SHORT COMMUNICATION

Biologically relevant doses of mixed aflatoxins B and G up-regulate MyD88, TLR2, TLR4 and CD14 transcripts in human PBMCs

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Abstract

Context: Aflatoxins (AFs) are highly hazardous carcinogenic mycotoxins originated from very common fungi present in the environment. Their effect on key immune-surveillance molecules is unclear.

Objective: We aimed to examine the effect of mixed AFs on immunologically relevant molecules and on viability in human peripheral blood mononuclear cells (PBMCs), in conditions similar to those occurring naturally, i.e. using a mixture of environmentally relevant levels of AFB1, AFB2, AFG1, and AFG2.

Materials and methods: We evaluated the mRNA expression of MyD88, toll-like receptor (TLR)-2, TLR4 and CD14, in human PBMCs treated with a mixture of AFB1, AFB2, AFG1, and AFG2 at different doses for 2, 12 and 24 h. We used qRT-PCR to assess changes in transcripts of MyD88, TLR2, TLR4 and CD14 in PBMCs. We also evaluated the viability of PBMCs exposed to AFs.

Results: Biologically relevant levels of mixed AFs elicited early immune modulation in human PBMCs. qRT-PCR results showed several folds increase of MyD88, TLR2, TLR4 and CD14 transcripts in PBMCs as early as 2 h post-exposure to mixed AFs. Kinetics and dose–response of the up-regulation differed for mentioned gene transcripts. Further, prolonged exposure to mixed AFs decreased PBMCs viability.

Conclusion: Immunoxicity of AFs on PBMCs may be mediated by up-regulation of key immune-surveillance molecule transcripts. The description of these effects induced by AFs on PBMCs are novel and should be taken into account when considering AF-related infectious and noninfectious diseases in areas highly exposed to AFs.

Introduction

There has been a huge interest in defining the role of pattern recognition receptors (PRRs), in immunity, inflammation and cancer1. Toll-like receptors (TLRs), the best characterized PRRs, have been extensively investigated in terms of cell stimulation and survival, oxidative stress, inflammation, immunotoxicity and cancer. Exposure of immune cells to environmental toxins likely initiates unnecessary inflammation through PRRs stimulation2–5. Even in our super-sanitized environment, many potential carcinogenic mycotoxins are still present, in particular, aflatoxins (AFs)6–8. These potent environmental carcinogens are naturally produced by Aspergillus sp. and contaminate feed and food, thus hugely affecting public health. Structurally, AFs are classified according to their ability to emit fluorescence under UV light. Depending on the wave length, those reflecting blue and green lights are called AFB (e.g. AFB1, AFB2) and AFG (e.g. AFG1 and AFG2), respectively9. All these AFs are found in common agricultural products, even as aerosol. Once uptaken, other highly reactive metabolites of AFs are formed (i.e. AFM, aflatoxical, etc.), causing even more oxidative stress in immune cells2,8.

It is widely assumed that both animals and humans consume a mix of different AFs by fungal contaminated feed and foods2,6. Consequently, meat and dairy products consumption may be related, in humans, to increased exposure to AFs; this might be more relevant in developing countries2,6. We have recently shown that exposure to even very low dose of AFs disrupts redox balance and causes homoeostasis alteration in key circulating innate immune cells1 and thus weakens public health. There is poor knowledge about the effects of naturally occurring levels of mixed AFs (mAFs) on the key molecules of the innate immune system in humans. To mimic natural exposure of the immune system to mAFs in humans, we prepared a mixture of environmentally relevant
levels of AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ and investigated their impact on human peripheral blood mononuclear cells (PBMCs), containing both lymphoid cell lineage, (i.e. B and T lymphocytes) and myeloid cell lineage (i.e. monocytes). We determined cell viability and measured the transcription of some key immune-surveillance molecules, such as MyD88, TLR2, TLR4 and CD14, by real-time quantitative reverse transcription PCR (qRT-PCR). Our results contribute to better understanding the impact of environmentally relevant levels of mAFs on general health.

