RESEARCH ARTICLE

Aflatoxins of type B and G affect porcine dendritic cell maturation in vitro

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Abstract

The toxic effects of highly carcinogenic mycotoxins, especially aflatoxins (AF), on key antigen-presenting cells, such as dendritic cells (DC), are largely unknown. To elucidate the effect of AF on DC function, porcine monocyte-derived DC (MoDC) were treated with a mixture of several AF (i.e., AFB_1, AFB_2, AFG_1, and AFG_2) and the phagocytic capacity, the membrane expression level of several DC activation markers, the T-cell proliferation-inducing capacity, and the cytokine secretion pattern were assessed. As compared to untreated MoDC, AF significantly up-regulated the expression of the co-stimulatory molecules CD25 and CD80/86. However, the phagocytic activity of MoDC was not affected by AF treatment. While the cytokine secretion pattern of AF-treated MoDC was similar to control MoDC, the T-cell proliferation-inducing capacity of MoDC was increased upon aflatoxin treatment. The results indicate that a mixture of naturally occurring AF enhances the antigen-presenting capacity of DC, which could explain the observed immunotoxicity of AF by breaking down tolerance and further emphasizes the need to reduce the admissible level of AF in agricultural commodities.

Introduction

Even in our super-sanitized world, many potential carcinogenic mycotoxins are present, in particular aflatoxins (AF) (Reddy et al., 2009; Wild & Turner, 2002). These potent carcinogens are mainly produced by *Aspergillus* sp., and frequently contaminate many agricultural products, such as corn and cereals. The consumption of AF-contaminated food/feed affects animal and public health. AF are classified into four types based on structure: AFB_1, AFB_2, AFG_1, and AFG_2 (Figure 1), all of which are concomitantly present on *Aspergillus*-infested crops. Although the exact proportion of AFB_1, $-B_2$, $-G_1$, and $-G_2$ in *Aspergillus*-contaminated feed and foods is unclear, these molds predominantly produce AFB_1 and to a lesser extent the other three AF in feed (Cervino et al., 2008; Joubrane et al., 2011; Li et al., 2009). AFB_1 is the most immune-disruptive aflatoxin, as a lower bioavailability and less toxic nature for AFB_2, $-G_1$, and $-G_2$ have been reported (Cusumano et al., 1990; Hoogenboom et al., 2001; Mehrzad et al., 2011). Although the occurrence of AF-contaminated food/feed has decreased substantially in more affluent, but not in developing, countries due to the presence of extensive monitoring programs, the contamination of agricultural commodities by AF-producing fungi nevertheless remains a serious threat for animal and public health (Martins et al., 2007; Hernández Hierro et al., 2008; Pukkala et al., 2009).

Besides their known carcinogenicity, AF cause immunosuppression in domestic animals and humans by impairing the innate and cell-mediated immunity (Chaytor et al., 2011; Gong et al., 2004; Luongo et al., 2013). Both cytolysis by natural killer (NK) cells and several macrophage functions, including phagocytosis, production of reactive oxygen species (ROS), and intracellular killing, are impaired by AF (Qureshi et al., 1998; Liu et al., 2002). In addition, AF dysregulate neutrophil functions in a bovine model, resulting in a decreased phagocytosis and intracellular ROS production (Mehrzad et al., 2011). Further, pathogen recognition is impaired in human leukocytes by AF treatment (Malvandi et al., 2013). Exposure of pigs to AF-contaminated feed increases their susceptibility to infection and reduces vaccine-induced protection, mainly through the dysregulation of T-cell polarization (Meissonnier et al., 2008; Venturini et al., 1996). This led to the assumption that AF interfere with the function of antigen-presenting cells, such as dendritic cells (DC), as these cells drive the polarization of naïve T-cells (Meissonnier et al., 2008). The knowledge concerning the impact of these AF on DC-T-cell co-operation and, thus, immune regulation is very limited.

