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RESEARCH ARTICLE

miR-155 influences cell-mediated immunity in Balb/c mice treated with aflatoxin M_1

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ABSTRACT

Aflatoxin M_1 (AFM_1) is a 4-hydroxylated metabolite of aflatoxin B_1 (AFB_1). It induces various toxicological effects including immunotoxicity. In the present study, we investigated the effects of AFM_1 on immune system and its modulation by MicroRNA (miR)-155. AFM_1 was administered intraperitoneally at doses of 25 and 50 µg/kg for 28 days to Balb/c mice and different immune system parameters were analyzed. The levels of miR-155 and targeted proteins were evaluated in isolated T cells from spleens of mice. Spleen weight was reduced in mice exposed to AFM_1 compared to negative control. Proliferation of splenocytes in response to phytohemagglutinin-A was reduced in mice exposed to AFM_1. IFN-γ was decreased in mice exposed to AFM_1, whereas IL-10 was increased. Concentration of IL-4 did not change different in mice exposed to AFM_1 compared to negative control. Exposure to AFM_1 reduced the expression of miR-155. Significant upregulation of phosphatidylinositol-3, 4, 5-trisphosphate 5-phosphatase 1 (Ship1) and suppressor of cytokine signaling 1 (Socs1) was observed in isolated T cells from spleens of mice treated with AFM_1, but the transcription factor Maf (c-MAF) was not affected. These results suggest that miR-155 and targeted proteins might be involved in the immunotoxicity observed in mice exposed to AFM_1.

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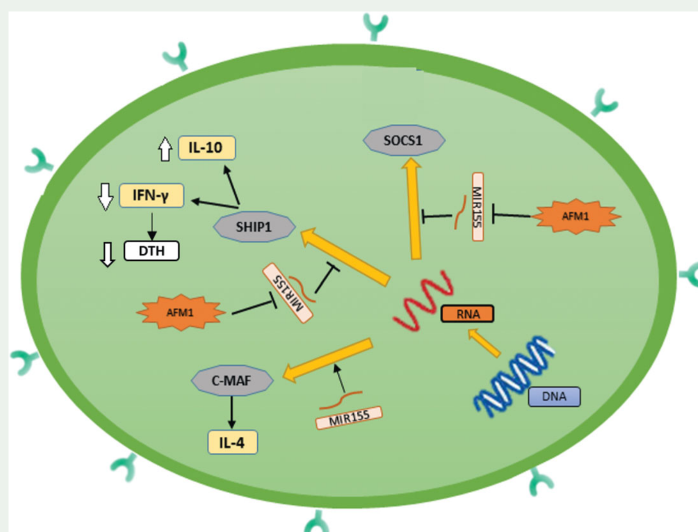
KEYWORDS
Immunotoxicity; Ship1; Socs1; micro-RNA; aflatoxin M1

GRAPHICAL ABSTRACT



The Immunotoxic effects of AFM_1 on T cell functions. Exposure to AFM_1 reduced the expression of miR-155. Significant upregulation of Ship1 and Socs1 was observed in isolated T cells from spleens of mice treated with AFM_1, but the c-MAF was not affected. Since Ship1 is a functional target of miR-155 that modulates production of IFN-γ, miR-155 may play a role in AFM_1-induced Th_1 response suppression (DTH) through targeting of this protein (↑ increase; ↓ decrease).

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HIGHLIGHTS

- AFM_1 inhibited cell-mediated immunity.
- Exposure of mice to AFM_1 resulted in a significant decrease in expression of miR-155.
- Exposure of mice to AFM_1 resulted in up-regulation of Ship1 and Socs1.

1. Introduction

The aflatoxins are a group of secondary metabolites produced by several *Aspergillus* species (Mohsenzadeh *et al.* 2016, Komsky-Elbaz *et al.* 2018). To date, at least 16 aflatoxins have been identified with various chemical structures, biosynthetic origin, and biological effects (Boutrif 1998, Zain 2011). Aflatoxin M1 (AFM_1), the hydroxylated metabolite of aflatoxin B1 (AFB_1), is found in biological fluids such as milk and urine of mammals after ingestion of AFB_1 contaminated food (Tanaka *et al.* 2015, Mohsenzadeh *et al.* 2016). Some countries have set the maximum limits for concentration of AFM_1 in foods with a range of 0.01 to 0.5 μg AFM_1/L (Maleki *et al.* 2015, Peng *et al.* 2016).

