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Immunobiologically relevant level of aflatoxin B_1 alters transcription of key functional immune genes, phagocytosis and survival of human dendritic cells

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ABSTRACT

The effects of naturally occurring levels of aflatoxin (AF) B_1 on the expression of key molecules and function of dendritic cells (DCs) were investigated on human monocyte-derived DCs (MDDCs) by cell culture, RT-qPCR, and flow cytometry. An environmentally relevant level of AFB_1 remarkably impaired the phagocytic capacity of MDDCs. Furthermore, AFB_1 significantly affected the transcript levels of some key functional genes in MDDCs. It caused an up-regulation of key transcripts in cytochrome P450 (CYP) family, MyD88, NF-KB, TNF-α, TLR2, TLR4, COX-2, HLA-DR, CCR7, CD209, LFA3 and CD16. AFB_1 down-regulated the expression of AhR, TGF-β, CD11c and CD64 within 2–12 h post-exposure. In contrast, the transcription of some other key genes, including IL-10, IL-1β, AKR7A2, GSTM1, IL-6. IL-8 and C5aR in post-AFB_1 treated MDDCs was only slightly changed. The results indicate that an environmentally relevant level of AFB_1 impairs the phagocytosis capacity of MDDCs and dysregulates the key functions in these pivotal immune cells. This could provide a mechanistic explanation for the observed *in vivo* immunotoxicity associated with this mycotoxin, and further emphasize the essential need for reduction of AFB_1 levels in agricultural commodities.

1. Introduction

Aflatoxins (AFs), secondary metabolites produced mainly by *Asperogillus spp*, are capable of causing various (non)infectious diseases in humans and animals [1–6]. AFB_1 is the most toxic form among AFs family and has a broad range of toxicities leading to mutagenicity, carcinogenicity, and immunosuppression. AFB_1 has been shown to affect hepatocytes [7–10], kidney [11], respiratory system [12], reproductive system [13], leukocytes [3,14–16] and neural cells [4]. To avoid exposure to AFs, authorities employ extensive measurements to monitor its presence in feedstuffs and foods; however, complete remove AFB_1 from foods/feed resources appears to be challenging. This is partly due to the limitation of analytical techniques in detecting naturally occurring levels of AFs in agricultural commodities, secretions and sera. As such, the AFB_1 levels in feedstuffs can be far higher than that detected by available analytical tools, which is associated with (para) clinical symptoms in animals and humans [17–19].

Further, lipophilic nature of AFB_1 facilitates its bioavailability, easily entering into the bloodstream and immune cells. Inside the cells, AFB_1 is metabolically bio-transformed to a highly active form, AFB_1-epoxide, through the activity of mainly cytochrome p450 (CYP) family enzymes, particularly CYP1A1 and CYP3A4 in monocytes and lymphocytes [2–4,14,16,20]. AFB_1-epoxide easily binds to DNA, RNA and proteins and forms respective adducts [21]. Our previous study has demonstrated that in human monocytes and lymphocytes a member of glutathione-S-transferase superfamily, GSTM1, is more active in AFB_1 detoxification [14].

Monocytes originated from myeloid precursors emigrate to tissues

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in order to differentiate to macrophage and dendritic cells (DCs) [15,22]. DCs constitute only about 1% of human peripheral blood mononuclear cells (PBMCs) [23]. They are localized in other peripheral tissues where they act as key immune sentinels that continually patrol and sample environmental antigens [24]. DCs bridge innate and adaptive immunity, playing a pivotal role in initiation of several immune responses including phagocytosis, antigen processing and presenting, exclusively T-cell activation and cytokine secretion. Therefore, recognition, engulfing, processing and presenting an antigen are key parameters, which enable DCs to regulate immune responses [25,26]. The pivotal phagocytosis/endocytosis empowers DCs to acquire and present foreign antigens, as well as to stop and suppress immune responses after engulfing a self-antigen, thereby appropriately dictating other key immune cells, especially T-cells [27]. Pathogen associated molecular patterns (PAMPs) *via* pattern recognition receptors (PRRs) are precisely recognized and internalized into DCs [28,29]. Complement systems and some key immune-molecules like CD64, CD16 and CCR7 also promote phagocytosis capacity of DCs after DCs licensing. This capacity is reduced and DCs migrate to lymph nodes with higher antigen presenting power [30–33]. DCs use different molecules of classes 1 and 2 of major histocompatibility complexes (MHCs) encoded by one gene complex of the cells to stimulate various subsets of T cytotoxic lymphocytes and T helpers, respectively [34]. These monocyte-derived DCs (MDDCs) efficiently bind and present antigen to naive T-cells by up-regulating MHCs I and II with co-stimulatory molecules and cytokine secretion, creating immunological synapses with naive T-cells causing differentiation of naive T-cells into various effector or regulatory T-cell subsets. This in turn drives the ensuing immune response to protect the host from invading pathogens [2,16,24,35].

We have previously shown that exposure to very low and non-cytotoxic concentrations of AFB_1 could stimulate MDDCs toward overexpression of TLR4 and TLR2 and a pro-inflammatory microenvironment, mistakenly leading to the activation of innate and adaptive immunity potentiating leukocytes disarmament and improper immunity [2,16,18]. Pure MDDCs can massively be generated by treating monocytes with granulocyte macrophage stimulating factor (GM-CSF) and interleukin 4 (IL-4) [2,16,18,36]. The MDDCs generation is also facilitated by bacterial lipopolysaccharide, IL-10, tumor necrosis factor alpha (TNF-α) treatment [37], MAPK, and specially NF-κB [38]. Overexpression of TLRs potentially triggers a signaling pathways that alters DCs phenotype, decreases DCs phagocytosis capacity, and facilitates their migration to lymphoid organs [35,39].

