Immunomodulatory effects of individual and combined mycotoxins in the THP-1 cell line

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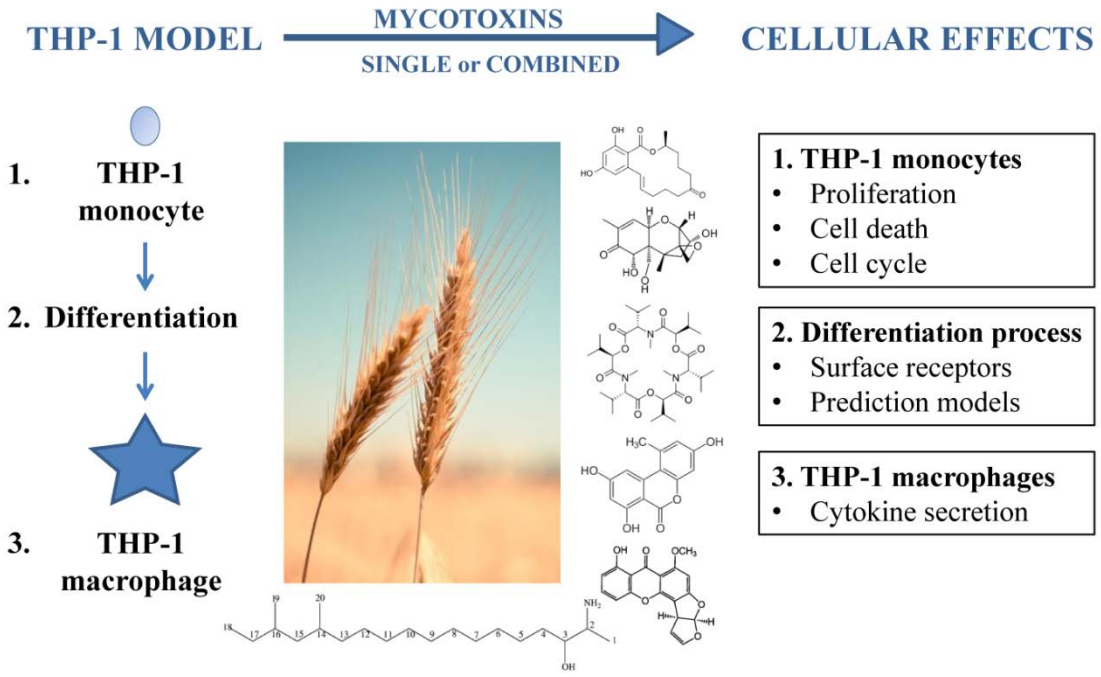
Graphical abstract



Highlights

- Mycotoxins differently affected proliferation and survival of THP-1 monocytes.
- AOH, DON and ZEA, reduced differentiation from monocytes into macrophages.
- The combined effects of these mycotoxins were mainly additive.
- Prediction models: concentration addition, independent action and isobolograms.
- AOH, DON and ZEA affected LPS induced macrophage activation.

Abstract

Mycotoxins commonly contaminate food and may pose a risk for disease in humans and animals. As they frequently co-occur, mixed exposures often take place. Monocyte function, including differentiation into active macrophages, is a central part of the immune response. Here we studied effects of naturally co-occurring mycotoxins in grain on monocyte function, and effects of individual and combined exposure on the differentiation process from monocytes into macrophages. The THP-1 cell line was used as a model system. The mycotoxins 2-amino-14,16-dimethyloctadecan-3-ol (AOD), alternariol (AOH), enniatin B (ENNB), deoxynivalenol (DON), sterigmatocystin (ST) and zearaleone (ZEA) differently affected cell viability in THP-1 monocytes, with DON as the most potent. AOH, ZEA and DON inhibited differentiation from monocytes into macrophages. Using this differentiation model, combined exposure of AOH, ZEA and DON were mainly found to be additive. However, the combination AOH+ZEA had somewhat synergistic effect at lower concentrations. Furthermore, alterations in macrophage functionality were found, as single exposure of AOH and ZEA inhibited lipopolysaccharide (LPS) induced TNF-α secretion, while DON increased this response. Overall, the mycotoxins affected monocyte viability and differentiation into macrophages differently. Combined exposures affected the differentiation process mainly additively.

Key words: mycotoxins, macrophages, differentiation, combinations, TNF-α

Abbreviations

AOD, 2-amino-14,16-dimethyloctadecan-3-ol; AOH, alternariol; BSA, bovine serum albumin; CA, concentration addition; CRC, concentration-response curve; DON, deoxynivalenol; DSBs, DNA double stranded breaks; EC, effect concentration; ELISA, enzyme linked immunosorbent assay; ENNB, enniatin B; IA, independent action; LPS, lipopolysaccharide; MFI, median fluoresce intensity; MOA, mode of action; NOAEC, no observed adverse effect concentration; PBS, phosphate buffered saline; PI, propidium iodide; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxidant species; SSBs, DNA single stranded breaks; ST, sterigmatocystin; TNF-α, tumor necrosis factor α; ZEA, zearaleone.

1. Introduction

Contamination of agricultural crops by fungi and their associated mycotoxins is a serious problem worldwide. Mycotoxins are secondary metabolites produced by fungi under appropriate environmental conditions, without being essential to fungal growth. Fungi of the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* are the main producers (Streit et al., 2012; Uhlig et al., 2013). Some of the mycotoxins are potent toxins naturally occurring in cereals/cereal based food (Uhlig et al., 2013) and may pose a potential health threat to humans and animals (Wu et al., 2014). *Fusarium* fungi produce deoxynivalenol (DON), one of the most frequently found mycotoxins in grain (Brera et al., 2015; Uhlig et al., 2013), and belongs to a family of mycotoxins called trichothecenes. The toxicological sequelae resulting from low dose exposure of DON include pro-inflammatory effects, anorexia, impaired weight gain and growth hormone dysregulation, whereas acute high dose exposure evokes gastroenteritis, emesis, leukocytosis, hemorrhage and shock-like syndrome (Pestka, 2010). The initiating mechanism of DON-induced toxicity is considered to be inhibition of the protein synthesis (Pestka, 2010). Simultaneously, DON increases gene transcription and release of important pro-inflammatory cytokines and chemokines involved in innate immunity. Other common *Fusarium* toxins found in grain are zearaleone (ZEA), enniatin B (ENNB) and the more recently identified mycotoxin 2-amino-14,16-dimethyloctadecan-3-ol; AOD (Uhlig et al., 2013; Uhlig et al., 2005). ZEA is estrogenic, and competes with 17β-estradiol for binding to the estrogen receptor, leading to problems in fertility and reproduction ability (Takemura et al., 2007). Furthermore, ZEA is reported to be genotoxic *in vitro* (Zinedine et al., 2007), but IARC found limited evidence of ZEA carcinogenicity in animal models and has classified it together with DON in group 3, not classifiable (IARC, 2002). ZEA has also been shown to inhibit protein and DNA synthesis, induce lipid peroxidation, ROS generation, cell death (Abid-Essefi et al., 2004; Kouadio et al., 2005) and to modulate pro-inflammatory responses (Pistol et al., 2015; Pistol et al., 2014). ENNB is cytotoxic in several *in vitro* cell systems (Dornetshuber et al., 2007; Gammelsrud et al., 2012; Ivanova et al., 2006) and has been found to exert pro-inflammatory properties (Gammelsrud et al., 2012). The mechanism of action is most likely linked to its ability to induce lysosomal instability (Gammelsrud et al., 2012; Ivanova et al., 2012) due to its ionophoric properties (Kamyar et al., 2004). Furthermore, ENNB is found to induce ROS, but seems not to be genotoxic (Dornetshuber et al., 2009; Gammelsrud et al., 2012). AOD is characterized as a

sphingosine analog (Uhlig et al., 2005), however in contrast to the well-known sphingosine analog and mycotoxin, fumonisin B1, AOD has no apparent effects on ceramid synthase (Uhlig et al., 2008). AOD is, however, cytotoxic at somewhat higher concentrations, but the mechanisms leading to the toxicity is unknown (Uhlig et al., 2008). The most prevalent *Alternaria* toxin found in grain is alternariol (AOH). Several *in vitro* studies have reported that AOH induces DNA-damage (Pfeiffer et al., 2007; Solhaug et al., 2012), which is linked to its ability to act as a topoisomerase poison (Fehr et al., 2009; Solhaug et al., 2016). Cellular effects such as generation of ROS, cell cycle arrest, appearance of abnormal nuclei, autophagy, senescence (Solhaug et al., 2012, 2013, 2014), differentiation, secretion of cytokines (Solhaug et al., 2015) and estrogenic potential (Frizzell et al., 2013) has also been reported. A number of *Penicillium* and *Aspergillus* metabolites such as sterigmatocystin (ST) can also be found in grain (Uhlig et al., 2013). ST share several structural similarities with aflatoxin B1, and is classified as a possible human carcinogen, 2B (IARC, 1987). ST binds covalently to DNA and form DNA adducts after metabolic activation (EFSA, 2013; Essigmann et al., 1979). Furthermore, ST induces ROS and oxidative DNA damage (Gao et al., 2015) and is found to have negative immunomodulary effects through the down-regulation of cytokine expression and secretion (Zhang et al., 2012).

The intestine and the associated immune system provide the first barrier to protect against ingested mycotoxins. The immune system is particular sensitive to mycotoxins (Corrier, 1991; Maresca, 2013; Wu et al., 2014). Macrophages are one of the most abundant immune cells in the intestinal mucosa where they are essential for maintaining homeostasis. Unlike most other tissue macrophages, which derive from primitive precursors that subsequently self-renew, the intestinal macrophage pool requires continual renewal from circulating blood monocytes (Bain and Mowat, 2014). Under steady state conditions, the monocytes differentiate locally into anti-inflammatory macrophages that express scavenger receptors and are highly phagocytic and hypo-responsive to pro-inflammatory stimuli (Bain and Mowat, 2014). Resending in close proximity to the gut microbiota, the specific adaptions enables the macrophages to ingest and destroy dying tissue cells and bacteria that penetrate the epithelial barrier without provoking inflammation. Inflammation and/ or invading pathogens may disrupt the normal pattern of monocyte differentiation, leading to accumulation of potent pro-inflammatory macrophages. In addition to

their own pro-inflammatory effects, they recruit other innate effector cells such as eosinophils (Bain and Mowat, 2014).

Humans and animals are often exposed to numerous mycotoxins simultaneously. Different fungi may produce several types of mycotoxins. Furthermore, if fungi contaminate the food most often different species of fungi are present. The potential health risks of exposure to naturally co-occurring mycotoxins in food have lately received more attention (Alassane-Kpembi et al., 2016). The toxicity of the individual mycotoxin does not always predict the toxicity of the mixture, as there may be potentiation, synergistic or antagonistic effects. It is of major concern if mixtures have synergistic effects, but even additive effects of mycotoxins may be of concern. Theoretically, unwanted effects may then occur even if all the mycotoxins alone are below the safety level (VKM, 2013). The effects could then exceed the safety levels set by the government.

