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RESEARCH ARTICLE

Cytochrome P450 isoforms are differently up-regulated in aflatoxin B_1-exposed human lymphocytes and monocytes

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Abstract

Context: Aflatoxins (AFs) are highly hazardous mycotoxins with potent carcinogenic, mutagenic and immune disregulatory properties. Cytochrome P450 (*CYP*) isoforms are central for enhanced AFB_1 toxicity *in situ*. It remains to be seen whether and how these AFB_1 activators work in human leukocytes.
Objective: To investigate the involvement of *CYP* isoforms in AFB_1 toxicity of circulating mononuclear cells, we examined the impact of environmentally relevant levels of AFB_1 on lymphocytes and monocytes.
Materials and methods: Very low and moderate doses of AFB_1 with/without *CYP* inducers on transcription of key *CYP* isoforms and toll-like receptor 4 (*TLR4*) were examined in human lymphocytes, monocytes and HepG2 cells; cell cycle distribution and viability were also analyzed in AFB_1-exposed lymphocytes and monocytes.
Results: Only *CYP1A1*, *CYP1B1*, *CYP3A4*, *CYP3A5* and *CYP3A7* expressed in lymphocytes and monocytes. *TLR4* much more expressed in monocytes than in lymphocytes, but HepG2 showed little *TLR4* transcription. While *CYP1A1*, *CYP1B1* and *CYP3A4* were highly induced by AFB_1 in monocytes, in lymphocytes only *CYP1A1* was induced. Among *CYP1A1*, *CYP1B1* and *CYP3A4* only *CYP1A1* responded to low and moderate levels of AFB_1. Enhanced transcripts of *CYPs* by AFB_1 yielded little synergies on *TLR4* transcription in lymphocytes and monocytes. Cell cycle arrest and necrosis were also detected in AFB_1-exposed lymphocytes and monocytes.
Conclusions: Our novel findings indicate that AFB_1 more intensively stimulates *CYP* genes expression in monocytes than in lymphocytes. Mechanistically, this could explain a more pronounced immunotoxicity of AFB_1 in myeloid than in lymphoid lineage cells *in vitro/situ/vivo*.

Keywords

Aflatoxin B_1, CYP, lymphocytes, monocytes, qPCR, toll-like receptor 4

History

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Introduction

As secondary metabolites and food contaminants, aflatoxins (AFs), are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* containing different types, including AFB_1, AFB_2, AFG_1, AFG_2, etc.[1,2] AFB_1 is the most common and toxic form of mycotoxins, and classified as group 1 carcinogens by International Agency for Research on Cancer (IARC). AFB_1 causes cancer in many organs such as liver, lungs and colon[3–7]. It is also a potent immunosuppressive[8]. Human and animal are frequently exposed to AFB_1 worldwide. The metabolic and biological behavior of AFB_1 in the body is poorly understood, and efforts to mitigate its harmful effects by finding metabolic pathways are valuable. The toxicity of AFB_1 depends on a biotransformation to its reactive hydroxylated derivatives, mediated by cytochrome p450 (*CYP*) enzymes. AFB_1-epoxide, which can bind to DNA, forms AF-N7 guanine and thus leads to irreparable DNA damage. One of the most critical examples of AFB_1 mutagenic effects is the alteration of the third base of codon 249 in P53 protein from G:C to T:A[4].

CYP families play key roles in metabolic pathways of AFB_1 in the body. Main role in AFB_1 activation is played by *CYP3A4*, *CYP1A2* and *CYP1A1*[9], and *CYP1B1*, *CYP*2A13 and *CYP2A6* can form AFB_1-epoxide[7]. *CYP3A4* and *CYP1A2* can also change AFB_1 to less toxic forms, AFQ_1 and AFM_1, respectively[10]. Only exo-isomer of AFB_1-epoxide, produced by *CYP*s, can bind to N7-guanine thus causing mutation[11]. Also, investigations have demonstrated that *CYP* inhibitors like oltipraz can cause a protection against AFB_1 toxicity[11]. Some chemicals induce expression of *CYP*s through pregnane X receptors (*PXRs*). These receptors form

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heterodimer with retinoid X receptors (*RXRs*) and bind to the xenobiotic responsive enhancer module (XREM) region in *CYP* promoters, amplifying *CYP*s transcription[12–14], thereby potentiating AFB_1 cytotoxicity. Dexamethasone (DEX) and rifampicin (RIF) are two potent inducers of *CYP1A1* and *CYP3A4*[15–18] and augment AFB_1 toxicity in immune cells[19]. Furthermore, various effects of DEX on the activity of different genes in monocytes have been reported[20].

Whether and how differently *CYP* family members are co-expressed in human immune cells remain controversial. While the majority of investigations suggest that *CYP3A4*, *CYP1A2*, *CYP1B1* and *CYP1A1* are expressed in human lymphocytes, others have reported that *CYP1A2* is confined to only hepatocytes[21–24]. Immunosuppressive effects of AFB_1 have been investigated and some of the reports show that AFB_1 inappropriately interferes with (1) complement system activation (2) bovine and rat neutrophils' free radicals production[25,26] (3) porcine and human antibody production[27], (4) transcription of toll-like receptors (TLRs) in human peripheral blood mononuclear cells (PBMCs)[28] and dendritic cells (DCs), and (5) porcine DC activation and T-cell polarization (unpublished observation). In our unpublished study, we have observed a high response of *TLR4* to very low dose of AFB_1-exposed PBMCs and DCs, leading us to assume that *TLR4* up-regulation is a useful biomarker for AFB_1 toxicity.

Although a lot of studies on AFB_1 metabolism has long been done in human and animals, almost all of these studies have been on hepatocytes, leading to the concept that lymphocytes and monocytes devoid of those metabolic pathways. To the best of our knowledge, no research has ever been conducted on the molecular metabolic pathways (activation and deactivation) of AFB_1 in human leukocytes. Finding *CYP* isoforms, which are responsible for activation of AFB_1 in human key immune cells, would therefore be valuable to broaden the side effects of environmentally acceptable levels of AFB_1, especially immunotoxic part of the effect. In this study, the potential *in vitro* effects of environmentally relevant doses of AFB_1 were examined at the transcriptomic levels of key *CYP*, *PXR* and *RXR* genes involved in AFB_1 toxicity in human lymphocytes and monocytes. Cell cycle analyses were also assessed to find the relationship between the expression of *CYP*s and the status of the cell division.

