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Aflatoxin B₁ modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats

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Abstract

Aflatoxin B₁ (AFB₁) is immunotoxic to animals and a suspected immunosuppressant in humans. In this study, we investigated the effects of AFB₁ on splenic lymphocyte phenotypes and the inflammatory cytokine expression in male F344 rats. Exposure of animals to AFB₁ (5–75 µg/kg body weight) for 1-week showed dose-dependent decreases in the percentage of splenic CD8⁺ T cells and CD3⁺CD8a⁺ NK cells. A general inhibition of the expression of IL-4 and IFN-γ by CD4⁺ T cells, IL-4 and IFN-γ by CD8a⁺ cells, and TNF-α expression by NK cells was also found; however, no concurrent histological changes in spleen tissue were present, suggesting acute immunosuppression without overt toxicity. Five-week exposure with AFB₁ significantly increased the percentages of CD3⁺ and CD8⁺ T cells, especially at low doses (25 µg/kg). AFB₁ treatment significantly decreased the anti-inflammatory cytokine IL-4 expression by CD4⁺ T cells and significantly increased the pro-inflammatory cytokine IFN-γ expression by CD4⁺ T cells and TNF-α expression by NK cells. These results indicated that repeated AFB₁ exposure promotes inflammatory responses by regulating cytokine expression. Our data provides novel insights into the mechanisms by which AFB₁ exposure differentially modulates the cell-mediated immune responses and suggests the involvement of an inflammatory response upon repeated exposure.

Keywords

aflatoxin B₁; spleen; lymphocyte; cytokine; surface marker expression

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INTRODUCTION

Aflatoxins (AFs) are produced predominately by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. AFs are frequent contaminants of food staples, such as maize and groundnuts, and AF exposure is a significant public health issue in Southeast Asia and Sub-Saharan Africa (Kensler *et al.*, 2011; Wogan *et al.*, 2012). Aflatoxin B₁ (AFB₁) is the most potent aflatoxin that is activated in the liver to its reactive metabolite, aflatoxin B₁-8,9-epoxide. This metabolite can rapidly bind with biological macromolecules such as DNA and protein to exhibit adverse effects in animals and humans (IARC, 1993). The immune toxic effects of AFB₁ have gained great attention due to chemical-viral interactions in the developing world where such co-exposure is common (Jiang *et al.*, 2008).

Mycotoxins, especially AFB₁ and trichothecenes, have been shown to affect the immune system in domestic and laboratory animals (Bondy and Pestka, 2000). A considerable amount of evidence from animal studies has demonstrated that AFB₁ possesses immunosuppressive activities (Bondy and Pestka, 2000; Sharma, 1993). Cell-mediated immunity is a particular target affected by AFB₁ exposure. AFB₁ decreased splenic helper T cells and antibody response to sheep red blood cells (SRBC) in C57BL/6 mice (Hatori *et al.*, 1991), and inhibited production of IL-2, IL-3, and IFN- γ by splenic macrophages (Dugyala and Sharma, 1996). AFB₁ inhibited murine macrophage function with regard to phagocytosis and production of nitric oxide and TNF- α both *in vivo* and *in vitro* (Moon *et al.*, 1999a, b). Dietary exposure of rats to AFB₁ (0.01-1.6 ppm) produced inhibitory effects on splenic T and B lymphocytes as well as IL-1, IL-2, and IL-6 production by splenocytes (Hinton *et al.*, 2003). The mechanisms behind the impaired cell-immunity have been proposed to involve the inhibition of gene expression (Dugyala and Sharma, 1996; Reddy and Sharma, 1989) and the installment of the cell cycle at G₂/M (Scott *et al.*, 1991).

The observation of AFB₁-induced inhibitory effects on cytokines in particular, however, deserves careful examination because of the inconsistent findings from different reports. For example, AFB₁ exposure has shown stimulating effects on the expression of pro-inflammatory cytokines such as TNF- α , IFN- γ , and IL-6 in pigs (Meisssonier *et al.*, 2008) while the inhibition of pro-inflammatory (IL-1 β , TNF- α) and stimulation of anti-inflammatory (IL-10) cytokine mRNA expression by AFB₁ exposure had also been reported in pigs (Marin *et al.*, 2002). Even in a single feeding assay, AFB₁ containing diets show differential effects based on treatment duration and doses (Hinton *et al.*, 2003). The model (spleen vs. white blood cells, e.g.) and dose selection and differences in exposure duration might play a role in the final outcomes.