Today the knowledge of effects caused by mixed exposure of mycotoxins on the immune system is limited. Accordingly, the aim of this study was to elucidate effects of naturally co-occurring mycotoxins on monocyte function, and more specifically analyze effects of individual and mixtures of mycotoxins on the differentiation process from monocytes into macrophages. As a model system, we used the human leukemia monocytic cell line THP-1. These cells express characteristic markers of immature monocytes in suspension and can differentiate into adherent macrophage-like cells by treatment with phorbol-12-myristate-13-acetate (PMA) (Daigneault et al., 2010; Park et al., 2007). This cell model is extensively used to study monocyte and macrophage functions and are at several aspects such as cytokine production, cell morphology and to some extend cell surface markers comparable with human peripheral mononuclear blood-derived monocytes/macrophages (Chanput., et al 2014). The following mycotoxins tested in this study are: AOH, DON, ENNB, ST, ZEA and AOD, which all are found to co-occur in natural infected grain (Uhlig et al., 2013). These mycotoxins are structurally highly different. Different interaction effects are therefore likely, and thus very interesting to explore. An important part of the study is to elucidate the experimental design taken for analysis of chemical interactions.

2. Materials and methods

2.1 Reagents and chemicals

Alternariol (AOH, #A1312), deoxynivalenol (DON, #0156), enniatin B (ENNB, #E5411), sterigmatocystin (ST, #S3255) and zearalenone (ZEA, #Z2125) were purchased from Sigma-Aldrich (MO, USA) and were of high purity grade, typical 98%. 2-amino-14,16-dimethyloctadecan-3-ol (AOD) was purified by Silvio Uhlig (98% purity) as described in (Uhlig et al., 2005), Trypsin-EDTA solution (2.5 g/l) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (MO, USA). RNAase (PureLink™ RNase A), bovine serum albumin (BSA) and Alamar Blue were purchased from Invitrogen (Life Technologies, CA, USA). Propidium iodide (PI) was bought from Molecular Probes (Life Technologies, CA, USA). Phosphate Buffered Saline (PBS), RPMI 1640 with L-glutamine, Penicillin/streptomycin and fetal bovine serum (FBS; EU standard) were purchased from Lonza (Verviers, Belgium). CellTox™ Green Cytotoxicity Assay was purchased from Promega Corporation (Wisconsin, USA). Phorbol-12-myristate-13-acetate (PMA) was purchased from Merck KGaA (Darmstadt, Germany). CD71-PE (#555537) was ordered from BD Biosciences. CD11b-Alexa Fluor 488 (#301318), CD14-Alexa Fluor 647 (#325612) and Human TruStain FcX™ (Fc Receptor Blocking Solution, #422302) were ordered from BioLegend (CA, USA). ELISA, human TNF-α Antibody Pair (#CHC1753) was purchased from Invitrogen (Life Technologies, CA, USA). Real Time ready cell lysis kit (#06366821001) and Transcriptor Universal cDNA master (#05893151001) were from Roche (Mannheim, Germany). Maxima SYBR Green/ROX qPCR Master Mix (#0253) was from Thermo Fisher Scientific (MA, USA). Primers for TNF-α (qHsaCED0037461) and β-actin (qHsaCED0036269) were from Bio-Rad (CA, USA).

2.2 Cell culture and treatments

The human acute monocyte leukemia cell line (THP-1) was obtained from European collection of cell cultures (ECACC) and grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured at 37 °C under 5% CO_2 in a humidified incubator and kept in a logarithmic growth phase at 5–15 × 10^5 cells/ml through routine sub-culturing, according to standard ECACC protocol. The passage number was kept below 20. The mycotoxins were dissolved in DMSO and the final concentration of solvent in cell culture was maximum 0.2%. Appropriate controls containing the same amount of solvent were included in each experiment.

2.3 Cellular toxicity of THP-1 monocytes upon mycotoxin exposure

THP-1 monocytes were seeded (125 000/cm^2) with subsequent treatment with mycotoxins for 24 h. Following treatment, the cellular toxicity was examined by measuring the metabolic activity, cell death and alterations in cell cycle as followed:

Alamar Blue assay: Metabolic activity of the cells was measured using the Alamar Blue assay according to the manufacturer's protocol. The dark blue oxidized form of Alamar Blue is reduced to a highly fluorescent form in functional mitochondria and the measured fluorescence intensity is thus proportional to the number of viable cells (Springer et al., 1998). The fluorescence (585 nm) was quantified using Victor2 Multi-label Counter (Perkin Elmer, Boston, MA, USA).

Cell death: Necrotic and late apoptotic cells have impaired membrane integrity and cannot exclude PI like viable cells do. 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, BD Biosciences, San Jose, CA, USA). Single cells were gated and a minimum of 10 000 cells acquired and analysed. Data acquired were analysed by Accuri CFlow Plus.

Cell cycle: For cell cycle analysis, the cells were washed with PBS and fixed with ice-cold 70% EtOH overnight at -20°C. The cells were then washed with PBS, incubated with 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 analysed. Data acquired were analysed by Accuri CFlow Plus.

2.4 Analysis of cell surface markers

THP-1 cells were seeded at 125 000/cm^2 and differentiated into adherent macrophages by treatment with PMA (50 ng/ml) for 48 h. In order to evaluate the effects of mycotoxins on the differentiation process, the mycotoxins were added simultaneously with PMA. The cells were collected by trypsination following exposure and washed twice with flow cytometry staining buffer (FSB; 0.5% BSA in PBS, 0.05% sodium azide), 500xg for 10 min. The cells were re-suspended in 50 µl FSB and Fc-blocker (TruStain FcX^{TM}) and incubated 30 min on ice. The directly conjugated antibodies CD14-Alexa Fluor 647, CD11b-Alexa Fluor 488 and CD71-PE were added to the blocking solution and the cells incubated further for 30 min. The cells were then washed twice with FSB, re-suspended in 200 µl PBS and analyzed by flow cytometry

(Accuri C6). Single cells were gated and a minimum of 10 000 cells acquired and analysed. Data acquired were analysed by Accuri CFlow Plus.

2.5 Mixture design

In order to evaluate the combined effects of the mycotoxins on the differentiation from monocyte to macrophage, the most affected cell surface receptor tested (CD14, Figure 2B) were chosen. Based on the initial concentration-response experiments, EC_{20} were calculated by doing nonlinear regressions (GraphPad Prism 6, San Diego, CA USA; Supplementary. Figure S1) by using equation 1.

$$resp = b + \frac{t-b}{1+10^{s \cdot \log(EC20)}} \qquad \text{(Equation 1)}$$

Equation 1 contains the variables, the response (resp), which is the CD14 expression, the monocyte control defined as the bottom value (b), the macrophage control defined as the top value (t), and the slope (s) expresses the steepness of the CRC. The ratios between the EC_{20}-values were used to calculate equal effect concentrations (EC) of the mycotoxins. Single, binary and tertiary combinations of the mycotoxins were then tested simultaneously, in four equal ECs expected to give effects in the range of 0-30% inhibition (Table 1).

2.6 Calculation of predictions

Concentration addition (CA)

The concentration addition (CA) model is based on the idea of similar mode of action of mixture components, thus all the components in the mixture behave as if they are simple dilutions of one another. CA can be mathematically formulated as outlined in equation 2.

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p^i}{ECx_i} \right)^{-1} \qquad \text{(Equation 2)}$$

Here n represents the number of mixture components, p^i the relative fraction of chemical i in the mixture, and x a common effect level (e.g. 5% inhibition) (Kortenkamp et al., 2012). ECx_{mix} denotes the total concentration of a mixture that causes an effect of x percent. ECx_i denotes the individual effect concentration of chemical i that causes an effect of x percent. This equation provides an explicit prediction of any effect concentration of a mixture under the hypothesis of

CA (Faust et al., 2001). Prerequisites for their calculation are the knowledge of the individual EC (ECx_i) and the relative concentration p^i of the constituents. The ECx_i were calculated from the individual concentration-response curve (CRC) obtained for the mycotoxins. The relative concentration p^i equals of the ratios were calculated as described in 2.5. For every mixture, EC_5, EC_{10}, EC_{15} and EC_{20} were calculated from the fitted curve obtained from the individual mycotoxin CRC (Figure 4). EC_{20} was the maximum inhibitory concentration that could be predicted from the CRC of DON (Figure 4B). A fitted CRC with 95% confidence intervals of the predicted EC-values was then drawn. The calculations were accomplished using the statistical program R (R Core Team 2014, version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria).

Independent action (IA)

In contrast to CA, the independent action (IA) model calculates combined effects rather than combined EC. IA is based on the idea of a different mechanism of action (MOA), that the primary interactions of toxicants have different molecular target sites. Under these assumptions, the effects of the individual mixture constituents are independent of the other constituents in a probabilistic sense. This means that the relative effect of a toxicant (e.g. 50% mortality) remains unchanged in the presence of another chemical. As the mycotoxins inhibited the expression of CD14 during differentiation, an IA model that predicts decreasing effects with increasing concentrations was used. This IA model is described in (Kortenkamp et al., 2012) and can be mathematically formulated as outlined in equation 3.

$$E(c_{mix}) = \prod_{i=1}^{n} E(c_i) \qquad \text{(Equation 3)}$$

Here c_i and c_{mix} are the concentrations of the individual constituents and the total concentration of the mixture, respectively. $E(c_i)$ represents the corresponding effects of the individual constituents and $E(c_{mix})$ the total effect of the mixture. Effects *E* are expressed as fractions (x percent) of a maximum possible effect (Faust et al., 2001). The predicted combined effect at concentration 1 (Table 1) was found by multiplying their individual effects $E(c_i)$ at concentration 1, and so forth for concentration 2-4. A CRC with 95% confidence intervals was fitted to the predicted data points using the statistical program R.

Isobolographic analysis

In an isobologram, concentrations of each agent that give similar effect (e.g. EC_{10}) are represented on the x- and y-axes. The straight line connecting the equally effective concentrations is assumed to represent the set of concentration pairs that give additivity. If the experimental data lie below or above the additivity line, the results are considered to be synergistic or antagonistic, respectively (Alassane-Kpembi et al., 2016).

The isobolograms were drawn for binary combinations of the mycotoxins at EC_{10} by using the statistical program R. The EC_{10} for single and combined toxins were estimated from the CRC shown in Figure 4 and 5 respectively. When possible, the 95% confidence interval obtained in Figure 4 and 5 were used to draw error bars.

2.7 Cytotoxicity, celltox green assay

CellTox green is a non-toxic dye that stains DNA of cells with impaired membrane integrity. The binding interactions with DNA produce a fluorescence signal that is proportional with cytotoxicity (necrotic, late apoptotic cells). CellTox^{TM} Green Dye was added to the cells as described by the manufacturer (Promega) and fluorescence (485_{ex}:520_{em}) quantified by Viktor2 multi-label counter (Perkin Elmer).