Materials and methods

Blood cells and reagents

Blood samples were taken and pooled from 10 healthy male volunteers with no history of using any of known inducer/inhibitor of *CYP* drugs and almost homogenous (18–21 years old). From those volunteers, four times (each time 2, 3, 2 and 3 individuals) heparinized blood samples were pooled together and applied to PBMCs isolation as previously[29] described, with some modification. Briefly, each pooled blood sample was diluted 1:4 in Dulbecco's phosphate buffered saline (DPBS) (without Mg^{++} and Ca^{++}) and then layered atop 15 ml Ficoll-Plaque plus (Lympholyte®, Zierikzee, the Netherlands) and finally centrifuged ($1100 \times g$, 20 °C, 40 min). The cloudy white layer was transferred to sterile 50 ml falcon tube then 10 ml PBS was added and finally centrifuged ($500 \times g$, 4 °C, 5 min). The isolated PBMCs from each sample were then dispensed in 3 ml (10^7 PBMCs/ml Roswell Park Memorial Institute medium-fetal bovine serum (RPMI-FBS)) volumes into culture plates (3 cm diameter) and incubated for 2 h in a 37 °C chamber containing 95% humidity and 5% CO_2. Non-adherent and adherent cells were separately used as lymphocytes and monocytes, respectively. The number of viable cell was achieved using trypan blue (≥98%). The monocytes and lymphocytes were separately counted, transferred and seeded (at a density of 5×10^6 cells/ml medium) in polystyrene 24-well tissue culture plates containing RPMI medium with 10% heat-inactivated FBS, 2 mM l-glutamine and 100 U/ml pen-strep.

Cells were treated, in duplicate, with two doses of AFB_1 (final concentration of 10 and 100 ng/ml) and two types of *CYP*s inducers, DEX and RIF (final concentrations of 50 and 10 μM), respectively. After 2 h incubation with treatments, the monocytes and lymphocytes in the wells were separately collected by centrifugation ($3000 \times g$, 4 min, 4 °C). Cells were then washed once in diethylpyrocarbonate (DEPC)-treated PBS and stored at −80 °C for RNA isolation. Also viability of lymphocytes and monocytes after treatments were measured using the propidium iodide (PI) exclusion method with flow cytometry (FC) assay, based on binding to nucleic acid and detection by FC. HepG2 cells used as control for *CYP*s expression were cultured in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 10% FBS, and were then sub-cultured with 70% confluency. The cells were kept in a humidified 37 °C incubator with 5% CO_2. The above procedure was repeated in four biological repeats among the pooled blood samples.

RNA purification and cDNA synthesis

Total RNA was purified using RNeasy Mini Kit (QIAGEN, Hilden, Germany) RNA integrity, quality and quantity were measured using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). First strand cDNA was prepared with RevertAid First Strand cDNA Synthesis (Fermentas, Finland), using oligodT primer, according to the protocol as follows. After removing of genomic DNA from extracted RNA, a mixture containing 30 ng oligodT primer and 1 μg extracted RNA in total 12 μl volume were mixed and incubated at 65 °C for 5 min and chilled on ice, then 2 μl of 10 mM dNTPs, 200 unit RevertAid M-MuLV reverse transcriptase, 20 unit RiboLock RNase Inhibitor and 1X reaction buffer were added in final volume of 20 μl then incubated (60 min, 42 °C) and were finally heated (5 min, 70 °C) to stop reaction.

qPCR assays

All members of family 1 and 3 of *CYP*s family were aligned using CLC main workbench software (clcbio Co., Aarhus, Denmark) to design primers based on un-conserved region; also, primers for *PXR* and *RXR* genes were designed using Allele ID 7.5 (Premierbiosoft, Palo Alto, CA) (Table 1). Exon junction or separated exons strategies were used to design primers in order to avoid mispairing during PCR, and 5× HOT FIREPol® EvaGreen®qPCR Mix Plus (Solis BioDyne) was used for qPCR. Final volume for each reaction was 20 μl,

Table 1. Primer sequences, annealing temperature (T_a) of designed primer sets (°C), expected PCR fragment sizes (bp) and accession (Acc) numbers.

Gene symbol (Acc number)	Primer sequence (5' → 3')	cDNA PCR product (bp)	T_a (°C)
TLR4 (NM_003264.3)	F: CAGTGAGGATGATGCCAGGAT R: ATGCCCCATCTTCAATTGTC	144	58
ACTB (NM_001101)	F: TGAAGATCAAGATCATTG R: TAACGCAACTAAGTCATA	179	50
GAPDH (NM_002046)	F: GAGCCACATCGCTCAGACAC R: CATGTAGTTGAGGTCAATGAAGG	150	60
CYP3A4 (NM_001202855)	F: TTGAAGTATTAAATATCTG R: TTGAAGTATTAAATATCTG	144	55
CYP1A1 (NM_000499)	F: CCCAGGGTACAGAGAAAGA R: GAAGGGGACGAAGGAAGA	135	53
CYP1A2 (NM_000761)	F: ACCAAGCCTGAGATACAGA R: GAAGGGCAAGAAGGAGGA	144	56
CYP3A43 (NM_057096)	F: GTAAAGAGACTCAGATCCCA R: ACAAAGTGGAAGTCCTTAG	150	56
CYP1B1 (NM_000104)	F: GTCAATGTCACTCTCAGA R: TTGCCTCTTGCTTCTTAT	95	52
CYP3A7 (NM_000765)	F: CTGTTTTGATCATGTCGGGA R: TGGGAAATGCTTTGTCCTTC	138	52
CYP3A5 (NM_001190484)	F: CGGCATCATAGGTAGGTGGT R: TATGAACTGGCCACTCACCC	94	59
PXR (NC_0136821)	F: TGGAAGACACTGCAGGTGG R: TGGGGAGAAGAGGGAGATGG	131	60
RXRa (NM_021976.3)	F: ATCTTTGACAGGGTGCT R: TTGGAGTCAGGGTTAAAG	110	60

containing 30 ng of specific forward and reverse primers, 4 µl of 5× evagreen master mixes and 1 µl of cDNA. The reaction was carried out in Rotter gen 6000 (QIAGEN, Hilden, Germany) with cycling program, including holding 15 min at 95 °C followed by cycling 45 times at 94 °C, 53 °C and 72 °C and 20 s for each temperature. Also melting was included ramping from 50° to 99°, rising by 0.5° in each step and wait 10 s for each step afterward. In melting curve analysis for each PCR product reaction, single peak was obtained. All reactions were run in duplicate. The absence of non-specific PCR products was confirmed using melting curve analysis accompanied by agarose gel electrophoresis. To confirm *β-actin* in human lymphocytes and monocytes as housekeeping gene, a second reference gene, *GAPDH* was used to compare *β-actin* gene expression in control and AFB_1-treated samples. We finally understood that exposure to AFB_1 did not affect on *β-actin* expression in monocytes, lymphocytes and HepG2, allowing *β-actin* to be used reliably as an internal control.