Currently available animal data are mainly focused on the overall cytokine production from a cell mixture. The relevance to the cell-specific function is not known, due to the fact that these cytokines can be produced by various cells (Elser *et al.*, 2002; Kurokouchi *et al.*, 1998). There exists a lack of data on how AFB₁ affects cell-specific cytokine expression in animal studies. Whether AFB₁-induced histological changes in liver and spleen are related to modulatory effects on splenic lymphocyte subsets and cell-specific cytokines are largely unknown. Aims of this study are to demonstrate the modulatory effects of AFB₁ on splenic lymphocyte phenotypic markers and cell-specific cytokines in F344 rats. This study also

holds the potential to provide evidence for immunosuppressive effects for future human epidemiological studies.

MATERIALS AND METHODS

Materials

Aflatoxin B₁ and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc (St. Louis, MO). Antibodies of CD28 and CD3 and fluorescence conjugated antibodies, including CD3 phycoerythrin (PE) clone G4.18, CD4 allophycocyanin (APC) clone OX-35, CD8 fluorescein isothiocyanate (FITC) clone OX-8, CD8a peridinin chlorophyll protein complex (PERCP) clone OX-8, CD45RA phycoerythrin cyanin 5 (PE-Cy5) clone OX-33, IL-4 PE clone OX-81 and INF- γ FITC clone DB-1 were purchased from BD Biosciences (San Jose, CA). Fix/perm and Perm/Wash Buffer were also purchased from BD Biosciences (San Jose, CA). TNF- α FITC clone TN3-19 antibody was purchased from Abcam (Cambridge, MA). Phorbol 12-myristate 13-acetate (PMA) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium was purchased from ATCC (Manassas, VA). All other solvents were of the highest grade available.

Animals

Male F344 rats (five weeks old, 120-130g) were purchased from Harlan Laboratory (Indianapolis, IN). Animals were individually housed under a 12 h/12 h light/dark cycle at 22 $\pm$ 2°C. Purified AIN 76A diet and tap water were provided *ad libitum*. Animals were acclimatized for 1-week before treatment. Animal care and use and the animal protocol were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

Experimental Design

Male F344 rats were randomly allocated into four groups: control, 5, 25, and 75 μ g AFB₁/kg body weight (BW). Rats (5 per treatment group) were gavaged daily with control vehicle (DMSO) or AFB₁ in DMSO (50 μ L/100 g BW) for a duration of 1-week or 5-weeks. Animals were dosed five days per week and were sacrificed at 24 h following the last dose. Upon euthanasia, spleens were aseptically removed and a single cell suspension prepared, which was used for cell-surface marker and intracellular cytokine staining. The parameters tested in this study are shown in Table 1. The selection of these markers was based on previous studies (Liu *et al.*, 2010; Morris and Komocsar, 1997).

Serum Aflatoxin B₁-lysine Adduct (AFB-Lys) Measurement

To monitor AFB₁ exposure, the levels of serum AFB-Lys were measured from individual animals at each time point. The method was adopted as previously reported (Qian *et al.*, 2010). Briefly, 150 μ L serum was digested with pronase (pronase:total protein, 1:4, w:w) for 3 h at 37°C. The digest was further purified by a solid phase cartridge, evaporated, reconstituted, and injected into an Agilent 1200 HPLC system for quantification by fluorescence signal. Excitation and emission wavelengths were 405 and 470 nm. The limit of detection is 0.4 pg/mg albumin. Serum AFB-Lys adduct level was adjusted by serum albumin content.