2.8 Cytokine measurement, ELISA

THP-1 cells were seeded at 125 000/cm^2 and differentiated into macrophages by treatment with PMA (50 ng/ml) simultaneously with mycotoxins for 48 h. The medium was then replaced and the cells exposed for LPS (0.1 ng/ml) for 3 h. The medium was harvested and centrifuged (500×*g*, 4 °C, 10 min) to remove cell debris. Levels of TNF-α in supernatants were then measured by enzyme linked immunosorbent assay (ELISA), according to the manufacturers' guidelines (Life technologies, Invitrogen). The absorbance was measured using a plate reader Victor2 Multi-label Counter (Perkin Elmer).

2.9 qPCR

THP-1 cells were seeded at 125 000/cm^2 in 96 well plates and differentiated into macrophages by treatment with PMA (50 ng/ml) simultaneously with mycotoxins for 48 h. Total RNA was

extracted using RealTime ready Cell lysis Kit according to manufacturers' recommendations. cDNA synthesis was done by using Transcriptor Universal cDNA Master and was performed using the following program: 29°C for 10 min, 55°C for 10 min, 85°C for 5 min with a final hold on 4°C (T100 Thermal Cycler, Bio-Rad). qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix and primers for TNF-α and β-actin. All PCR reactions were set up in triplicates and analyzed using StratageneMx3005P Real Time PCR machine (Agilent Technologies, CA, USA) equipped with MxPro v4.10d software. The following PCR program was used: 95°C for 10 min, then 40 cycles of 95 °C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. Gene expression of the housekeeping gene β-actin was used for normalization.

2.10 Statistical analysis

Statistical significance ($p<0.05$) was assessed using 1-way ANOVA, followed by Dunnett's post hoc test. Data analyses were performed using Sigma Plot version 13.0.

The CRC of single or combined toxins were modeled with a regression. The mycotoxins concentration was the independent variable while the expression of the surface receptor, e.g. CD14, was the response. The independent variable were tested to be linear, log-linear or modeled with a b-spline with 3 degrees of freedom (Hastie, 1992). The best fit was defined to be the model with lowest akaike information criterion (AIC). When two fits were equally good, biological assumptions were used to determine which fit to use further. To calculate the predicted CA and IA values, the fitted CRC (EC vs. CD14) were used. The 95% confidence interval for the EC and CD14 expression were predicted from the fitted curve in order to predict confidence intervals for CA and IA. As the effects obtained from the CRC was limited (Figure 4), only between 0 – 20%, both the predicted CA curve and its confidence interval do not cover the whole concentration interval. The isobologram also used the EC values; hence, not all confidence interval could be calculated from the curve. The data analyses were performed by using the statistical program R.

3. Results

3.1 Cellular toxicity on THP-1 monocytes upon mycotoxin exposure

First, we characterized the toxic effects of some co-occurring natural mycotoxins on THP-1 monocytes by examine their effects on viability, cell cycle distribution and cell death. As can be seen in Figure 1, the various mycotoxins exerted a quit different toxicity profiles in the THP-1 model. Reduced viability as a response to mycotoxin exposure was determined by the Alamar Blue assay (Figure 1 A-F, first column). By calculating the inhibitory concentrations giving a 20% reduction (EC_{20}) of viable cells, the relative potencies of the toxins were found to be: DON > ENNB > AOD > AOH > ST > ZEA (Supplementary Table S1). As both increased cell death as well as cell cycle arrest can contribute to the reduction of viable cells, we further characterized the cell death by analyzing for apoptosis and necrosis. At higher concentrations, several of the toxins induced both necrosis and apoptosis, with DON and ENNB being the most potent (Figure 1 and Supplementary Table S1). Furthermore, some of the toxins induced a specific cell cycle arrest; AOH in S- and G2/M-phase, ST in the S-phase and ZEA in the G2/M-phase (Figure 1).

3.2 Effects on the monocyte to macrophage differentiation after single exposure

To characterize possible effects of the mycotoxins on the differentiation process from monocyte to macrophage, THP-1 monocytes were differentiated into macrophages by PMA and simultaneously exposed to mycotoxins. When the THP-1 monocytes differentiate into THP-1 macrophages, they go through several changes; they lose their ability to proliferate, get adherent in addition to several morphological and phenotypical changes. The effects (viability, cytotoxicity, cell cycle) of the mycotoxins on THP-1 monocytes after 24h exposure (Figure 1) can therefore not be directly compared to THP-1 cells that go through a 48h differentiation process into THP-1 macrophages with the presence of one or several mycotoxins. However, the concentrations of mycotoxins are selected at the lower end of the scale, were an effect on the differentiation process, without any cytotoxicity (necrosis), is likely to occur. The differentiation process was characterized by expression of the cell surface markers CD14, CD11b and CD71 after 48 h. By comparing the level of the surface receptors on untreated monocytes with the PMA differentiated macrophages, both CD14 and CD11b were clearly up-regulated, while CD71 was down-regulated (Figure 2). This suggests that these markers are suitable to evaluate effects of mycotoxins on monocyte-macrophage differentiation. Interestingly, AOH, DON and ZEA added during the differentiation process markedly reduced the PMA-induced up-regulation of CD14 (Figure 2A). Furthermore, AOH and DON also reduced the enhancement of CD11b (Figure 2B)

while only AOH affected the down-regulation of CD71 (Figure 2C). In contrast, the other mycotoxins tested, AOD, ENNB and ST had no effects (Figure 2). However, light microscopic evaluation did not reveal any clear effect of the mycotoxins on the PMA-induced morphological changes (data not shown).

To ensure that the concentrations of the tested mycotoxins still were in the non-toxic range, cytotoxicity was tested by using the CellTox Green cytotoxicity assay. In agreement with microscopic evaluation (data not shown), none of the tested concentrations of the mycotoxins induced cytotoxicity during the differentiation process (Figure 3).

3.3 Effects on the monocyte to macrophage differentiation after combined exposure

Since AOH, DON and ZEA reduced the PMA-induced up-regulation of CD14 during differentiation into macrophages (Figure 2A), we wanted to investigate possible interaction effects. EC_{20} (calculated as described in materials and methods 2.5), were found for the different mycotoxins; AOH 1.67 µM, DON 0.047 µM and ZEA 2.56 µM. The ratios between the EC_{20}-values were thus: 7:10 for AOH-ZEA, 3:100 for DON-AOH, 1:50 for DON-ZEA and 70:100:2 for AOH-ZEA-DON. Based on these ratios, equal EC were calculated. Concentrations expected to give inhibitory effects between 0-30% of CD14 expression compared to the macrophage control were selected (Table 1). The cells were then exposed to PMA and equal EC of mycotoxins; single, binary and tertiary exposures. The CRC for CD14 expression after single exposure to AOH, DON and ZEA by using equal effect-concentrations (Table 1) are shown in Figure 4.

To calculate the IA/CA predictions for the combined exposures, we used the values obtained from single exposures plotted in a CRC with a fitted trend line (materials and methods 2.6 and 2.10). The most optimal model for the experimental data for AOH and ZEA were a linear model, showing that the PMA-induced up-regulation of CD14 is reduced with increasing concentrations of the mycotoxins (Figure 4A, C). For DON, a spline model was chosen (Figure 4B), even though this model not was significant better compare to the linear model (Supplementary Figure S2). By choosing this regression model, the trend line seems to include the values better compared to the linear model, which might underestimate the effects of DON at lower

concentrations. In support of this, results obtained in the initial experiment are comparable regarding the effects of DON at the lower concentrations (Figure 2A). This experiment also point out the importance of doing single and combined exposure simultaneously, as minor changes regarding effects may occur over time, especially when working with cells (Reid, 2011).

The CRCs for CD14 expression after binary and tertiary exposure to AOH, DON and ZEA by using equal EC (Table 1) are shown in Figure 5. The CA and IA model predictions were transposed on top of the experimental data to determine whether the models could predict the interactions occurring in the mixtures. The experimental curve was considered to be significantly different from the predicted, and thus a synergistic or antagonistic response, if its confidence interval did not overlap with the model-predicted confidence intervals, as described in Petersen and Tollefsen (2011). The IA and CA models predicted quite similar results. The binary (AOH+DON and DON+ZEA and AOH+ZEA) and the tertiary (AOH+DON+ZEA) combinations were mainly found to be not significant different from the predicted models. The combined effects are therefore considered to be additive. By using the linear DON model (Figure S2), the results ended up similarly (data not shown). Interestingly, at the lowest concentration it seems that the combination of AOH+ZEA has a synergistic effect as the confidence intervals of the predicted CA model does not overlap with the observed values (Figure 5B). Due to the overlapping confidence intervals from the IA and CA models, we could not evaluate which of the models that made the best predictions of the experimental results.

In addition to the IA/CA approach, isobolograms for the binary combinations (AOH+DON, AOH+ZEA and DON+ZEA) were drawn at EC_{10} (Figure 6). Here, additive effect follows the diagonal line between the effective concentrations of each single toxin. If the measured combined effect of two toxins is above or below the diagonal line, it indicates an antagonist or a synergistic effect of the combination respectively. In agreement with the IA/CA approach, additive interactions with the binary combinations AOH+DON and DON+ZEA were found, while a synergistic response were found for the combination AOH+ZEA at EC_{10}.

None of the tested mycotoxins, alone or in combinations, at the selected concentrations (Table 1) induced cytotoxicity during the differentiation process as measured by the CellTox Green assay (data not shown).

3.4 The effects of mycotoxins on macrophage activation

Next, we wanted to investigate if the inhibitory effects of AOH, DON and ZEA on PMA-induced up-regulation of CD14 could affect macrophage activation. LPS, a ligand for the CD14 receptor induced secretion of the pro-inflammatory cytokine TNF-α in PMA-differentiated THP-1 macrophages (Supplementary Figure S3). When THP-1 monocytes were differentiated into macrophages by treatment with PMA simultaneously with the mycotoxin exposures, AOH and ZEA were found to reduce LPS induced secretion of TNF-α, while DON led to an increased secretion (Figure 7). To further investigate if this was due to effects on TNF-α gene expression induced by the mycotoxins, qPCR was performed. As shown in Figure 8, AOH and ZEA reduced TNF-α gene expression. In contrast, DON exposure seems to increase this expression, which is in accordance with previous findings (Pestka 2010). Thus, in accordance with reduced CD14 expressions, both AOH and ZEA reduced the LPS induced TNF-α secretion. In contrast, DON increased this response, most probably due to a direct effect of DON on TNF-α gene expression.