DC link innate and acquired immunity and as such are pivotal in the induction of immune responses to control and eliminate pathogens (Joffre et al., 2009). These professional antigen-presenting cells (APC) are localized at peripheral tissues where they act as immune sentinels continuously patrolling and sampling environmental antigens (Huang et al., 2001; Joffre et al., 2009). Upon antigen encounter and processing, DC mature up-regulating MHC-II and co-stimulatory molecules to efficiently present antigen to naïve T-cells. Moreover, their cytokine secretion pattern dictates the polarization of naïve $CD4^+$ T-cells

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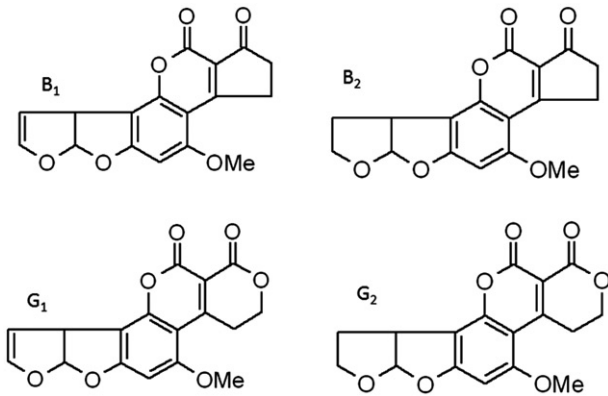
Figure 1. Chemical structures of the four tested aflatoxins (AF). Note that AFB_2 and AFG_2 are derivatives of AFB_1 and AFG_1, respectively. OMe = methoxy.

into T-helper (T_H)- 1, T_H2, or T_H17 effector cells or regulatory cells, which in turn drives the ensuing immune response against invading pathogens (Huang et al., 2001; Joffre et al., 2009). Little is known about the effect of mixed AF on DC function. To mimic the natural exposure of the immune system to AF, we prepared a mixture of naturally-occurring levels of AFB_1, AFB_2, AFG_1, and AFG_2 (mAF) and examined their impact on function of swine monocyte-derived DC (MoDC). Swine were used here, as pigs represent an important economically relevant animal model, whose immune system closely resembles that of humans (Dawson et al., 2013; Fairbairn et al., 2011; Meurens et al., 2012).

Materials and methods

Aflatoxins

AFB_1, AFB_2, AFG_1, and AFG_2 (mAF) were separately purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany); all were *Aspergillus*-derived, >98% pure, and free of lipopolysaccharide and contaminant chemicals (according to supplier). Each was first separately dissolved in 96% ethanol (at 0.1 mg/ml) according to Mehrzad et al. (2011). Further dilutions were then made with sterile, endotoxin-free Dulbecco's phosphate-buffered saline (DPBS, pH 7.3; Gibco, Merelbeke, Belgium). Aliquots of the AFB_1, AFB_2, AFG_1, and AFG_2 solutions were then combined and 10 µl of this AF mixture added to the cell cultures to obtain final overall concentrations of each agent in the well of, respectively, 2.0, 1.0, 0.5 and 0.5 ng/ml. The mixture of these environmentally relevant levels of AF was chosen to mimic as closely as possible *in vivo* situations (see Discussion).

Generation of porcine monocyte-derived dendritic cells (MoDC)

Heparinized blood samples were obtained from the external jugular vein of three separate Belgian Landrace piglets (female, 8–20-weeks-old) kept as blood donors under standard conditions at the Faculty of Veterinary Medicine (Merelbeke, Belgium). Blood sampling was performed from each of the three piglets over the span of 1-month (i.e., 1 week between each host). All animal experiments were in accordance with the local animal welfare regulations and were approved by the Ethical Committee of the Faculty of Veterinary Medicine.

Peripheral blood mononuclear cells (PBMC) were isolated from each whole blood sample by lymphoprep density gradient centrifugation (Axis-Shield, Oslo, Norway). Monocytes were further enriched to a purity of >95% by immuno-magnetic bead

selection (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) using anti-CD172a monoclonal antibody (mAb; clone 74-12-15a; Pescovitz et al., 1984) and goat anti-mouse microbeads together with LS separation columns (Miltenyi Biotec). The $CD172a^+$ monocytes were subsequently cultured in 24-well plates (Nunc, Thermo Fisher Scientific, Langenselbold, Germany) at a density of 5.0×10^5 cells/ml in phenol-red free Dulbecco's modified Eagle's Medium (DMEM; Gibco), supplemented with 10% (v/v) fetal calf serum (FCS; Greiner, Wemmel, Belgium), 100 U/ penicillin/ml (Gibco), 100 µg streptomycin/ml (Gibco), recombinant porcine (rp) GM-CSF (17.5 ng/ml; Inumaru et al., 1998) and rpIL-4 (5 ng/ml; R&D Systems, Minneapolis, MN), and then incubated at 37 °C in a humidified atmosphere at 5% CO_2 to generate MoDC as previously described (Devriendt et al., 2010). On Day 3 of the culture period, MoDC were fed by addition of fresh medium supplemented with rpGM-CSF and rpIL-4 at the same concentrations. On Day 4 or 5 of the culture period, cells with long membrane protrusions, a typical feature of immature DC, dominated the cell culture.