Single Splenic Cell Suspension Preparation

The procedure for preparing a single splenic cell suspension was modified as previously reported (Kizaki *et al.*, 1991). Approximately one half of the spleen tissues taken at necropsy were minced into 1 mL cold complete RPMI-1640 medium (RPMI-1640 containing 2 mM L-glutamine (ATCC), supplied with 10% FBS and 100 IU/mL Penicillin and 100 IU/mL streptomycin), then gently mashed through a 70 nylon mesh cell strainer and collected into 5 mL cold complete RPMI-1640 medium. The collected cells were pelleted by centrifuge at 400 g for 5 min at 4°C and supernatants were discarded. Then red blood cells were removed by incubating with lysing buffer for 5 min at room temperature (RT). The remaining cells were washed once and collected into 3 mL complete RPMI-1640 medium. Cell counting was done in a hemocytometer and was adjusted to 2×10^7 /mL by volume.

Splenic Lymphocyte Surface Marker Staining

Four splenic lymphocyte surface markers tested were CD3, CD4, CD8 and CD45RA. An aliquot of 1.0×10^6 splenic cells were first blocked in 100 μ L block buffer (CD32, 1:100) for 10 min at RT. Then cells were pelleted by centrifugation and supernatants were discarded. These cells were incubated with staining buffer (PBS, including 10% FBS and 0.1% NaN_3) containing appropriately diluted fluorescence conjugated antibodies (CD3 PE: 1:200; CD4 APC: 1:100; CD8 FITC: 1:100; CD45RA PE-CY5: 1:200) for 30 min at 4°C in the dark, 100 μ L per tube. Single fluorescence antibody controls were included. After incubation, the cell mixtures were washed once with staining buffer and then fixed in 250 μ L cold 2% formaldehyde for 12 h. The fixed cell mixtures were pelleted and resuspended in 500 μ L staining buffer in plastic tubes before flow cytometric analysis within one day.

Stimulation of Cytokine Expression

Duplicates of 1.0×10^6 cells for each sample were transferred to a 96-well flat bottom plate pre-coated with anti-CD3 antibody (1:100). The cells were pelleted by centrifuging at 250 g for 4 min at 4°C and supernatants were discarded. 100 μ L of stimulating buffer (complete RPMI-1640 medium supplied with anti-CD28 antibody (1:100), brefeldin A (1:100), PMA (50 ng/mL), and ionomycin (500 ng/mL)) was added to stimulate cytokine production for 6 h in a humidified incubator (37°C, 5% CO_2).

Intracellular Cytokine Staining

To determine cell-specific intracellular cytokine expression, both surface markers and intracellular cytokines were stained. The combination of cell-surface markers and cytokine markers were (1) CD4 APC + CD8a PERCP + IL-4 PE + IFN- γ FITC, and (2) CD3 PE + CD8a PERCP + TNF- α FITC. The method was modified from a previous protocol (Yui, 2000). Briefly, cells in each well were washed twice with 200 μ L cold PBS after stimulation, then were resuspended in 100 μ L staining buffer containing either CD4 APC (1:100) + CD8a PERCP (1:100) antibodies or PE CD3 (1:200) + CD8a PERCP (1:100) antibodies and incubated for 30 min at 4°C in the dark. Cells were pelleted by centrifuging at 250 x g for 4 min at 4°C, then washed once with 250 μ L staining buffer. Next, 100 μ L Fix/Perm buffer was added into each well and mixed quickly and thoroughly prior to incubation for 30 min at 4°C in dark. The cells were washed twice with 250 μ L 1 $\times$ Perm/Wash buffer (500 g, 4°C,

5 min) prior to intracellular cytokine staining. Conjugated anti-cytokines were prepared in 1× Perm/Wash buffer as follows: IL-4 PE: 1:100 and IFN- γ FITC: 1:5; TNF- α FITC: 1:100. Cells were incubated in a mixture of IL-4 PE and IFN- γ FITC or TNF- α FITC for 30 min at 4°C in dark. After intracellular staining, cells were washed twice with 1× Perm/Wash buffer (500 μ L 4°C, 5 min), followed by fixation with 200 μ L cold 2% formaldehyde. Lastly, cells were washed once with staining buffer and resuspended in 500 μ L staining buffer in plastic tubes before flow cytometric analysis within one day.