FC analyses of cell cycle with PI

Lymphocytes and monocytes were stained with fluorescein isothiocyanate (FITC), permeabilized and fixed accordingly[30] for cell cycle analysis. Briefly, cells were fixed in 3% formaldehyde and washed with PBS for 10 min at 4 °C then permeabilzed with 0.2% tween-20 for 10 min at 37 °C and washed again with PBS. Ten microgram per milliliter PI and 30 U/ml RNase were added to 1×10^6 cells. A minimum of 10 000 cells/events was acquired and collected for cell cycle analysis and gated based on size of lymphocytes and monocytes by excluding debris and dead cells.

Statistical analysis

Normalization and analysis of the qPCR data were calculated using GenEX version 5 software (MultiD Co, Göteborg, Sweden) and Relative Expression Software Tool, REST (QIAGEN, Hilden, Germany). Cycle of threshold (CT) values were means of duplicates in each qPCR run and three experimental repeats after interplate calibration. Relative differences in RT-qPCR among each plots of experiments were determined using the Pfaffl method[31]. Optimization experiments were also performed to ensure that the efficiency of the target and the internal control gene (β-actin) was approximately equal. The Pfaffl equation was first used to calculate the relative gene expression ratio. The obtained results were expressed as means ± standard error of means (SEM). Relative data (Pfaffl-based fold change) for AFB_1 and inducers were transformed to achieve analysis of variance (ANOVA) assumptions and final analysis of mean comparison (least significant difference) were done, using SPSS version 21 (SPSS Inc., Chicago, IL), between all groups (2×4 groups, two AFB_1 concentrations and four interactions between inducers. Value of $p < 0.05$ was considered significant.

Results

Profile of *CYPs* family transcripts in human monocytes and lymphocytes and HepG2

Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses revealed the presence of the specifically corrected bands and size in agarose gel electrophoresis (Figure 1a). Detection of isoforms of *CYP*1 and *CYP*3, *TLR4* and *ACTB* mRNAs in monocytes, lymphocytes and HePG2 revealed transcription of neither *CYP1A2* nor three variants of *CYP3A4*3 in monocytes and lymphocytes; but all studied *CYP* isoforms transcripts highly expressed in HepG2 cells. In contrast, HepG2 cells expressed little *TLR4*, which was interesting, but the observed sharp *TLR4* bands in lymphocytes and monocytes was not surprising (Figure 1a).

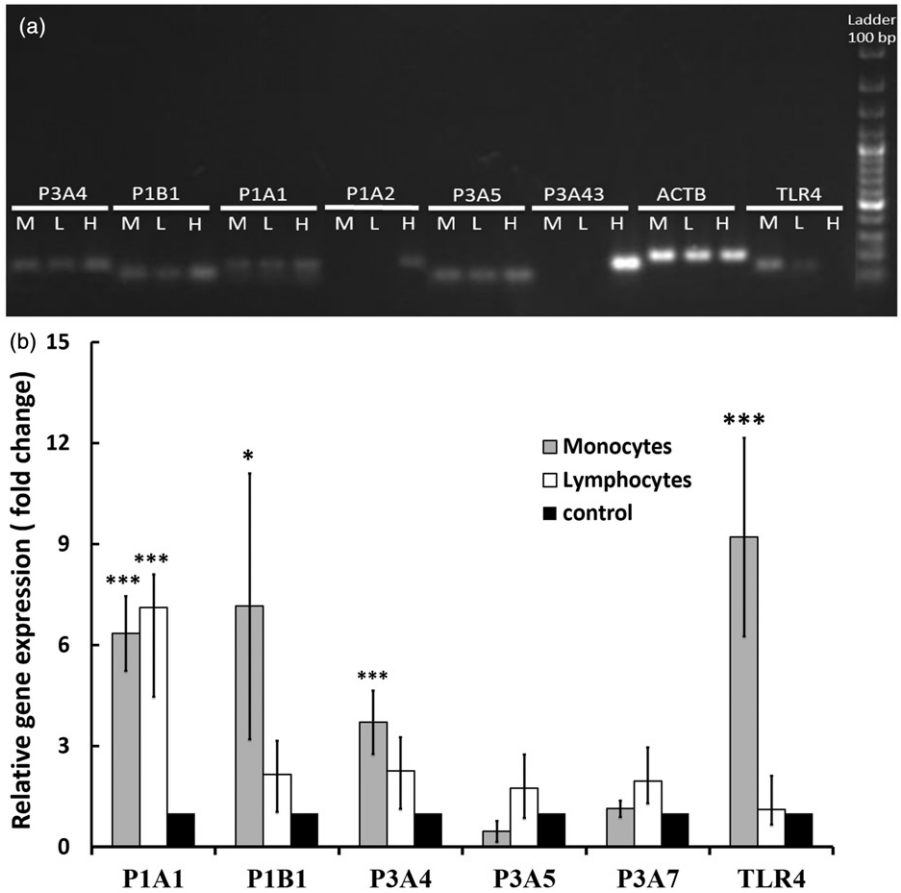


Figure 1. Detection of *CYP* transcript isoforms in human monocytes (M), lymphocytes (L) and HepG2 (H) and their responses to 10 ng/ml of AFB_1. (a) Detection of 10 CYP isoforms, *TLR4* and *ACTB* mRNAs in monocytes (M), lymphocytes (L) and HepG2 by RT-qPCR in agarose gel electrophoresis. (b) Comparative transcription of key *CYP* family 1 and 3 and *TLR4* (as control marker) in monocytes and lymphocytes exposed to 10 ng/ml of AFB_1 for 2 h. *CYP1A1*, *CYP1B1*, *CYP3A4* and *TLR4* were remarkably expressed in very low dose of AFB_1 in monocytes and only *CYP1A1* in lymphocytes. Transcription of *TLR4* in AFB_1-exposed monocytes was much higher than that of in lymphocytes. All data were normalized with *ACTB* as an internal control. The relative expression level of each gene in control for both monocytes and lymphocytes was 1 according to explanation describes in methods. The data are presented as the mean ± SEM ($n = 4$). *$p < 0.05$; ***$p < 0.001$; n.s., non-significant.

