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

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23 **Abstract**

24 Alternariol (AOH), a mycotoxin produced by *Alternaria* fungi, is frequently found as a

25 contaminant in fruit and grain products. Here we examined if AOH could modify macrophage

26 phenotype and inflammatory responses. In RAW 264.7 mouse macrophages AOH changed the

27 cell morphology of from round to star-shaped cells, with increased levels of CD83, CD86,

28 CD11b, MHCII and endocytic activity. TNFα and IL-6 were enhanced at mRNA-level, but only

29 TNFα showed increased secretion. No changes were found in IL-10 or IL-12p40 expression.

30 Primary human macrophages changed the cell morphology from round into elongated shapes

31 with dendrite-like protrusions in response to AOH. The levels of CD83 and CD86 were

32 increased, HLA-DR and CD68 were down-regulated and CD80, CD200R and CD163 remained

33 unchanged. Increased secretion of TNFα and IL-6 were found after AOH exposure, while IL-8,

34 IL-10 and IL-12p70 were not changed. Furthermore, AOH reduced macrophage endocytic

35 activity and autophagosomes. AOH was also found to induce DNA damage, which is suggested

36 to be linked to the morphological and phenotypical changes. Thus, AOH was found to change the

37 morphology and phenotype of the two cell models, but either of them could be characterized as

38 typical M1/M2 macrophages or as dendritic cells (DC).

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40 **Keywords:**

41 Macrophages, differentiation, DNA damage, mycotoxins, alternariol

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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, 20111). 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

90 DNA damage (SSBs, DSBs and oxidative DNA damage). Cells accumulated in G_2-phase (4N),

91 with diploid or abnormal partly divided nuclei. Furthermore, the AOH-induced cell cycle arrest

92 was accompanied by increased autophagy and senescence, which were suggested to be a

93 consequence of DSBs.

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95 Macrophages play a key role in innate and adaptive immunity. Their main function is to perform

96 phagocytic clearance of pathogens and dying cells and to modulate the adaptive immune response

97 through antigen processing and presentation and by cytokine secretion. Both tissue-resident

98 macrophages and monocyte-derived macrophages are recruited during inflammation (Hume *et*

99 *al.,* 2008; Mowat and Bain, 2010). Monocytes develop into various forms of macrophages

100 according to the nature of environmental signals (Hume, 2008; Bain and Mowat, 2014). The main

101 macrophage polarization states are referred to as classically activated macrophages (M1) and

102 alternatively activated macrophages (M2), thus mirroring the Th1/Th2 differentiation paradigm

103 (Biswas *et al.*, 2012; Martinez and Gordon, 2014). Th1-related cytokines such as interferon-γ

104 (INF-γ), as well as microbial stimuli such as lipopolysaccharide (LPS) polarize macrophages to

105 an M1 phenotype. These cells produce pro-inflammatory cytokines such as TNFα, IL-12/23 and

106 IL-8; and have inducible nitric oxide synthase (iNOS). They are able to ingest (endocytic

107 activity) and kill pathogens rapidly; however, the use of ROS and NO will also result in tissue

108 damage (Laskin *et al.*, 2011; Biswas *et al.*, 2012; Mills and Ley, 2014). M1 macrophages have

109 increased antigen presenting capacity and display increased levels of major histocompatibility

110 complex class II (MHCII) and B7 co-stimulatory molecules such as CD80 and CD86 (Ambarus

111 *et al.*, 2012; Biswas *et al.*, 2012). The integrin CD11b is considered being a pan-macrophage

112 marker, associated with adherence and phagocytosis. M2 polarization is broader and includes IL-

113 4/IL-13 stimulated macrophages (M2a), IL-10 induced macrophages (M2c) and immune

114 complex-triggered macrophages (M2b) (Mantovani *et al.*, 2004). M2 macrophages produce anti-

115 inflammatory cytokines such as IL-10, and are generally involved in tissue regeneration

116 (Mantovani *et al.*, 2004; Biswas *et al.*, 2012). Typical surface receptors expressed by M2 cells are

117 the membrane glycoprotein CD200r, which is expressed on M2a cells and the scavenger receptor

118 CD163, which is expressed on M2c cells (Koning *et al.*, 2010; Ambarus *et al.*, 2012).

119 Interestingly, proliferation of M2 macrophages rather than recruitment from the blood has

120 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

152 Mowiol were from Millipore (Hayward, CA, USA) and Upcell plates were purchased from Nunc

153 (Rochester, NY, USA). H_2DCFDA-CM, DHE, N-acetyl-L-cysteine (NAC), propidium iodide,

154 Hoechst and RNase were from, Life technologies (Grand Island, NY, USA).

155 *Antibodies:* Anti-human CD163 Alexa Fluor 647, Alexa Fluor 488 Mouse IgG2b (κ Isotype Ctrl),

156 anti-human CD83 PE, anti-human CD86 PerCP/Cy5.5, anti-human CD80 Alexa Fluor 488, anti-

157 human CD200r PE, anti-human CD68 Alexa Fluor 488, anti-mouse CD83 PE, Rat igG1 PE,

158 human Trustain FcX (Fc Receptor Blocking Solution) and TruStain fcX anti-mouse CD16/32

159 (mouse Fc receptos blocker), were from BioLegend (San Diego, CA, USA). anti-human HLA-

160 DR Alexa Fluor 674 were from Bioss (Woburn, MA, USA), anti-mouse CD80 FITC, Armenian

161 Hamster igG FITC isotype Ctrl, anti-mouse MHCII FITC, Rat igG2b FITC isotype Ctrl, anti-

162 mouse CD11b Alexa Fluor 488, Rat IgG2b Alexa Fluor 488 isotype Ctrl, anti-mouse CD86 APC

163 and Rat IgG2aκ Iso Control APC were from eBioscience (San Diego, CA, USA). γH2AX and

164 LC3B were from cell signaling (Beverly, MA, USA) and anti-rabbit Alexa Fluor 647 were from

165 molecular probes (Life Technology, Grand Island, NY, USA).

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2.2. Cell cultures

168 *RAW 264.7 macrophages:* The mouse macrophage cell line RAW 264.7 was obtained from

169 European Collection of Cell Cultures (ECACC) and grown in DMEM supplemented with 10%

170 heat inactivated fetal bovine serum (FBS; EU standard, Lonza), penicillin (100 U/ml), and

171 streptomycin (100 μg/ml). The cells were cultured at 37 °C with 5% CO_2 in a humidified

172 atmosphere and kept in logarithmic growth phase at 1×10^6 - 10×10^6 cells/75 cm^2 through

173 routine sub-culturing by scraping, according to standard ECACC protocol. Cells were plated (0.3

174 $\times 10^6$ cells/cm^2) 24 h prior to the experiment, which resulted in approximately 70% confluence at

175 the day of exposure. Medium were refreshed before exposures. For measurement of cell surface

176 receptors, endocytosis and apoptosis/necrosis, the cells were grown on temperature-responsive

177 UpCell plates (Nunc, Rochester, NY, USA) from which cells detach at temperatures below 32 ºC.

178 Otherwise the cells were harvested by scraping, as recommended by ECACC.

179 *Primary human macrophages:* Peripheral blood mononuclear cells (PBMCs) were isolated from

180 buffy coats obtained from healthy donors according to the international ethical guidelines

181 (CIOMS) (Østfold Hospital Trust, Norway) by Ficoll density gradient centrifugation. About 10%

182 of the PBMC isolated from human blood is monocytes (Auffray *et al.*, 2009). The PBMCs were

183   cultivated in RPMI 1640 supplemented with 10% heat inactivated FBS and penicillin (100
184   U/ml)/streptomycin (100 µg/ml), at 37 °C under 5% CO_2, and plated at a density of 1.0 x
185   10^6 cells/cm^2. The PBMCs were cultivated for 24 h to allow the monocytes to adhere to the
186   plastic. The non-adherent PBMC were washed off with PBS and the monocytes were allowed to
187   differentiate into macrophages in the presence of granulocyte macrophage colony-stimulating
188   factor (GM-CS, 50 ng/ml) for 7 days. The cells were approximately 70% confluent at the day of
189   exposure. The medium was replenished on day four and on the day of exposure. Cells from
190   different donors were used for the biological replicates of the experiments. Macrophage purity
191   and differentiation were verified by flow cytometric analysis of CD68 (Supplementary, Fig. S1).
192   Positive controls for M1 and M2 differentiation were generated by treatment with GM-CSF (50
193   ng/ml) alone for the first 4 days, then INF-γ (50 ng/ml) and IL-10 (50 ng/ml) were added,
194   respectively. For the generation of DCs, the cells were stimulated with GM-CSF (50 ng/ml) + IL-
195   4 (25 ng/ml) for 6 days and LPS (100 ng/ml) were added and the cell incubated further for 24 h.
196   The supplemented RPMI 1640 media was exchanged on day four and six. Cell differentiation
197   was verified by flow cytometric analysis of specific markers associated with the cell type as M1
198   express CD80, M2 express CD163 and CD200r and DCs express CD83 and DC86 (data not
199   shown). The cells were harvested by trypsination.

200   *Primary mouse peritoneal macrophages:* Female B6C3F1 mice (5 weeks old) weighing 16 to 18
201   g were obtained from Charles River Laboratories, Inc (Wilmington, MA, USA) or Harlan
202   (Indianapolis, IN, USA). Housing, handling and sample collection procedures conformed to the
203   policies of the Michigan State University All-University Committee on Animal Use and Care in
204   accordance with NIH guidelines. Mice were injected ip with 1.5 ml of sterile 3% (w/v)
205   thioglycollate broth. After 4 days, mice were euthanized and macrophages collected by peritoneal
206   lavage with ice-cold Hank's BSS (Invitrogen Corporation, Carlsbad, CA, USA). Cells were
207   pelleted by centrifugation at 1100 g for 5 min. Cells were washed with PBS once and re-
208   suspended in RPMI-1640 containing 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml)/
209   streptomycin (100 µg/ml) and cultivated cultured at 37 °C under 5% CO_2 in a humidified
210   incubator (0.4 x 10^6 cells/cm^2). After 3 h incubation, non-adherent cells were removed. The cells
211   were then cultivated further for 24 h before treatment. The cells were approximately 70%
212   confluent at the day of exposure. The cells were harvested by trypsination.