**Methods**

Blood samples were collected by sterile venipuncture from 10 healthy young men (aged 20–25 years); homogenous male individuals were used to increase results consistency. PBMCs were isolated from heparinized blood by lympholyte®-H (Cederlane Laboratories Ltd., Zierikzee, The Netherlands) through gradient centrifugation, re-suspended in RPMI-1640 supplemented with 10% fetal calf serum (FCS) 1% penicillin/streptomycin (10,000 U/μl) HEPES buffer and 1-glutamine, all from Gibco (Carlsbad, CA), and 2 × 10$^6$ cells/ml were seeded in polystyrene 24-well plates (JET (Guangzhou) Bio-Filtration Products, Co. Ltd, China).

AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$ (mAFs) were separately purchased from Sigma-Aldrich Chemie (Saint Louis, MO); all of them were pure and free of lipopolysaccharide (LPS) and other chemicals. They were first separately dissolved in 96% ethanol (0.1 mg/ml). Further dilutions were made with sterile endotoxin-free Dulbecco’s PBS (DPBS) (Gibco, Carlsbad, CA). The isolated PBMCs were then exposed to mAFs (mix of AFB$_1$ 0.5 ng/ml, AFB$_2$ 0.25 ng/ml, AFG$_1$ 0.125 ng/ml and AFG$_2$ 0.125 ng/ml) or double concentration (mix of AFB$_1$ 1.0 ng/ml, AFB$_2$ 0.5 ng/ml, AFG$_1$ 0.25 ng/ml and AFG$_2$ 0.25 ng/ml) for 2, 12 and 24 h. All procedures were done in sterile conditions under biosafety level II laminar flow.

Total RNA was extracted using TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer instruction. After treatment with DNase I kit (Fermentas, Vilnius, Lithuania), RNA was quantified using NanoDrop 3300 (Thermo scientific, Wilmington, DE). A quantity of 1 μg total RNA from each sample was used as template for the RT-PCR assays. cDNA was routinely synthesized using oligo-dT primers and M-Mulv reverse transcriptase (Fermentas, Vilnius, Lithuania). Briefly, cDNA (2 μl) was reacted with 250 mM of dNTPs, 1x reaction buffer (Fermentas, Vilnius, Lithuania), forward and reverse 10 μM primers (Table 1) and 0.4 U Taq polymerase (Fermentas, Vilnius, Lithuania) in a 25-μl final reaction volume. PCR reactions were performed with 35 cycles using annealing temperatures of 51–56°C (depending on primers). PCR products were also visualized under UV gel documentation (Biorad, Hercules, CA) after running on 2% agarose gel (Merek, Darmstadt, Germany).

Using the same primers (Table 1), the qRT-PCR was performed by Absolute™ QPCR SYBR® Green Mix kit (Thermo Fisher Scientific, Illkirch-Cedex, France) according to manufacturer’s instruction on the Swift SpectrumTM 48 Real Time Thermal Cycler PCR machine (Esco Micro Pte Ltd, Singapore). The synthesized cDNA from all 10 individuals were heated at 95 °C for 10 min, and then subjected to 44 cycles of amplification by melting at 95 °C and annealing at 51–56 °C (depending on the primers) for 1 min. Experimental samples were run in duplicate. To check for amplicon contamination, reaction mixtures without cDNA (RT-minus) and non-template control were always conducted in triplicate for each probe. Data were normalized using GAPDH and transformed using the comparative Ct method$^{11}$. The relative quantification of gene expression changes (2$^{-ΔΔCt}$ and/or folding) were then calculated using the formula: $\Delta \Delta C_t = \Sigma C_{(C_{GOI} - C_{HKG})_{control}} - (C_{GOI} - C_{HKG})_{AFs-treated}$, where GOI is gene of interest, HKG is the housekeeping gene and 2$^{-ΔΔC_t}$ is the fold-change of the mAFs-treated GOI expression relative to that in non-treated PBMCs.