4. Discussion

The ability of natural toxins such as mycotoxins to modulate the immune response may have serious implications for mounting an appropriate response to infectious agents. All of the tested mycotoxins reduced the viability of THP-1 monocytes. Further study showed that while AOD, DON, ENNB and ST induced cell death, AOH and ZEA primarily induced cell cycle arrest. This is likely because the various toxins differentially affect cellular targets. The differentiation from monocytes into macrophages prepares the cells to actively participate in the immune responses (Takashiba et al., 1999) and involves morphological as well as functionally changes (Daigneault et al., 2010). The differentiation process is often evaluated by changes of surface receptor expression (Mittar et al., 2011). The surface receptor CD14 acting as a co-receptor of TLR4, and is expressed at varying extent on both monocytes and macrophages. Another macrophage marker is CD11b, which is induced during differentiation of monocytes into macrophages (Schwende et al., 1996). CD11b is an integrin that is involved in cell adhesion, migration as well as cellular activation and phagocytosis (Solovjov et al., 2005). The third surface receptor used to characterize the differentiation process in this study is CD71, also known as the transferrin

receptor. It is involved in the uptake of iron from plasma (Ponka and Lok, 1999) as well as in proliferation (Testa et al., 1993). Furthermore, it is found to be a useful marker for immature, proliferating cells, such as the THP-1 cell line, and its expression is reduced upon PMA-induced macrophage differentiation (Booker et al., 2015). However, it should be noted that the PMA-induced changes in markers seen in the THP-1 monocyte model are somewhat different when compare to a corresponding model of primary human monocytes. In primary cells, CD11b and CD14 are down-regulated during differentiation into macrophages (Ambarus et al., 2012; Daigneault et al., 2010; Prieto et al., 1994; Wahlström et al., 1999), while CD71 are found to be up-regulated. Nevertheless, these markers are often used for characterizing the differentiation-process of the THP-1 cells (Booker et al., 2015; Daigneault et al., 2010; Spano et al., 2013). In agreement with this, we found that PMA-induced differentiation increased the expression of CD14 and CD11b, while CD71 was decreased. Simultaneous with the changes in cell surface receptors, the morphology of the THP-1 cells changed from small and round monocytic like cells into flat and more adherent cells with apparent increased cytoplasmatic volume, typical of macrophages.

Mycotoxins have shown to affect immune cells in a way that might give immune modulatory effects in several studies. DON is able to reduce maturation of dendritic cells (Luongo et al., 2010) and alter macrophage activation as measured by the expression of several cell surface receptors (Waché et al., 2009). Furthermore, both T-2 toxin (Hymery et al., 2009) and cyclopiazonic acid (Hymery et al., 2014) have been found to disturb human monocyte differentiation into macrophages. Here we report that AOH reduced the PMA-induced macrophage differentiation, evaluated by a reduced CD14 and CD11b expression with a subsequent increase in CD71. In contrast, DON did not affect the changes in CD71 and ZEA only reduced the effect on CD14. This may suggest that the mycotoxins have different primary molecular targets and then follow different signaling pathways to reduce the PMA-induced differentiation. The fact that AOH attenuates the reduction of CD71, may indicate that the differentiation process is reduced to such an extent that the cells still holds some proliferative activity, as CD71 also are considered as a proliferation marker (Testa et al., 1993).

PMA mimics the function of the second messenger diacylglycerol (DAG), an activator of signaling kinases in the protein kinase C pathway (Liu et al., 1998; Schwende et al., 1996.). Via its activation of PKC, PMA increase auto-phosphorylation of the double-stranded RNA-dependent protein kinase (PKR), resulting in its degradation. (Zhou et al., 2005). PKR is a key mediator of interferon (IFN) induced action against certain viruses and plays an important role in signal transduction, and regulates proliferation, differentiation, and survival of murine hematopoietic stem/progenitor cells (Liu et al., 2013). Interestingly, PKR activity is also suggested to be a critical upstream mediator of ribotoxic stress response induced by DON (Zhou et al., 2014). Thus, it could be hypothesized that DON though its activation of PKR, reduce PMAs´ activation of PKC and macrophage differentiation. This suggestion, however, remains to be tested. The main mechanism of action for ZEA is considered to be through its binding to estrogen receptors, thereby generating an estrogen-like response. However, independent of being an estrogen agonist, it has been suggested that ZEA inhibits PMA induced Ca^{2+} release and the following PKC activation (Murata et al., 2002), thus giving a possible link of ZEA mediated effects on the differentiation process. AOH exert it effects mainly by acting as a topoisomerase poison/ inhibitor and thus generating DNA damage. In addition, it generates ROS and has been found to act as a weak estrogen. These events are shown to induce several signaling pathways, including the DNA damage response (γH2AX, p53, CHK) autophagy (sestrin 2, AMPK, mTOR), senescence as recently reviewed (Solhaug et al., 2016). However, how to connect these, or other jet undefined events to AOH effects on PMA-induced differentiation still remain unclear.

Concerns are expressed on whether mixtures, in which each of the substances is present at low doses, may have negative health effects (VKM, 2013). If substances act by a similar mechanism, CA is assumed. As a result, adverse effects of the mixture may occur even if the individual substances are present at doses below their respective no-observed-adverse-effect-concentrations (NOAEC). In contrast, if the substances act by a dissimilar/independent mechanism (IA), effect-addition is assumed and adverse effects are considered as unlikely if the individual substances do not affect the toxicity of each other and are present at doses below zero effect level (VKM, 2013). Here we employed the IA/CA- approach and isobologram analysis to analyze possible interaction effects between AOH, DON and ZEA on the effect of CD14 expression during differentiation. In

contrast to several other models, the IA/CA-models use results based on CRCs, including an evaluation of the shape and slope of the combined mixtures CRCs in order to verify possible antagonistic or synergistic effects (Alassane-Kpembi et al., 2016). Interestingly, the combination AOH+ZEA seem to have somewhat synergistic effects at lower concentrations. Beside this, the observed data did mainly not diverge from the predicted CRC for either model and the effects of the combinations are therefore considered to be additive. The confidence interval in the two prediction models overlapped and we could not distinguish between the models ability to predict the effect of the mixtures. The asymmetric shapes of the isobolograms (EC_{10}) of the AOH+DON and DON+ZEA mixtures may suggest that the effect of each mycotoxins is independent of the other, however, this is not statistical significant. Nevertheless, an independent action of these mycotoxins may imply that it is sufficient to evaluate their effects as single toxins only. This can be done since no combination effect is expected from two compounds with independent action when both compounds are present below the threshold of effect. Furthermore, the isobologram analysis verified the CA/IA-approach and found the binary combination AOH+ZEA to have a slightly synergistic effect. AOH, DON and ZEA are in general found to induce quite different signaling pathways. This is likely the explanation of the additive or lack of synergistic effects seen upon combination. Interestingly, the combination AOH+ZEA were found to have somewhat synergistic effect at EC_{10}. The reason for this effect could be several. Both AOH (Frizzell et al., 2013) and ZEA (Takemura et al., 2007) are found to be estrogenic and interact with estrogen receptors. As human macrophages, including THP-1 cells are found to express estrogen receptors (Subramanian et al., 2009) it is tempting to speculate that this might be a common interaction point and/or signaling pathway, that could somehow explain the synergistic effect observed at the differentiation process. Interestingly, at a recent mycotoxin meeting (Vejdovszky et al., 2016), a study of the combination AOH and ZEA were reported to have synergistic effect measured by estrogen-dependent activation of alkaline phosphatase and cell proliferation, giving some support of this suggestion. However, interactions on metabolic processes affecting the transformation of xenobiotics seem to be far the most common mechanism of synergy (Cedergreen 2014).

Several weaknesses by applying the CA-/IA- prediction models on our dataset were observed. We chose to study the combined effects in the lower range of the CRC (0-20%), as possible adverse combined effects that occur below the respective single mycotoxins NOAEC are of most importance for regulated toxins. However, the CA-model is generated by the obtained EC-values,

and thus no estimation of values above EC_{20} could be made. Regarding the IA-model, gradual end points such as receptor expression do not precisely fit the theoretical assumptions, as it only fit binomial endpoints such as cell death (Cedergreen et al., 2008). However, even when binomial endpoints are measured, it is likely that a cellular process to some degree alter the responsiveness of another. Furthermore, complexity regarding identification of MOA may also challenge the IA-model as a mechanistic concept (EFSA, 2015). The definition of MOA is ambiguous as it often is dependent on the concentration (McCarty and Borgert, 2006). However, in practice this model provides good predictions of mixtures of chemicals with different MOA (Cedergreen et al., 2008), as also demonstrated in this study. Another source of concern by using the CA- and IA-model is that they both heavily relay on the generated CRCs with fitted curves, which will require numerous data points to be correct. When fewer data points are used, the curve with the best fit may not necessarily be the curve that that describes the response in the most correct/biological manner. Furthermore, a higher degree of variation in the response is normal when using values that are on the lower end of the CRC (0-20% effect) and might complicate the conclusions. Our dataset is rather limited, due to quite extensive workload to prepare each sample. The fitted curves are therefore only indicative. Thus, not surprisingly, neither the CA- nor the IA model was found to be significant better than the other to predict the results. This conclusion also holds true when comparing the molecular structure and the so far known targets and effected cell signaling systems, as these mycotoxins seems to act on the cells by highly different mechanisms.

Here we find that AOH, DON and ZEA reduced PMA-induced up-regulation of CD14 during differentiation from monocytes to macrophages, which may suggest that they have immunosuppressive effects. CD14 is an important co-receptor of TLR4 and then central in LPS-induced activation of macrophages (Plociennikowska et al., 2015). Furthermore, increased expression of CD14 on PMA differentiated THP-1 macrophages has been associated with increased sensitivity for LPS compare to THP-1 monocytes, probably through an accumulation of NFkB in the cytoplasm during the differentiation process (Park et al., 2007; Takashiba et al., 1999). In our study, AOH and ZEA added during PMA-macrophage differentiation reduced the response to LPS, measured by the release of TNF-α. However, AOH and ZEA reduced TNF-α expression regardless of the LPS stimulation, suggesting additional explanations. Interestingly, ZEA has been reported to reduce expression of NFκB followed by decreased secretion of TNF-α in pig liver (Pistol et al., 2014). A ZEA mediated reduction of NFκB expression may thus be a

possibly explanation of the reduced response towards LPS stimulation and TNF-α secretion. Even though AOH-induced signaling pathways has been thorough studied the last years (Solhaug et al., 2016), a possibly explanation to the decreased secretion of TNF-α remains to be elucidated. In contrast to ZEA and AOH, LPS treated cells pre-treated with DON enhanced secretion of TNF-α, despite of the reduced level of CD14. DON are known to induce both NFκB activation and TNF-α expression, however, the signaling pathway is different from the LPS/CD14/TLR-4 pathway (Pestka, 2010). Thus explaining why DON exposure resulted in increased release of TNF-α following LPS exposure, despite reducing the levels of CD14 on the macrophages.

5. Conclusions

The tested mycotoxins differently affected cell proliferation and cell survival of THP-1 monocytes. Furthermore, AOH, DON and ZEA, reduced the differentiation process from monocytes into macrophages at non-toxic concentrations. The combined effects of these mycotoxins were mainly not significant different from the predicted additive effects using the CA/IA approach. However, the combination AOH+ZEA were slightly synergistic at lower concentrations. Isobolographic analysis suggests that the effects of AOH+DON and DON+ZEA are independent of each other, in contrast to AOH+ZEA. The mycotoxins also induced alterations in macrophage functionality in response to LPS.