Flow Cytometric Analysis of Splenic Lymphocyte Phenotypes and Intracellular Cytokine Expression

A total of 20,000 cells for either surface markers or different combinations of cytokine markers were counted on a HyperCyAn flow cytometer (Beckman Coulter Inc., Fullerton, CA) for analysis of splenic lymphocyte phenotype or cytokine expression. Data were analyzed by FlowJo software (Tree Star Inc., Ashland, OR).

Histopathological Examination of Spleen Tissues

Dissected rat spleen tissues were fixed in 4% neutral formalin and then embedded in paraffin. Tissue sections (5 μ m) were cut and routinely processed for H.E. staining and examined under light microscopy. Photographs were taken on an Olympus XC30 microscope with an Olympus UC30 digital camera (Olympus America Inc., Center Valley, PA) linked to a Cellsense image analysis system (Celsense Inc., Pittsburgh, PA). The apoptotic cells in splenic tissue sections were defined by the presence of heterochromatic cell fragments or apoptotic bodies, as previously described (Elmore, 2006).

Statistical Analysis

Serum levels of AFB-Lys adduct, the percentages of surface marker and cytokine expression were expressed as mean $\pm$ standard deviation (SD). Comparisons of these parameters between treated groups and the control group were conducted using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Serum Levels of AFB-Lys Adduct

As shown in Table 2, serum levels of AFB-Lys adduct in animals were measured to reflect the internal doses. Dose-dependent increases of serum AFB-Lys adduct levels were found after 1- and 5-week treatment. The adduct levels increased by 134.95% and 98.41% after 5-weeks of treatment for the 5 and 25 μ g/kg groups, respectively. However, only a slight change in the adduct level was found for the 75 μ g/kg group compared to that after 1-week treatment (7.32 vs. 7.18 ng/mg albumin). Typical chromatograms are shown in Figure 1.

Effects of 1-week Exposure of AFB₁ on the Expression of Splenic Lymphocytes Phenotypic Markers

The effects of AFB₁ on the percentage of splenic lymphocyte subsets after 1-week treatment are shown in Figure 2. Dose-dependent decreases in the percentages of cytotoxic T cells (CD8⁺) and CD3⁺CD8a⁺ NK cells were found after 1-week treatment, and statistical significance was found for the 25 and 75 µg/kg groups as compared to the control ($P < 0.05$). No statistically significant effects on T lymphocytes (CD3⁺), helper T lymphocytes (CD4⁺), or B lymphocytes (CD45RA⁺) were found after 1-week treatment (data not shown).

Effects of 1-week AFB₁ Exposure on the Expression of Splenic Lymphocyte Intracellular Cytokines

Without stimulation, the levels of intracellular cytokines are very low (e.g. IL-4), which makes the comparison among groups nearly impossible. In this study, we use the data in control group without stimulation as background to define the positive values. Any values higher than the background were considered positive. AFB₁ treatment produced a dose-related and significant reduction of IL-4 expression by CD4⁺ T cells at all dose levels after *ex vivo* stimulation ($P < 0.05$, Figure 3A). Dose-dependent inhibition of IFN-γ expression by CD4⁺ T cells was significant only in the 75 µg/kg group ($P < 0.05$, Figure 3B). A significant inhibition of IL-4 expression by CD8a⁺ cells occurred in the 25 and 75 µg/kg groups ($P < 0.05$), with 39% and 58% inhibition, respectively (Figure 3C). Inhibited IFN-γ expression by CD8a⁺ cells also appeared in the 25 and 75 µg/kg groups ($P < 0.05$, Figure 3D). The effects of AFB₁ on IL-4 and IFN-γ expression by CD8a⁺ cells also exhibited a dose-related change. Dose-dependent inhibition in the expression of TNF-α by CD3⁺CD8a⁺ NK cells occurred with the statistically significant difference observed in the 75 µg/kg group ($P < 0.05$, Figure 3E).