213 *Exposure:* AOH was dissolved in DMSO and the final concentration of solvent in cell culture

214 was 0.1%. Appropriate controls containing the same amount of solvent were included in each

215 experiment. For experiments with the antioxidant N-acetyl-L-cysteine (NAC); NAC was

216 dissolved in complete medium complemented with HEPES (25 mM) and pH adjusted to 7.2. The

217 cells were pre-treated with NAC for 1 h before exposure to AOH.

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220 ### *2.3. Evaluation of cell morphology*

221 *Fluorescence microscopy:*

222 The cells were seeded and cultivated on Millicell EZ slides. After exposure to AOH, the cells

223 were fixed in 4% PFA for 10 min at ambient temperature followed by permeabilization and

224 blocking in 3% BSA/PBS, 0.05% saponin for 1 h. The cells were then stained with Phalloidin

225 Alexa Fluor 555, diluted in 3% BSA / PBS / 0.05% saponin for 1 h, and washed 3 times for 5 min

226 with 3% BSA / PBS / 0.05% saponin. The nuclei were stained with Hoechst 33342 (1µg/µl) and

227 coverslips mounted with mowiol. Pictures were captured with a fluorescence microscope (Nikon

228 Eclipse 80i, equipped with a DS-Ri1 camera, Amsterdam, Netherlands).

229 *Scanning electron microscopy (SEM):*

230 SEM was done as previously described (Christoffersen *et al.*, 2015). Samples were washed and

231 fixed with 5% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 45

232 min; then replaced with 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4) for 30 min.

233 Samples were then washed, dehydrated in graded ethanol series and dried using a critical-point

234 dryer (CDP 030, BAL-TEC GmBH, Germany). Dry samples were mounted on aluminum stubs

235 using double-faced carbon tape (Agar Scientific, UK), and coated with approximately 500 Å

236 platinum using a sputter coater (Polaron SC7640, Quorum Technologies, UK). Microscopic

237 analyses were performed using an EVO-50 Zeiss microscope (Carl Zeiss AG, Germany).

238 *Light microscopy:*

239 Cell morphology was evaluated by light microscopy (Leica DMIL. Solms, Germany). Random

240 pictures were taken by Moticam 1000 (Motic, Hong Kong, China).

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242 ### *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_m1for 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 10^6 cells) with propidium iodide (PI, 10 µg/ml) and Hoechst 33342 (5

305 µg/ml) for 30 min. Stained cells suspended in 10 µl FBS were applied to slides and air-dried.

306 Nuclear morphology associated with necrosis and apoptosis was determined using a Nikon

307 Eclipse E400 fluorescence microscope. A minimum of 300 cells were counted per slide.

308 *Flow cytometry:* Necrotic and late apoptotic cells have impaired membrane integrity and cannot

309 exclude PI like viable cells. Cells were harvested and stained with PI (5 µg/ml) for 10 min in the

310 dark at ambient temperature and analyzed immediately by flow cytometry (Accuri C6) using

311 $488_{ex}:585/42_{em}$.

312 *Alamar Blue:* Viability of the primary mouse peritoneal macrophages was measured by Alamar

313 blue as described in Solhaug *et al.,* 2012.

314

315 *2.9. ROS measurements*

316 ROS production was detected using oxidation-sensitive fluorescent probes. H_2DCFDA-CM (1

317 µM) was used to detect H_2O_2 and DHE (5 µM) to detect $O_2^{.-}$. The cells were harvested and

318 loaded with H_2DCFDA-CM or DHE for 20 min at 37 °C under 5% CO_2. The cells were washed

319 twice with ice cold PBS and analyzed by flow cytometry (Accuri C6). H_2DCFDA-CM was

320 measured by using $488_{ex}:530/30_{em}$ and DHE using $488_{ex}:585/42_{em}$. Relative expression was

321 expressed as MFI. The pro-oxidant H_2O_2 (1 mM, 10 min) was used as a positive control.

322

323 *2.10. Measurement of γH2AX and LC3II by flow cytometry*

324 Following treatments, cells were harvested, washed once in PBS, fixed in 2% paraformaldehyde

325 (PFA) in PBS for 10 min on ice, and post-fixed/permeabilized in 90% ice-cold methanol for 24 h

326 or more at -20 °C. For staining with antibody, 5×10^5 cells were washed twice with 0.5% BSA in

327 PBS and then incubated with γH2AX or LC3B antibody in 0.5% BSA/PBS overnight at 4 °C.

328 Cells were then rinsed twice in 0.5% BSA/PBS and incubated with secondary antibody

329 conjugated to Alexa Fluor 647 for 2 h at room temperature in the dark. Finally cells were rinsed

330 twice and analyzed by flow cytometry (Accuri C6) using $633_{ex}:675/25_{em}$. For analysis of cell

331 cycle distribution in addition to protein expression, the cells were washed with PBS, incubated

332 with propidium iodide (PI; 10 µg/ml) / RNase A (100 µg/ml) in PBS for 30 min at 37 °C before

333 analyses on a flow cytometer (Accuri C6). Single cells were gated and a minimum of 10,000 cells

334 acquired and analyzed using $488_{ex}:585/40_{em}$ vs $647_{ex}:675/25_{em}$.

335

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 polyploidy (Fig. 1). Little or no cell death was observed after AOH exposure (Supplementary, Fig. S2), which is in agreement with that AOH reduce the proliferationrate 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

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367   activation, were all up-regulated in RAW 264.7 cells after exposure to AOH (30 µM). Similarly,

368   also the expression of the integrin CD11b was found to be up-regulated by AOH. In contrast,

369   CD83 which is associated with DC maturation was not affected. To investigate if AOH induced

370   changes of pro-inflammatory cytokines, we measured the expression of TNFα, IL-6 and IL-

371   12p40 by RT-PCR. Both TNFα and IL-6 mRNA expression were found to be up-regulated in

372   RAW 264.7 cells (Fig. 3A). IL-12 is composed of two heterologous chains; p40 and p35, which

373   together form IL-12p70 and is a key cytokine released from immature and mature DCs (Steinman

374   *et al.,* 2007; Shortman *et al.,* 2007). AOH was not found to induce expression of IL-12p40 in

375   RAW 264.7 cells (Fig 3A). At the protein level, ELISA confirmed an AOH-induced up-

376   regulation of TNFα release (Fig. 3B). In contrast, IL-6 could not be detected (data not shown),

377   which might be caused by effects linked to post-transcriptional changes/ processes induced by

378   AOH. To further characterize the AOH induced differentiation of the RAW 264.7 cells, we

379   employed qPCR to measure expression of iNOS and Arg-1 as their up-regulation is commonly

380   used as indicators for M1 or M2 macrophage polarization in mice, respectively (Stout *et al.*,

381   2005; Kigerl *et al.*, 2009)**.** However, AOH (30 µM, 12-72 h) did not increase the expression of

382   either iNOS or Arg-1 (data not shown). Endocytosis is required for the intracellular processing

383   and presentation of exogenous antigenic fragments, and is a crucial function of macrophages as

384   well as immature DCs. Endocytic capacity of the AOH treated cells was investigated by the

385   uptake of FITC-dextran. A significantly higher amount of FITC-dextran was taken up by

386   macrophages treated with AOH (30 µM) for 48 h (Fig. 4) compared to untreated cells, indicating

387   a higher endocytic activity. This was not the case when cells were stimulated with lower

388   concentrations of AOH (15 µM), which corresponds well with the morphological changes

389   described above.

390

391   *3.2. AOH induces morphological and phenotypically changes in human primary macrophages*

392   To further study the effects of AOH on macrophage morphology, we next investigated if AOH

393   induced similar morphological changes in primary human macrophages. Human blood

394   monocytes were differentiated into macrophages by GM-CSF for 7 days, followed by exposure to

395   AOH (30 µM) for 24 h. A dramatic change in morphology was observed in AOH-treated cells

396   compared to the untreated control after 24 h (Fig. 5A) and 48 h exposure (Fig. 5B). Most cells

397   appeared elongated with dendrite-like protrusions after AOH-exposure, while untreated cells

14

398 remained round. The morphological changes were similar after 24 and 48 h AOH (30 µM)
399 exposure. No cell death was observed (Supplementary, Fig. S4). To further characterize the
400 prominent changes in morphology induced by AOH (30 µM, 24 h), we measured the expression
401 of several surface receptors, commonly associated with M1 (CD80, CD86), M2 (CD200r,
402 CD163) or DCs (CD83, CD86, HLA-DR) in addition to the more common macrophage marker
403 CD68. Interestingly, both CD83 and CD86 were found to be up-regulated in response to AOH
404 treatment while HLA-DR and CD68 were down-regulated (Fig. 6). No significant changes were
405 detected for CD80, CD200r and CD163 (Fig. 6). To assess the effects of AOH on pro-
406 inflammatory cytokine production in primary macrophages, the secretion of TNFα, IL-6, IL-8,
407 IL-1β, IL-10 and IL-12p70 was measured using cytokine bead assay (CBA). AOH induced
408 increased secretion of TNFα and IL-6, but had no effects on IL-8, IL-1β, IL-10 or IL-12p70
409 levels (Fig. 7). In RAW 264.7 macrophages AOH were found to increase the endocytic capacity
410 (Fig. 4). In contrast, the primary macrophages had lower uptake of FITC-dextran when treated
411 with AOH compared to control (Fig. 8). Interestingly, similar effects of AOH on morphology and
412 uptake of FITC-dextran, with no effects on cell viability, were observed in AOH-treated primary
413 peritoneal macrophages isolated from mouse (Supplementary, Fig. S5).