The occurrence of AFM_1 in dairy products, its toxic potency, and resistance to high temperature in pasteurization presses are important public health problems. AFM_1 can cause mutation of genes and anomalies to chromosomes and other effects via mechanisms similar to AFB_1, but with lower potency (Erkekoğlu *et al.* 2008, Wild and Gong 2010). AFM_1 is classified as a group 2B (possible human carcinogen) by the International Agency for Research on Cancer (IARC). Exposure to aflatoxin can impair both the innate and the acquired immune systems, especially cell-mediated immune responses (Abia *et al.* 2013, Pierron *et al.* 2016). AFB_1 suppresses cell-mediated responses by deactivation of proliferation and function of T or B lymphocytes. It also inhibits the activity of natural killer cells, impairs macrophage/neutrophil effector function and modulates secretions of inflammatory cytokines. Information about the effects of AFB_1 on the immune system is more than AFM_1 (Mohsenzadeh *et al.* 2016).

MicroRNAs (miRs) are small non-coding RNA molecules of approximately 20–30 nucleotides in length that posttranscriptionally repress expression of genes by degradation or repression of mRNA. (Anglicheau *et al.* 2010, Razavi-Azarkhiavi *et al.* 2017). miRs have unique characteristics including tissue-specific, highly stable, non-post process modified, and quantifiable which make them ideal biomarkers for diagnosis of some types of diseases (Khoo *et al.* 2012, Schraml *et al.* 2017). Recently, it has been suggested that circulating miRs play important roles in regulation of the immune responses including differentiation of cells as well as their immunological functions (Chen *et al.* 2013, Motawi *et al.* 2016). miR-155 may modules a variety of immunoregulatory signaling molecules such as phosphatidylinositol-3, 4, 5-trisphosphate 5-phosphatase 1 (Ship1), suppressor of cytokine signaling 1 (Socs1), and transcription factor Maf (c-Maf) in (Baumjohann and Ansel 2013, Lind and Ohashi 2014). Changes in the profiles of miRNA are associated with exposure to AFB_1; however, no such information is currently available for AFM_1 (Rieswijk *et al.* 2016). The association between miRs and AFB_1 suggests that miRs might be used as potential biomarkers for the toxicological effects of the aflatoxins (Liu *et al.* 2014, Yang *et al.* 2014, Zhu *et al.* 2015). In the present study, to understand the role of miR-155 and targeted proteins in the immunotoxicity of AFM_1, T cells were isolated from spleens of mice and the amount of miR-155 measured by RT- PCR. Also, the expression of targeted proteins of miR-155 was analyzed by Western blot.

2. Materials and methods

2.1. Animals

Six- to eight-week old, male, inbred, Balb/c mice between 19 and 21 g were purchased from the School of Pharmacy, Mashhad University of Medical Sciences (Mashhad, Iran). Animals were acclimatized for 1 week prior to use. Mice were housed in polystyrene cages [five per cage] with free access to feed and water with an ambient room temperature of 20–25 °C and a relative humidity of 50% and a light/dark cycle of 12/12 h. The protocol was approved by the Mashhad University of Medical Sciences Institutional Animal Use and Care Committee.

2.2. Doses and exposure schedules

One milligram of AFM_1 (Apollo Scientific Ltd, Manchester, UK, purity 99.50%) was dissolved in 1 ml of methanol, divided into aliquots of 8 μl and refrigerated at 4 °C until used. Animals were injected with suitable volumes of AFM_1 in normal saline to provide the desired dose. Doses selected were based on the predicted no observed adverse effect level (NOAEL) which was estimated to be 2.5 μg AFM_1/kg. Doses that were 10- and 20-fold greater than the NOAEL were used to evaluate potency (Kuiper-Goodman 1990, Neishabouri *et al.* 2004).

Sixty mice were divided into three groups ($n = 20$). Each group was further divided into four subsets ($n = 5$) and treated as follows: (1) IP injection with 25 μg AFM_1/kg, 5 days/week for 28 days; (2) IP injection with 50 μg AFM_1/kg, 5 days/week for 28 days; (3) IP injection with 20 mg cyclophosphamide (CTX)/kg for 5 days as the positive control; (4) IP injection with normal saline and methanol, 5 days/week for 28 days, as the negative control.

2.3. Spleen weight

On day 28 of treatment, 2 h after the last dose, mice were killed by cervical dislocation. Spleen weight was determined for each mouse.

Table 1. Effect of 28 days intraperitoneal injection of AFM_1 on some immune system parameters of Balb/c mice.