Little is known about the molecular mechanisms associated with the functioning of AFB_1-exposed humDCs (Fig. 1). The study described herein was designed to explore AFB_1-metabolizing enzymes which are responsible for activation and deactivation of AFB_1 in MDDCs, and thus to better understand the pattern of activation and deactivation of AFB_1 in DCs. In addition, we evaluated the phagocytosis capacity of AFB_1-exposed MDDCs and explored the expression pattern of key functional genes involved in migration, antigen presentation, cell survival, and T-cell activation in AFB_1-exposed MDDCs.

2. Materials and methods

2.1. Reagents

AFB_1 was obtained from Sigma-Aldrich chemie (Taufkichen, Germany) and was dissolved (under a class 2 laminar flow/hood) in 96% ethanol to make a 0.1 mg/ml concentration. Then, it was further diluted in phosphate-buffered saline (PBS). All tissue culture, media and their supplements including RPMI (Roswell Park Memorial Institute), fetal bovine serum, L-glutamine, penicillin/streptomycin, non-essential amino acids and also PBS were purchased from Invitrogen, Life Technologies (Waltham, Massachusetts, USA). Recombinant human cytokines GM-CSF and IL-4 were obtained from R&D system (R&D, Minneapolis, Minnesota). In addition, FITC-loaded polystyrene microparticles with 0.1 diameters and Annexin-V PI staining kit were obtained from Sigma-Aldrich and Santa Cruz Biotechnology (USA, California) respectively. Ficoll-Paque plus was purchased from Lympholyte (Zierikzee, the Netherlands).

2.2. Blood sampling and experimental design

All experiment procedures for blood sampling and cell culture were approved by the ethical committee of Ferdowsi University of Mashhad. Sodium-heparinized blood was collected and pooled from 20 healthy male volunteers (18–21 years old), from which pool of 2–3 (totally n = 8 pools) were used for PBMCs isolation and then DCs production. PBMCs were isolated as previously described [1–3,14,16,18] with some modifications. Briefly, blood samples were diluted fourfold with PBS and after adding the same volume of Ficoll, they were centrifuged (1100*g*, 20 °C, 40 min). The PBMCs layer was collected and washed twice with PBS (500 × *g*, 4 °C, 5 min). Finally, 3 ml of 10^7 PBMCs/ml of complete RPMI medium (with > 98% viability) was seeded in a 3 cm

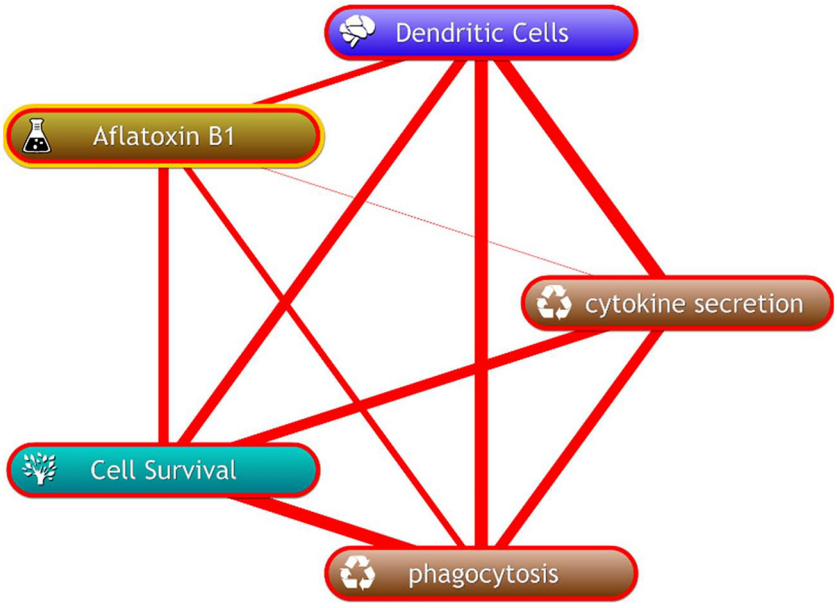

Fig. 1. Associations between AFB_1 and dendritic cells (DCs), phagocytosis, cytokine secretion and cell survival. A search in COREMINE Medical (http://www.coremine.com) shows that in total approximately 160 papers have been published to provide evidence for a correlation between AFB_1 and DCs' phagocytosis, cytokine secretion and survival. However, the majority of above papers have been assessing the effect of AFB_1 on cell (mainly non-immune cells) survival with huge controversies, and only 6 papers have been published on mammals' innate myeloid lineage cells specially human DCs and AFB_1 [1–4,14,16,18,40].

diameter culture plate and was incubated for 2 h in a 37 °C incubator (Memmert, Germany) with 95% humidity and 5% CO_2. Adherent cells (more than 95% monocytes) were re-suspended in warm RPMI medium containing 10% FBS, 3 mmol/l L-glutamine, 1% penicillin/streptomycin, 800U GM-CSF/ml, 1000U IL-4/ml, and 50 mM 2-mercaptoethanol (2-ME) as DCs medium and were incubated for 6 days (at 37 °C, 5% CO_2, 95% humidity) [2,16,18]. Transformation of monocytes to DCs was confirmed with CD11c marker. Harvested immature MDDCs yielded 95% $CD11^+$ MDDCs with > 90% viability.

In day 5, immature MDDCs with long membrane protrusions (washed with DCs culture media without phenol red, and seeded at 3×10^6 cells/ml in culture plates), were treated with 0 or 10 ng of AFB_1/ml for 2 and 12 h (37 °C, 5% CO_2, 95% humidity). Phenol red-free media were used to avoid any possible AFB_1-phenol red interactions during DCs exposure and also any potential fluorescence emission from phenol red in flow cytometric assays [2,16,18,40]. The suspensions of MDDCs were then centrifuged (350*g*, 5 min, 4 °C) and the cells were used for cellular and molecular analyses.