414

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

420

421 ***3.3 AOH induced ROS, DNA damage and cell cycle arrest in human primary macrophages***
422 AOH-induced ROS was found to be associated with SSBs and oxidative DNA damage (Solhaug
423 *et al.*, 2012). Thus, in order to further elucidate possible mechanisms involved in AOH-induced
424 morphological changes, we next analyzed intracellular ROS levels using H_2DCFDA-CM and
425 DHE probes detecting H_2O_2 and $O_2^{.-}$, respectively. In contrast to the RAW 264.7 macrophages
426 (Solhaug *et al.*, 2012), AOH (30 - 60 µM) showed only a slight up-regulation (not significant) of
427 H_2O_2 production after 2 h, 6 h and 24 h exposure (Fig. 10AB). No $O_2^{.-}$ production was detected
428 after 24 h of AOH exposure (30 – 60 µM, data not shown). Furthermore, in accordance with

429   observations in RAW 264.7 cells, addition of the ROS scavenger NAC (5 mM; 24 h) did not

430   change the AOH-induced morphological changes as evaluated by light microscopy

431   (Supplementary, Fig. S6).

432

433   AOH's effects on topoisomerase (Fehr *et al.,* 2009) have been suggested to be linked to an

434   increased level of DSBs (Solhaug *et al.*, 2012). As increased phosphorylation of H2AX (γH2AX)

435   is indicative of DSBs, we next examined γH2AX expression vs. cell cycle by flow cytometry

436   (Sordet *et al.*, 2009). As expected, the primary macrophages had only a limited proliferation rate

437   as only approximately 2% of the cells were found to be in S phase (Fig. 11AD). Despite the low

438   level of proliferation in these cells, AOH exposure markedly enhanced cell numbers in G_2 (Fig.

439   11ACD). Most importantly, increased levels of γH2AX were seen following exposure to AOH

440   (Fig. 11AB), suggesting an enhanced level of DSBs possibly via its known effect on

441   topoisomerase. Interestingly, γH2AX was enhanced in all phases of the cell cycle, suggesting that

442   inhibition of topoisomerase during replication as well as transcription could cause DSBs (Fig.

443   11A).

444

445   **4. Discussion**

446   Our previous studies show that AOH induces ROS and DNA damage followed by G_2 arrest in

447   RAW 264.7 macrophages (Solhaug *et al.*, 2012, 2013). We have also identified AOH as an

448   inducer of autophagy as well as senescence in these cells (Solhaug *et al.*, 2014). These cellular

449   effects were suggested to be linked to an AOH-initiating effect on topoisomerase thereby causing

450   DSBs, rather than enhanced ROS (Solhaug *et al.*, 2012, 2014). In the present study, we find that

451   AOH modified the phenotype of proliferating RAW 264.7 and the slowly proliferating primary

452   human macrophages. The AOH-induced changes on morphology and inflammatory cytokine

453   responses were rather similar in the two models. In contrast, the effects of AOH with regard to

454   endocytosis, autophagy as well as expression of CD-markers were markedly different (Table 1).

455   The phenotypes did neither match with typical M1/ M2 macrophages nor with DC.

456

457   Plasticity and functional polarization are hallmarks of macrophages. Here we find that AOH

458   induced star-shaped morphology of the RAW 264.7 cells. Similar morphological changes have

459   been previously reported and associated with macrophage differentiation into DCs (Saxena *et al.*,

460   2003; Lee *et al.*, 2004). In this study, the levels of CD80, CD86, MHCII and CD11b were

461   increased, as here also seen in AOH-exposed cells. In contrast, the expression of CD83, a marker

462   of DC differentiation and maturation, was found to be unchanged after AOH exposure.

463   Furthermore, AOH did not induce expression of IL-12p40, which is a key cytokine released from

464   immature and mature DCs (Steinman *et al.,* 2007; Shortman *et al.,* 2007). Although, the changes

465   in cell surface marker expression and enhanced endocytic activity shows that AOH exposure

466   induced marked phenotypical changes in the macrophages. The AOH treated RAW 264.7

467   macrophages displayed several of the M1 characteristics, such as increased expression of MHCII,

468   CD80, CD86 and increased secretion of TNFα. On the other hand, AOH did not enhance the

469   expression of iNOS or release of IL-12p70, and the differentiated macrophages can therefore not

470   be characterized as classical M1 macrophages. Similarly, the typical characteristics of M2

471   macrophages, such as elongated morphology (Bolling *et al.*, 2012) and expression of IL-10 and

472   Arg-1, were not fulfilled. Thus, the RAW 264.7 cells obtained after AOH exposure cannot be

473   categorized as classical DC or as M1/ M2 macrophages.

474

475   In the primary human macrophage model, AOH induced even more DC-like morphology with

476   elongated cells showing dendrite-like protrusions. Similar to what was seen in the RAW 264.7

477   cells, AOH also modifies the phenotype of the primary macrophages. More specifically, the

478   AOH-exposed primary human macrophages show increased expression of CD83 and CD86

479   together with decreased CD68 levels, increased secretion of TNFα and IL-6 and decreased

480   endocytosis, which may represent modifications into a more DC-like phenotype. On the other

481   hand, rather a down regulation of HLA-DR and no increased secretion of IL-12p70 were found.

482   As seen with the RAW 264.7 model, the phenotypically characteristic of the AOH treated

483   primary macrophages did not fit with DC, M1 nor M2 polarized macrophages.

484

485   It is important to note that the DC and M1/M2 classification scheme of macrophages is generally

486   considered to be an oversimplification of the true spectrum of macrophage phenotypes (Wermuth

487   and Jimenez, 2015). Thus, it is not surprising that the changes seen following exposure to a

488   cellular stressor like the mycotoxin AOH could not be clearly categorized. The interesting and

489   important part is that AOH did change the morphology and the macrophage phenotype in the

490   experimental models tested, RAW 264.7 mouse macrophages, primary human macrophages and

491 primary mouse peritoneal macrophages (Supplementary, Fig. S5). Although the macrophages

492 exposed were in different stage of differentiation due to their diverse origin. The macrophages

493 response to AOH could have potentially important implications as the proper function of tissue

494 macrophages and DCs are essential for the health of specialized parenchymal and stromal cells

495 (Mills and Ley, 2014). Possible theoretical implications could be a decrease of immune response

496 in case of infections and/or a disturbed balance of the adaptive immune system.

497

498 There have been reports linking exposure to various mycotoxins to changes in macrophage

499 phenotype and/or effects on the differentiation processes of monocytes to macrophages (Wache

500 *et al.*, 2009; Gammelsrud *et al.*, 2012; Ficheux *et al.*, 2013). More specific, deoxynivalenol, a

501 *Fusarium* mycotoxin, inhibits INF-γ mediated macrophage activation, assessed by the expression

502 of several surface receptors, when the cells are exposed during the differentiation process from

503 monocytes to macrophages (Wache *et al.*, 2009). Furthermore, monocytes exposed to enniatin B,

504 another *Fusarium* mycotoxin, during the differentiation process into macrophages presented a

505 decrease of endocytosis and an increase of CD71 (Ficheux *et al.*, 2013). Interestingly, enniatin B

506 exposed to RAW 264.7 macrophages were also found to induce expression of CD163, a marker

507 for M2 macrophages (Gammelsrud *et al.*, 2012). To our knowledge, this is the first study

508 describing morphological and phenotypical changes induced by AOH on macrophages.

509

510 Various cellular processes have been associated to the macrophage differentiation development,

511 from specific interactions with specific cellular receptors (Martinez and Gordon*,* 2014) to more

512 unspecific processes including DNA damage responses (So *et al.*, 2013) and autophagy (Jacquel

513 *et al.*, 2012). Less is known with regard to important initial molecular events triggering these

514 changes. There are reports suggesting a crucial role for ROS (Nakanishi *et al.*, 2013) and NO

515 (Nogueira-Pedro *et al.*, 2014). We recently reported that AOH could be a potent inducer of ROS

516 in the RAW 264.7 macrophage model (Solhaug *et al.*, 2012). However, here in this study,

517 we–found that a potent anti-oxidant (NAC) did not reduce the AOH-induced morphological

518 changes. Furthermore, AOH induced differentiation of primary humane macrophages in the

519 apparent absence of ROS formation. Hence, ROS does not seem to be an obligatory part of

520 macrophage differentiation induced by AOH.

521

522 The other primary initiating molecular event of AOH presently known is an interaction with

523 topoisomerase (Fehr *et al.*, 2009). In general, interactions with topoisomerase often result in

524 DSBs as a consequence of the delayed replicative and/or transcriptional synthesises (Durand-

525 Dubief *et al.,* 2014, Sordet *et al.*, 2009). In our recent study, we observed that AOH exposure

526 resulted in increased γH2AX, and suggested that AOH-induced DSBs were an important

527 triggering signal for G_2 arrest and autophagy (Solhaug *et al.*, 2012, 2014). Also in the primary

528 human macrophage model, AOH is suggested to increase γH2AX as a DNA damage response

529 caused by DSBs. γH2AX was enhanced in all phases of the cell cycle, suggesting that inhibition

530 of topoisomerase during replication as well as transcription could cause DSBs. Most

531 interestingly, differentiation has been suggested as an outcome in response to DSBs (Sherman *et*

532 *al.*, 2011). Furthermore, DNA damaging agents is found to alter the differentiation-process of

533 monocytes to favour the generation of M2 macrophages (Dijkgraaf *et al.*, 2013). DNA damage

534 has also been suggested to be an important mediator in the decision of hematopoietic stem cells

535 to exit quiescence and to differentiate (Weiss and Ito, 2015). Differentiation is typical tightly

536 linked to cell cycle withdrawal (Rots *et al.,* 1999). Thus, we first hypothesized that increased

537 DSBs could result in a G_2 arrest, linked to a changed phenotype. However, in contrast to AOH-

538 exposed RAW 264.7 cells, the primary human macrophages were found to be only slowly

539 proliferating and the majority of the cells were in G_1 phase. Thus, there are obviously no direct

540 link between the AOH-induced macrophage differentiation and a specific cell cycle arrest.

541 However, a link between AOH induced DNA damage and changes in macrophage morphology

542 and phenotype is still plausible.

543

544 Autophagy (macro-autophagy) is a re-cycling mechanism by which cells through lysosomal

545 degradation reuse amino- and fatty acids. Interestingly, autophagy has been reported to be

546 essential during the differentiation process from monocytes to macrophages, which require

547 marked architectural remodelling (Jacquel *et al.*, 2012; Zhang *et al.*, 2012). Furthermore,

548 autophagy has been found to be important in differentiation and polarization of macrophages.