Conflicts of interest:

The authors declare that there are no conflicts of interest

Acknowledgements

The authors wish to thank Randi Faller and Søren Grove (Norwegian Veterinary Institute) for skillful help regarding qPCR analysis. The Research Council of Norway has supported the work through the project MYCOMIX, grant nr. 213087. We also want to thank FUNtox at the Norwegian Veterinary Institute for additional funding.

Figure Legends:

Figure 1: Cytotoxicity induced by mycotoxins in THP-1 monocytes. Viability, necrosis, and cell cycle distribution of THP-1 monocytes after 24 h exposure of (A) AOD, (B) AOH, (C) DON, (D) ENNB, (E) ST and (F) ZEA. Viability were measured by Alamar Blue, necrosis by the uptake of PI followed by flow cytometry and cell

cycle distributing by fixing the cells followed by PI-staining and flow cytometry. The results represent mean ± SEM of 3-6 independent experiments. * indicates significantly different ($p<0.05$) from control.

Figure 2: Effects of the mycotoxins on the monocyte to macrophage differentiation evaluated by expression of the surface receptors CD14, CD11b and CD71. THP-1 monocytes were simultaneously treated with PMA and single mycotoxins for 48 h. Untreated THP-1 monocytes were included as negative control. The results represent mean ± SEM of 4 independent experiments. *indicates significantly different ($p<0.05$) from macrophage control. Mo= monocytes, Ma= macrophages.

Figure 3: Cytotoxic effects of single mycotoxins during the monocyte to macrophage differentiation. THP-1 monocytes were simultaneously treated with PMA and single mycotoxins at the indicated concentrations for 48 h. Cytotoxicity were evaluated by the CellTox Green assay. The results represent mean ± SEM of 3 independent experiments. *indicates significantly different ($p<0.05$) from macrophage control. Mo = monocytes, Ma = macrophages, PC = positive control, 100% lysis of cells.

Figure 4: Effects of single mycotoxins at equal effect concentration on CD14 expression. THP-1 monocytes were simultaneously treated with PMA and single mycotoxins (A) AOH, (B) DON and (C) ZEA at equal effect-concentrations (Table 1) for 48 h. The results represent 4-6 independent experiments and are presented as experimental data (gray dots) and a fitted curve with 95% confidence interval. * indicates significantly different ($p<0.05$) from control.

Figure 5: Effects of mixed mycotoxins at equal effect concentration on CD14 expression with applied CA and IA predicted models. THP-1 monocytes were simultaneously treated with PMA and binary and tertiary combinations of AOH, DON and ZEA at equal effect-concentrations (Table 1) for 48 h. (A) AOH+DON (B) AOH+ZEA (C) DON+ZEA (D) AOH+DON+ZEA. The results represent 4-6 independent experiments and are presented as experimental data (small gray dots) and a fitted black curve with 95% confidence interval. * indicates significantly different ($p<0.05$) from control.

Figure 6: Isobolographic analysis of binary mycotoxins combinations on CD14 expression. The isobolograms, (A) DON+AOH, (B) AOH+ZEA and (C) ZEA+DON were made by using EC_{10} values obtained from CRC of single (Figure 4) and binary combined (Figure 5) mycotoxin exposure. When possible, error bars (gray dashed line) were made on basis of the 95% confidence interval observed in figure 4 and 5. Circle indicates single exposure while square indicates binary combined exposure.

Figure 7: AOH, DON and ZEA affect LPS induced TNF-α secretion. THP-1 monocytes were differentiated into macrophages by treatment with PMA simultaneously with AOH (15 µM), DON (0.5 µM) and ZEA (15 µM) for 48 h**.** TNF-α secreted by the differentiated macrophages during a 3 h LPS-challenge (0.1 ng/ml) were analyzed by ELISA. The result represents mean values ± SEM of 4 independent experiments. * indicates significantly different from control, $p<0.05$.

Figure 8: AOH, DON and ZEA affect TNF-α gene expression. THP-1 monocytes were differentiated into macrophages by treatment with PMA simultaneously with AOH (15 µM), DON (0.5 µM) and ZEA (15 µM) for 48 h**.** Gene expression of TNF-α were then measured by qPCR. The result represents mean values ± SEM of 3 independent experiments. * indicates significantly different from control, $p<0.05$.

Table 1: Calculated equal EC of AOH, DON and ZEA on CD14 expression.

Supplementary

Figure S1: Non-linear regression analysis of data presented in figure 2, effects of the mycotoxins on the monocyte to macrophage differentiation evaluated by expression of CD14. The CRC relationship was assumed to follow a sigmoidal curve. Sigmoidal CRCs for (A) AOH, (B) DON and (C) ZEA are shown with 95% confidence intervals. The data points represent mean ± SEM of 4 independent experiments. The top value was fixed to the macrophage control, while the bottom value was fixated to the monocyte control. The top horizontal line shows EC_{20}, while the lower horizontal line shows EC_{50} of PMA induced CD14-expression. IC_{20} for AOH, DON and ZEA were found to be 1.67, 0.047 and 2.56 µM, respectively. These values were then used to calculate the ratios ratio between the mycotoxins.

Figure S2: Effect of DON on CD14 expression, analyzed by a linear model displaying a decreasing trend. THP-1 monocytes were simultaneously treated with PMA and DON for 48 h. The results are presented as median (black dots) and a fitted line (linear model) with 95% confidence interval of 4-6 independent experiments. None of the different concentrations of DON tested were significant different ($p<0.05$) from control.

Figure S3: LPS induced secretion of TNF-α. THP-1 monocytes were differentiated into macrophages by treatment with PMA for 48 h. The cells were then treated with LPS at the concentrations indicated for 3 h and the cell supernatant analyzed for secreted TNF-α by ELISA. The result represents mean ± SEM of 4 independent experiments. *indicates significantly different ($p<0.05$) from macrophage control.

Table S1: Effects of the mycotoxins on THP-1 monocytes viability, necrosis and apoptosis. The concentration of the indicated mycotoxin that led to 20% reduction of proliferation (IC_{20}), 10% increase (EC_{10}) in the amount of necrotic cells or the amount of apoptotic cells induced by the highest concentration tested. The IC_{20} and EC_{10} calculations were done by curve fitting analysis based on the data presented in Figure 1 as described in Material and Methods. * indicates significantly different ($p<0.05$) from control.

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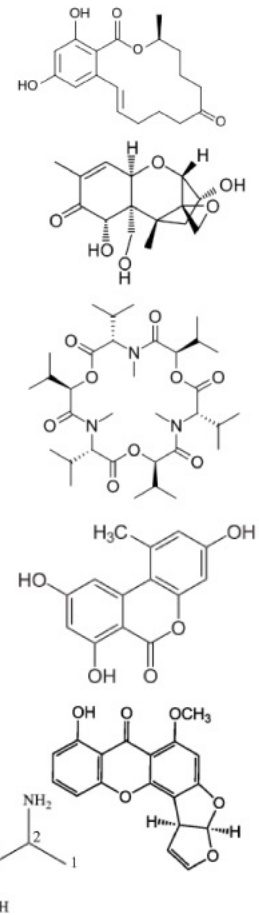
THP-1 MODEL → MYCOTOXINS (SINGLE or COMBINED) → CELLULAR EFFECTS