2.3. Quantification of the expression of gene families in DCs

After appropriate AFB_1 exposure, total RNA was extracted from 2×10^6 MDDCs. Briefly, RNA was purified from treated and control MDDCs using TriPure isolation reagent (Roche Diagnostics, Indianapolis, IN). The purified RNA was dissolved in nuclease-free water (Promega, Madison, WI) and immediately stored at −80 °C for later PCR analyses. Before freezing, the concentration and quality of the RNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Langenselbold, Germany) and gel electrophoresis, respectively. About 2 µg of the isolated RNA from each cell group was used for first strand cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Finland) with 10 pmol of oligo-dT primers. Exon junction or intron-spanning primers were designed for 25 pairs of primers for AFB_1 metabolism, cytokines, function, and TLRs related genes in MDDCs (see details at Table 1). ACTB was used as the reference gene throughout this experiment. Each RT-qPCR reaction was performed in a 20 µl final volume containing 10 pmole of forward and reverse primers, 4 µl 5× HOT FIREPol® EvaGreen®qPCR master Mix Plus (Solis BioDyne, Tartu, Estonia), and 1 µl (500 ng) cDNA template in CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, USA). To optimize the qPCR assays, amplification efficiency was calculated for each gene using ten-fold serial dilutions of its cDNA. qPCR conditions for all genes were carried out (in duplicate) with a cycling program including holding for 15 min at 95 °C, followed by cycling 45 times at 94, annealing temperature (Ta) of 53–62 °C (dependent on the primers, see Table 1), and 72 °C (20 s for each temperature). Fluorescent signal was acquired during each extension phase. Melting curve analyses included ramping from 50 − 99 °C, rising 0.5 °C/step and waiting 10 s for each step. A single peak was obtained in each qPCR product reaction which was accompanied by agarose gel electrophoresis to ascertain the specificity of the reactions or the absence of non-specific PCR products (data not shown).

Normalization and analyses of qPCR data were calculated using GenEX Version 5 software (MultiD, Göteborg, Sweden) and Relative Expression Software Tool (REST; QIAGEN, Hilden, Germany). In each qPCR reaction, the cycle number at which the fluorescence rose appropriately above the background was determined as the crossing point (CP). Means of CP duplicates in each qPCR and eight biological repeats were calculated. Optimization experiments were also performed to ensure that the efficiency of the target and the reference genes was approximately equal. A Pfaffl equation [41] was first used to calculate relative gene expression ratio, *i.e.,* the change in each target gene expression divided by the change in *ACTB* expression. A slope was determined from the exponential phase. Amplification efficiency (E) was calculated based on slope, where $E = 10^{[-1/slope]}$. The expression of each target gene was calibrated to that of *ACTB* using the formula: Relative fold change (RCF) = {$((E_{target\ gene})$ (Control $CP_{target\ gene}$ − treated $CP_{target\ gene}))/((EACTB)$ (Control CP_{ACTB} − treated CP_{ACTB}))}.

2.4. Flow cytometry-based assessment of AFB_1 impact on phagocytosis capacity of DCs

To determine the effect of AFB_1 on the functional activity of MDDCs, 10^7 fluorescein isothiocyanate (FITC)-loaded polystyrene microparticles (1.0-µm, 20 beads/MDDCs; Sigma) were added to 10^6 MDDCs that had been treated/incubated with/without AFB_1 for 2 and 12 h at 37 °C in 6-well culture plates. After 3 h of culture at 37 °C, the MDDCs were then harvested on ice, washed with ice-cold PBS, and were assessed by flow cytometry [minimum event count/sample = 10,000] for internalization of particles. Results were recorded as mean fluorescence intensity (MFI, intensity of phagocytosed FITC-labeled microparticles). The number of $FITC^+$ DCs reflected the number of DCs that could phagocytize microparticles and so reflected the phagocytic ability of the MDDCs.

2.5. Flow cytometry-based analyses of apoptosis and necrosis of DCs

The MDDCs survival assay was performed as previously described with some modifications [2,16,40]. Annexin V-FITC and propidium iodide (PI) [Anexin V-PI kit (Sc-45552, Santa Cruz)] were applied on suspension of MDDCs to detect phosphatidylserin, an early apoptotic marker, and to label permeable cells as necrotic cells. Briefly, 10^5 MDDCs which were incubated for 2 and 12 h at 37 °C in the presence of the aforementioned concentration of AFB_1 in culture medium were incubated with 1 µg Annexin V in 100 µl assay buffer (included in kit) and 10 µl PI for 15 min at room temperature. Flow cytometry control samples were prepared by exposing the cells to Annexin V or PI alone, or neither. After incubation, 400 µl 1X assay buffer was added to each sample and the annexin V/PI apoptosis assays were conducted using single laser emitting light at 488 nm for FITC and PI. 10000 cells were gated using a FACSCalibur System (Beckton Dickinson, Franklin Lakes, New Jersey). To create quadrants, flow cytometry data were analyzed with FCS Express 5 Plus and flowing software (De Novo software).

2.6. Statistical methods

Mean fluorescent intensity for phagocytosis, apoptosis and necrosis were pre-analyzed for normality and homogeneity tests using SPSS 22. ANOVA and LSD multiple comparison tests were used to compare phagocytosis capacity between treatments. Pre-processing qPCR data and ANOVA were analyzed in GenEx and SPSS ver22, respectively. Results were normalized to ACTB gene expression. Three groups of qPCR data were analyzed using one-way ANOVA and Dunnetts'multiple comparison test after data were tested for normality and variance homogeneity. $P \leq 0.05$ was considered significant.

3. Results

3.1. Effects of AFB_1 on the expression of gene families in DCs

The effects of naturally occurring levels of aflatoxin (AF) B_1 on the expression of key molecules and function of dendritic cells (DCs) were investigated on human monocyte-derived DCs (MDDCs) by cell culture, RT-qPCR, and flow cytometry. An environmentally relevant level of AFB_1 remarkably impaired the phagocytic capacity of MDDCs. Furthermore, AFB_1 significantly affected the transcript levels of some key functional genes in MDDCs. It caused an up-regulation of key transcripts in cytochrome P450 (CYP) family, MyD88, NF-KB, TNF-α, TLR2, TLR4, COX-2, HLA-DR, CCR7, CD209, LFA3 and CD16. AFB_1 down-regulated the expression of AhR, TGF-β, CD11c and CD64 within 2–12 h post-exposure. In contrast, the transcription of some other key

Table 1
Primer sequences and characteristics for expression analysis of different categories of genes involved in AFB_1 pathogenesis and effects on monocyte-derived dendritic cells (MDDCs) (for detailed information please see the methodology of the qPCR).