Effects of 5-week AFB₁ Exposure on the Expression of Splenic Lymphocyte Phenotypic Markers

The effects of AFB₁ treatment on splenic lymphocyte phenotypes after 5 weeks of treatment are shown in Figure 4. Significant increases in the percentages of CD3⁺ and CD8⁺ T lymphocytes were observed in the 5 and 25 µg/kg groups ($P < 0.05$) but not the 75 µg/kg group ($P > 0.05$). Other splenic lymphocyte subsets, including CD4⁺ T cells, B cells and NK cells, were not significantly affected after 5-week treatment with AFB₁ (data not shown).

Effects of 5-week Exposure of AFB₁ on the Expression of Splenic Lymphocyte Intracellular Cytokines

Effects of AFB₁ on cytokine expression are shown in Figure 5. Differential effects on IL-4 and IFN-γ expression by CD4⁺ T cells were found. Significant decreases in percentages of IL-4 expressing CD4⁺ T cells were found at all dose levels ($P < 0.05$, Figure 5A) while a significant increase in IFN-γ expressing CD4⁺ T cells (37.8%) occurred only in the 25 µg/kg group ($P < 0.05$, Figure 5B). A significant inhibition of IL-4 expressing CD8a⁺ cells was also found in the 25 µg/kg group ($P < 0.05$, Figure 5C). Although the inhibition in the 75 µg/kg group was not statistically significant, the fluorescence intensity of IL-4 in CD8a⁺ cells was significantly decreased by 58% ($P < 0.05$, data not shown). In addition, there was

also a significant elevation of TNF- α expression by CD3⁻CD8a⁺ NK cells (85.9%) in the 75 μ g/kg group ($P < 0.05$, Figure 5D).

The pattern changes of splenic lymphocyte phenotypes and cell-specific cytokine expressions after 1-week and 5-weeks of AFB₁ exposure are summarized in Table 3. IL-4 expression by CD4⁺ T cells is consistently decreased following both 1-week and 5-week exposures; IFN- γ expression by CD4⁺ T cells and TNF- α expression by NK cells experienced a temporal inversion from being inhibited to elevation.

Effects of AFB₁ on Spleen Histology

No apparent histopathological changes of the splenic microstructures were found after 1-week or 5-weeks of treatment with AFB₁ at all dose levels (Figure 6) except that treatment with 75 μ g/kg AFB₁ caused slightly increased apoptosis of lymphocytes after 5-weeks of treatment (Figure 6D).

DISCUSSION

Results of this study demonstrated that short-term exposure (1-week) to AFB₁ could dose-dependently decrease the percentages of splenic CD8⁺ lymphocytes and NK cells and the production of cytokines by CD4⁺, CD8⁺ lymphocytes and NK cells; prolonged exposure (5-weeks) to low levels of AFB₁ (25 μ g/kg) had stimulatory effects on splenic lymphocytes, but not at the high dose (75 μ g/kg). Prolonged 5-week exposure also inhibited IL-4 expression while stimulating IFN- γ and TNF- α expression activities. Splenic B lymphocytes were not significantly affected throughout the experimental period at all dose levels. Data from this study provides evidence of modulatory effects of AFB₁ on splenic lymphocyte phenotypes and cell-specific cytokine expression. To our knowledge, this is the first animal study to investigate the effects of AFB₁ on lymphocyte specific functions in cytokine production. Results of this study demonstrate that both splenic lymphocyte phenotypes and cell specific cytokine production can be modulated by AFB₁, and prolonged exposure may play a role in chronic inflammation through regulating cytokine expression.

Doses selected in this study were equivalent to 0.175 ppm, 0.875 ppm and 2.625 ppm, respectively, in contaminated food. These doses are relevant to human exposure, especially for high-risk populations in developing countries where food contamination with aflatoxins is a common problem. For example, individual daily exposures can be expected to range from 1 to more than 50 mg AFB₁ in Southern China and Kenya (Daniel *et al.*, 2011; Groopman *et al.*, 1992).

