymal and stromal cells (Mills and Ley, 2014). Possible theoretical implications could be a decrease of immune response in case of infections and/or a disturbed balance of the adaptive immune system.

There have been reports linking exposure to various mycotoxins to changes in macrophage phenotype and/or effects on the differentiation processes of monocytes to macrophages (Wache et al., 2009; Gammelsrud et al., 2012; Ficheux et al., 2013). More specific, deoxynivalenol, a Fusarium mycotoxin, inhibits INF-γ mediated macrophage activation, assessed by the expression of several surface receptors, when the cells are exposed during the differentiation process from monocytes to macrophages (Wache et al., 2009). Furthermore, monocytes exposed to enniatin B, another Fusarium mycotoxin, during the differentiation process into macrophages presented a decrease of endocytosis and an increase of CD71 (Ficheux et al., 2013). Interestingly, enniatin B exposed to RAW 264.7 macrophages were also found to induce expression of CD163, a marker for M2 macrophages (Gammelsrud et al., 2012). To our knowledge, this is the first study describing morphological and phenotypical changes induced by AOH on macrophages.

Various cellular processes have been associated to the macrophage differentiation development, from specific interactions with specific cellular receptors (Martinez and Gordon, 2014) to more unspecific processes including DNA damage responses (So et al., 2013) and autophagy (Jacquel et al., 2012). Less is known with regard to important initial molecular events triggering these changes. There are reports suggesting a crucial role for ROS (Nakanishi et al., 2013) and NO (Nogueira-Pedro et al., 2014). We recently reported that AOH could be a potent inducer of ROS in the RAW 264.7 macrophage model (Solhaug et al., 2012). However, here in this study, we–found that a potent anti-oxidant (NAC) did not reduce the AOH-induced morphological changes. Furthermore, AOH induced differentiation of primary humane macrophages in the apparent absence of ROS formation. Hence, ROS does not seem to be an obligatory part of macrophage differentiation induced by AOH.
The other primary initiating molecular event of AOH presently known is an interaction with topoisomerase (Fehr et al., 2009). In general, interactions with topoisomerase often result in DSBs as a consequence of the delayed replicative and/or transcriptional syntheses (Durand-Dubief et al., 2014, Sordet et al., 2009). In our recent study, we observed that AOH exposure resulted in increased γH2AX, and suggested that AOH-induced DSBs were an important triggering signal for G2 arrest and autophagy (Solhaug et al., 2012, 2014). Also in the primary human macrophage model, AOH is suggested to increase γH2AX as a DNA damage response caused by DSBs. γH2AX was enhanced in all phases of the cell cycle, suggesting that inhibition of topoisomerase during replication as well as transcription could cause DSBs. Most interestingly, differentiation has been suggested as an outcome in response to DSBs (Sherman et al., 2011). Furthermore, DNA damaging agents is found to alter the differentiation-process of monocytes to favour the generation of M2 macrophages (Dijkgraaf et al., 2013). DNA damage has also been suggested to be an important mediator in the decision of hematopoietic stem cells to exit quiescence and to differentiate (Weiss and Ito, 2015). Differentiation is typical tightly linked to cell cycle withdrawal (Rots et al., 1999). Thus, we first hypothesized that increased DSBs could result in a G2 arrest, linked to a changed phenotype. However, in contrast to AOH-exposed RAW 264.7 cells, the primary human macrophages were found to be only slowly proliferating and the majority of the cells were in G1 phase. Thus, there are obviously no direct link between the AOH-induced macrophage differentiation and a specific cell cycle arrest. However, a link between AOH induced DNA damage and changes in macrophage morphology and phenotype is still plausible.

Autophagy (macro-autophagy) is a re-cycling mechanism by which cells through lysosomal degradation reuse amino- and fatty acids. Interestingly, autophagy has been reported to be essential during the differentiation process from monocytes to macrophages, which require marked architectural remodelling (Jacquel et al., 2012; Zhang et al., 2012). Furthermore, autophagy has been found to be important in differentiation and polarization of macrophages. However, the precise role of autophagy is still uncertain and it has been suggested to be a both a positive and negative regulator of M1 differentiation (Jung et al., 2010; Chen et al., 2014; Liu et al., 2015). Here we find that AOH-induced morphological changes in the RAW 264.7 cells, previously shown to have increased DSBs and increased autophagy; whereas AOH reduced
autophagy in the primary human macrophages. This supports the notion that there is no clear relationship between autophagy and macrophage differentiation/ polarization process. There seems to be rather complex signalling interactions on-going between autophagy and the macrophage differentiation process, as inhibition of AOH-induced autophagy caused increased cell death rather than influence the degree of morphological changes in RAW 264.7 cells (Solhaug et al., 2014).