Phagocytosis assay

Samples of immature MoDC isolated from each of the piglets were incubated with mAF for 1, 2, 12, and 24 h at 37 °C in the humidified 5% CO_2 atmosphere in six separate wells; after the treatment period these wells were then pooled to collect the MoDC for phagocytosis assays. At the end of each respective period, 5.0×10^6 fluorescein isothiocyanate (FITC)-loaded polystyrene micro-particles (1.0 µm diameter; Sigma) were added to the stimulated MoDC (10 microparticles/DC) and the cells incubated for 3 h at 37 °C under 5% CO_2. Subsequently, the cells were harvested on ice by flushing, washed with ice-cold PBS, and microparticle internalization was assessed by flow cytometry with a FACSCanto flow cytometer (BD Biosciences, Erembodegem, Belgium) with a minimum event count of 20 000/ sample. For each sample, flow cytometric analyses of phagocytosis were performed by assessing the fluorescence intensity of 20 000 cells within the live, singlet cell gate. The mean fluorescent intensity (MFI) was calculated using FACSDiva 6.1.3 software (BD Biosciences). The results were presented as the percentage of phagocytosed microparticles relative to that in mock-stimulated MoDC (control MoDC). Relative phagocytic activity was calculated as $100 \times$ (phagocytosis by AF-treated MoDC/phagocytosis by control [0 ng AF/ml] MoDC).

Cell surface expression of co-stimulatory molecules and activation markers

Cell surface expression of co-stimulatory molecules and activation markers upon stimulation were assessed in samples of each piglet's MoDC with medium in six separate wells and the abovementioned levels of mAF for 12 and 24 h using flow cytometry (i.e. after the treatment period these wells were then pooled to collect the MoDC for staining). These assays were performed using mAb against MHC-II (MSA3, IgG_{2a}; Lunney et al., 1994), CD40 (G28-5, IgG_1, anti-human; Bimczok et al., 2007), CD25 (K231.3B2, IgG_1; Bailey et al., 1992), and a human CTLA4-muIgG_{2a} fusion protein (all from Ancell, Bayport, MN), respectively, followed by R-phycoerythrin- and AlexaFluor-647-conjugated isotype-specific anti-mouse secondary antibodies (Life Technologies, Merelbeke, Belgium).

In brief, the MoDC were harvested after the culturing with the AF and washed in staining medium (DMEM + 1% FCS) and then incubated with pre-titrated saturating concentrations of each primary Ab for 20 min at 4 °C. Cells stained with isotype-matched irrelevant mAb (Life Technologies) were used to assess non-

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specific binding. After washing, the cells were stained for 20 min at 4 °C with the secondary Ab in staining medium. The cells were then washed and propidium iodide (5 µg/ml) was added to help in exclusion of dead cells during analyses. Flow cytometry data were then acquired and analyzed as above. Expression of each marker was defined by quantifying the fluorescence intensity of 20 000 cells within the live, singlet cell gate. Relative marker expression (%) was calculated as $100 \times [(MFI_{treatment} - MFI_{control})/MFI_{control}]$.