549 However, the precise role of autophagy is still uncertain and it has been suggested to be a both a

550 positive and negative regulator of M1 differentiation (Jung *et al.*, 2010; Chen *et al.*, 2014; Liu *et*

551 *al.*, 2015). Here we find that AOH-induced morphological changes in the RAW 264.7 cells,

552 previously shown to have increased DSBs and increased autophagy; whereas AOH reduced

19

553   autophagy in the primary human macrophages. This supports the notion that there is no clear
554   relationship between autophagy and macrophage differentiation/ polarization process. There
555   seems to be rather complex signalling interactions on-going between autophagy and the
556   macrophage differentiation process, as inhibition of AOH-induced autophagy caused increased
557   cell death rather than influence the degree of morphological changes in RAW 264.7 cells
558   (Solhaug *et al.*, 2014).

559

560   The concentrations of AOH used in this study are in the same range that causing DNA damage as
561   previously published by us (Solhaug *et al.,* 2012) and others (Brugger *et al.,* 2006; Fehr *et al.,*
562   2009; Pfeiffer *et al.,* 2007). Current knowledge concerning adverse effects of AOH in humans
563   and animals are still limited. An *in vivo* mouse study done by Schuchardt and co-workers (2014)
564   showed that uptake of AOH over the gut were somewhat poor. However, this can be different for
565   other species and/or with other prolonged feeding trails. An ongoing inflammation in the gut may
566   also increase the uptake as well as the effect. Thus an uptake of AOH at levels that may affect the
567   immune system cannot be excluded.

568

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

575

576

588

589 **Figure legends:**

590

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

595

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

601

602 **Figure 3: Effects of AOH on TNFα, IL-12 and IL-6 cytokine production in RAW 264.7 cells.**
603 (A) RAW 264.7 cells were treated with AOH (30 µM) or left untreated for 48 h. Gene expression
604 of TNFα, IL-12p40 and IL-6 were analyzed by qRT-PCR .Two independent experiments using
605 biological triplicates were performed. The results were statistically assessed using one-way
606 ANOVA with Dunnett`s post-test. (B) RAW 264.7 cells were treated with AOH (30 µM) or left
607 untreated for 6 - 48 h and analyzed for TNFα secretion by ELISA. The results represent the mean
608 values ± SD of 3 independent incubations.* indicates significantly different from control, $p <$
609 0.05 (1-way-ANOVA with Dunnett`s post-test).

610

611 **Figure 4: Alterations in endocytic activity induced by AOH in RAW 264.7 cells.** RAW 264.7
612 cells were left untreated or treated with AOH at the concentrations indicated for 48 h and
613 analyzed for FITC-dextran uptake by flow cytometry (A). The results represent mean values ±

614 SEM of 3 independent experiments (B). * indicates significantly different from control, p > 0.05

615 (1-way-ANOVA with Dunnett`s post-test).

616

617 **Figure 5: AOH induces morphological changes in human primary macrophages.** (A) Human

618 primary macrophages were exposed to AOH (30 µM) or left untreated for 24 h and the actin

619 filaments were stained with Phalloidin Alexa Fluor 555 (upper) and the nuclei stained with

620 Hoechst (lower) prior to fluorescence microscopic analysis. (B) Human primary macrophages

621 were exposed to AOH (30 µM) or left untreated for 48 h and analyzed by SEM.

622

623 **Figure 6: Phenotypic characterization of AOH-treated human primary macrophages.** Cells

624 were treated with AOH (30 µM) or left untreated for 24 h and analyzed for the expression of

625 CD80, CD200r, CD163, CD83, CD86, HLA-DR or CD68 by flow cytometry (A). The relative

626 expression is quantified as MFI and the results represent mean values ± SEM of 3-5 independent

627 experiments (B). * indicates significantly different from control, $p < 0.05$ (1-way-ANOVA with

628 Dunnett`s post-test).

629

630 **Figure 7: AOH induces cytokine secretion in human primary macrophages.** Cells were

631 treated with 30 µM AOH or left untreated for 24 h and the supernatant analyzed for cytokine

632 secretion (IL-12p70, TNFα, IL-10, IL-6, IL-8 and IL-1β) by CBA. The results represent mean

633 values ± SEM of 3 independent incubations, representative of 9 different experiments. * indicates

634 significantly different from control, $p < 0.05$ (1-way-ANOVA with Dunnett`s post-test).

635

636 **Figure 8: Endocytic activity induced by AOH in human primary macrophages.** Cells were

637 treated with 30 µM AOH or left untreated for 24 h and analyzed for FITC-dextran uptake by flow

638 cytometry (A). The relative expression is quantified as MFI. The results represent mean values ±

639 SEM of 4 independent experiments (B). * indicates significantly different from control, $p < 0.05$

640 (1-way-ANOVA with Dunnett`s post-test).

641

642 **Figure 9: Expression of LC3II in response to AOH in human primary macrophages.** Cells

643 were treated with 30 µM AOH or left untreated for 24 h and analyzed for LC3II expression by

644 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): H_2O_2 (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/macrophage 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 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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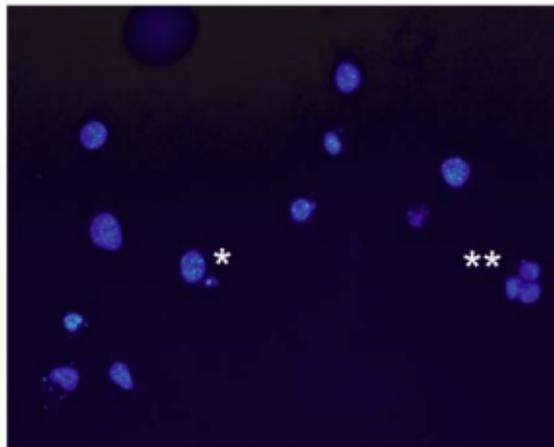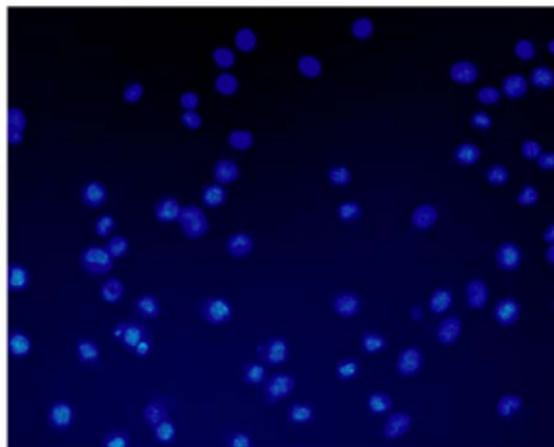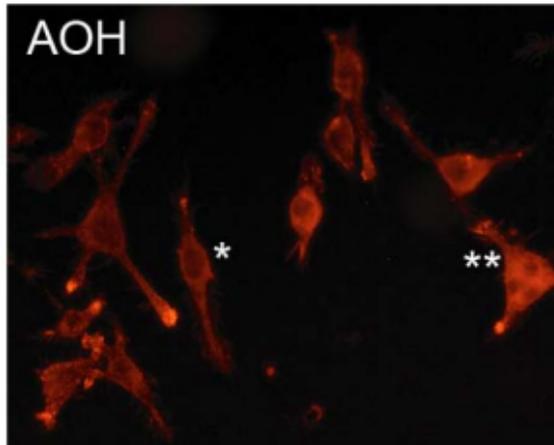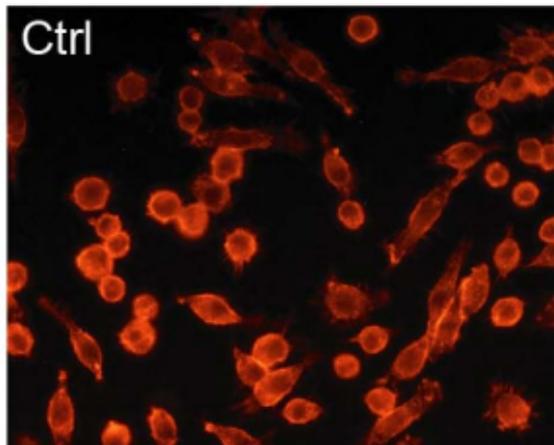
Fig. 1, single column

Fig 3, single colunm

Fig 4, single column

A

Counts

Ctrl
AOH 30 μM

FITC-Dextran uptake

B

FITC-Dextran uptake
(% of untreated control)