Serum AFB-Lys adduct is an established biomarker for AFB₁ exposure (Sabbioni *et al.*, 1987), and it was used to monitor the dosimetry in the animal study. Serum AFB-Lys adduct levels exhibited different patterns: at doses 25 μ g/kg, it increased by about 2-fold after 5-weeks of treatment compared to 1-week of treatment; there was no obvious change for the 75 μ g/kg group. This observation may indicate that a plateau was reached for this biomarker or that the metabolic balance for liver enzyme functions was affected in the study animals. The periportal necrosis and bile duct proliferation found in liver at this dose group

(data not shown) supports that liver injury influenced AFB₁ metabolic activation and adduct formation.

CD4⁺ and CD8⁺ T lymphocytes are important effector cells for cell-mediated immune responses (Jiang and Chess, 2000). NK cells serve as a defensive barrier to kill pathogens and foreign cells and participate in the innate and adaptive immune mechanisms (Vivier *et al.*, 2011). CD8⁺ T cells from spleen of normal rats almost exclusively express the alpha/beta isoform while NK cells carry only CD8 alpha chains (alpha/alpha) (Liu *et al.*, 2010; Torres-Nagel *et al.*, 1992). Therefore, splenic CD8a⁺ cells could be a mixture of both CD8⁺ T cells and CD3⁻CD8a⁺ NK cells, with the majority being CD8⁺ cells. Due to the limited commercial availability of conjugated antibodies for rat, the CD8a⁺ cells were chosen for assessing cytokine expression in this study. These lymphocytes secrete several different cytokines, such as IL-4, IFN- γ and TNF- α , which are important coordinators of immune and inflammatory responses (Arai *et al.*, 1990). In this study, we found that acute exposure to AFB₁ for 1-week decreased the percentages of CD8⁺ lymphocytes and NK cells. The impaired cell-mediated immunity was similarly observed in other species such as mice (Reddy *et al.*, 1987) and chickens (Thaxton *et al.*, 1974). In agreement with this, B lymphocytes (CD45RA⁺) were not significantly affected by AFB₁ after 1-week. Although the percentage of CD4⁺ T lymphocytes was not reduced, the expression of IL-4 and IFN- γ by CD4⁺ T lymphocytes was significantly inhibited at the high dose (75 μ g/kg). On the other hand, both the percentage of CD8⁺ T lymphocytes and the intracellular expression of IL-4 and IFN- γ were significantly decreased after 1-week treatment, indicating that CD8⁺ T lymphocytes might be more susceptible to AFB₁ exposure. TNF- α expression by NK cells was also found to be inhibited after 1-week of exposure to AFB₁. Apparently, these immune effects may not be relevant to overt toxicity in spleen as no obvious histological changes were observed in spleen at all dose levels. Instead, the inhibitory effects could result from gene expressions or a restricted cell cycle at G₂/M, which has been reported by other studies (Dugyala and Sharma, 1996; Reddy and Sharma, 1989; Scott *et al.*, 1991).

Dose-dependent effects on splenic lymphocyte phenotypes were found after 5-weeks of prolonged exposure, e.g., lower doses of AFB₁ (5 and 25 μ g/kg) increased percentages of CD3⁺ and CD8⁺ T lymphocytes, while the highest dose (75 μ g/kg) had no apparent effect. This finding was similar to deoxynivalenol (DON) induced immune modulation where low dose DON exposure upregulates cytokines and inflammatory genes with concurrent immune stimulation. A high dose enhances leukocyte apoptosis with concurrent immune suppression (Pestka, 2008). Earlier studies have also demonstrated that repeated treatment with AFB₁ for 4-weeks could enhance protein and DNA synthesis in splenic lymphocytes at a low dose (30 μ g/kg body weight), but not at the high dose (145 μ g/kg body) (Reddy and Sharma, 1989), which is in agreement with our findings.