Next, to evaluate the viability of mAFs-exposed and non-exposed or control PBMCs in cell culture dishes, we performed trypan blue exclusion assay$^{12}$ by counting the cells with a hemocytometer.

Results were expressed as the mean ± standard error of mean (SEM). Statistical differences were determined using Student’s t-test for differences between the two groups and one-way analysis of variance with Tukey assay for multiple comparisons. The significance level was set at $p<0.05$. All tests were performed using PASW18 statistical software (IBM Corp., Armonk, NY).

**Results and discussion**

We examined key PRRs expression in PBMCs exposed to very low doses of mAFs, similar to those that may be obtained by assumption of environmentally contaminated feed and food in Europe or North America as well. Based on the chemical structure and properties of AFs group B and G, they can easily pass through plasma membrane and convert immediately into

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<thead>
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<th>Table 1. Primer sequences, annealing temperature of primer sets, expected PCR fragment sizes and accession numbers.</th>
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<tr>
<td><strong>Gene symbol</strong> (Acc number)</td>
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Acc, accession numbers; T$_m$, annealing temperature of primer sets.
their highly oxidative epoxides, aflatoxicol, AFM1 and AFM2 in the body, binding to proteins, DNA and RNA, causing oxidative stress to PBMCs, thus disturbing physiological process of leukocytes. Therefore, inflammation and oxidative stress tend to be sequential states of mAFs-exposed PBMCs. As shown in Figure 1, in PBMCs exposed to a low dose of mAFs, we detected a significant increase in the gene expression of both TLR2 (Figure 1, left panel, upper right) and TLR4 (Figure 1, left panel, lower left) (p < 0.001) as early as 2 h after treatment. After 12 h the expression of TLR2 and TLR4 was still very high although decreased as compared to that after 2 h. At 24 h after mAFs stimulation, TLR2 and TLR4 gene expression increased over the 12 h levels (significantly in the case of TLR4), but not reaching the levels of 2 h (Figure 1, left panels). In general, TLR4 fold increase was higher than that of TLR2.

To confirm the effect of mAFs on TLR2 and TLR4, we performed gene expression quantification for other molecules involved in TLRs action and signaling. We chose CD14 for its cooperation with TLR4, and MyD88 as a key protein of TLRs signaling in immune cells. Results revealed that CD14 (Figure 1, left panel, lower right) expression level, increased steadily starting 2 h after mAFs exposure till 24 h post-treatment. MyD88 (Figure 1, left panel, upper right) was also significantly over-expressed at 2 h post-treatment and the over-expression consistently increased after 12 and 24 h of PBMCs exposure to mAFs.

We thus observed several folds over-expression of MyD88, TLR2, TLR4 and CD14 genes after only 2 h of PBMCs exposure to mAFs. TLR2 and TLR4, and similarly CD14 and MyD88, are transcribed in response to the oxidative conditions caused by mAFs. TLRs recognize their ligands with cooperation of other surface proteins such as CD14 and MD2; e.g. LPS recognition efficiently occurs after heterodimerization of TLR4 with MD2 and with CD14. Compared to TLR2, TLR4 is more responsive to mAFs-induced stimulation. CD14 and MyD88 on the other hand, constantly increase during the first 24 h after mAFs stimulation. Our findings highlight that mAFs may act as danger signals similar to those triggering TLRs activation.