1. **THP-1 monocyte**
2. **Differentiation**
3. **THP-1 macrophage**

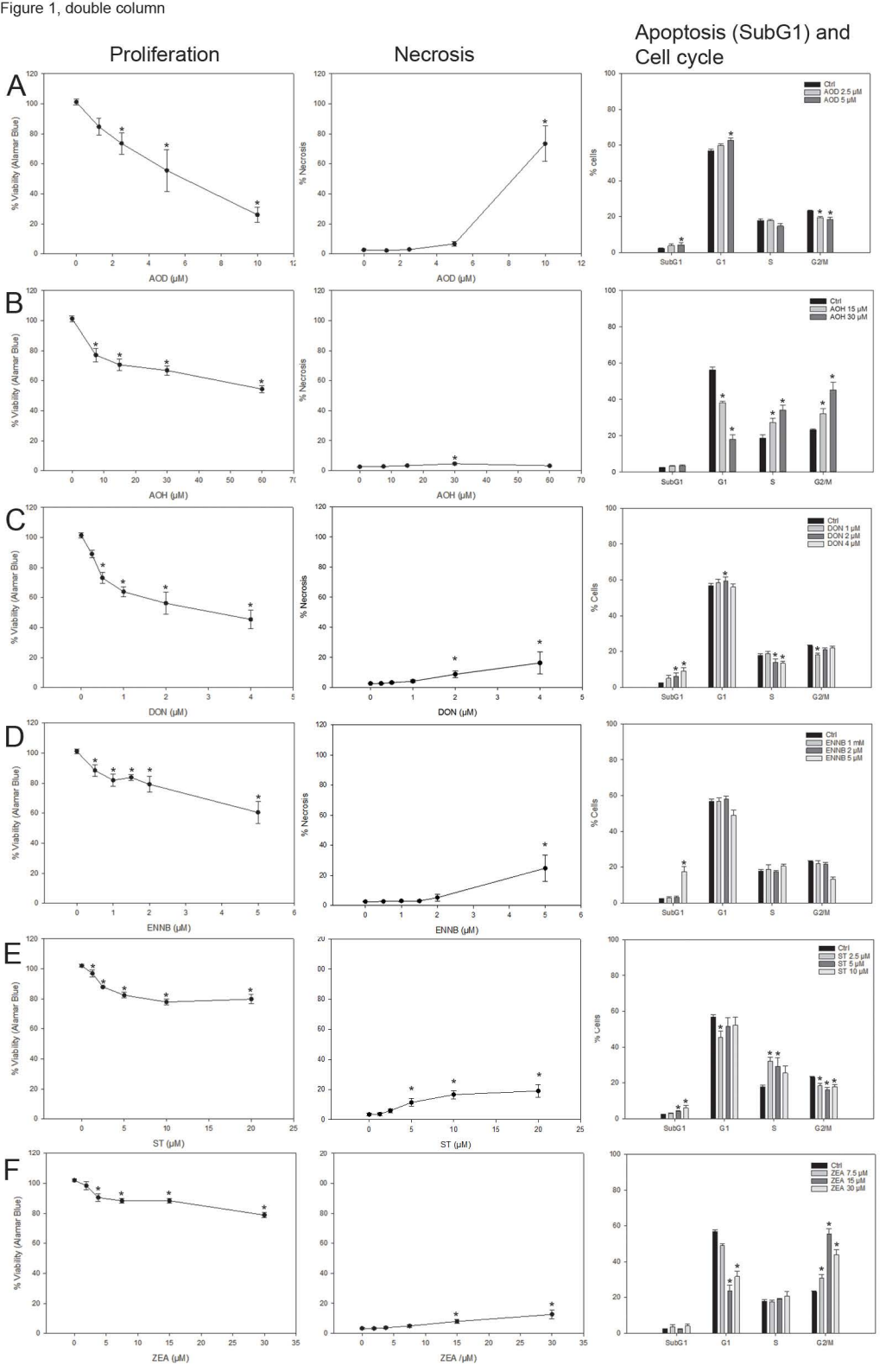


1. THP-1 monocytes
- Proliferation
- Cell death
- Cell cycle

2. Differentiation process
- Surface receptors
- Prediction models

3. THP-1 macrophages
- Cytokine secretion

Figure 1, double column



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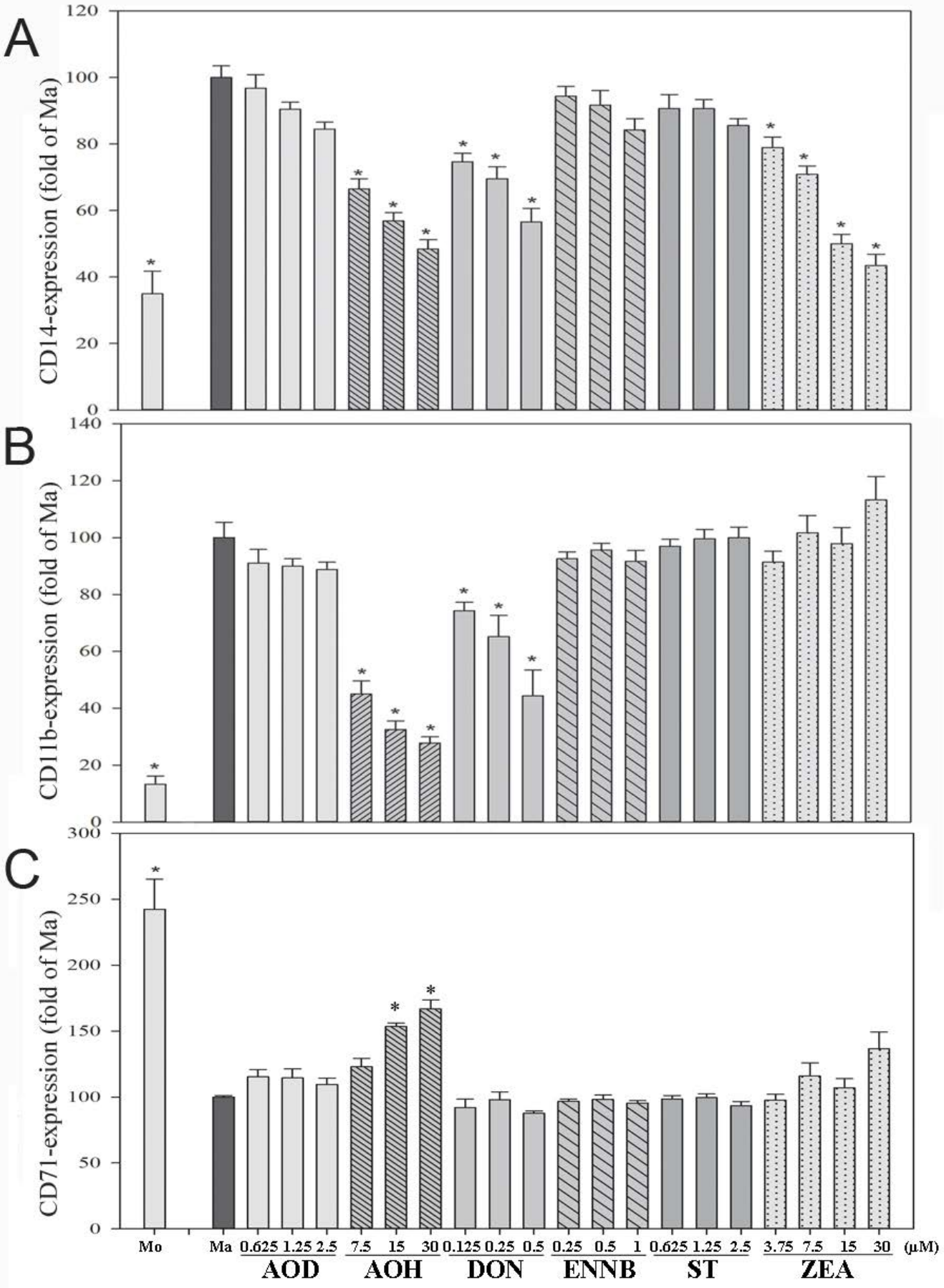

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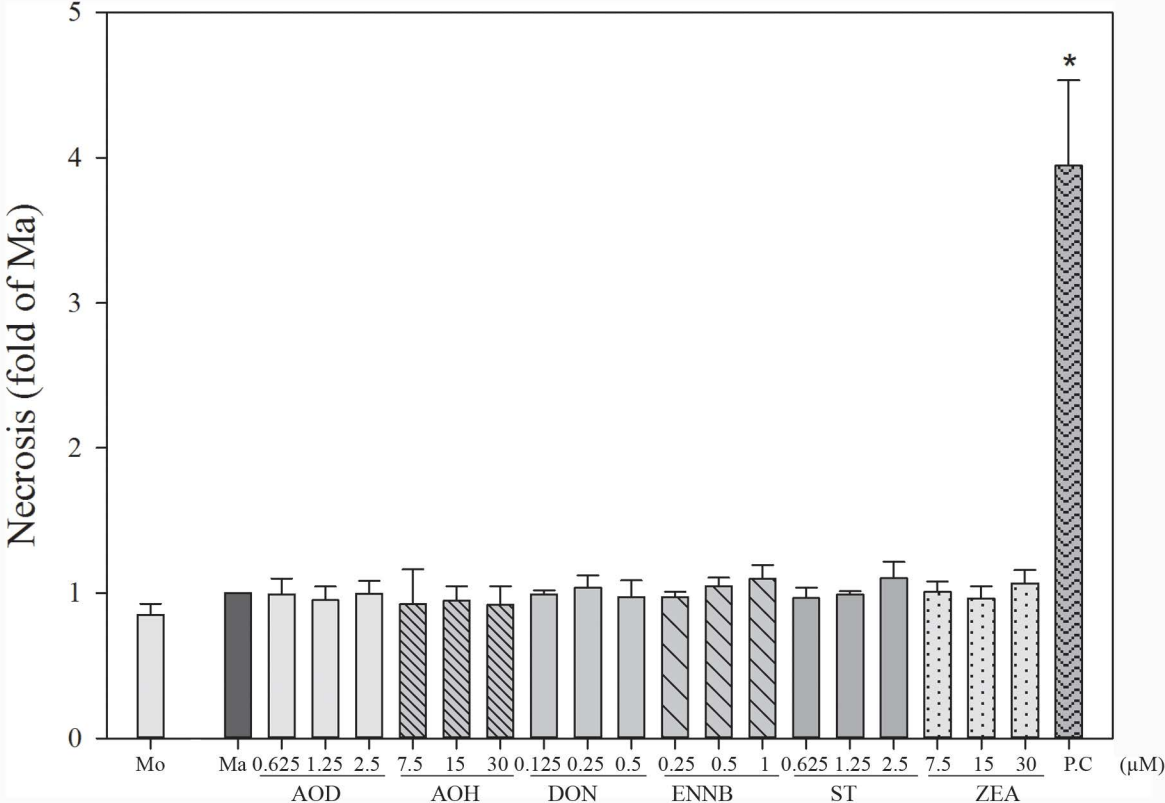

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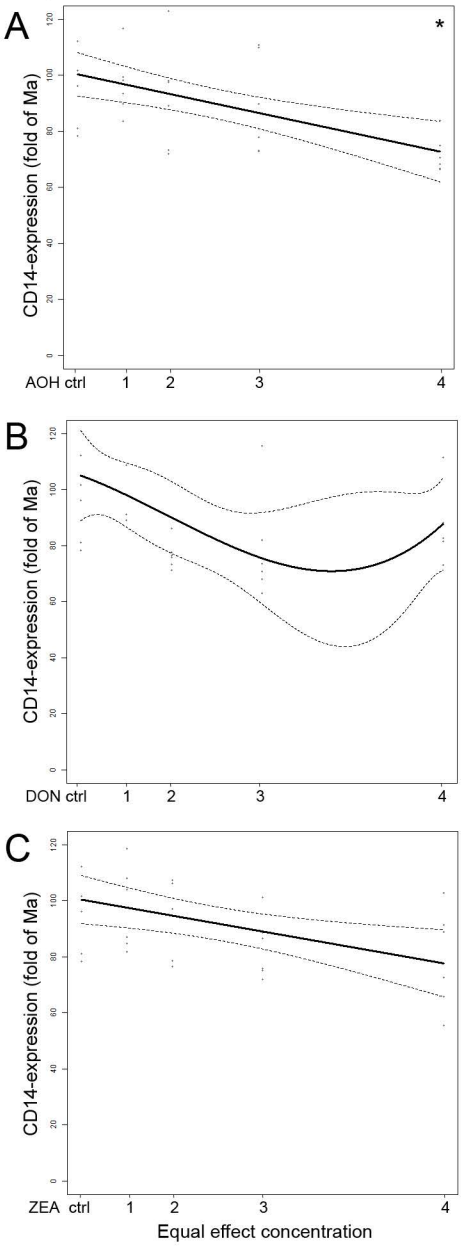

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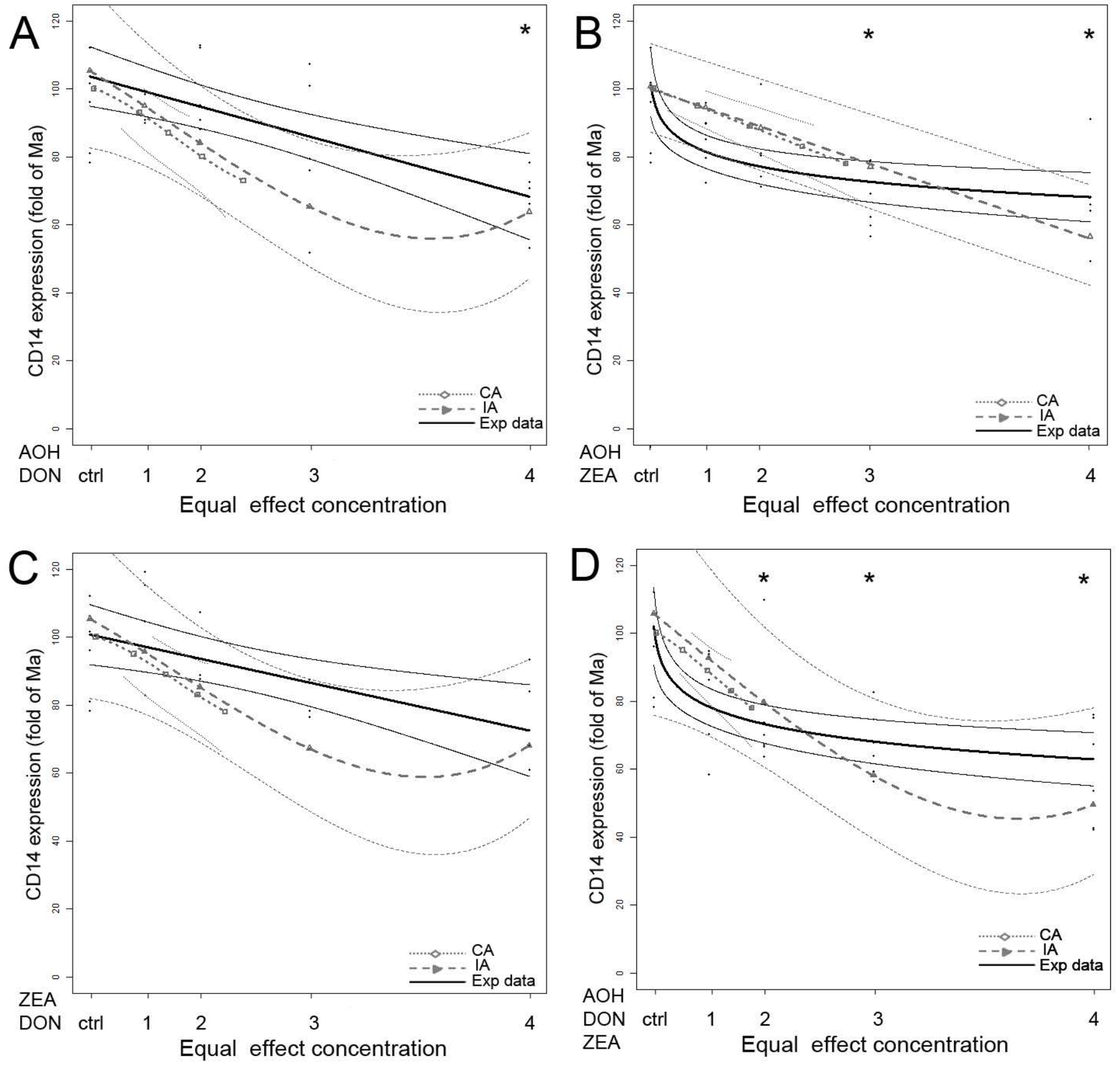