Different patterns of *CYP* family transcripts in AFB_1-exposed monocytes and lymphocytes

To determine if AFB_1 can change the steady-state mRNA levels of *CYP* genes in selected immune cells, we exposed monocytes and lymphocytes to only 10 ng/ml AFB_1 for 2 h to quantify the transcription patterns of five *CYP*s and *TLR4*. Although AFB_1 up-regulated all targeted genes, but it was statistically significant only for *CYP1B1*, *CYP3A4* and *CYP1A1* in monocytes, and only *CYP1A1* in lymphocytes (Figure 1b); therefore, further detailed qPCR analyses were performed only on *CYP1B1*, *CYP3A4* and *CYP1A1* as well as *TLR4* in lymphocytes and monocytes.

Effects of AFB_1 and *CYP* inducers on transcription of *CYPs* in monocytes and lymphocytes

In monocytes, all three targeted *CYP* genes were up-regulated in the presence of both doses of AFB_1 with/without *CYP* inducers (Figure 2). Presence of AFB_1 in monocytes differently modulated *CYP1A1* and *CYP3A4* expression from *CYP1B1* and *TLR4* (Figure 2; $p < 0.05$). Presence of 100 ng/ml AFB_1 with *CYP*-inducers resulted in a significant up-regulation in *CYP1A1* ($p < 0.05$). Transcription of *CYP1B1* did not obey AFB_1-*CYP* inducers interaction. Treatment with either 100 ng/ml of AFB_1 or DEX resulted in a significant up-regulation in *CYP3A4* ($p < 0.05$). Minimal transcription of *CYP3A4* was observed in a treatment of 10 ng/ml of AFB_1 with no inducers (Figure 2). In the presence of the *CYP* inducers, transcript of *TLR4* behaved in a concentration-dependent manner to the increasing levels of AFB_1 (Figure 2). Also, pattern of up-regulation in *TLR4* gene was consistent with up-regulation of *CYP1A1*, *CYP1B1* and *CYP3A4*.

In lymphocytes, transcripts of *CYP1A1* and *TLR4* were up-regulated in the presence of both doses of AFB_1 with/without *CYP* inducers (Figure 3). Both RIF and DEX significantly increased transcription of *CYP1A1*, but not of *TLR4*, in lymphocytes (Figure 3). Treatment of lymphocytes with either RIF or 100 ng/ml had similar up-regulatory effects on *CYP1A1* in lymphocytes.

Effects of AFB_1 on transcription of *PXR* and *RXR* in monocytes and lymphocytes

We sought to assess the effect of AFB_1 (10 ng/ml for 2 h) on the transcription of *PXR* and *RXR* genes in monocytes and lymphocytes. As shown in Figure 4, AFB_1 failed to enhance transcription of *PXR* and *RXR* genes in monocytes and lymphocytes.

Cell cycle analysis

In histograms obtained from WINMDI software, untreated lymphocytes and monocytes showed cell cycle distributions of 76 ± 2.1% and 74 ± 1.7% in G1/G0, 6 ± 0.3% and 5 ± 0.7% in S, 7 ± 0.3% and 3 ± 1.1% in G2/M, respectively. At concentration of 10 and 100 ng/ml AFB_1 led to a non-significant increase in the percentage of cells in G2/M in monocytes, but G2/M significantly increased in lymphocytes treated with 100 ng/ml of AFB_1 (Figure 5e). Other cell cycle parameters did not change the duration of the cell cycle in S and G0/G1 phases. These data indicated that 100 ng/ml of