Contrary to the overall inhibition of cytokine production after 1-week of exposure to AFB₁, significant inhibition of IL-4 expression by CD4⁺ and CD8⁺ cells and stimulation of IFN- γ expression by CD4⁺ cells and TNF- α expression by NK cells was found after 5-weeks of exposure to AFB₁. Stimulation of IFN- γ production by CD4⁺ cells could be a direct response or a feedback caused by inhibition of IL-4 given the interaction between IL-4 and INF- γ (Elser *et al.*, 2002). The stimulation of TNF- α production by NK cells might take part

in chronic inflammation, which is consistent with the elevated IL-6 production as reported previously (Hinton et al., 2003) since TNF- α can stimulate splenic production of IL-6 in rat (Teti *et al.*, 1993). Given the anti-inflammatory effect of IL-4 and pro-inflammatory effects of IFN- γ and TNF- α (Niir *et al.*, 1997), the inhibition of IL-4 and stimulation of IFN- γ and TNF- α might lead to a net result of inflammatory responses. Data from *in vitro* studies also support this finding. Murine macrophages exposed to AFB₁ showed decreased secretion of anti-inflammatory IL-10 and increased pro-inflammatory IL-6 (Bruneau *et al.*, 2012). Similar effects were found in fumonisin B₁ treated pig peripheral blood mononuclear cells, which demonstrated an inhibitory expression of IL-4 and a stimulation of IFN- γ at both protein and mRNA levels (Taranu *et al.*, 2005). Considering the rather short turnover time of these cytokines (Sander *et al.*, 1993), it seems unlikely that the observed pattern of cytokine release is due to this natural turnover. However, further research is warranted to clarify the potential mechanisms involved and the complications associated with cytokine interactions, such as direct versus indirect effects.

Findings in this study indicated that the functions of CD4⁺ and CD8⁺ cells were impaired by AFB₁, which supports the finding in humans that CD8⁺ lymphocyte function was negatively correlated with AFB₁ exposure levels (Jiang et al., 2008); however, different effects were found in regard to expression of IFN- γ and IL-4 by CD4⁺ cells. The variation might be due to different experimental designs and exposure periods as well as different comparison groups (non-treated rats versus low dose exposed humans). An *in vitro* study has shown that IL-4 acts synergistically with IL-5 to increase IgA production by LPS activated murine B cells, while IFN- γ inhibits IL-5 mediated up-regulation of IgA secretion (Eckmann *et al.*, 1992). Therefore, the observed inhibition of IL-4 and stimulation of IFN- γ in this study supports the finding of decreased saliva IgA in Gambian children with high AFB₁ exposure (Turner *et al.*, 2003).

To summarize the findings in the current study, a flow chart was constructed as shown in Figure 7. Based on previous knowledge and our data, short-term exposure to AFB₁ may elicit immunosuppressive effects through inhibitory effects on gene expression while prolonged exposure may upregulate cytokines and proinflammatory genes to enhance inflammation and apoptosis. The data provides novel insight into AFB₁ exposure induced alterations of cell-mediated immune responses. However, exact mechanisms for the complex regulation of cytokine production, as well as for the transition from short-term to prolonged effects warrants further study.

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List of abbreviations

AFB₁	aflatoxin B ₁
AFB-Lys	aflatoxin B ₁ -lysine adduct

APC	allophycocyanin
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
INF-γ	interferon γ
IL	interleukin
LPS	lipopolysaccharide
NK cell	natural killer cell
PERCP	peridinin chlorophyll protein complex
PMA	phorbol 12-myristate 13-acetate
PE	phycoerythrin
PE-Cy5	phycoerythrin cyanin 5
ROS	reactive oxygen species
SRBC	sheep red blood cell
TNF-α	tumor necrosis factor α

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Figure 1.

Typical chromatograms of AFB-Lys adduct. (A) An authentic standard, 1.0 ng/mL. (B) A diluted rat serum sample (5-fold) from 5 $\mu\text{g/kg}$ AFB₁ exposure. (C) A diluted rat serum sample (10-fold) from 25 $\mu\text{g/kg}$ AFB₁ exposure. Arrows indicate the peak for AFB-Lys adduct.

Figure 2.

Effects of AFB₁ on splenic lymphocyte phenotypes after 1-week exposure. Dose-dependent decreases of the percentages of CD8⁺ T cells (A) and CD3⁻CD8a⁺ NK cells (B) were found.

*Indicates $P < 0.05$ compared with control group.

Figure 3.

Effects of AFB₁ on cytokine expression in splenic lymphocytes after 1-week exposure.