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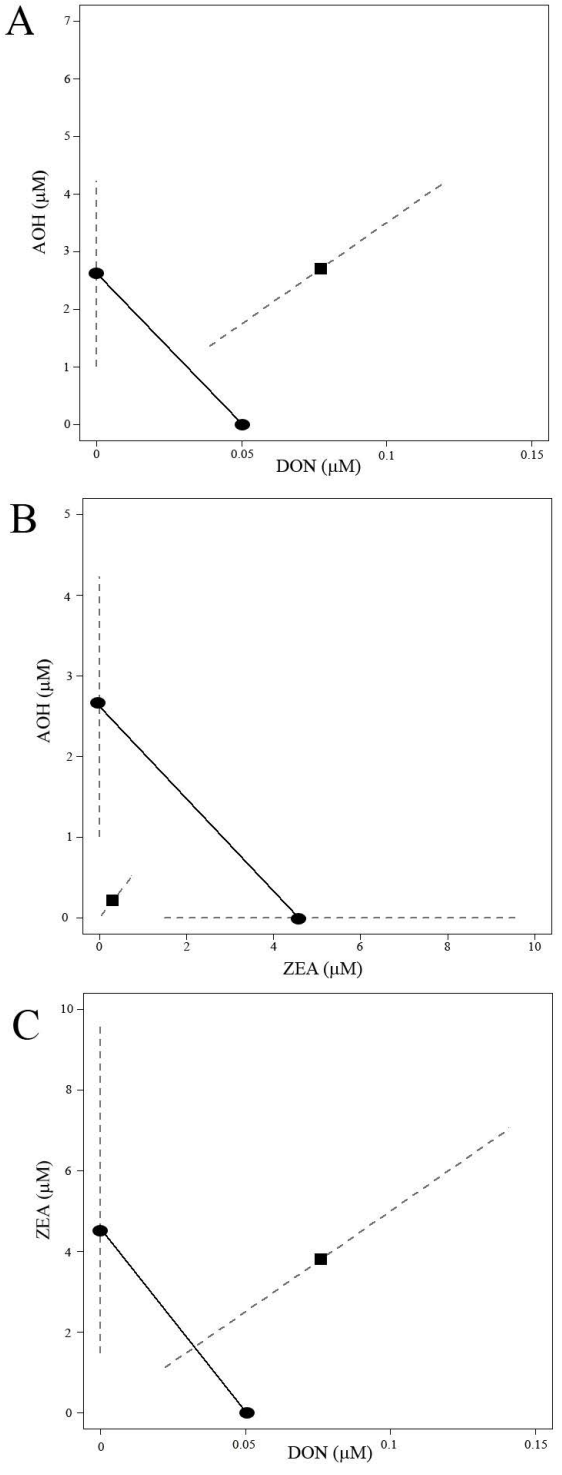

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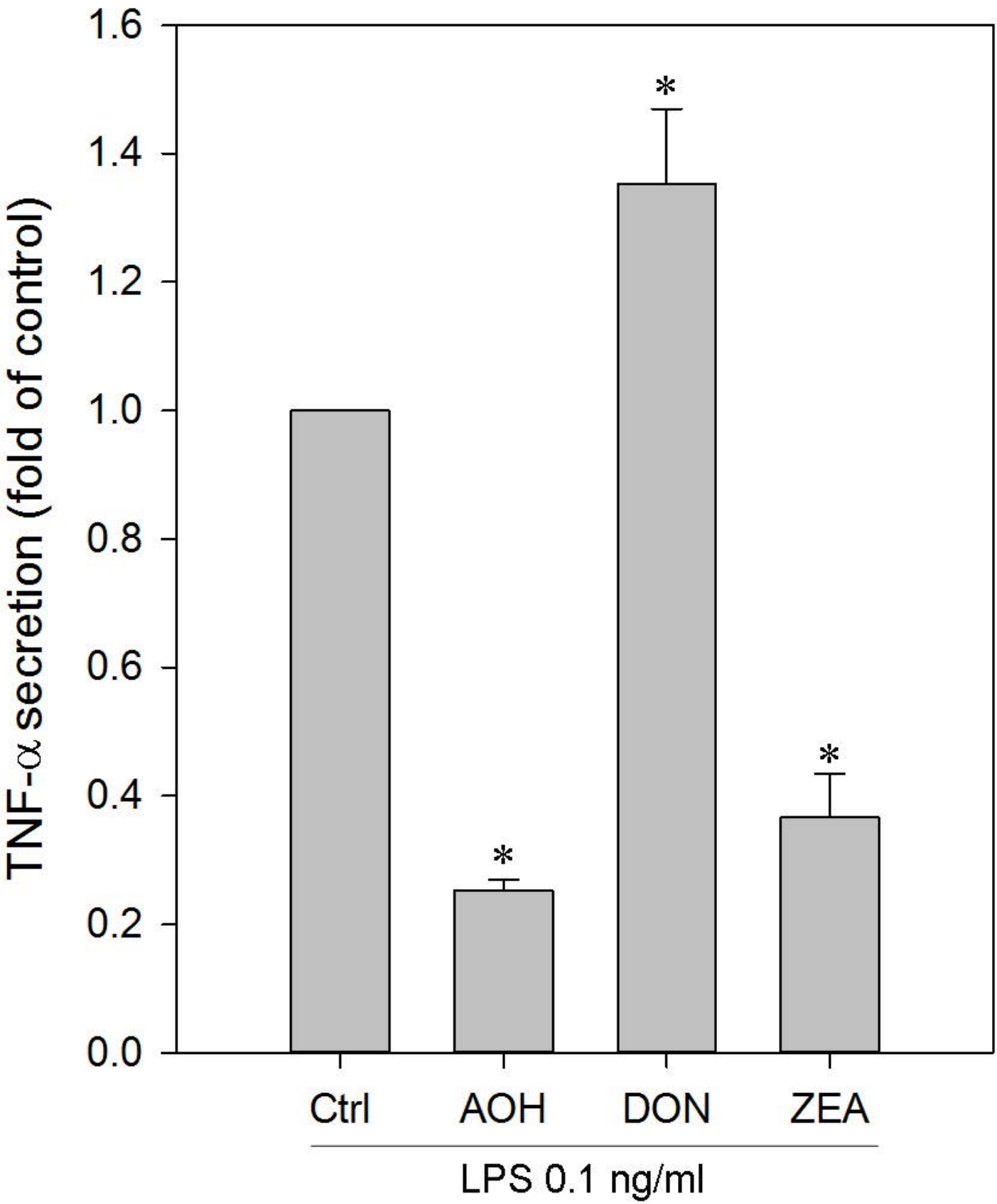

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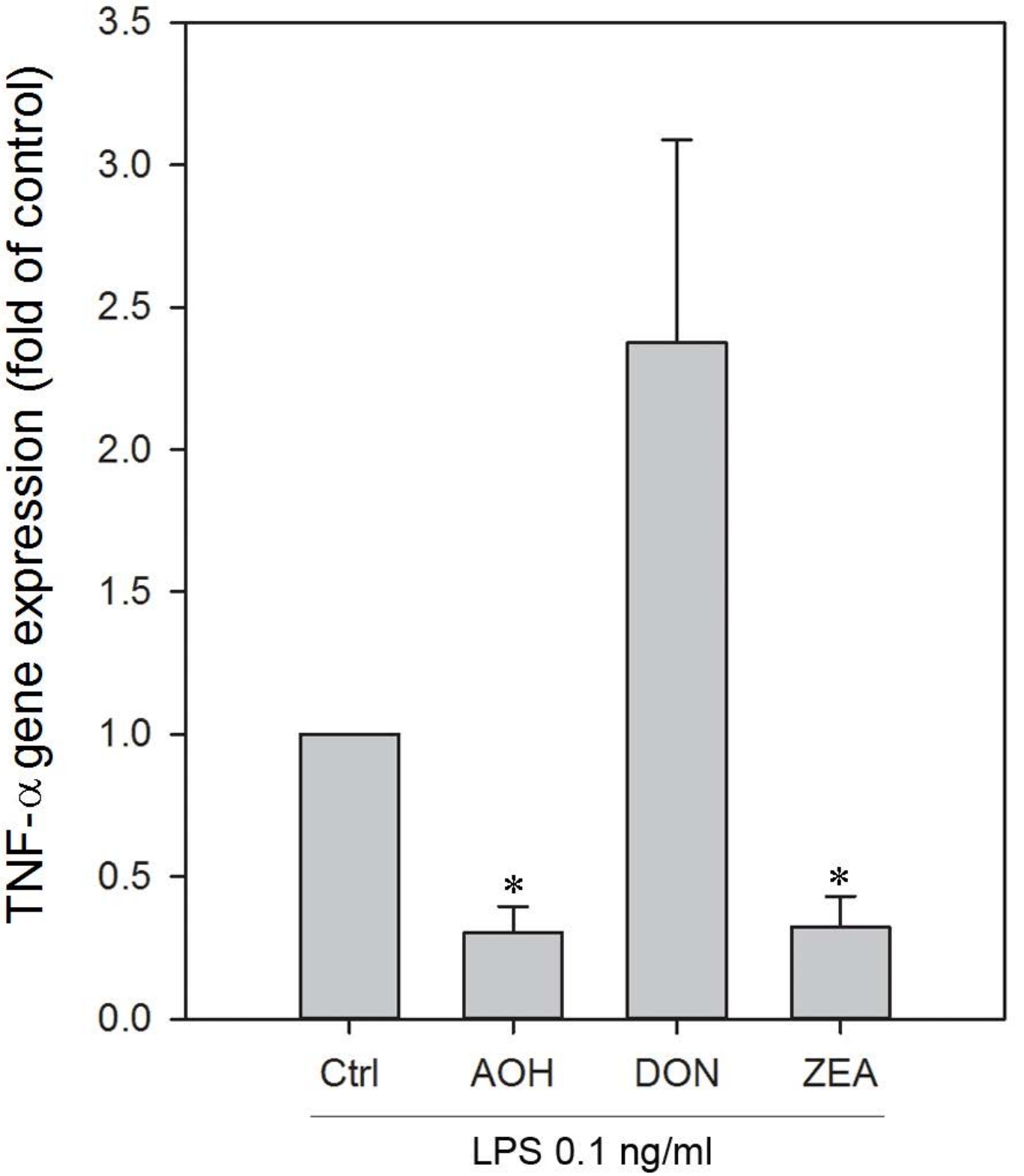
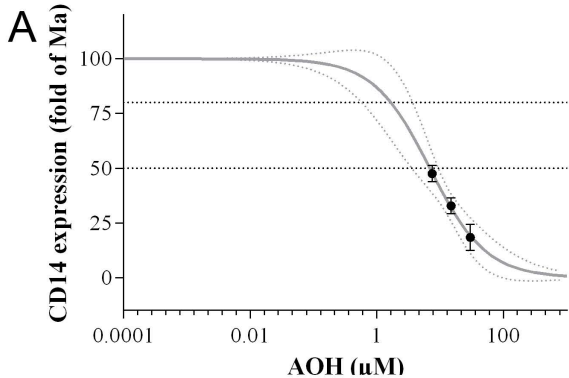
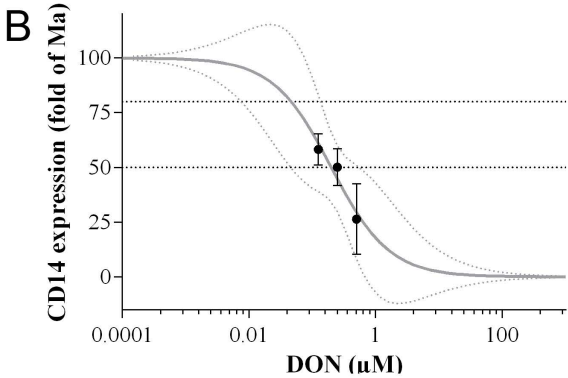