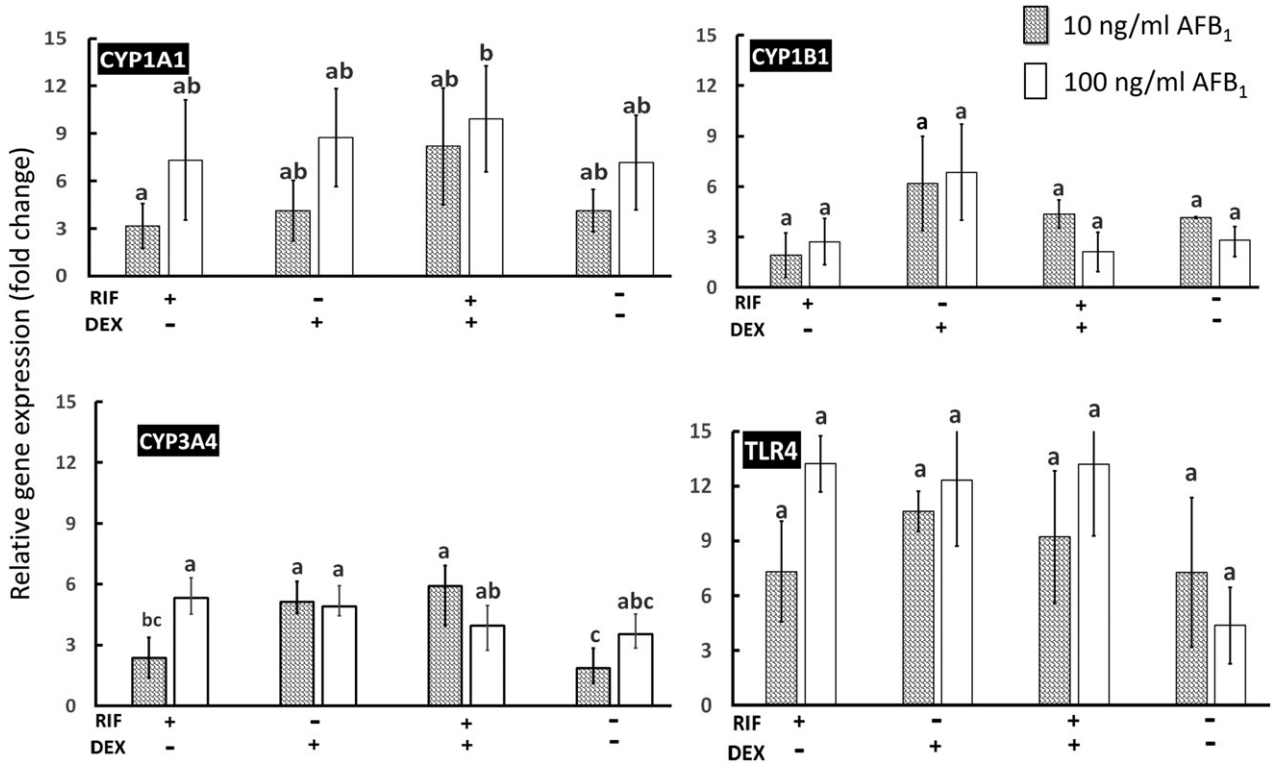


Figure 2. Gene expression quantification of four *CYP* isoforms (*CYP1A1*, *CYP1B1* and *CYP3A4*) and *TLR4* in monocytes exposed to different doses of AFB_1 with/without *CYP* inducers (RIF and DEX). The monocytes were exposed to 10 and 100 ng/ml of AFB_1 separately and simultaneously with/without RIF and DEX for 2 h. Transcripts of all four genes were up-regulated in the presence of both doses of AFB_1 with/without *CYP* inducers. Presences of AFB_1 in monocytes cultures differently modulated *CYP1A1* and *CYP3A4* expression from *CYP1B1* and *TLR4* ($p < 0.05$). In the presence of the *CYP* inducers, transcript of *TLR4* behaved concentration dependently manner to AFB_1. The relative expression level of each gene in control for both monocytes and lymphocytes was 1 according to explanation describes in methods. All data were normalized with *ACTB* as an internal control. The data are presented as the mean ± SEM ($n = 4$). Different letter indicates significant difference between the groups ($p < 0.05$).

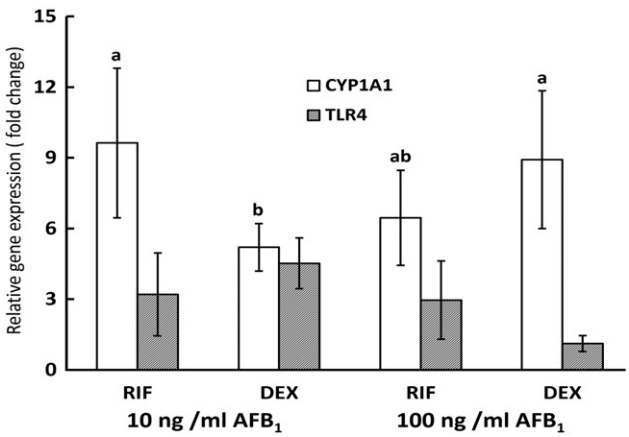


Figure 3. Gene expression quantification of *CYP1A1* and *TLR4* in lymphocytes exposed to different doses of AFB_1 with *CYP* inducers (RIF and DEX). The lymphocytes were exposed to 10 and 100 ng/ml of AFB_1 separately and simultaneously with RIF and DEX for 2 h. Transcript of *CYP1A1* up-regulated in the presence of both doses of AFB_1 with CYP inducers. Presences of AFB_1 in lymphocytes cultures differently modulated *CYP1A1* ($p < 0.05$). In the presence of the CYP inducers, transcript of TLR4 did not obey the concentration of AFB_1. The relative expression level of each gene in control lymphocytes was 1 according to explanation describes in material and methods. All data were normalized with *ACTB* as an internal control. The data are presented as the mean ± SEM ($n = 4$). Same letter indicates no significant difference between the groups.

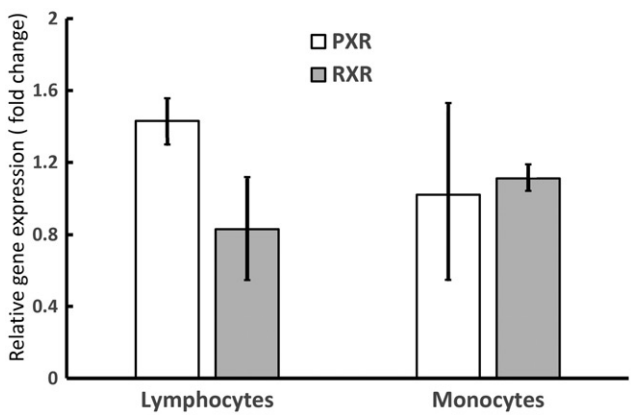


Figure 4. AFB_1 fails to enhance the transcription of *PXR* and *RXR* genes in monocytes and lymphocytes. The cells were treated with AFB_1 (10 ng/ml) for 2 h. The relative expression level of each gene in control for both monocytes and lymphocytes was 1 according to explanation describes in methods. All data were normalized with *ACTB* as an internal control. The data are presented as the mean ± SEM ($n = 4$).

AFB_1 could arrest immune cells particularly lymphocytes growth in G2/M phase. As shown in Figure 5, neither low dose nor moderate dose of AFB_1 could affect on apoptosis and viability of lymphocytes and monocytes, except on lymphocytes with moderate dose of AFB_1 (Figure 5e).