Group of genes	Gene symbol (Acc number)	Primer sequence (5′ to 3′)	Amplicon (bp)	Annealing temperature (°C)
AFB_1 metabolism	CYP1A1 (NM_000499)	F: CCCAGGGTACAGAGAAAGA R: GAAGGGGACGAAGGAAGA	144	56
	CYP1B1 NM_000104	F:GTCAATGTCACTCTCAGA R:TTGCCTCTTGCTTCTTAT	95	60
	CYP3A4 NM_017460	F:TGGAGATGTGTTGGTGAG R:TGTGGATTGTTGAGAGAG	144	54
	AKR7A2 NM_003689	F:GCCAGTCCGAGACCATCC R:TGCAGCCTCTTCAATGACG	154	53
	GSTM1 NM_000561	F:GCAGGAAACAAGGGCTTGGA R:CCTACTTGTTGCCCCAGACA	103	58
TLRs-related genes	TLR4 NM_138554	F:GAGGCCATTATGCTATGT R:TTTCTCCCTTCCTCCTTT	143	55
	TLR2 NG_016229	F:AACTTCAATCCCCCCTTC R:CACCACTCACTCTTCACA	146	59
	Myd88 NM_001172569	F:ACAGAGAGAGGAAGAGAG R:GAAGGAGAGAGGGAGAGA	77	57
	NF-kb NM_003998	F:TGCTGGAGTTCAGGATAAC R:GGATGATTGCTAAGTGTAAGAC	179	58
	Cox-2 NM_000963	F:TTGAAGAACTTACAGGAGAA R:GCAGGAGAACATATAACATTAC	196	58
MDDCs functional genes	CD64 NM_000566	F:CCCCCAGCTACAGAATCA R:ACACCAGCTTATCCTTCCA	198	55
	CD16 NM_001271036	F:ACAAACCTCTCCACCCTC R:TCCTCCTTGAACACCCAC	92	58
	AHR NM_020731	F:ATATCCGAATGATTAAGACT R:CTTCCTCATCTGTTAGTG	162	57
	NQO1 NM_000903	F:AGGAGTTGATGATTTGGG R:GGCATAGGGCTTAGACGT	142	56
	HLA-DRB5 NM_002125	F:GACTTCACCCAACAGGACTC R:AAGAATAAGAGCCAAGCAGGAA	111	62
	LFA-3 NM_001779	F:TATGTGCTTGAGTCTCTTCC R:CGATGGCTGTTGTAATGC	101	60
	CD209 NM_021155	F:CTGAGGAGCAGAACTTCC R:GCCATTGCCACTAAATTCC	200	61
	C5aR1 NM_001736	F:ACCAGAACATGAACTCCTT R:GCAGCGTGTTAGAAGTTT	108	57
MDDCs cytokines	IL-6 NM_000600	F:CACCTCTTCAGAACGAATTG R:GGCAAGTCTCCTCATTGA	194	55
	IL-10 NM_000572	F:TGGAGGACTTTAAGGGTTAC R:GATGTCTGGGTCTTGGTT	104	54
	IL-8 NM_000584	F:TTTGAAGAGGGCTGAGAAT R:ACAATACATGAAGTGTTGAAGT	122	62
	TNF-alpha NM_000594	F:CTCAGCCTCTTCTCCTTC R:GGGTTTGCTACAACATGG	111	58
	TGF-beta NM_000660	F:TTTGATGTCACCGGAGTTGT R:TAGTGAACCCGTTGATGTCC	130	56
	IL-1b NM_000576	F:GGCTTATTACAGTGGCAATGA R:TAGTGGTGGTCGGAGATTC	135	61
Reference	ACTB NM_001101	F:TGAAGATCAAGATCATTG R:TAACGCAACTAAGTCATA	179	58

genes, including IL-10, IL-1β, AKR7A2, GSTM1, IL-6. IL-8 and C5aR in post-AFB_1 treated MDDCs was only slightly changed. The results indicate that an environmentally relevant level of AFB_1 impairs the phagocytosis capacity of MDDCs and dysregulates the key functions in these pivotal immune cells. This could provide a mechanistic explanation for the observed *in vivo* immunotoxicity associated with this mycotoxin, and further emphasize the essential need for reduction of AFB_1 levels in agricultural commodities (Fig. 2).

AFB_1 metabolizing genes, specifically AFB_1 activation isoforms, were markedly upregulated in cells exposed to 10 ng/ml AFB_1 for 12 h (Fig. 2A). These genes were CYP3A4, CYP1A1 and CYP1B1. However, no significant changes were detected for the expression of AFB_1 detoxification genes, GSTM1 and AKR7A2. AhR gene was up-regulated after 2 h AFB_1 exposure, but unexpectedly down-regulated after 12 h exposure (Fig. 2A).

The expression of some key TLR-related genes in AFB_1-exposed MDDCs suggested a pro-inflammatory microenvironment. Comparative transcription of AFB_1-exposed MDDCs for two key PRRs, *TLR2* and *TLR4*, and two TLR signaling pathway molecules, *NF-kb* and MyD88 along with cyclooxygenase 2 (*Cox-2*) revealed that 2 h exposure has caused a remarkable upregulation of these genes, except Cox-2, while 12 h exposure upregulated all genes (Fig. 2B).

Genes involved in the function of DCs, CD16, HLA-DR, CCR7, CD209 and LFA3 were significantly up-regulated in MDDCs after exposure to AFB_1, but CD11c and CD64 genes were down-regulated with only slight change on C5aR gene (Fig. 2C).