We next challenged PBMCs with a double dose of mAFs corresponding approximately to what may be found in contaminated foods such as nuts, pistachio, hazelnuts, fig, spice, corn and copra, etc. Experimental procedure was the same as for the first (lower) dose. We found that doubling the dose of mAFs, although this concentration of mAFs is classified as a low concentration, also resulted in over-expression of MyD88, TLR2, TLR4 and CD14 genes in the PBMCs after 2 h of exposure, but at lower levels than the first dose. The pattern of over-expression was in a descending direction, especially for TLR2 and TLR4 (Figure 1, left panel, upper left and lower left). For TLR2, the over-expression level was significantly less than that of first dose both at 2 and 12 h post-treatment, but surprisingly, TLR2 in mAFs-exposed
PBMCs was significantly down-regulated at 24 h post-exposure (Figure 1, left panel, upper left). For TLR4, CD14 and MyD88, the data showed a relatively similar descending over-expression during 24 h post-mAFs exposure and their expression levels were not down-regulated compared to control group. Similar to the first dose, the over-expression of these genes in PBMCs was significantly different from those in non-treated PBMCs. The aim of using duplicated concentration of mAFs was from our and others’ recent reports indicating that even at very low concentrations of AFs, increase in concentrations will impose more oxidative stress in the cells. In other words, if the detected over-expression is only due to oxidative stress, we would likely detect increase in gene expression of TLRs in the second dose as well. Interestingly, we detected less pronounced over-expression compared to the first dose. This might be due to poisoning the PBMCs contributing to the decrease of cell viability which is discussed bellow. Nonetheless, the observed response in PBMCs’ immune-surveillance molecules induced by mAFs would unequivocally lead us to consider AFs B and G as danger signals in the blood stream.

Modulation of transcription of MyD88, a key adaptor protein for downstream TLR signaling pathways, in mAFs-treated PBMCs observed in our study reveals pro-inflammatory status of the cells. All TLR (except TLR3) pass their signals to nucleus via MyD88-dependent cascade. Once a TLR is activated by its ligand(s), specific repertoire of the toll/interleukin-1 receptor domain adaptors like MyD88 can result in activation of a downstream pathway like NF-κB and eventually lead to secretion of pro-inflammatory cytokines or cell proliferation regulation. Thus, it is indisputable that any change in MyD88 gene expression is associated with change in TLR responses.

The viability of PBMCs was unchanged in nontreated groups; in contrast, the viability of PBMCs exposed to mAFs decreased remarkably (Figure 1, right panel, lower). The increased viability of PBMCs was significant (p<0.01) at 24 h post-mAFs exposure for the first dose, and 12 and 24 h post mAFs exposure of the second dose, i.e. doubling the first dose. Prolonged over-expression of TLR2/TLR4 transcripts in the first dose and TLR4 in the second dose of mAFs till 24 h indicates some other contributing factors to triggering the over-expression of the stated immunosensor molecules. One possible mechanism is the response to damage-associated molecular patterns (DAMPs) released form necrotic PBMCs. In this case, there are some reports showing the ability of mAFs for induction of apoptosis/necrosis and DNA damage in some cell types. In this study, we used PBMCs, which contain mainly a heterogeneous population of T and B lymphocytes and monocytes. The PBMCs are sensitive to abnormal conditions by showing apoptosis and DNA damages. When PBMCs are exposed to mAFs, they start becoming necrotic. This might be due to oxidative metabolism of AFs and PBMCs damages (i.e. DNA damage). It is therefore conclusive that mAFs possess potentially necrotic/apoptotic properties for human PBMCs.

The different responses of TLR2 and TLR4, CD14 and MyD88 might be due to the fact that TLR2 plays a role in controlling cell viability/death. It is also supported by our data especially at 12 h post-treatment with the second dose.

Moreover, detected down-regulation of TLR2 and CD14 transcripts at 24 h is consistent with other recent reports in post-AFs-exposed murine macrophages. Consistently, our results clearly demonstrated that human PBMCs could sense the presence of mAFs and immediately turn to the pro-inflammatory condition.

Altogether, our novel findings clearly showed that even environmentally relevant levels of AFs could disrupt gene expression of immune-surveillance molecules in human PBMCs. The outcome of these immunotoxic effects can have a huge implication in our super-sanitized environment and public health.

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**Declaration of interest**

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

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**References**