	AFM_1 25 (μg/kg)	AFM_1 50 (μg/kg)	CTX 20 (mg/kg)	Negative control
Spleen weight	135 ± 11***	140 ± 10***	110 ± 13.1***	176 ± 9.23
DTH (%)[a] (24h)	24.86 ± 3.46**	18.15 ± 8.20**	16.86 ± 9.14***	38.95 ± 6.17
DTH (%)[a] (48h)	23.66 ± 5.01**	17.20 ± 5.14**	15.77 ± 7.12**	36.8 ± 6.30
Proliferation response to PHA[b]	1.72 ± 0.03*	1.78 ± 0.03*	1.52 ± 0.05***	1.84 ± 0.03
IFN-ɣ (pg/ml)	231 ± 32	178 ± 24***	151 ± 13***	267 ± 15
IL-10 (pg/ml)	184 ± 13**	193 ± 12**	95 ± 15***	162 ± 24
IL-4 (pg/ml)	122 ± 16	114 ± 13	80 ± 11***	117 ± 9

Data showed as mean ± SD. AFM_1: Aflatoxin M_1; CTX: Cyclophosphamide; DTH (%): Delayed- type hypersensitivity; PHA: Phytohemagglutinin; IFN-γ: Interferon-γ; IL10: Interleukin 10.
[a]Percent of increase in paw thickness.
[b]Proliferation index (absorbance of the sample stimulated by PHA divided by the sample without stimulation by PHA).
*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$ 1 versus negative control (NS).

2.4. Delayed-type hypersensitivity response (DTH)

On day 23 of treatment, animals (five mice/group) were sensitized by IP injection of 1×10^9 sheep red blood cells (SRBC) in complete Freund's adjuvant (sensitization phase). After 5 days of immunization, sensitized mice were challenged with a booster dose of 1×10^8 SRBCs in the left hind footpad (effector phase). The right hind footpad was injected with the same volume of incomplete Freund's adjuvant to serve as the trauma control for nonspecific swelling. The increase in volume of both footpads was measured 24 and 48 h after the challenge with SRBCs by a pressure sensitive micrometer screw gauge (Mitutoyo, Kawasaki, Japan). Differences between the volume of the left and right hind footpads were calculated (Zimecki and Kruzel 2000).

2.5. Spleen cell subtyping

Single-cell suspensions of splenocytes were prepared and evaluated for subset distribution by 3-color flow cytometry. Briefly, single-cell suspensions of splenocytes in RPMI-1640 (1×10^6 cells/ml) were prepared and spleen cellularity was determined using the trypan blue dye exclusion method. Then CD^{3+}, CD^{4+}, and CD^{8+} cell subtypes were measured by use of a BD FACS Calibur™ flow cytometer and a mouse T lymphocyte subset antibody cocktail kit (with isotype control) (PE-Cy 7 CD^{3e}, PE CD^4, FITC CD^8, BD Pharmingen) according to the manufacturer's protocol (Mahmoudi *et al.* 2016).

2.6. Lymphocyte proliferation test

Proliferation of lymphocytes was determined in triplicates as previously described (Mosmann 1983). One hundred microliter aliquots of splenocytes, standardized at 2 million cell/mL, were pipetted into a 96-well microtiter plate. Phytohemagglutinin-A (PHA) was added to each well at a final concentration of 5 μg/ml. After incubating for 48 h at 37 °C and 5% CO_2, cell proliferation was determined by the MTT-based assay. A 10% solution of 3-(4, 5-diamethyl-2-thiazolyl) 2, 5-diphenyl-2*H*-tetrazolium (MTT; 5 mg/ml) was added to each well and incubated 4 h at 37 °C in a 5% CO_2 atmosphere. The blue formazan precipitate was then dissolved in acidic isopropanol and the optical density was measured at 570 nm in a Stat-Fax™ Elisa Reader. The index of proliferation (PI) was calculated as follows (Equation (1); Riahi *et al.* 2010).

$$PI = Absorbanceofstimulatedcells/Absorbanceofunstimulatedcells \quad (1)$$

2.7. Production of cytokines

Cells were stimulated by exposure to PHA for 48 h at 37 °C in a 5% CO_2 atmosphere. Supernatant fractions were collected and kept at −70 °C until analyzed. Concentrations of IFN-ɣ, IL-4, and IL-10 were measured in the supernatant fractions by use of a commercially available ELISA kit (Koma biotech) according to the manufacturer's protocol (Mahmoudi *et al.* 2016).

2.8. Isolation of primary T cells

Spleens were disrupted in Hanks' Balanced Salt Solution containing 2% fetal bovine serum (FBS). Aggregates and debris were removed by passing the cell suspension through a 70 μm mesh nylon strainer. The cell suspensions were centrifuged at 300 x *g* for 10 min and resuspended in PBS containing 2% FBS and 1 mM EDTA. T cells were isolated by negative selection using an EasySep™ Mouse T Cell Isolation Kit (StemCell Technologies, Vancouver, BC).Viability of cells was determined by trypan blue exclusion (Quispe Calla *et al.* 2015).