Table 1

Mycotoxin	**Concentrations**			
	1	**2**	**3**	**4**
AOH (µM)	0.875	1.75	3.5	7
DON (µM)	0.025	0.05	0.1	0.2
ZEA (µM)	1.25	2.5	5	10





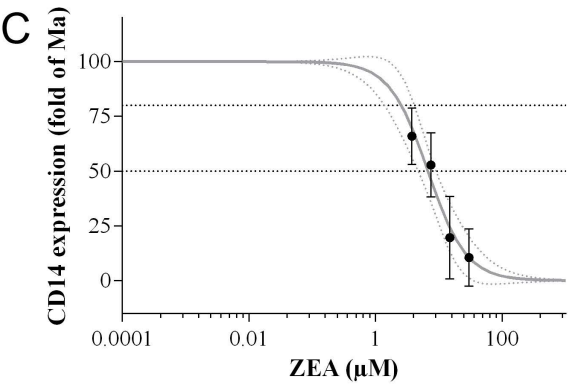


Figure S1: Non-linear regression analysis of data presented in figure 2, effects of the mycotoxins on the monocyte to macrophage differentiation evaluated by expression of CD14. The CRC relationship was assumed to follow a sigmoidal curve. Sigmoidal CRCs for (A) AOH, (B) DON and (C) ZEA are shown with 95% confidence intervals. The data points represent mean ± SEM of 4 independent experiments. The top value was fixed to the macrophage control, while the bottom value was fixated to the monocyte control. The top horizontal line shows EC20, while the lower horizontal line shows EC50 of PMA induced CD14-expression. IC20 for AOH, DON and ZEA were found to be 1.67, 0.047 and 2.56 µM, respectively. These values were then used to calculate the ratios ratio between the mycotoxins.

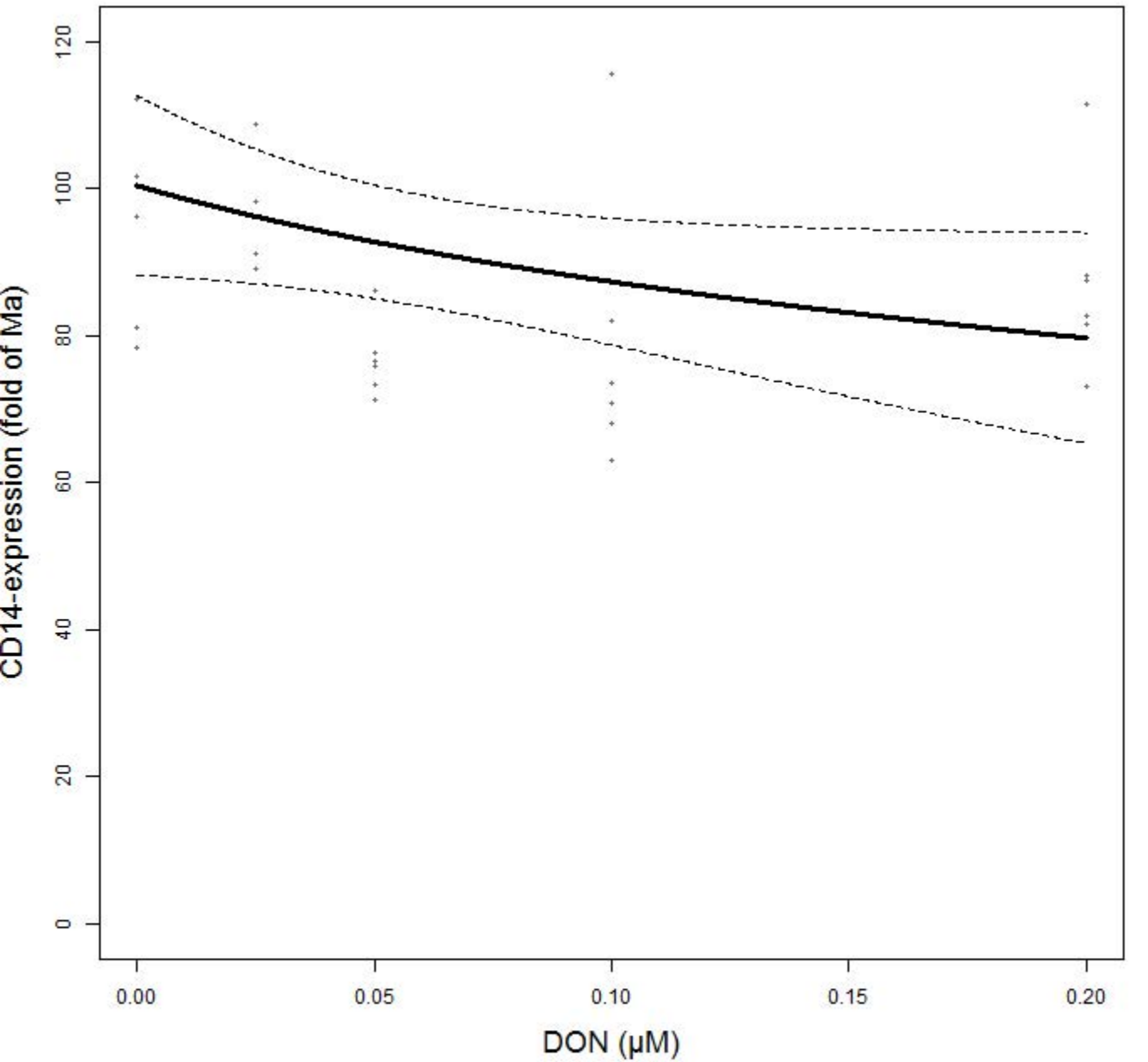


Figure S2: Effect of DON on CD14 expression, analyzed by a linear model displaying a decreasing trend. THP-1 monocytes were simultaneously treated with PMA and DON for 48 h. The results are presented as median (black dots) and a fitted line (linear model) with 95% confidence interval of 4-6 independent experiments. None of the different concentrations of DON tested were significant different ($p<0.05$) from control (one-way ANOVA with Dunnett's post hoc test).

Figure S3

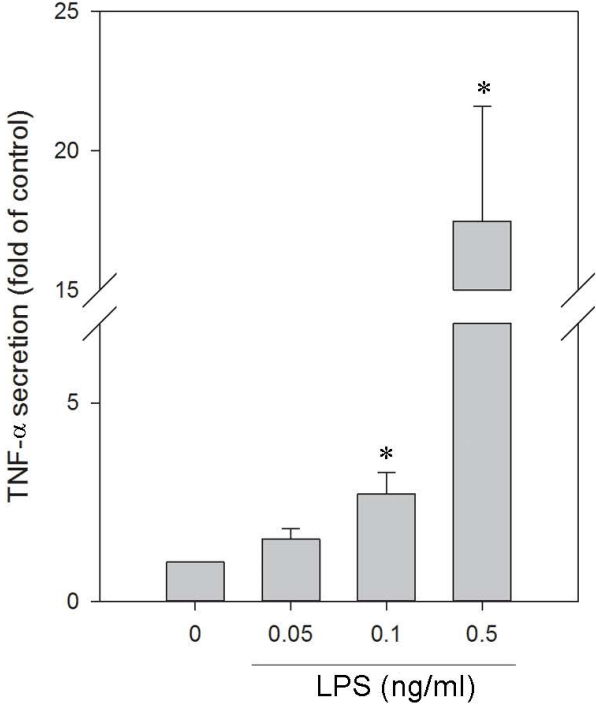


Figure S3: LPS induced secretion of TNF-α. THP-1 monocytes were differentiated into macrophages by treatment with PMA for 48 h. The cells were then treated with LPS at the concentrations indicated for 3 h and the cell supernatant analyzed for secreted TNF-α by ELISA.

Table S1

Viability		**Necrosis**		**Apoptosis**		
Mycotoxin	IC_{20}	Mycotoxin	EC_{10}	Mycotoxin	Cons.	% apoptosis
DON	0.342 µM	DON	2.39 µM	Ctrl		2.40
ENNB	1.21 µM	ENNB	3.89 µM	DON	4 µM	7.63*
AOD	2.61 µM	ST	4.62 µM	AOD	5 µM	4.33*
AOH	3.02 µM	AOD	7.64 µM	ENNB	5 µM	14.90*
ST	13.8 µM	ZEA	22.01 µM	ST	10 µM	4.95*
ZEA	29.8 µM	AOH	n.d	AOH	30 µM	3.50*
				ZEA	30 µM	3.03