T-cell proliferation assay

The T-cell stimulatory capacity of MoDC was analyzed in an allogenic T-cell proliferation assay. T-cells were isolated from each piglet's PBMC fraction by enriching CD6+ cells to a purity of >95% by positive immunomagnetic selection with anti-CD6 mAb (IgG$_1$, clone a38b2; Saalmüller et al., 1994) and goat anti-mouse microbeads, together with LS columns (Miltenyi Biotech). The MoDC were then treated with the above-mentioned levels of mAF (or left untreated) for 12 and 24 h. The MoDC were then harvested by flushing, washed, counted with a counting chamber (Neubauer) in nigrosin solution, and co-cultured at titrated numbers with 2.0×10^5 allogenic CD6+ T-cells in round-bottom 96-well microtiter plates (Nunc). In all cases, a 1:30 (DC:T) ratio was used for the proliferation assays; each DC:T sample was analyzed in triplicate. CD6+ T-cells stimulated with 5 µg/ml ConA (Sigma) only were used as a positive control. All cultures were performed in proliferation medium (DMEM supplemented with 10% FCS, penicillin/streptomycin, and 50 µM 2-mercaptoethanol) at 37 °C in a 5% CO$_2$ humidified atmosphere. After 5 days of culture, the cells were pulse-labeled with 1 µCi/well [³H]-thymidine (Amersham ICN, Bucks, UK) for an additional 18 h. Cells were then harvested onto glass fiber filters (Perkin-Elmer Life Sciences, Brussels, Belgium) and [³H]-thymidine incorporation measured using a β-scintillation counter (Perkin-Elmer). Results were calculated in terms of a stimulation index (SI) wherein SI = mean cpm of co-cultures DC$_{treatment}$/mean cpm of co-cultures DC$_{control}$.

Analysis of the cytokine secretion profile by ELISA

MoDC from each piglet were generated and stimulated with mAF as above. After 12 and 24 h, cell-free culture supernatants were collected and levels of porcine tumor necrosis factor (TNF)-α, interleukin (IL)-1β, -6, -8, and -10 cytokines then determined with commercially available ELISA kits (TNFα, IL-1β, IL-6, and IL-8: R&D Systems; IL-10: Life Technologies, Merelbeke, Belgium) according to manufacturer instructions. All samples were diluted by ½ (TNFα, IL-1β, IL-6, IL-10) or ¼ (IL-8) in reagent diluent prior to use in the assays. Optical densities were then measured in an ELISA plate reader (MTX Lab systems, Vienna, VA) at 450 nm. Cytokine concentrations in the medium were calculated using DeltaSOFT JV 2.1.2 software (BioMetallics, Princeton, NJ) with a 4-parameter curve-fitting algorithm applied for standard curve calculations. The limits of detection of the TNFα, IL-1β, IL-6, IL-8, and IL-10 kits were, respectively, 125, 62.5, 4.3, 125, and 3.0 pg/ml.

Statistical analyses

Statistical analyses were performed with SPSS 20 (IBM, Armonk, NY). Effects of mAF on phagocytosis of microparticles, DC marker expression, T-cell proliferation, and cytokine secretion were assessed with a non-parametric Mann-Whitney test. Significance was accepted at $p < 0.05$.

Results

mAF do not impair the phagocytic capacity of MoDC

As AF disrupts phagocytosis of particles by innate immune cells, this study sought to assess the effect of AF on DC-mediated phagocytosis of microparticles. Before the addition of polystyrene microparticles, MoDC from each piglet were treated for different lengths of time with a mixture of several AF. At the low concentrations used here, the mAF did not affect cell viability (data not shown). After 1 h of treatment, the mAF caused the cells to display a slight but not significant increase in internalization of the microparticles (Figure 2). However, as the treatment length increased (i.e. 2, 12, or 24 h), the mean phagocytic activity of the cells from the three piglets returned to a level unchanged as compared to that in mock-treated MoDC. Note: For the benefit of the Readers, all data from each piglet is also provided in Supplemental Table S1.

mAF enhance the expression of activation markers by DC

DC need to up-regulate the cell surface expression of peptide-MHC complexes and co-stimulatory molecules to efficiently present processed antigens to naïve T-cells. Thus, we wanted to elucidate the effect of mAF on the activation of porcine MoDC by assessing the expression of cell surface markers MHC-II, CD40, CD80/86, and CD25. The latter is considered an important marker for porcine DC activation (Devriendt et al., 2013). As indicated in Figure 3, mAF treatment had a clear time-dependent effect on surface expression of these markers on the cells isolated from the three piglets. After 12 h of mAF treatment, there was an increase in the mean expression of CD80/86 and CD25 as compared to that by control (i.e. 0 ng AF/ml) DC, although this was only significant for CD25 ($p = 0.037$) (Figure 3). Prolonged exposure (24 h) of the cells to the toxin mixture further increased the mean CD80/86 expression ($p = 0.019$), whereas the effect on CD25 expression disappeared (Figure 3). Note: For the benefit of the readers, all data from each piglet is also provided in Supplemental Table S1.