Analyses on six key cytokine transcripts in MDDCs, showed that AFB_1 was able to somehow affect inflammatory cytokines in DCS (Fig. 2D). A significant down-regulation in mRNA expression of TGF-β and up-regulation in mRNA expression of TNF-α was observed with a slight increase in mRNA expression of IL-1β, IL-6 and IL-8, but IL-10 was remained unchanged in post-AFB_1 treated MDDCs (Fig. 2C).

3.2. Effects of AFB_1 on the phagocytosis capacity of DCs

The flow cytometry-based phagocytosis assay revealed a significant

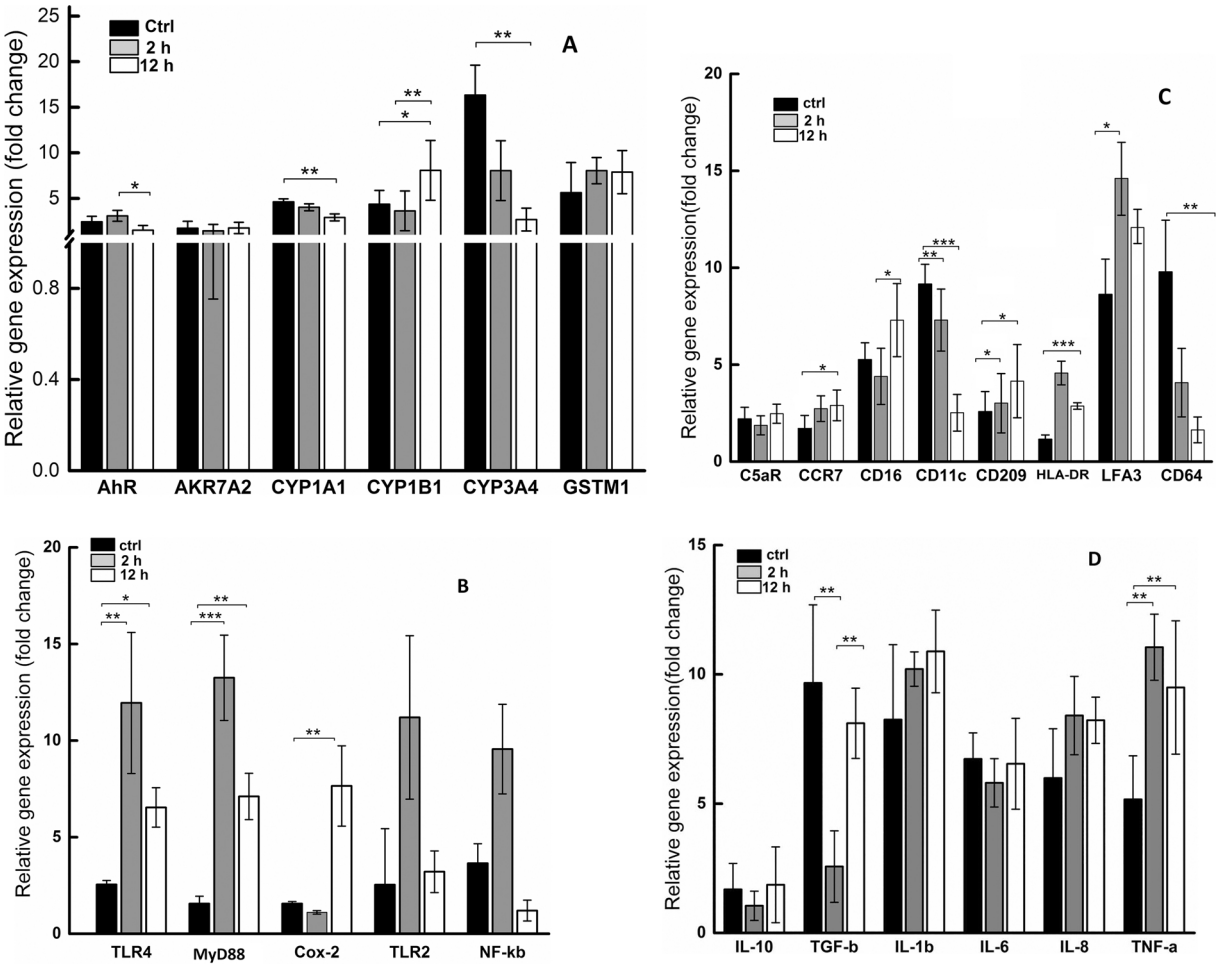

Fig. 2. Effects of AFB_1 on the expression of genes related to AFB_1 metabolism and xenobiotic-response genes (A), PRRs (B), key DCs functional genes (C), and some key cytokines (D) in monocyte-derived dendritic cells (MDDCs). The MDDCs were incubated without/with 10 ng/ml of AFB_1 for 2 h and 12 h. We can see a strong over-expression of some key genes induced by AFB_1, (though some unaffected and even down regulated). The relative level of the lowest expression of each gene between all replicates was considered as 1. All data were normalized with *ACTB* as internal control. The data are presented as the mean ± SD (n = 8). *, ** and *** are confidence level in 5, 1 and 0.1% respectively. Detailed information are available in the methodology of the study with the related References.