Short-term exposure significantly decreases the expression of IL-4 (A) and IFN-γ (B) by CD4⁺ T cells, IL-4 (C) and IFN-γ (D) by CD8a⁺ T cells, and TNF-α by CD3⁺CD8a⁺ NK cells (E). *Indicates $P < 0.05$ compared with control group.

Figure 4.

Effects of AFB₁ on splenic lymphocyte phenotypes after 5-weeks of exposure. Long-term exposure increased the percentages of CD3⁺ T cells (A) and CD8⁺ T cells (B), especially at low dose levels. *Indicates $P < 0.05$ compared with control group.

Figure 5.

Effects of AFB₁ on cytokine expression in splenic lymphocytes after 5-weeks of exposure. The expression of IL-4 by CD4⁺ T cells was inhibited at all doses (A) while IFN-γ expression was elevated at 25 µg/kg group (B). The IL-4 expression by CD8a⁺ T cells was inhibited at 25 µg/kg group (C) and TNF-α expression by CD3⁺CD8a⁺ NK cells was increased at 75 µg/kg group (D). *Indicates $P < 0.05$ compared with control group.

Figure 6.

Histological effects of AFB₁ on spleen after 5-weeks of exposure. (A) and (C) are spleen tissues from control group at low (×100) and high (×400) magnifications. Normal spleen tissue is composed of two major parts: white pulp and red pulp (RP). The white pulp has three characteristic components: periarteriolar lymphoid sheath (PALS), follicle and marginal zone (MZ). (B) and (D) are spleen tissues from 75 µg/kg group at low (×100) and high (×400) magnifications. Increased apoptosis was found in the PALS region at 75 µg/kg AFB₁. The arrows indicate small heterochromatic cell fragments, indicative of apoptotic bodies.

Figure 7.
Possible mechanisms of AFB₁ immunotoxic effects in F344 rats.

Table 1

Immune parameters examined

Groups (cellular expression)		Immune parameters
T-lymphocytes		CD3 ⁺
T cell subsets		CD4 ⁺ , CD8 ⁺
B cells		CD45RA ⁺
NK cells		CD3-CD8a ⁺
Cytokine expression	(IL-4-) expression by CD4 ⁺ or CD8a ⁺ T cells, (IFN- γ -) expression by CD4 ⁺ or CD8a ⁺ T cells, (TNF- α -) expression by CD3-CD8a ⁺ NK cells	

Table 2

Serum levels of AFB-Lys adduct

Dose (µg/kg)	Serum AFB-Lys levels (ng/mg albumin)	
	1-week	5-week
0	ND	ND
5	0.22±0.01	0.52±0.02 ^c
25	1.54±0.09 ^a	3.06±0.15 ^{a, c}
75	7.18±0.28 ^{a, b}	7.32±0.28 ^{a, b}

Note. Values are mean ± SD for 5 rats. ND, not detectable.

^a $P < 0.001$, compared to 5 µg/kg in the same column.

^b $P < 0.001$, compared to 25 µg/kg in the same column.

^c $P < 0.01$, compared to 1-week in the same row.

Table 3Correlation between AFB₁ and immune parameters changes

Immune parameters (%)	1-week (µg/kg)			5-week (µg/kg)		
	5	25	75	5	25	75
CD3 ⁺ T cells	-	-	-	↑	↑	-
CD8 ⁺ T cells	-	↓	↓	↑	↑	-
CD3 ⁺ CD8a ⁺ NK cells	-	↓	↓	-	-	-
CD4 ⁺ IL-4 ⁺ T cells	↓	↓	↓	↓	↓	↓
CD4 ⁺ IFN-γ ⁺ T cells	-	-	↓	-	↑	-
CD8a ⁺ IL-4 ⁺ T cells	-	↓	↓	-	↓	-
CD8a ⁺ IFN-γ ⁺ T cells	-	↓	↓	-	-	-
CD3 ⁺ CD8a ⁺ TNF-α ⁺ NK cells	-	-	↓	-	-	↑

Note.

↑*P* < 0.05, significant increase compared to control↓*P* < 0.05, significant decrease compared to control-*P* > 0.05, no significant difference compared to control.