mAF enhance the T-cell stimulatory activity of DC

To further assess if mAF-mediated phenotypic DC activation was linked to a functional activation, the effect of mAF on the
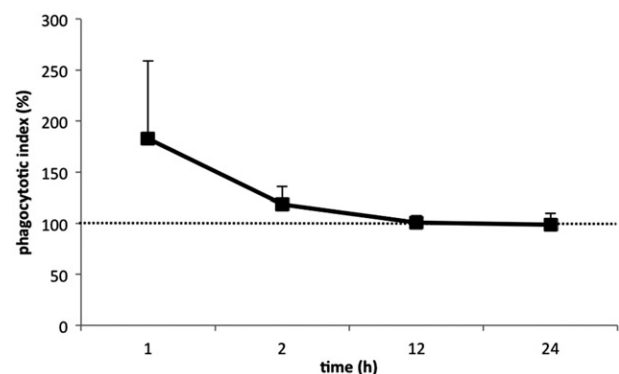

Figure 2. Effect of naturally occurring levels of AF on phagocytic activity of porcine monocyte-derived dendritic cells (MoDC). MoDC isolated from each of three piglets were separately treated for different periods of time with a mixture of several aflatoxins (mAF) containing AFB$_1$ (2.0 ng/ml), AFB$_2$ (1.0 ng/ml), AFG$_1$ (0.5 ng/ml), and AFG$_2$ (0.5 ng/ml). At the end of each period, 5.0×10^6 fluorescent 1.0-µm microparticles were added to each set of MoDC to assess if there were effects on phagocytic activity induced by the treatments. Data are presented as mean ($\pm$SEM, $n = 3$) percent (%) change in phagocytic activity relative to values for each piglet's corresponding untreated MoDC. Dashed line = phagocytic activity of control non-AF-treated MoDC.
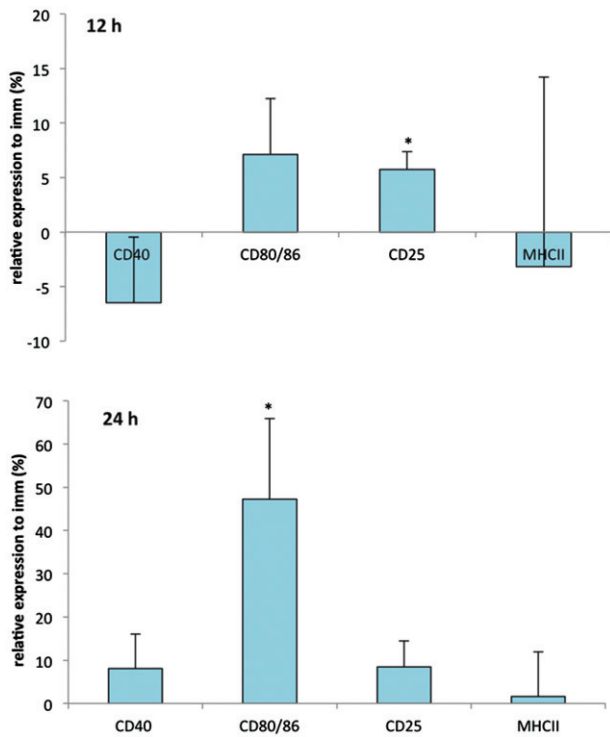
Figure 3. Aflatoxins affect the cell surface expression of DC activation markers. MoDC isolated from each of three piglets were separately treated with a mixture of several aflatoxins (mAF containing AFB$_1$ [2.0 ng/ml], AFB$_2$ [1.0 ng/ml], AFG$_1$ [0.5 ng/ml], and AFG$_2$ [0.5 ng/ml]) for 12 and 24 h and then analyzed for expression of CD40, CD80/86, CD25, and MHC-II by flow cytometry. Data are presented as mean (±SEM, $n = 3$) percent (%) change in expression relative to values for each piglet's corresponding untreated MoDC. $*p < 0.05$ to untreated cells. imm = immature (untreated or control) MoDC.
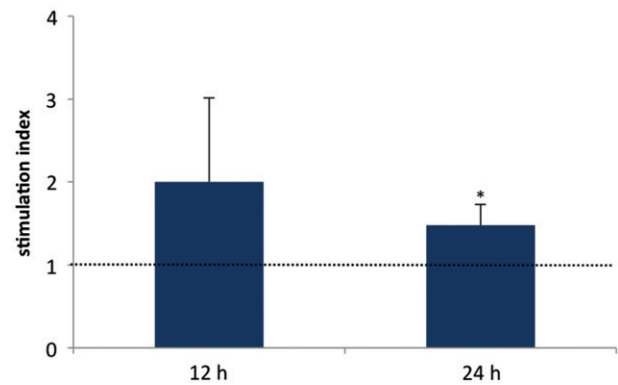