700
600
500
400
300
200
100
0

*

Ctrl   15   30

AOH (μM)
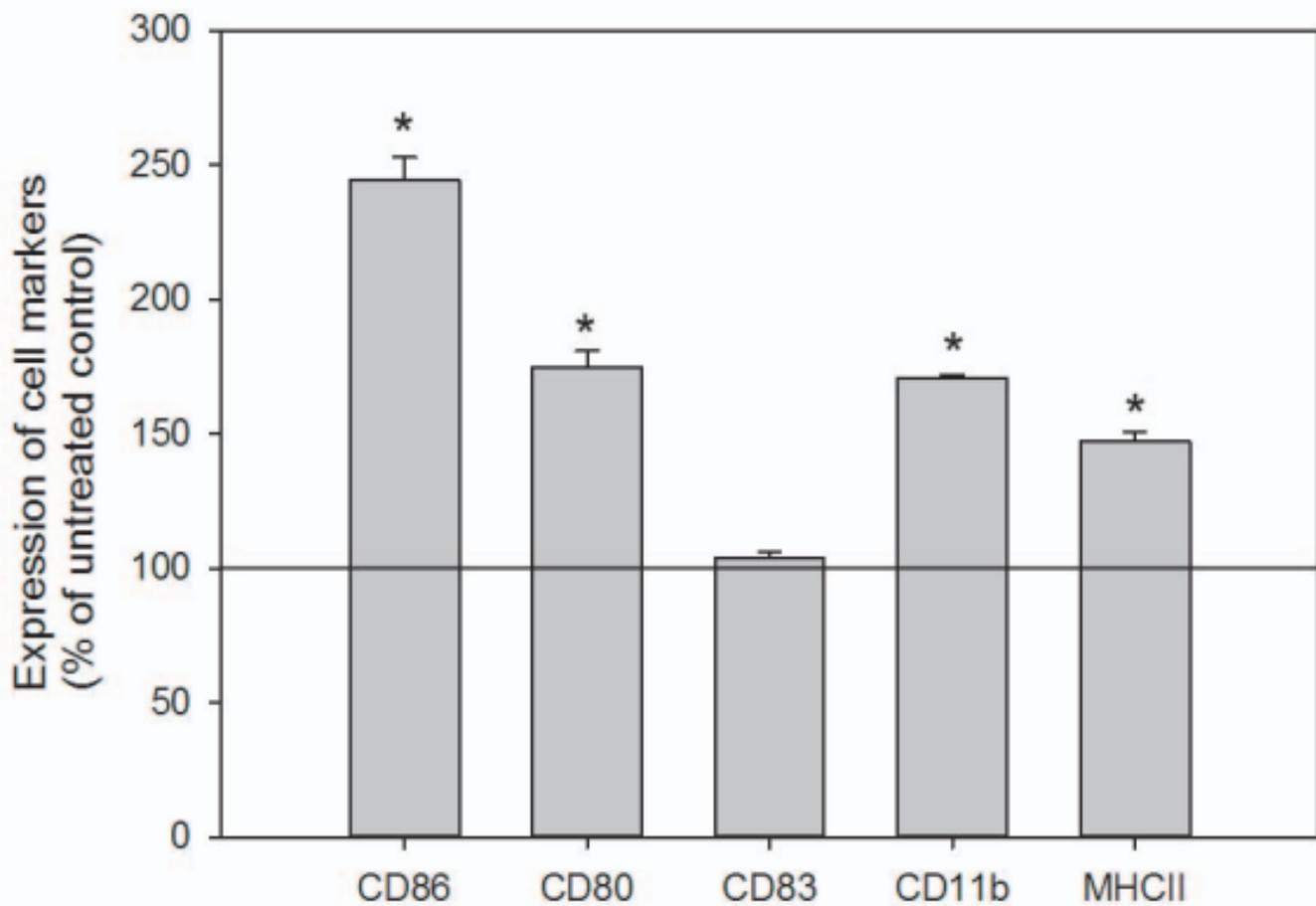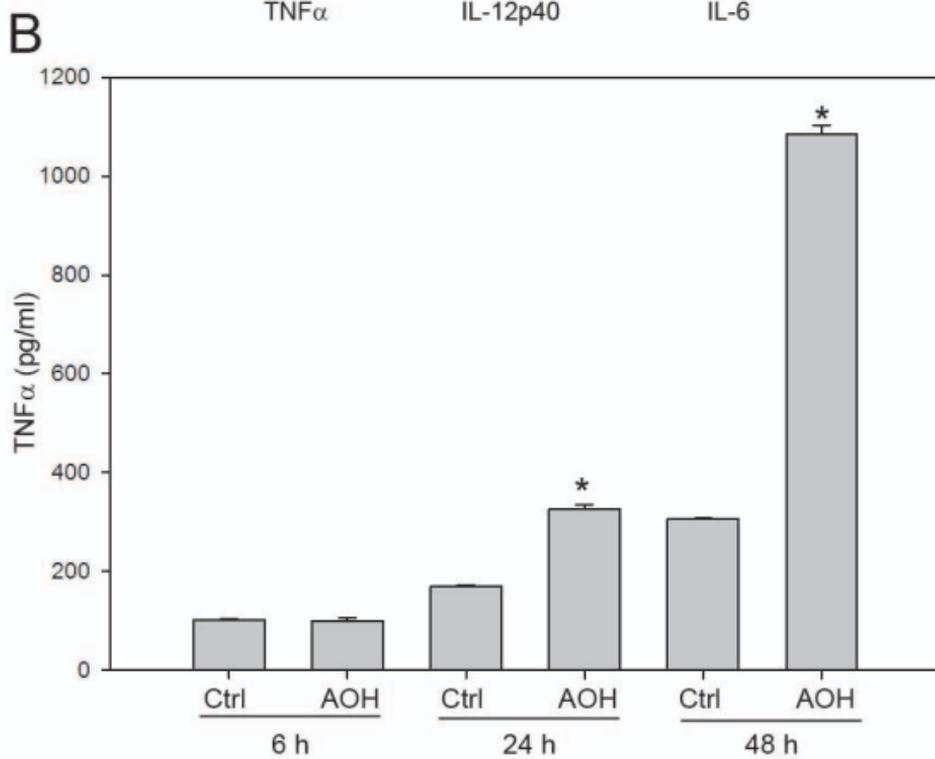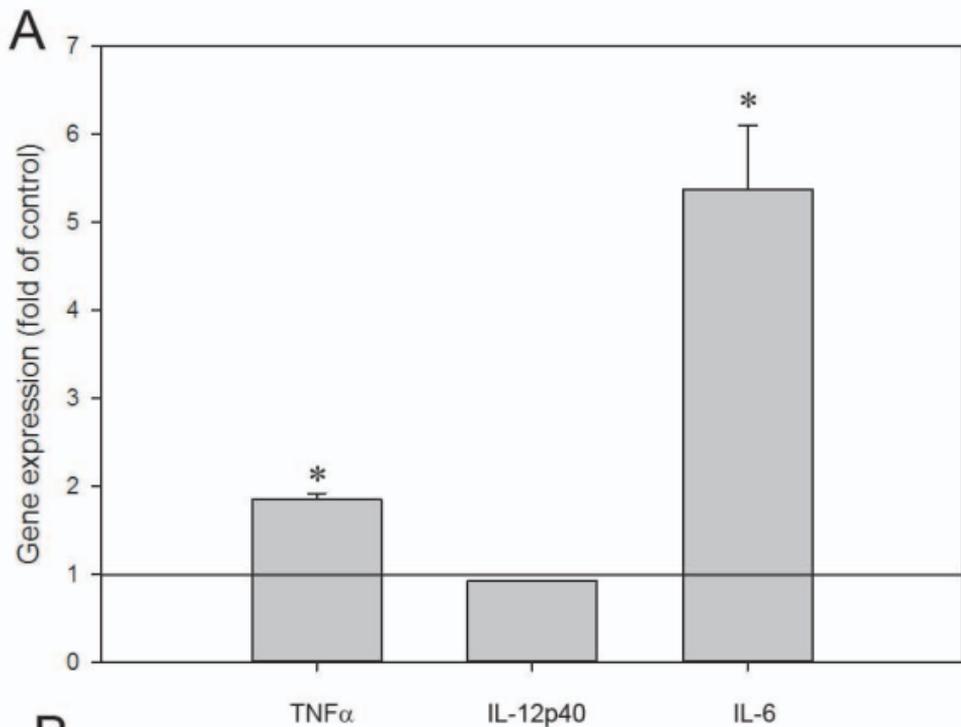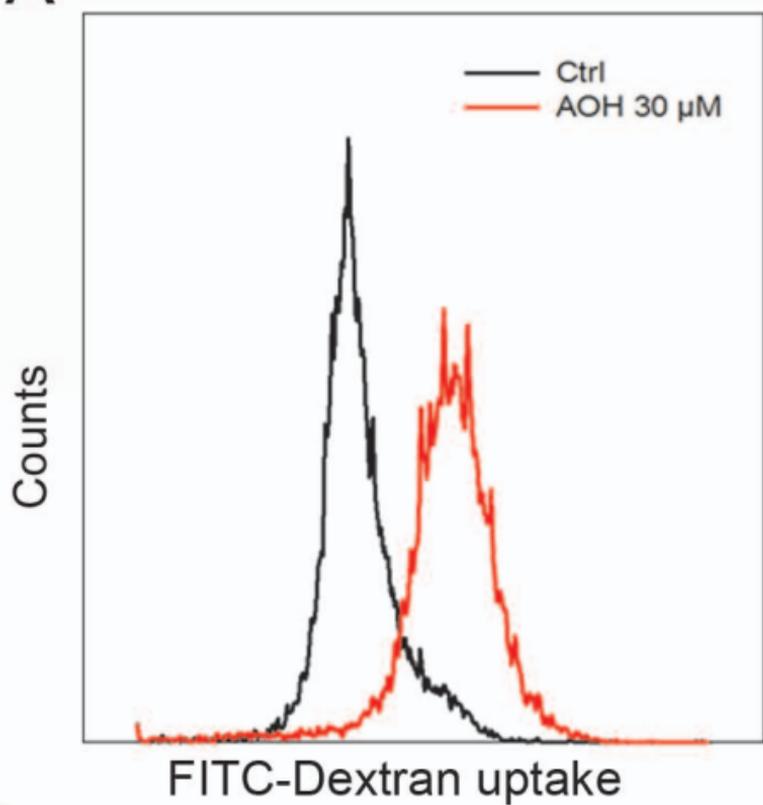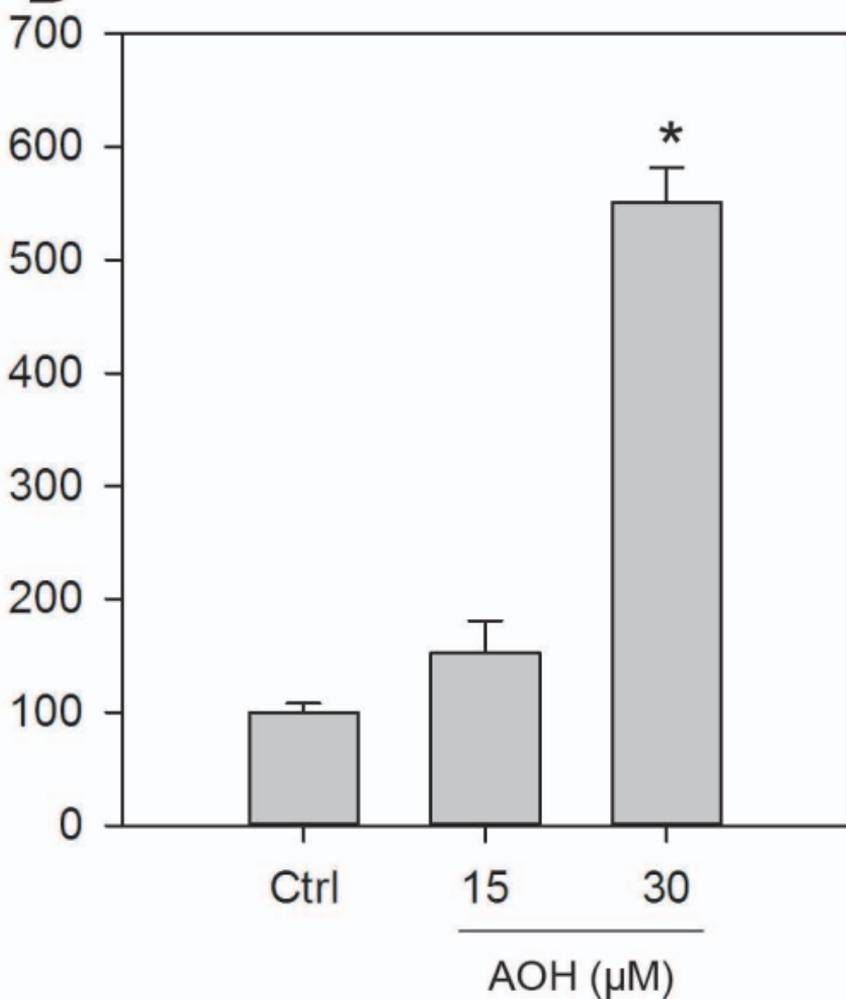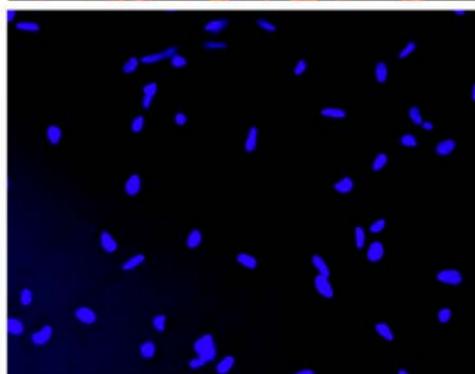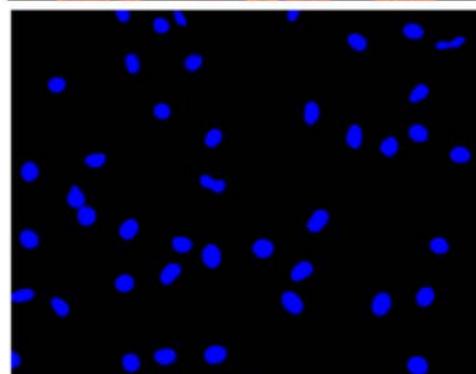