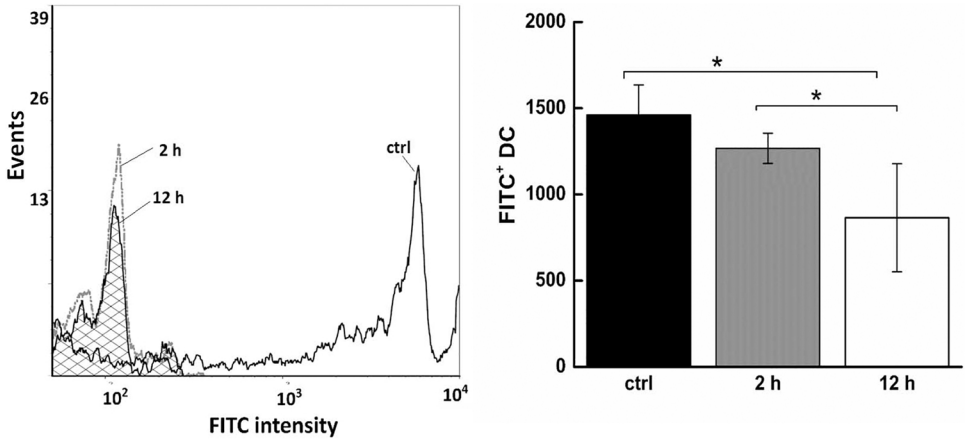

Fig. 3. Effects of AFB_1 on monocyte-derived dendritic cells (MDDCs) phagocytosis. Left panel: representative overlay histograms of MDDCs phagocytosis after 2 h (dotted line), 12 h (shaded line) and control (ctrl, filled line) exposing to 10 ng/ml AFB_1. Prolonged AFB_1 cause reduced the number of FITC-coated microbeads particle in the MDDCs. Right panel: overall changes in phagocytosis of AFB_1-exposed and non-exposed (ctrl) MDDCs. The data are presented as the mean ± SD (n = 8). * is confidence level in 5% (n = 8).

decrease in phagocytosis of FITC-labeled microparticles by post-AFB_1-treated MDDCs (Fig. 3); this finding was consistent with the results acquired on mRNA expression of the opsonin molecule (*i.e.,* CD64) (Fig. 2C). Indeed, AFB_1 caused DCs to be significantly less efficient/potent at uptaking particles/antigens/pathogens. Rate of engulfing particles in 2 and 12 h exposed MDDCs, was markedly lower (~15 and 25%, respectively) than that in control DCs.

3.3. Effects of AFB_1 on apoptosis and survival of DCs

To explore any potential effects of AFB_1 on DCs survival, 10000 MDDCs were analyzed per treatment regimen (Fig. 4). With little effects on DCs necrosis, low levels of AFB_1 significantly ($P < 0.05$) increased DCs apoptosis (Fig. 4). Induction of apoptosis was more intensive at 12 h post-AFB_1 exposure (Fig. 4, lower panel).

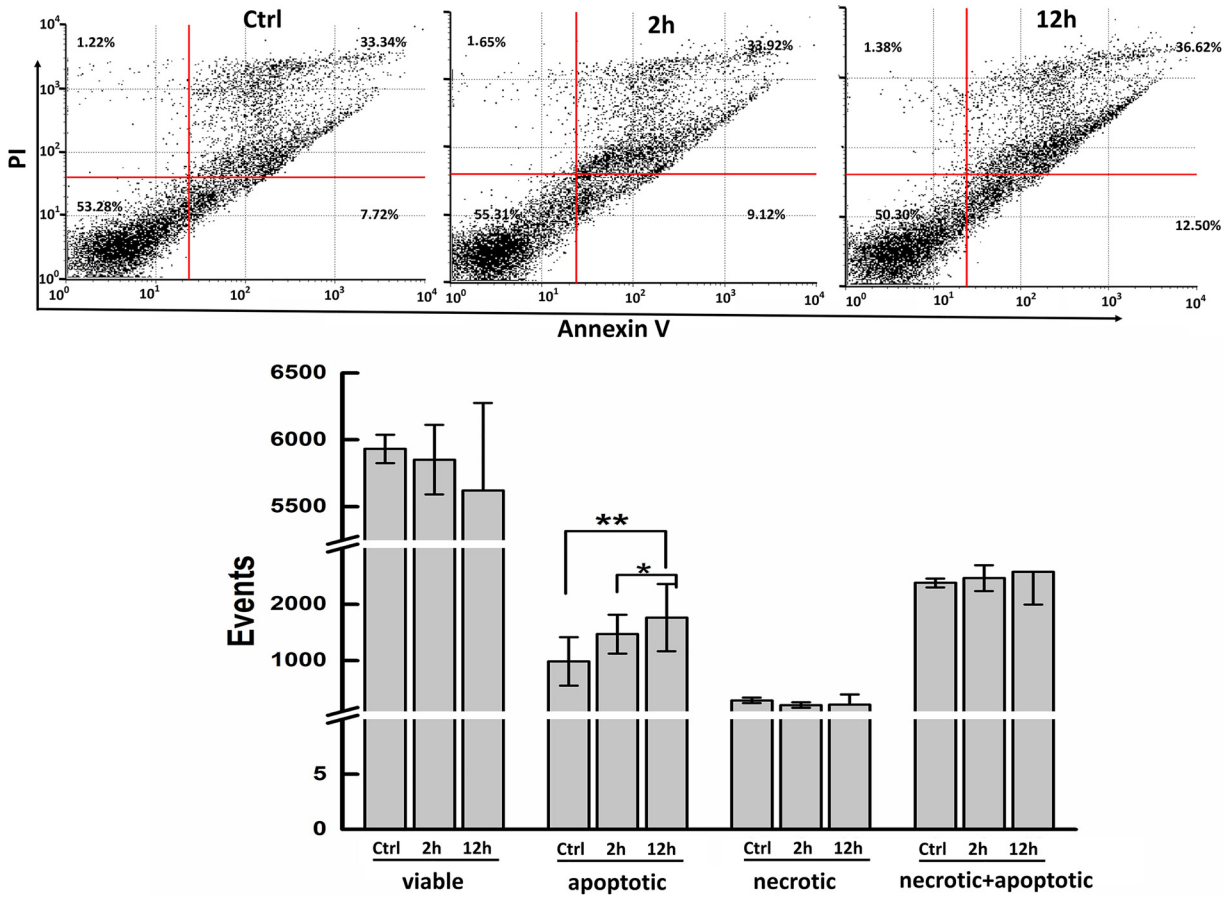

Fig. 4. Effects of AFB_1 on apoptosis and necrosis in monocyte-derived dendritic cells (MDDCs). Upper panel: representative flow-cytometric dot plots of MDDCs for control (ctrl), 2 h and 12 h of AFB_1 exposure. Four parts of quadrants show live (lower left), necrotic (upper left), early apoptotic (lower right) and apoptotic-necrotic (upper right) MDDCs. Lower part: overall number of MDDCs in each of quadrant part are showed as histograms. Data are presented as mean ± SD. Significant differences are indicated by * ($\alpha \leq 0.05$), and ** ($p < 0.01$), respectively (n = 8).