Figure 4. Aflatoxins affect the T-cell stimulatory capacity of porcine MoDC. MoDC isolated from each of three piglets were separately treated with a mixture of several aflatoxins (see Figure 3 legend) for 12 and 24 h. The mAF-treated MoDC were subsequently co-cultured with 2.0×10^5 CD6$^+$ T-cells at a 1:30 (DC:T) ratio. T-cell proliferation was measured via [^{3}H]-methyl thymidine incorporation. Data are presented as mean (±SEM, $n = 3$) stimulation index of the three individual sets of piglet cells analyzed. $*p < 0.05$ vs non-AF-treated MoDC.

ability of DC to induce T-cell proliferation was examined. Up-regulation of CD80/CD86 by mAF was accompanied by an enhanced T-cell stimulatory capacity of the DC (Figure 4). After 12 h of MoDC exposure to the mAF, the resultant mean increase in T-cell stimulatory ability still showed a large variation; after a 24 h incubation, this increase was more consistent, resulting in significantly enhanced T-cell stimulatory capacity as compared to that seen with non-AF-treated DC ($p = 0.002$). Note: For the benefit of the readers, all data from each piglet is also provided in Supplemental Table S1.

mAF do not affect cytokine secretion by MoDC

As the mAF increased the ability of DC to activate T-cells, and as cytokines play a crucial role in activation and polarization of T-cells, we next determined the effect of the mAF on DC cytokine secretion pattern. As shown in Figure 5, among the cells isolated from the three piglets, there was a small increase in secretion (i.e. mean levels in culture medium) of inflammatory IL-1β, IL-6, and TNFα, and a slight decrease in that of anti-inflammatory IL-10 (Figure 5). However, these concentrations were not significantly different from those in the culture medium of the mock-treated MoDC. Note: For the benefit of the readers, all data from each piglet is also provided in Supplemental Table S1.

Discussion

The study here examined the effects of a mixture of different aflatoxins (AF) at levels that may be obtained by consumption of environmentally-contaminated feed and foods. The AF and their toxic effects on key immune cells is a threat to animal and human health worldwide (CAST, 1989; Luongo et al., 2013; Reddy et al., 2009; Viegas et al., 2013). Recent data indicate that the chronic exposure of piglets to low mycotoxin levels still could affect the intestinal immune system and prolong infection with intestinal pathogens (Devriendt et al., 2009; Oswald et al., 2003). Based on the chemical structure and properties of Group B and G AF, they can easily pass through the plasma membrane of (for example) intestinal epithelial cells and rapidly appear in the blood circulation (Battacone et al., 2003; Gallo et al., 2008; Martins et al., 2007). Although the expression of major AF-metabolizing enzymes in intestinal epithelial cells is yet to be explored, detoxification of AF by these cells is minimal, and these highly carcinogenic AF can easily reach the blood stream. Thereafter, the AF are converted to highly oxidative epoxides that bind to proteins, DNA, and/or RNA, causing oxidative stress and disturbing normal cellular processes (Bernabucci et al., 2011; Mehrzad et al., 2011). Indeed, the toxicity of AF not only depends on their intact form but also on their biotransformation to reactive hydroxylated derivatives (Ayed-Boussema et al., 2012; Tulayakul et al., 2007). In human immune cells, cytochrome P450 (CYP) enzymes are involved in metabolism of AF and this CYP-dependent biotransformation of AF might be required for their effect on porcine DC (Bahari et al., 2014). DC are the most potent APC with the unique ability to activate naïve T-cells and elucidating the impact of mixed AF on DC functions key to dictating the nature of the immune response could help to understand the immunotoxicity caused by low doses of mycotoxins.