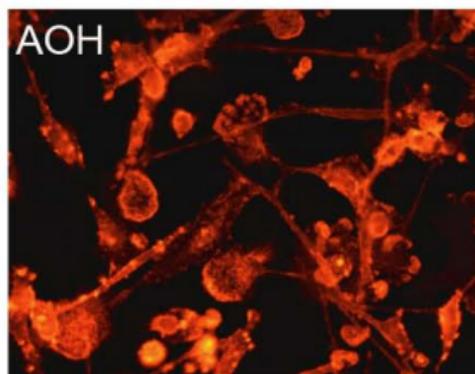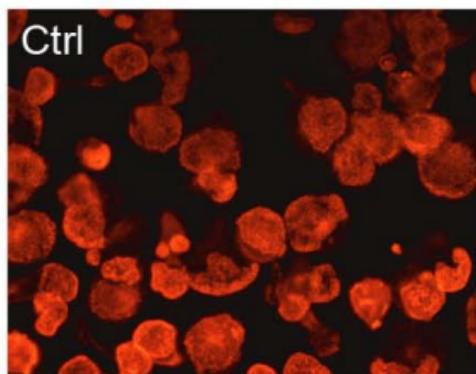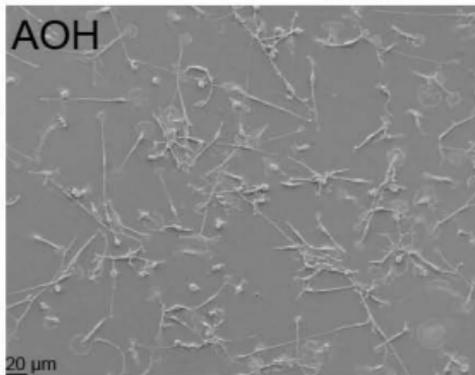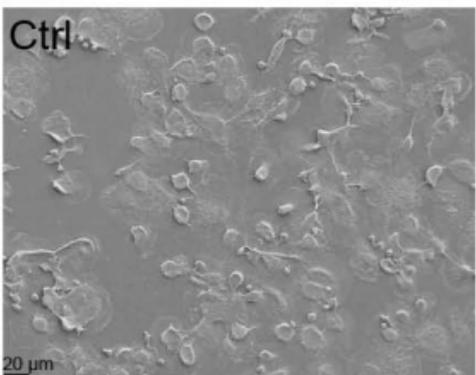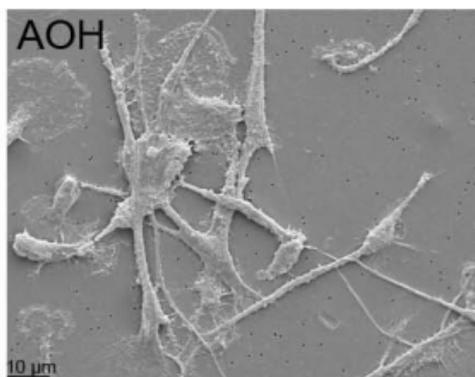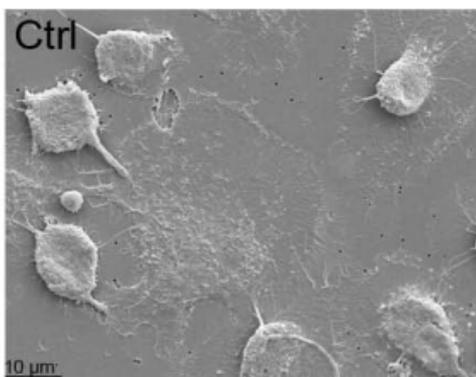
Fig 6, 2-column
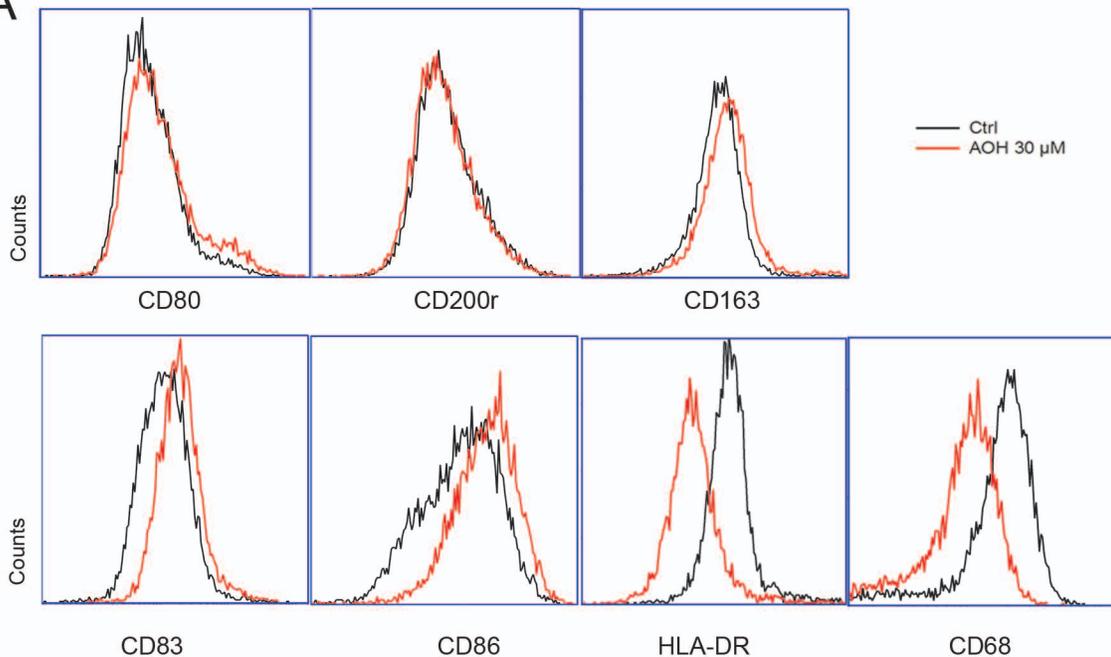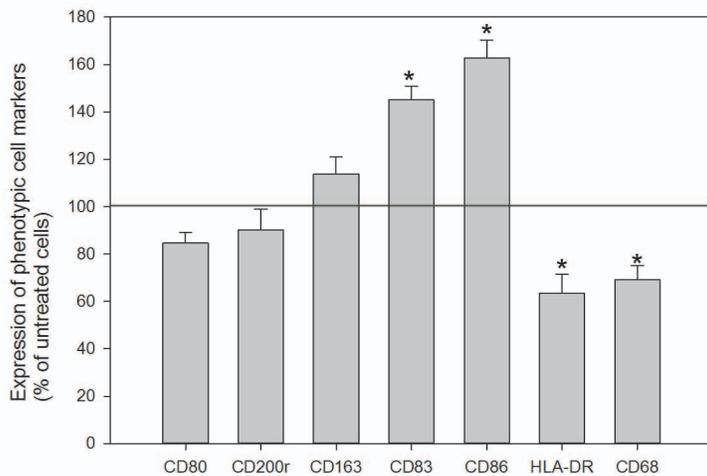
Fig 7, single column