The concentrations of AOH used in this study are in the same range that causing DNA damage as previously published by us (Solhaug et al., 2012) and others (Brugger et al., 2006; Fehr et al., 2009; Pfeiffer et al., 2007). Current knowledge concerning adverse effects of AOH in humans and animals are still limited. An in vivo mouse study done by Schuchardt and co-workers (2014) showed that uptake of AOH over the gut were somewhat poor. However, this can be different for other species and/or with other prolonged feeding trails. An ongoing inflammation in the gut may also increase the uptake as well as the effect. Thus an uptake of AOH at levels that may affect the immune system cannot be excluded.

Conclusion
The present study show that AOH induced marked phenotypic changes in macrophages. These changes could not be directly linked to an initial AOH-induced ROS production, cell cycle arrest or autophagy as seen as a consequence of AOH-induced DSBs. However, AOH-induced DSBs will result in a complex DNA damage response and a link towards macrophage differentiation is still a likely explanation.

Conflicts of interest statement
The authors declare that there are no conflicts of interest.

Acknowledgements
The authors wish to thank Dr. Charlotte Kleiveland (Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway) and Laura L. Vines (Michigan State University, Department of Food Science and Human Nutrition, East Lansing,
MI, USA) for practical assistance in the laboratory. The work has been supported by The Research Council of Norway through the project Toxicological characterization of selected secondary fungal metabolites in grain, grant nr.: 185622/V40. We also wish to thank the research group, FUNtox, at the Norwegian Veterinary Institute, for additional funding.

Figure legends:

**Figure 1: AOH induces morphological changes in RAW 264.7 cells.** Cells were exposed to AOH (30 µM) or left untreated for 24 h before staining the actin filaments and DNA with Phalloidin Alexa Fluor 555 (upper) or Hoechst (lower), respectively, followed by fluorescent microscopy. * Micronuclei. ** Polyploidy.

**Figure 2: Expression of cell surface receptors following AOH-exposure in RAW 264.7 cells.** RAW 264.7 cells were treated with 30 µM AOH for 48 h and analyzed for the expression of CD86, CD80, CD83, MHCII and CD11b surface molecules by flow cytometry. Mean values ± SEM of 4-6 independent experiments are presented. * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 3: Effects of AOH on TNFα, IL-12 and IL-6 cytokine production in RAW 264.7 cells.** (A) RAW 264.7 cells were treated with AOH (30 µM) or left untreated for 48 h. Gene expression of TNFα, IL-12p40 and IL-6 were analyzed by qRT-PCR. Two independent experiments using biological triplicates were performed. The results were statistically assessed using one-way ANOVA with Dunnett’s post-test. (B) RAW 264.7 cells were treated with AOH (30 µM) or left untreated for 6 - 48 h and analyzed for TNFα secretion by ELISA. The results represent the mean values ± SD of 3 independent incubations. * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 4: Alterations in endocytic activity induced by AOH in RAW 264.7 cells.** RAW 264.7 cells were left untreated or treated with AOH at the concentrations indicated for 48 h and analyzed for FITC-dextran uptake by flow cytometry (A). The results represent mean values ±
SEM of 3 independent experiments (B). * indicates significantly different from control, p > 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 5: AOH induces morphological changes in human primary macrophages.** (A) Human primary macrophages were exposed to AOH (30 µM) or left untreated for 24 h and the actin filaments were stained with Phalloidin Alexa Fluor 555 (upper) and the nuclei stained with Hoechst (lower) prior to fluorescence microscopic analysis. (B) Human primary macrophages were exposed to AOH (30 µM) or left untreated for 48 h and analyzed by SEM.