Indeed, ingestion of AF-contaminated feed by piglets resulted in an altered T-cell proliferation, presumably due to a direct effect of AF on DC (Meissonnier et al., 2008). Basically, T-cell activation by DC requires three inter-related signals: (1) the interaction of the T-cell receptor with peptide-MHC complexes on the DC surface; (2) DC have to up-regulate the expression of co-stimulatory molecules, such as CD40 and CD80/86, to fully activate naïve T-cells; and (3) secretion of cytokines which will influence the polarization of the activated T-cells. If one of those signals is absent, T-cell activation and consequently T-cell proliferation will be impaired (Huang et al., 2001). The observed broad effects of mAF on DC clearly suggested that AF did provide some signals in DC. To the best of our knowledge, this is the first study elucidating the effects of mAF on the function of porcine DC.
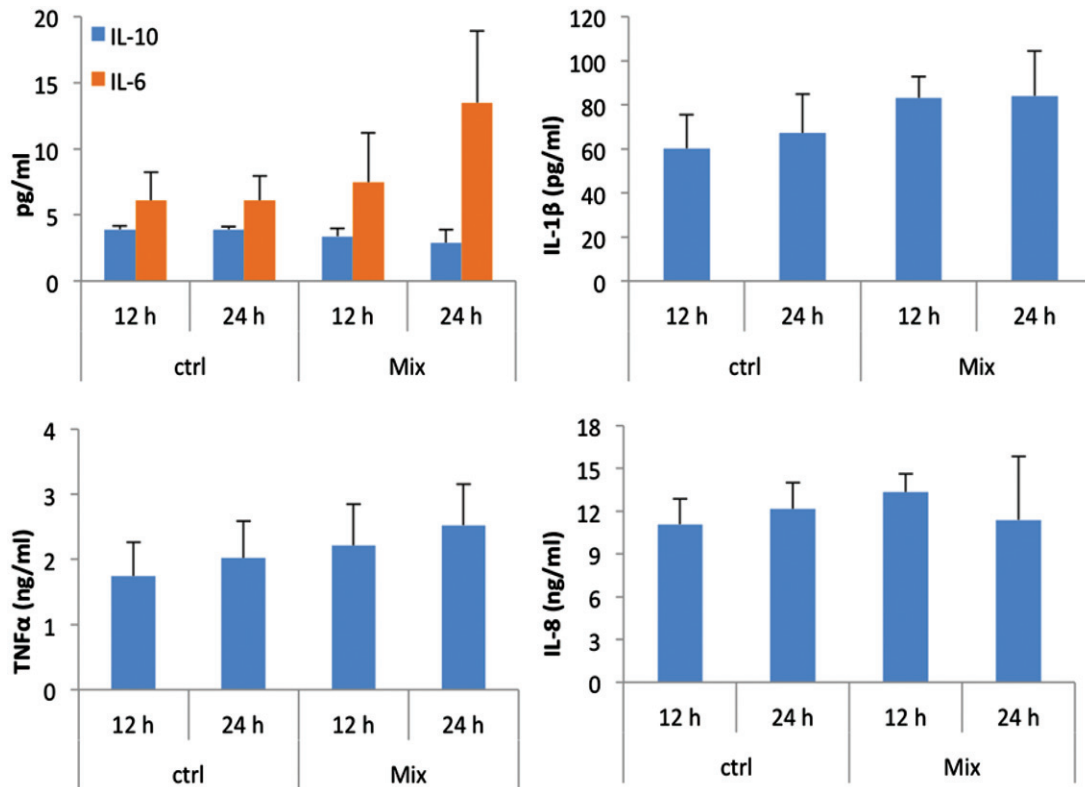
Figure 5. Cytokine expression pattern of AF-exposed MoDC. MoDC isolated from each of three piglets were separately treated with either 0 ng/ml (ctrl) or a mixture of aflatoxins (see Figure 3 legend) for 12 and 24 h. Cell culture supernatants harvested at the end of each incubation period were analyzed for the presence of IL-1β, IL-6, IL-8, IL-10, and TNFα by ELISA. Data are presented as mean ($\pm$SEM, $n = 3$) levels of each cytokine in the medium (at each timepoint) from the three individual sets of piglet cells analyzed. Ctrl = non-AF-treated MoDC.