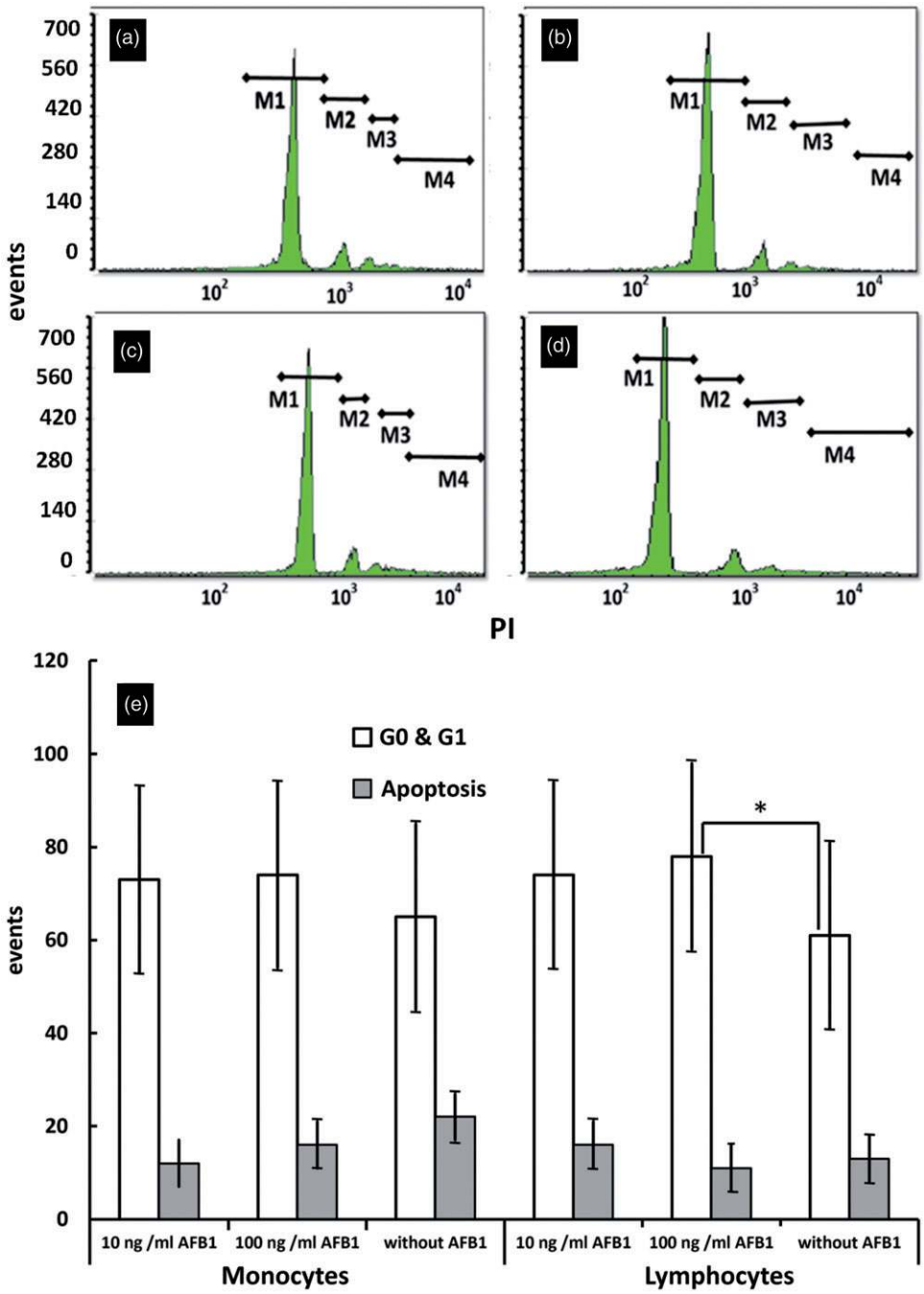


Figure 5. Flow cytometric analyses of cell cycle distribution of monocytes and lymphocytes after exposure to AFB_1 for 2 h. The four histograms representatively show the percentage of (a) monocytes without AFB_1, (b) lymphocytes without AFB_1 (c) monocytes exposed to 100 ng/ml of AFB_1, (d) lymphocytes exposed to 100 ng/ml of AFB_1. G0–G1, S and G2/M are M1, M2 and M3 plus M4 phases of cell cycle, respectively. Before permeabilization of the cells, formaldehyde was used to inhibit leakage of small mass of DNA fragments from the nucleus and only fragmented apoptotic DNA were included as DNA content. (e) Representative data from monocytes and lymphocytes exposed to 0, 10 and 100 ng/ml of AFB_1. AFB_1 remarkably arrested G0/G1 phases only in lymphocytes-exposed to high dose (100 ng/ml) of AFB_1; values are (mean ± SEM) of four individuals; $*p < 0.05$.

Discussion

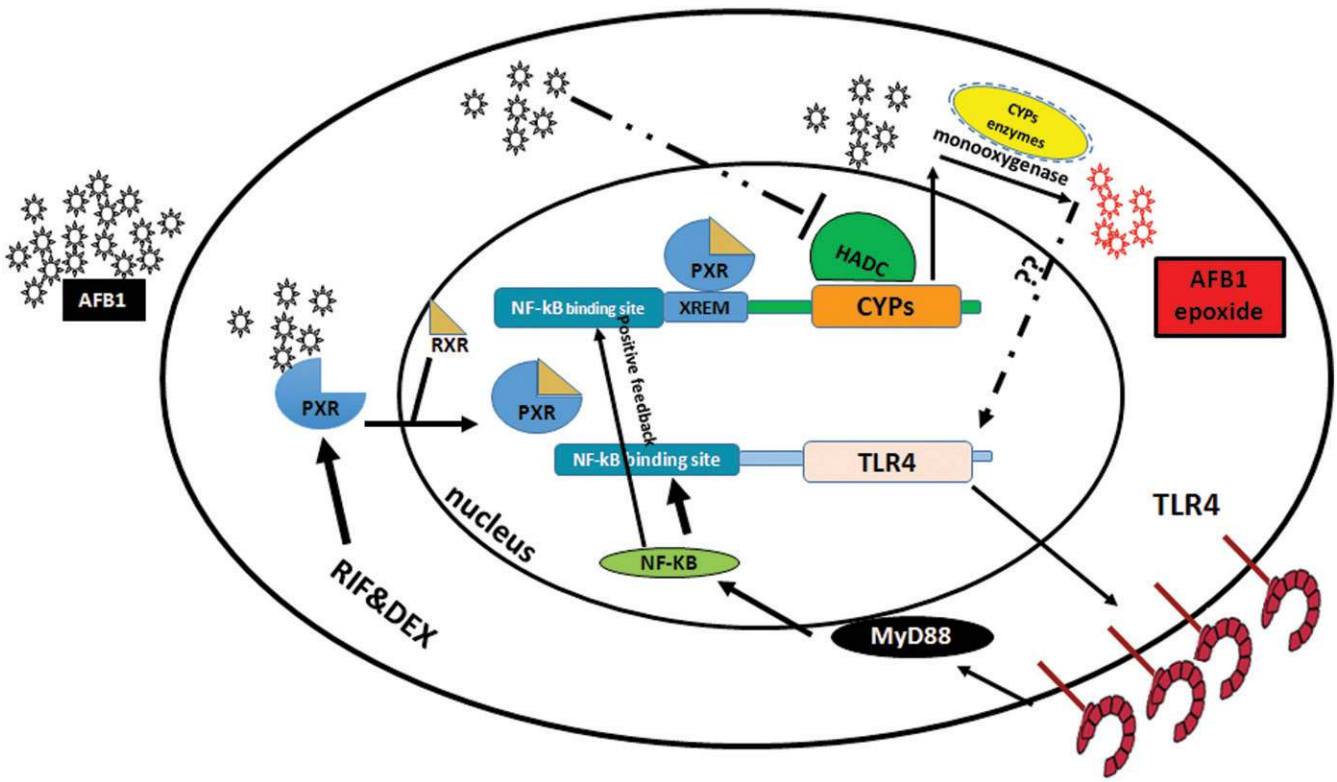
We showed for the first time that the environmentally relevant doses of AFB_1 in the presence of some *CYP*s inducers can change transcription patterns of two families of *CYP*s; we also demonstrated that the induction of various *CYP* enzymes' mRNAs by low and moderate doses of AFB_1 differently occurred in monocytes as compared with in lymphocytes. Indeed, there was the difference in toxic reaction against AFB_1 between lymphocytes and monocytes. *TLR4* was applied as a potential biomarker for AFB_1 exposure in the most accessible human circulating cells, lymphocytes and monocytes. In lymphocytes and monocytes, after detection of some key *CYP*s isoforms with RT-PCR, we first showed transcripts of seven members of *CYP* family 1 and 3, including *CYP1A1*, *CYP1B1*, *CYP3A4*, *CYP3A5*, *CYP3A7* and *TLR4* in both lymphocytes and monocytes. Since *CYP* isoforms are responsible for the metabolism of AFB_1 activation in liver, lungs and kidneys[11,32–35], we thus used liver cell line, HepG2, as a control for expression of the