4. Discussion

In order to assess the potential effects of AFB_1 on expression of key genes, phagocytosis capacity, and apoptotic/necrotic states of DCs, MDDCs were exposed to immunobiologically relevant level, ~10 ng/ml, of AFB_1 [4–6] within different timepoints in the current study. Because DCs have a high capacity to induce innate and adaptive immune responses, any external signals with a potential to alter the fundamental immune processes of these cells, are able to directly affect the efficacy of immune responses [24,34,35,42]. As DCs are potent to orchestrate adaptive and innate immune responses and induce all aspects of primary and secondary immune responses, small changes in their functions and stability causes major effect on essential immune responses. Here we demonstrate that AFB_1 significantly interferes with canonical functions of MDDCs. This is in line with our previous functional and proteomics studies in porcine [2,16] and human [18] MDDCs. Once activated by antigen, myeloid DCs undergo maturation into competent antigen-presenting cells and migrate to nearby organized lymphoid organs. In these lymphoid organs, T cells specifically recognize the processed antigen presented on the surface of DCs [35,43]. Naturally relevant level of AFB_1 [4–6] is able to deregulate these fundamental immune functions in mammalian DCs.

As expected we found that 2 h challenge of MDDCs with AFB_1 produced more pronounced effect on over-expression of TLR2 and TLR4. It seems prolonged exposure partially triggers immunotolerance disturbances towards more over-expression of PRRs. Our dynamic tests of AFB_1 on other immune cells and species also showed AFB_1 induced more responses in PRRs in early h of exposure. Indeed, AFB_1 interferes with many routine functions of immune cells through deregulation of PRRs genes expression, decrease of intracellular ROS production capacity, increase in apoptotic cells, cell arresting, and etc. [2,3,14,16,18,40,44].

TLRs and their related genes are highly expressed on the cell surface of DCs; thereby, their mRNA up-regulation could trigger inflammatory responses. In this study we focused on the effects of the AFB_1 on *TLR*-related mRNA abundance in human MDDCs. Nevertheless, the molecular pathways underlying these findings remain elusive.

Of interest to us was to assess the gene expression of DCs' key opsonin receptor, CD64, when challenged with AFB_1. The *in vitro* experiment herein confirmed that low and non-toxic level of AFB_1 down-regulated expression of pro-phagocytosis gene, CD64 mRNA, in human MDDCs. This might explain the impaired phagocytosis capacity in AFB_1-exposed mammalian DCs as has been shown in this study and also reported by others [2,16]. Until recently the effect of AFB_1 on the CD64 mRNA abundance in human DCs was unclear.

One of the most important factors regarding to efficacy of a toxic substances is their potency in inducing cell death, *i.e.,* necrosis/apoptosis, [40]. Therefore, we assessed flow cytometry-based necrosis/apoptosis in MDDCs induced by AFB_1 (see Fig. 4). We demonstrated for the first time that AFB_1 changes the maintenance of homeostasis of human MDDCs by inducing apoptosis. It also changes cytokines secretions and diminishes phagocytosis capacity and its related molecules. We also showed that 10 ng/ml AFB_1 affects the secretory properties of MDDCs by producing pro-inflammatory cytokine's mRNA, *TNF-α*, whereas it did not alter mRNA expression of IL-1β or IL-6, IL-8 and IL-10. Here we did not assess the protein levels of cytokines, but we

recently did such assessment on key PRRs and ILs in human DCs at both mRNA and protein levels [18], and also on key ILs at the protein levels in porcine DCs [2,16] supporting the role of AFB_1 in over-expressing key pro-inflammatory cytokines. We also, for the first time, observed a trend for mRNA over-expression of CCR7, CD209 and LFA-3, with little change on C5aR mRNA in AFB_1-exposeed MDDCs. It should be mentioned that we analyzed only one complement component, requiring further works on other complement components.

Cytochrome P450 possess mixed function of oxidation and has major roll in metabolism conversion of naturally occurring materials in dietary compounds above all AFB_1 [22,45,46]. Thus suppression of activation members of this superfamily enzymes has big impact on AFB_1 characterizations in immune system [3,14]. Our previous studies demonstrated that very low dose of AFB_1 increased gene expression of CYP1A1 in monocytes and CYP3A4 and CYP1B1 in lymphocytes mostly in 2 h of challenge (shorter time of exposure). Further, GSTM1 is transcribed more than AKR7A2 in AFB_1-exposed human monocytes and lymphocytes. Therefore, herein over-expression of CYP3A4, CYP1B1 and CYP1A1 in MDDCs after 12 h of AFB_1 exposure is not surprising. This up-regulation in CYP family causes a positive feedback in AFB_1 activation leading to more oxidative stress in immune cells [40,47].

Previously, it has been shown that TGF-β production and antigen presenting in DCs have indirect correlation. In this study, decrease in TGF-β gene expression in MDDCs due to AFB_1 exposure is in consistent with upregulation of HLA-DR and probably increase in MDDCs antigen presenting capacity [48].

DCs maturation and executive licensing require up-regulation of key chemokine receptor CCR7. CCR7 and IL-10 secretion by DCs can regulate migratory rate of DCs and also antigen presenting function of DCs in an autocrine manner [49]. Thus, AFB_1 could contribute to pseudo-licensing direction of immature MDDCs, leading to further DCs maturation involving in over-expression of CCR7, which in turn facilitates DCs migration to the lymphoid tissue. Indeed, one of the major signs for MDDCs licensing is reduction of their phagocytosis capacity. In this scenario they no longer are able to efficiently engulf antigens by receptor-dependent phagocytosis and micropinocytosis (see schematic Fig. 5). The observed prolonged AFB_1-exposure of MDDCs further deteriorated their phagocytosis capacity. Down-regulation of key phagocytic element, CD64 mRNA confirms the observed diminished phagocytosis in AFB_1-exposured MDDCs [50–52].