We challenged DC with a dose of mAF corresponding to approximately what can be found in mold-contaminated feed and, hence, could reflect naturally occurring levels in young piglets. Indeed, when animals are fed feedstuffs contaminated at the upper limit of 10 µg/kg feed on a dry matter basis which is tolerated by the EU Feed legislation (2002), and considering the daily feed intake of piglets, total AF levels in the blood stream could reach $\sim$10 ng/ml. Although there are differing proportions of AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$ in *Aspergillus*-contaminated feed, aflatoxinogenic molds predominantly produce AFB$_1$ and to a lesser extent the other three AF in feed (Cervino et al., 2008; Joubrane et al., 2011; Li et al., 2009). Worldwide, occupational exposure to AF is alarming; a level of even $\sim$8 ng AFB$_1$/ml in blood of swine 'workers' whose exposure was by the inhalation, oral, and/or dermal routes, has been reported (Viegas et al., 2013). Further, in countries where mycotoxin-monitoring programs are absent, livestock may encounter far higher levels of the AF than the doses used here. Moreover, as the four AF types (in varying proportions) are present in *Aspergillus*-infested feed/food, and a mixture of environmentally relevant levels of these AF were used here to investigate effects on DC function, the approach in this study closely mimicked *in vivo* conditions that reflect levels often encountered by piglets. Taken together, these factors make the dose rationale used here relevant.

The ability of DC to take up antigens is central to their function. Using flow cytometric assays we assessed the phagocytosis capacity of AF-exposed DC. Previous studies indicated that AF could decrease the phagocytotic activity of neutrophils and macrophages (Mehrzad et al., 2011; Moon et al., 1999). In contrast, the phagocytotic capacity of DC was modestly increased at short lengths of exposure tested here (i.e. 1 h), possibly reflecting a response of the DC to mycotoxin sensing. Intriguingly, a 12 or 24 h treatment period did not influence

microparticle uptake by the MoDC; this implied little interference by the AF with membrane and actin cytoskeleton dynamics, two well-known key mechanisms necessary for efficient phagocytosis (Goodridge et al., 2012).

To justify the broad effects of AF on DC, we demonstrated there was a relationship between the mAF and cell-surface marker expression in porcine DC and confirmed that the mAF modified DC phenotype. Indeed, expression of CD25 and co-stimulatory CD80/86 were each increased, indicating a phenotypical activation of the mAF-treated DC. Remarkably, the mAF enhanced the T-cell proliferation-inducing capacity of the MoDC. This is in contrast with a diminished cell-mediated immunity in AFB$_1$-fed piglets observed by Meissonnier et al. (2008). This increased T-cell proliferation-inducing activity might be due to the effect of mAF on the secretion of cytokines.

However, the DC cytokine secretion profile did not show a decreased IL-10 secretion. The increased T-cell stimulatory capacity of mAF-treated MoDC also did not seem to be a result of increased levels of pro-inflammatory cytokines, as mAF treatment only slightly increased the secretion of these cytokines by the porcine DC. This is in line with previous data wherein the mAF did not modulate IL-1β and TNFα expression in swine alveolar macrophages (Liu et al., 2002). However, as we assayed only a limited amount of cytokines, a potential effect of the mAF on other cyto-/chemokines cannot be precluded. Because the naturally-occurring level of mAF did not affect the monitored pro- and anti-inflammatory cytokine secretion pattern of the DC, one critical unanswered question is how mAF-treated MoDC mediated an increase in T-cell proliferation. Our results sufficiently support the point that the observed increased T-cell proliferation induced by mAF-treated DC could mainly result from the increased expression of the DC activation markers CD25 and CD80/86. The enhanced antigen-presenting capacity of

steady-state DC induced by naturally-occurring levels of AF could result in a breakdown of immunological tolerance and provide a mechanism for the observed immunotoxicity of AF in mammals. Further functional assays are in progress to elucidate molecular mechanisms behind this phenomenon.

In conclusion, the results here indicate that a mixture of naturally-occurring AF dysregulates the antigen-presenting capacity of porcine DC. This immunotoxic effect at low levels could impact animal and public health and further emphasizes the need to reduce the admissible level of AF in agricultural products.

Acknowledgements

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Table S1