targeted *CYPs*, which were highly detectable. Among transcripts of studied *CYP* family, *CYP3A43* and *CYP1A2* isoforms were detectable only in HepG2. Zero expression of *TLR4* in HepG2 was not surprising.

CYP1A1, *CYP1A2*, *CYP3A4* and *CYP3A5* play major role in AFB_1 epoxidation especially in hepatocytes[10,35]. We provided novel data on up-regulated *CYP1A1*, *CYP3A4* and *CYP1B1* transcripts in monocytes and *CYP1A1* in lymphocytes exposed to very low dose of AFB_1. The observed remarkable increase in *CYP1A1* transcripts in low dose of AFB_1-exposed human lymphocytes and monocytes is consistent with reports in other animals[36].

Although *CYP3A4* is key contributor to AFB_1 activity in human hepatocytes[12,37] in the lymphocytes and monocytes the pattern was different. Even between the lymphocytes and monocytes we observed different behaviors of *CYP*s to AFB_1 exposure. Indeed, *CYP1A1* and even *CYP1B1* had more response to low doses of AFB_1 in monocytes, but their expression in lymphocytes was insignificant. Based on our previous knowledge, AFB_1 up-regulates *TLR4* expression in human leukocytes[28], we therefore used *TLR4* as an indicator of AFB_1-dependent toxicity in lymphocytes and monocytes. The underlying mechanisms of AFB_1-induced *TLR4* up-regulation are not yet understood. One of the possible pathways of triggered *TLR4* in AFB_1-exposed lymphocytes and monocytes is the involvement of MyD88 signaling pathway[14,28] and therefore it is possible that activated form of AFB_1 could effect on downstream pathways of MyD88 such as interleukin-1 receptor-associated kinase 4 (IRAK4), TRAF6, IκB kinase (IKK) and nuclear factor kappa B (NF-κB) (Scheme 1).

The observed more pronounced increase in *TLR4* transcripts in monocytes, compared with lymphocytes, with and without AFB_1 exposure would be due to: (1) *TLRs* are expressed in multiple tissues predominantly in innate immune cells (myeloid lineage cells) especially monocytes[38], leading to the production of soluble and insoluble innate immune molecules and (2) higher expression of *CYP1A1* and *CYP1B1*



Scheme 1. Diagrammatic representation of predicted impact of AFB_1 with/without well-known *CYPs* inducers (DEX and RIF) on transcription of *CPY* isoforms in lymphocytes and monocytes as well as the consequences of the effects. With its potential lipophilic properties, AFB_1 rapidly enters the leukocytes; in the cytoplasm it potentially binds to xenobiotic sensing nuclear receptors like *PXR*, leading to translocation of *PXR* to the nucleus and attaching to the XREM at the upstream region of *CYPs*. This binding results in increased transcription and translation of *CYP* isofomrs. With the strong oxidation capacity of *CYPs*, AFB_1-epoxide is formed. The AFB-epoxide potentiates transcription of *TLR4* and MyD88, via unknown pathways, thus leading to NF-κB activity. The NF-κB possesses specific activation sites on both *CYPs* and *TLR4* promoters in lymphocytes and monocytes. Activation of these promoters potentiates transcription and translations of those targeted *CYPs* genes. Additionally, RIF and DEXA, via the same pathways, strongly bind to *PXR*, potentiating the transcription and translation of *CYPs* isoforms. RIF and DEX mainly activate *CYP3A4* and *CYP1A1*, respectively. In this study, we observed that both in lymphocytes and monocytes *CYP1A1* was predominant AFB_1 activator; furthermore, in monocytes *CYP1B1* and *CYP3A4* also predominantly contributed to AFB_1 toxicity/activator as well. Another potential pathways of AFB_1 toxicity is via inhibition of HDAC to *CYPs*, potentiating transcription of *CYPs*, thereby synergizing AFB_1 toxicity. Thickness of the arrows represents higher AFB_1 toxicity/activity. Hepatocytes, which express almost all *CYPs* but not *TLR4*, compared with leukocytes; the pathways depicted in lymphocytes and monocytes shed more light and encourage scientists to find broader toxicity assessment of AFB_1 on immune cells and molecules. We propose a detailed proteomic analyses in accordance to this scheme to deepen the molecular mechanisms of AFB_1 activation and deactivation in leukocytes and hepatocytes. This scheme, partially adapted from references[9,27–29,40,46,47] as well as some of our unpublished findings, is from our understanding of how broadly AFB_1 affects vital molecules inside and outside the immune cells. These events other than oxidative stress could trigger AFB_1-associated immunotoxicity in mammals' leukocytes.

causes more AFB_1 peroxidation in monocytes than in lymphocytes. Other research shows that lower expression of *CYP1A2* than *CYP3A4* in human hepatocytes is compensated with higher AFB_1 affinity to *CYP1A2*[39], underscoring dominant role of *CYP1A2* in activation of AFB_1 at the submicromolar level. On the other hand, though *CYP1A2* is constitutively highly expressed in hepatocytes, but it is originated from highly identical *CYP1A1*. Also, both *CYP1A1* and *CYP1A2* are oriented head to head containing bidirectional promoter; therefore, considering the similar role of *CYP1A1* and *CYP1B1* in AFB_1 metabolism is indisputable[40]. Some reports show CYP1A1 is the most critical CYPs in monocytes and macrophages[21]. But our study clearly shows *CYP1A2* far higher up-regulated than *CYP1A1* in AFB_1-exposed monocytes and lymphocytes. Also, there is positive correlation among *CYP1A1* induction and aryl hydrocarbon receptor (AhR) and transcriptional factors such as, ARNT and NFkB[24,41]. Thus, more sensitivity of monocytes to low dose of AFB_1 for *CYP1A1* and *CYP1B1* observed in our study can lead to almost 10-fold up-regulation of *TLR4*. We only exposed the cells under a single time point and two doses; to further confirm, it is worth examining the dynamics effects of AFB_1 with various doses. Since a kind of balance between induction and degradation of different *CYP* enzymes expressed in monocytes and lymphocytes by AFB_1 is apparent, it is worth testing the effect of *CYP* inhibitors and siRNA on quantification of AFB_1 metabolites.