Over-expression of TLRs-related genes, particularly MyD88 in AFB_1-exposed MDDCs might lead to activation of TLRs signaling pathway [2,16,18]. Though the molecular size of AFB_1 is very small and is hardly an immunogenic substance, it up-regulated and triggered TLR4 signaling pathways in MDDCs, similar to LPS-treated DCs [18]. LPS is a well-characterized TLR4 ligand that induces DCs maturation and has been shown to induce downregulation of MDDCs phagocytosis [53,54] as well. Also, it is well known that after detection of PAMP by PRR, large number of cytokines including TNF-α, IL-1β and IL-12 are released which is in line with our finding [2,16,54].

Key pro-inflammatory cytokines, TNF-α and IL-1β, play major roles in resistance against infections and tumors. IL-12 promotes T-cell activation, particularly T-helper proliferation [36,37,52]. Decrease in T-cell activation after AFB_1 exposure has been previously shown in porcine MDDCs [2,16]. Here, our results show that AFB_1 significantly up-regulated TNF-α, but not other cytokines. Also, *TGF-β* mRNA, as a key anti-inflammatory cytokine, was down-regulated in MDDCs after short time of exposure to AFB_1 with up-regulation of *TNF-α*, *TLR4*, *MyD88* and *Cox-2*. This further emphasizes on the pro-inflammatory micro-environment of AFB_1-exposed MDDCs. In this study, we did not assess the pro-inflammatory cytokines of AFB_1-exposed MDDCs at protein level. However, we have previously found that AFB_1 induces pro-inflammatory cytokines at protein levels in human [18] and porcine [2,16]; significant in-depth comparative works, nonetheless, is required to better undesrtand and interpret the current findings.

The finding herein also showed NF-kB gene expression was increased after 2 h of AFB_1 exposure. NF-kB possess two subunits, and when dimerizes their functions are identical. NF-kB is a major transcription factor in regulation of immune molecules [22,38]. Furthermore, it is a key element in the regulation of the development, maturation and function of DCs. Some reports show a positive co-expression of NF-kB and HLA-DR, CD80 and CD86 as part of APC function, which is in accordance to our finding. The stimulatory effect of AFB_1 on NF-kB could be important for modulation of DCs differentiation and function.

After 2 h of AFB_1 exposure, phagocytosis capacity of MDDCs and HLA-DR mRNA level, as indicators of APC function, was decreased and increased, respectively. Thus, it seems that AFB_1 potentiates DCs

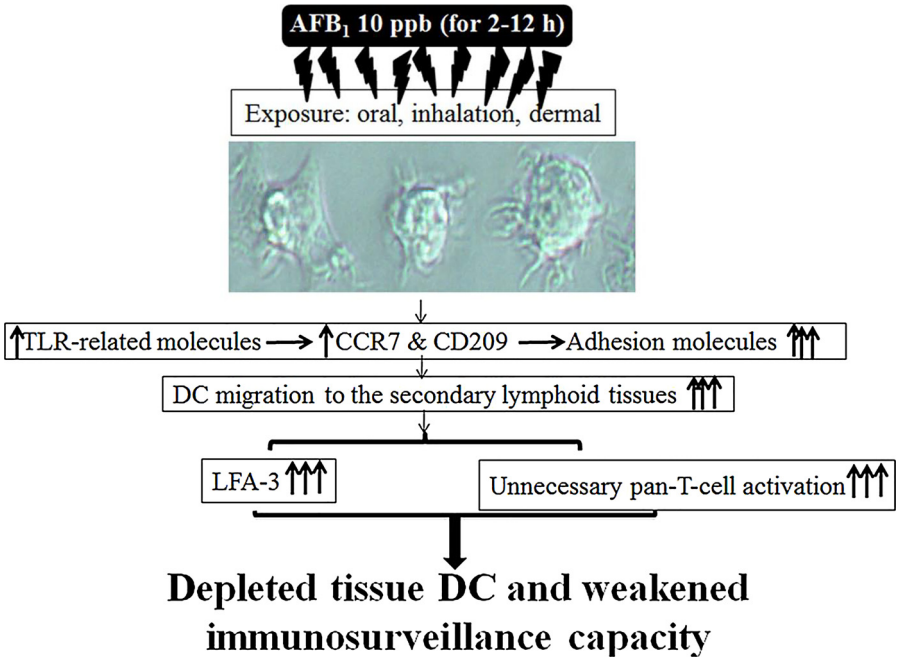

Fig. 5. Schematic diagram depicting how the proposed issue of pseudolicensing of AFB_1-exposed monocyte-derived dendritic cells (MDDCs) creates/leads to immunodisregulation *in vivo*. Though mRNA expression of CCR7, CD209 and LFA tended to increase/change, nevertheless the protein levels of those molecules should be evaluated for future prospective.

migration toward tissues and secondary lymphoid organs. This phenomenon potentially breaks immune tolerance and cause autoimmune and allergic problems, suggesting that AFB_1-induced MDDCs maturation might act as an immunogen to increase autoimmune and allergic diseases. However, further fundamental and immunoclinical studies are needed to confirm this.

Overall, the differences found in mRNA expression of candidate molecules in this study cannot be immediately considered as a full reflection of protein expression; thereby, more research at protein levels are needed. Although our findings are at transcriptional and functional levels, they show that AFB_1-exposed MDDCs fail to appropriately orchestrate bridging the innate and adaptive immune responses to various pathogens. Moreover, current molecular assays reveal that AFB_1 breaks the tolerance and fastens DCs maturation. Considering the broad roles of DCs in immunobiology, and the observed dysregulatory impacts of very low and non-toxic concentration of AFB_1 on several fundamental immune processes of DCs, this study provides another compelling reason to avoid AFB_1 exposure. As such, authorities should certainly lower the tolerable limits of AFs in agricultural commodities and foods. Our findings reveal several molecular mechanisms involved in immunodysregulation and immunotoxicity after AFB_1 exposure in humans.

Conflict of interest

None declared.

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