**Figure 6: Phenotypic characterization of AOH-treated human primary macrophages.** Cells were treated with AOH (30 µM) or left untreated for 24 h and analyzed for the expression of CD80, CD200r, CD163, CD83, CD86, HLA-DR or CD68 by flow cytometry (A). The relative expression is quantified as MFI and the results represent mean values ± SEM of 3-5 independent experiments (B). * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 7: AOH induces cytokine secretion in human primary macrophages.** Cells were treated with 30 µM AOH or left untreated for 24 h and the supernatant analyzed for cytokine secretion (IL-12p70, TNFα, IL-10, IL-6, IL-8 and IL-1β) by CBA. The results represent mean values ± SEM of 3 independent incubations, representative of 9 different experiments. * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 8: Endocytic activity induced by AOH in human primary macrophages.** Cells were treated with 30 µM AOH or left untreated for 24 h and analyzed for FITC-dextran uptake by flow cytometry (A). The relative expression is quantified as MFI. The results represent mean values ± SEM of 4 independent experiments (B). * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 9: Expression of LC3II in response to AOH in human primary macrophages.** Cells were treated with 30 µM AOH or left untreated for 24 h and analyzed for LC3II expression by flow cytometry. The relative expression is quantified as MFI. The results represent mean values ±
SEM of 3 independent experiments. * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 10: AOH induced ROS in human primary macrophages.** Cells were treated with AOH (30 and 60 µM) or left untreated and analyzed for ROS production after 2, 6 and 24 h by flow cytometry. Positive ctrl (PC): H2O2 (1 mM, 10 min). The results are representative for 3 independent experiments (A). The relative expression is quantified as MFI. The results represent mean values ± SEM of 3 independent experiments (B). * indicates significantly different from control, p < 0.05 (1-way-ANOVA with Dunnett’s post-test).

**Figure 11: AOH induced DNA damage in human primary macrophages.** The cells were treated with AOH at the indicated concentrations or left untreated for 6 or 24 h and analyzed for γH2AX expression and cell cycle by flow cytometry. (A) Events above the dotted line represent cells positive for γH2AX, while the x-axis represent DNA content (cell cycle distribution). (B) Quantification of γH2AX positive cells. (C) Cell cycle distribution in response to AOH 30 µM, 24 h exposure. (D) Quantification of cell cycle distribution. The results represent mean values ± SEM of 3 independent experiments. * indicates significantly different from control, p< 0.05 (B: 1-way-ANOVA with Dunnett’s post-test, D: paired t-test).

**Supplementary:**

**Figure S1: Human monocyte-macrophage differentiation.** (A) GM-CSF induced differentiation of primary human monocytes into macrophages. Pictures are taken after 1 or 7 days. (B) Flow cytometric characterization of monocyte/macrophase purity and differentiation after 1 (upper) and 7 days of GM-CSF treatment. Black line: Isotype control, Red line: CD68

**Figure S2: AOH induced cell death in RAW 264.7 cells.** RAW 264.7 cells were treated with AOH (60 µM) or left untreated for 48 h and analyzed for cell death; necrosis and apoptosis by PI/Hoechst 33342 staining and fluorescence microscopy. The data represent mean of 2 independent experiments.
Figure S3: Effects of NAC on AOH induced morphology in RAW 264.7 cells. The cells were treated with AOH (30 µM) for 24 h in the presence or absence of NAC (5 mM) or left untreated. The cell morphology was evaluated by light microscopy. The results are representative for 3 independent experiments.

Figure S4: AOH induced cell death in human primary macrophages. The cells were treated with AOH at the indicated concentrations or left untreated for 24 h before PI-staining and flow cytometry. The data represent one of three representative experiments, mean values ± SE of 3 independent incubations.

Figure S5: AOH induces morphological changes in primary mouse peritoneal macrophages. The cells were exposed to AOH at the concentrations indicated for 48 h or left untreated, and analyzed by light microscopy (A), for viability by Alamar Blue (B) and for endocytic activity (C). The data represent one experiment, representative of 2 independent experiments. The viability results represent mean ± SD of 3 independent incubations.

Figure S6: Effects of antioxidants on AOH induced morphology in human primary macrophages. The cells were treated with AOH (30 µM) for 24 h in the presence or absence of NAC (5 mM) or left untreated. The cell morphology was evaluated by light microscopy. The results are representative for 2 independent experiments.

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