Fig 10, single column

A

| | 2h | 6h | 24h |

Counts

H_2O_2 H_2O_2 H_2O_2

—— Ctrl   —— AOH 30 μM   —— AOH 60 μM   —— Positive ctrl

B

2h

ROS, H_2O_2 (% of untreated control)

1400
1200
1000
800
600
400
200
0

*

0   30   60   PC

AOH (μM)

Fig 11, 2-column

A

	Ctrl	AOH 15 μM	AOH 30 μM	AOH 60 μM
6 h	0,4%	0,8%	3,6%	3,4%
24 h	0,7%	3,9%	15,8%	34,7%

γH2AX

DNA content

B

γH2AX expression (fold of control)

24 h

Ctrl   AOH 30 μM

C

Counts

Ctrl
24 h

AOH 30 μM
24 h

G1   S   G2

DNA content

D

Cells (%)

24 h

■ Ctrl
▨ AOH 30 μM

G1   S   G2/M
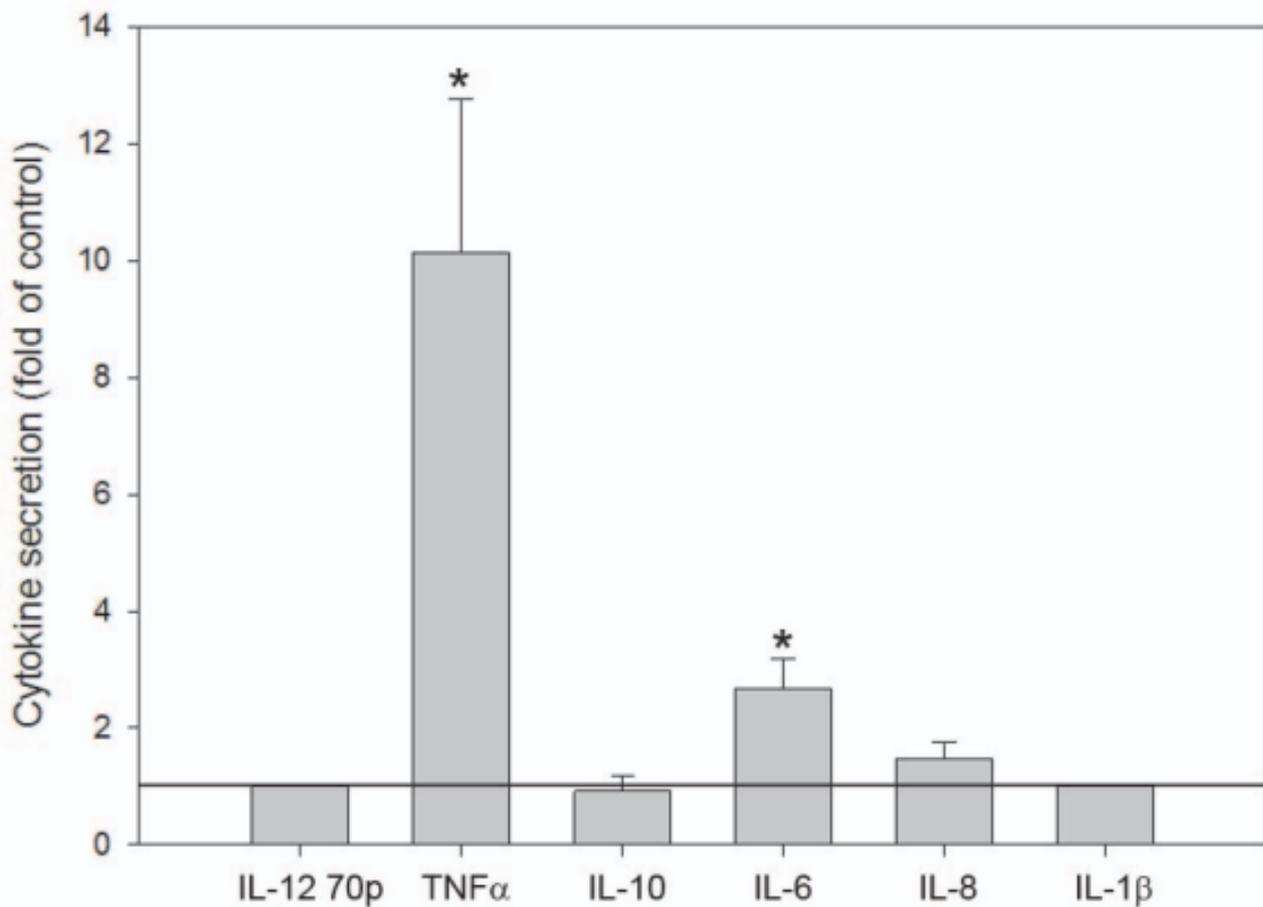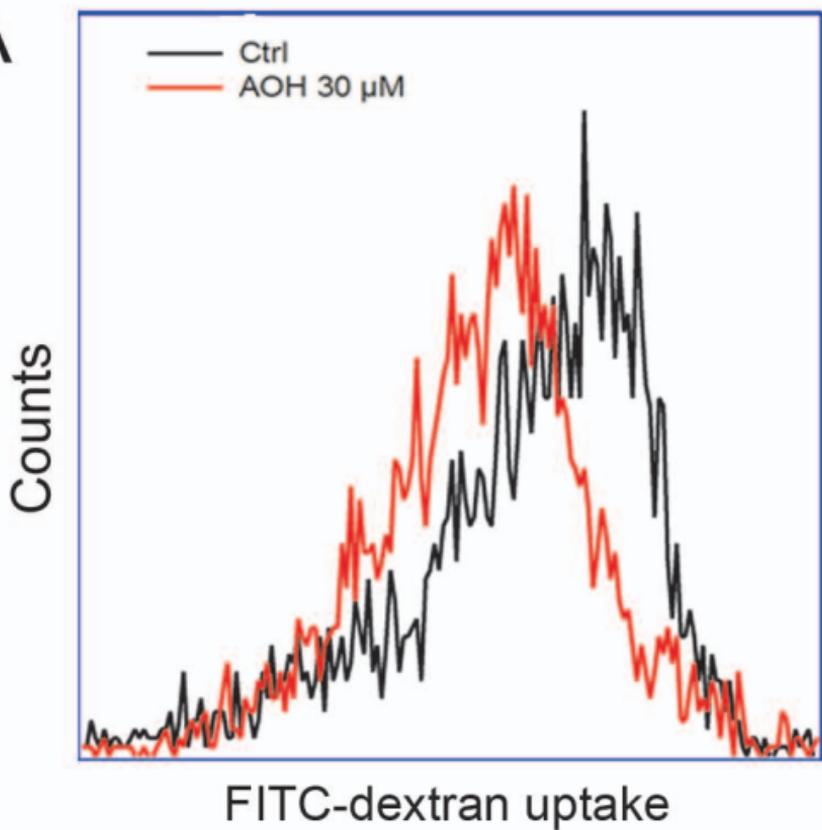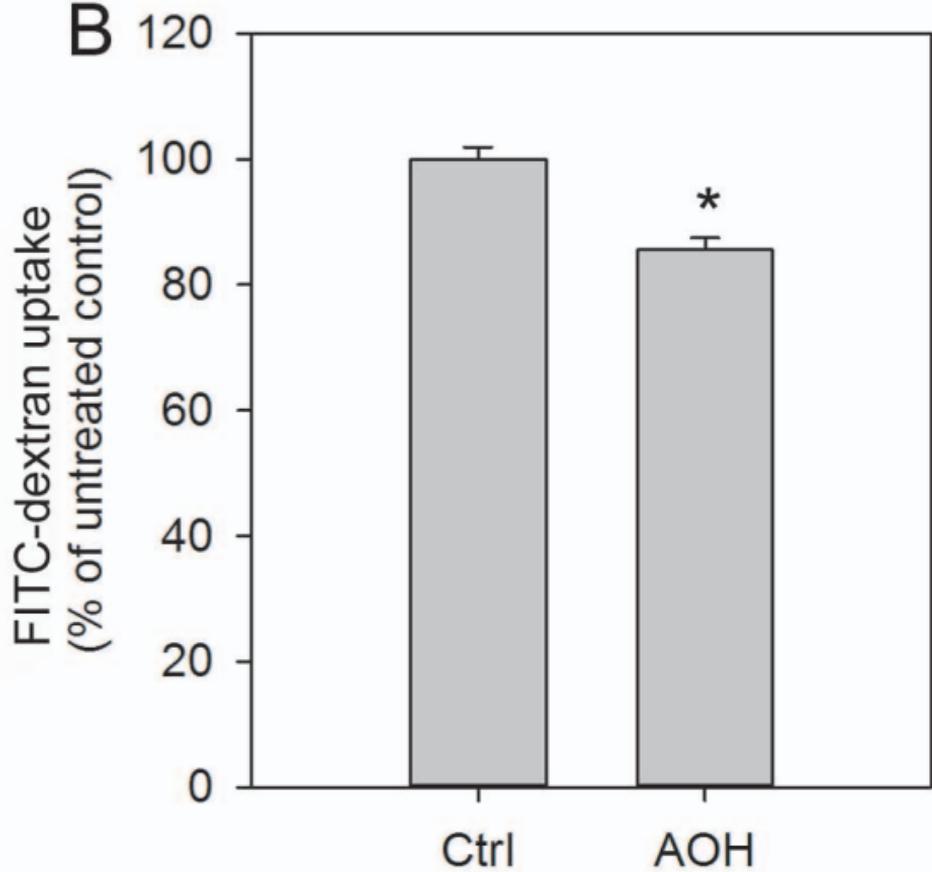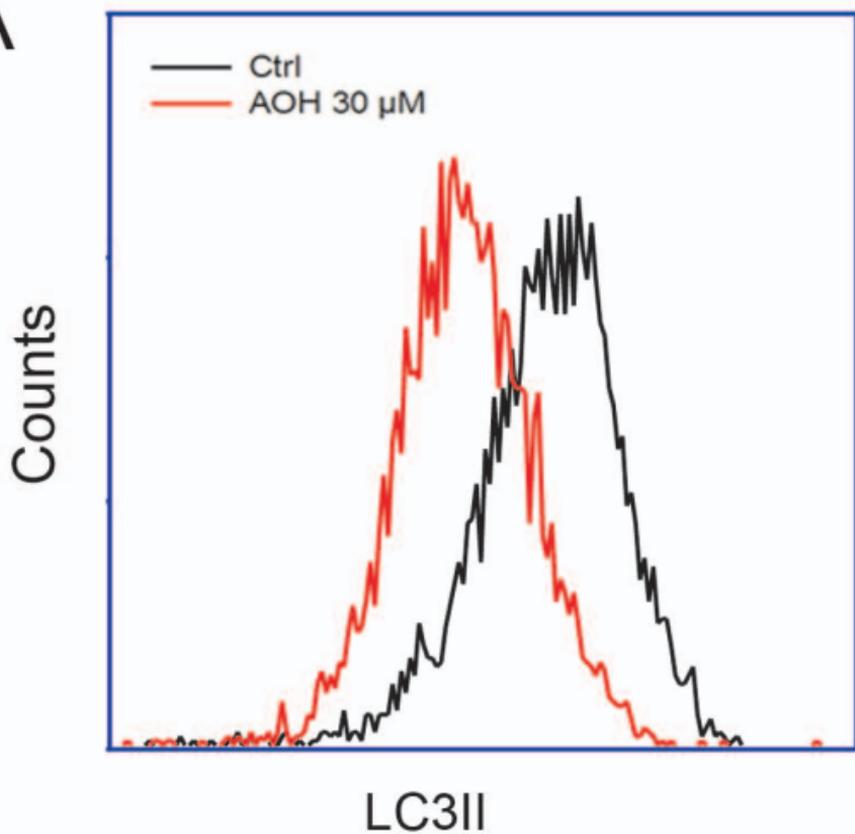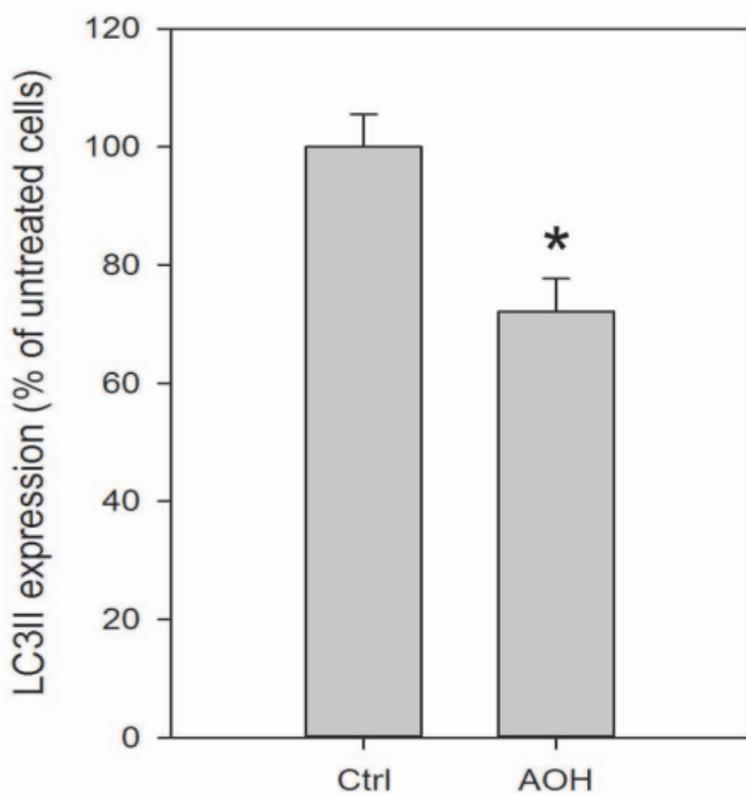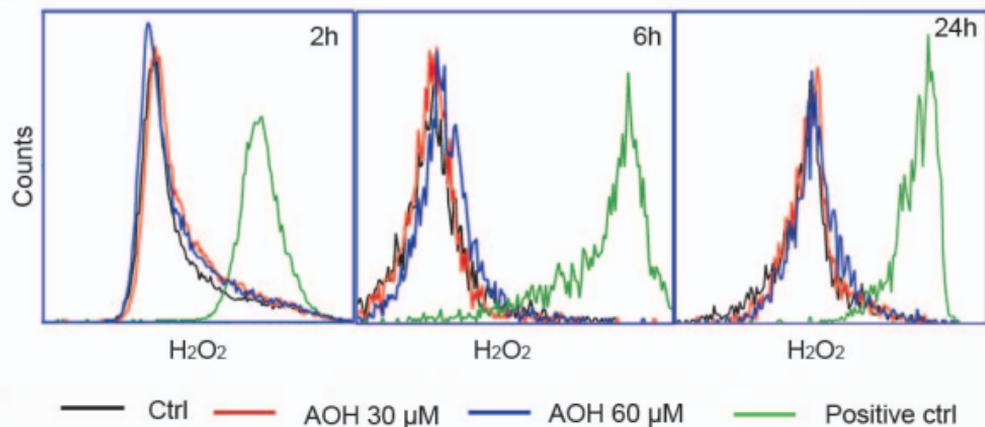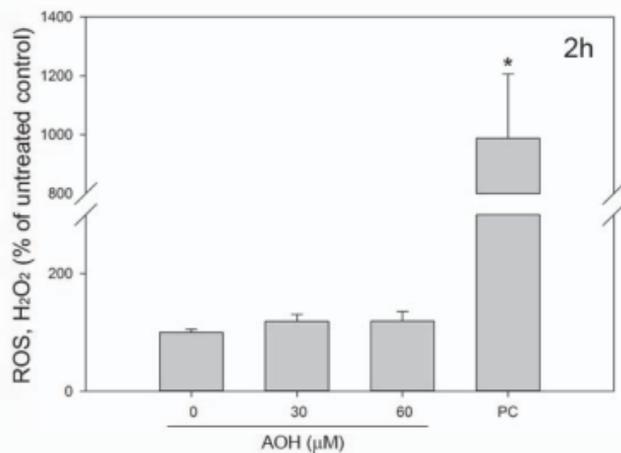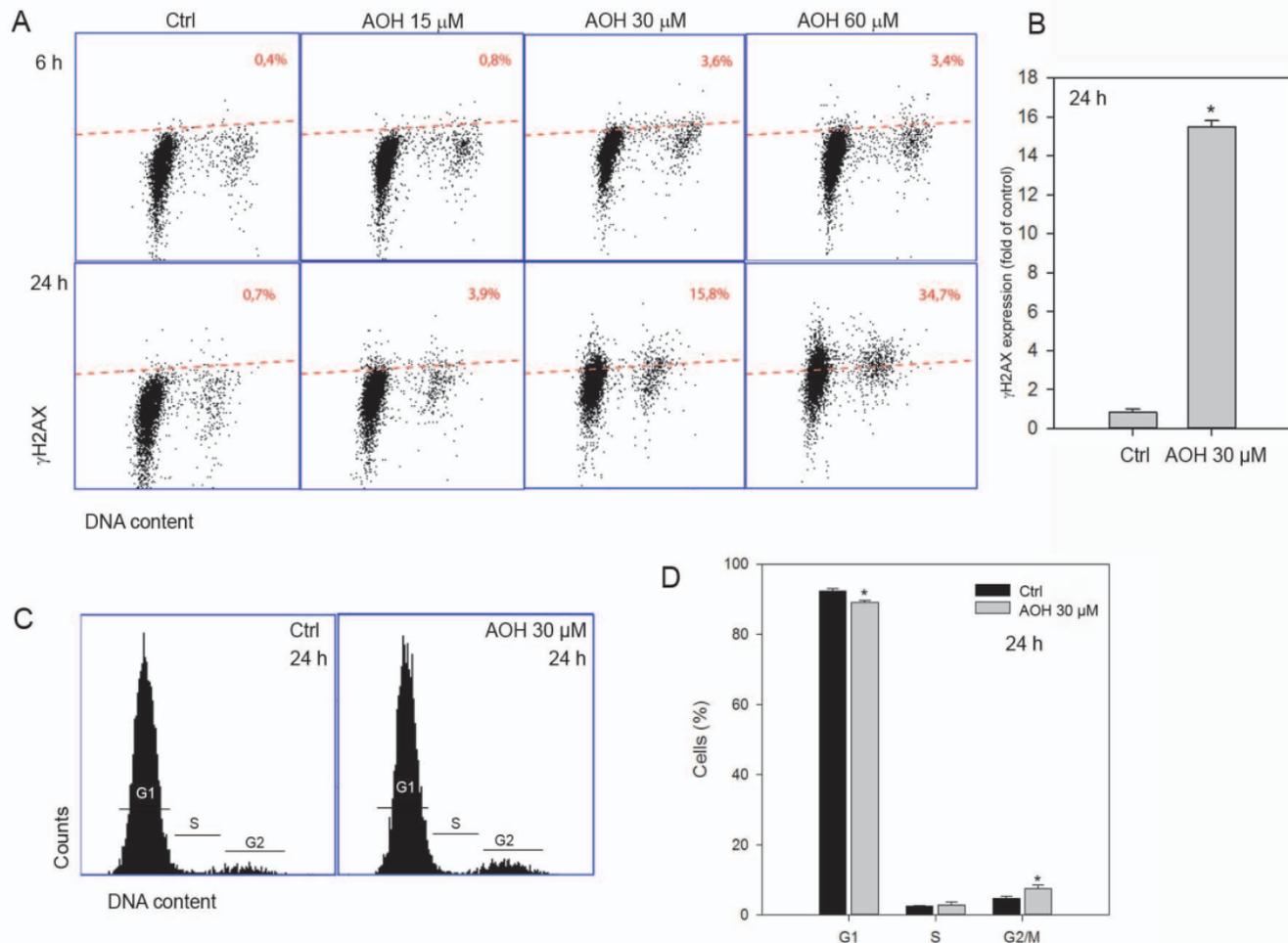
Fig S1, supplementary, single column

Ctrl   AOH   AOH+NAC

A

Ctrl   AOH, 60 µM

B

Viability (fold of control)

Ctrl   AOH 30 µM   AOH 60 µM

C

Counts

Ctrl
AOH 30 µM

FITC-Dextran

Ctrl   AOH   AOH + NAC