Expression of three *CYP*s (*CYP1A1*, *CYP1B1* and *CYP3A4*) and *TLR4* in monocytes showed different patterns with 10 and 100 ng/ml AFB_1 with and without RIF and DEX. Among transcriptional factors, *PXR* is major mediator of AFB_1-induced *CYP*s expression[9,42]. Though the expression of *PXR* and *RXR* genes in AFB_1-treated monocytes and lymphocytes was slight, sometimes a small change on such major genes might have huge effects. Examining post-transcriptional modifications of *PXR* and *RXR* in AFB_1-treated mononuclear cells is therefore valuable. DEX and RIF are potent transcriptional activator of *CYP*s, particularly *CYP3A4*, with binding to *PXR*[43]. We showed there were no significant differences in increasing in *CYP1A1* expression between the treatment with 10 or 100 ng/ml of AFB_1 plus RIF and/or DEX in monocytes. This finding is consistent with other report[39]. Thus, low dose of AFB_1 could play as *CYP1A1* inducers in monocytes, which can be important in drug metabolism in leukocytes, particularly in people living in high AFB_1-exposure area. The observed up-regulation of *CYP1A1* transcript induced by AFB_1 was similar to the effects of dioxin on *CYP1A1* in human leukocytes[44].

Among studied *CYP*s in monocytes, only *CYP3A4* maximally responded to only 100 ng/ml of AFB_1. This is supported by other study in hepatocytes[9]. The observed AFB_1-exposed *CYP*s up-regulated in lymphocytes and monocytes can be due, in part, to the conformational change on *PXR* caused by AFB_1 and its translocation into the nucleus[9,45–47], potentiating *PXR* transcriptional activity. Furthermore, other potential pathways of simultaneous up-regulation of *PXR* and *CYP* genes and thus AFB_1 toxicity resulted from inhibition of histone deacetylases (HDAC) activity on *CYP* genes; this will enhance transcription of *CYP*s, thereby synergizing AFB_1 toxicity observed in this study[45] (Scheme 1). Some known phenomena related to *CYP1A1* over-expression would be due to the fact that it is highly inducible with AhR, further augmenting AFB_1 toxicity[9,46].

We focused another key innate immune molecule, *TLR4*, in lymphocytes, monocytes and HepG2 as a marker for the *in vitro* effects caused by AFB_1 on the *CYP* genes. Although *TLR4* over-expressed about five-fold in AFB_1-treated lymphocytes and monocytes, but the pattern of over-expression surprisingly differed from those observed in *CYP* genes. These results led us to exclude *TLR4* expression as interpretable biomarkers of AFB_1-dose-dependent-cell toxicity and *CYP* inducers. This needs deeper comparative works on other enzymes with monooxygenase activity. Further the issue of experimentation on *CYPs* isoforms is their polymorphical properties, which dramatically interfere with *CYPs* transcription[10,11], thus requiring quantitative proteomics analysis to ascertain functional consequences of the AFB_1-induced effects. Though we have done some previously unpublished works on the impact of very low dose of AFB_1 on transcriptomics and proteomics aspects of *TLRs*, we propose detailed proteomic analyses in accordance to Scheme 1 to deepen the molecular mechanisms of AFB_1 activation and deactivation in leukocytes and hepatocytes and to eventually find a new biomarker for AFB_1 exposure in humans and animals. In our focused *in vitro* model metabolism-independent effects of AFB_1 might have been occurred, and use of *in vivo* model with CYPs-mediated transformation of AFB_1 is therefore warranted to better relate our observed *in vitro* results on *CYPs*, *TLR4* and cell cycles in human immune cells. Generally, AFB_1 instantly affects DNA in any cells *in vivo*[25,34,39], which promotes risk of cancer; it also impairs redox status of immune cells[26], boosting auto-inflammation and risks of many infectious-and-non-infectious diseases, especially cancer.

Despite of dramatic increased in *CYPs* transcription caused by AFB_1 and subsequently more oxidative stress in lymphocytes and monocytes, cell cycle of monocytes changed little. The lymphocytes and monocytes are hugely different in terms of their lineage and function, and in spite of their different properties, the impact of the AFB_1 on their division differed only when 100 ng/ml of AFB_1 was used (the cell division was arrested at G0 phase and did not follow S phase); that is why AFB_1-exposed animals have inappropriate Ab production[27,48] and inadequate Ag-presentation and T-cell polarization (unpublished observation), which is a kind of broad AFB_1-inducec immunotoxicity. Further research is needed to pinpoint the relationship between *CYPs* transcript and toxic effects of AFB_1, including *TLR4* expression and cell cycle arrest in immune cells.

In short, *CYP* isoforms are up-regulated in human lymphocytes and monocytes in response to AFB_1 exposure. Our novel findings indicate that the key *CYP* isoforms, *CYP1A1*, *CYP1B1*, *CYP3A4*, *CYP3A5* and *CYP3A7* as well as *TLR4* in human lymphoid and myeloid cell lineages differently respond to different levels of AFB_1 and its activators. Compared with lymphocytes, the AFB_1-exposed monocytes seem more intensively responsive especially for isoforms *CYP1A1*, *CYP1B1* and *CYP3A4*. Also, since *PXR* is the key regulator of CYP3A induction, proteomics analyses of *PXR* to confirm *CYPs* regulation in lymphocytes and monocytes is valuable. Mechanistically, this could explain a far more potent

immunotoxicity of AFB_1 in myeloid than in lymphoid lineage cells *in vitro/situ/vivo*. The little synergy between enhanced transcripts of the key *CYP* isoforms and *TLR4* is interestingly surprising and needs more detailed genomic and proteomic analyses in accordance to the proposed Scheme 1.

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Declaration of interest

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