2.9. Quantitative real time PCR

Total RNA was extracted from T cells using a mirVana miRNA Isolation Kit (Applied BioSystems). cDNA synthesis was performed by use of the stem-loop hybridization, based on the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following the instructions provided by the manufacturer. Cycling conditions included 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Specific primer (Assay ID No. 002571, Applied Biosystems) and Taqman Universal PCR Master Mix (Applied Biosystems) were used for amplification of miR-155. RT-PCR was performed with the Step One Plus Real-time PCR system (Applied Biosystems) during 40 cycles, including enzyme activation (95 °C for 10 s), denaturation (95 °C for 15 s), annealing and extension (60 °C for 60 s, each). The relative amount of miRs was normalized by U6 small nuclear RNA

Table 2. Effect of 28 days intraperitoneal injection of AFM_1 on the subtypes of spleen cells of Balb/c mice.

	AFM_1 25 (μg/kg)	AFM_1 50 (μg/kg)	CTX 20 (mg/kg)	Negative control
CD^{3+} cell (%)	52.31 ± 4.43***	55.11 ± 1.83***	57.54 ± 3.45***	67.2 ± 5.04
CD^{3+} content ($\times 10^7$)	3.82 ± 0.23***	3.88 ± 0.53***	3.59 ± 0.07***	5.43 ± 0.05
CD^{4+} cell (%)	36.28 ± 3.24	35.65 ± 6.21*	35.44 ± 6.21**	43.44 ± 3.66
CD^{4+} content($\times 10^7$)	2.66 ± 0.23**	2.51 ± 0.06***	2.14 ± 0.09***	3.51 ± 0.61
CD^{8+} cell (%)	15.45 ± 2.26***	14.91 ± 1.88***	15.84 ± 1.24***	22.64 ± 3.11
CD^{8+} content($\times 10^7$)	1.13 ± 0.05***	1.05 ± 0.11***	0.99 ± 0.04***	1.83 ± 0.23

Data showed as mean ± SD. AFM_1: Aflatoxin M_1; CTX: Cyclophosphamide.
*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$ versus negative control (NS).

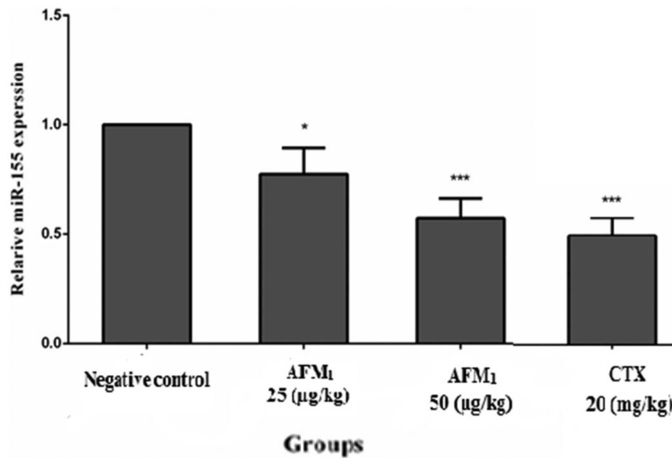

Figure 1. Effect of AFM_1 treatment on transcript levels of microRNA-155 (miR-155) in purified T cells isolated from spleen by qRT-PCR. Data are expressed as mean ± SD ($n = 5$). The experiment was performed in triplicate. *$p < 0.05$ and ***$p < 0.001$ versus negative control (NS) according to ANOVA followed by the Tukey post-hoc test. The level of miR-155 expression was normalized with U6. AFM1: Aflatoxin M1; CTX: Cyclophosphamide.

(Assay ID No. 001973; Applied Biosystems)(Razavi-Azarkhiavi *et al.* 2017).

2.10. Protein estimation

T cells were harvested and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate (Na3VO4), 10 mM β glycerophosphate, 0.2% *w/v* sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and a complete protease inhibitor cocktail. The protein content of T cells was quantified by the Bradford method. Briefly, 190 μl of Coomassie blue (G250) was added to 10 μl of each sample and the mixture was kept at room temperature for 10 min. Absorbance was measured at 595 nm in a spectrophotometer. By use of different concentrations (0.25 and 1 mg/ml) of bovine serum albumin, a standard curve was prepared (Razavi-Azarkhiavi *et al.* 2017).

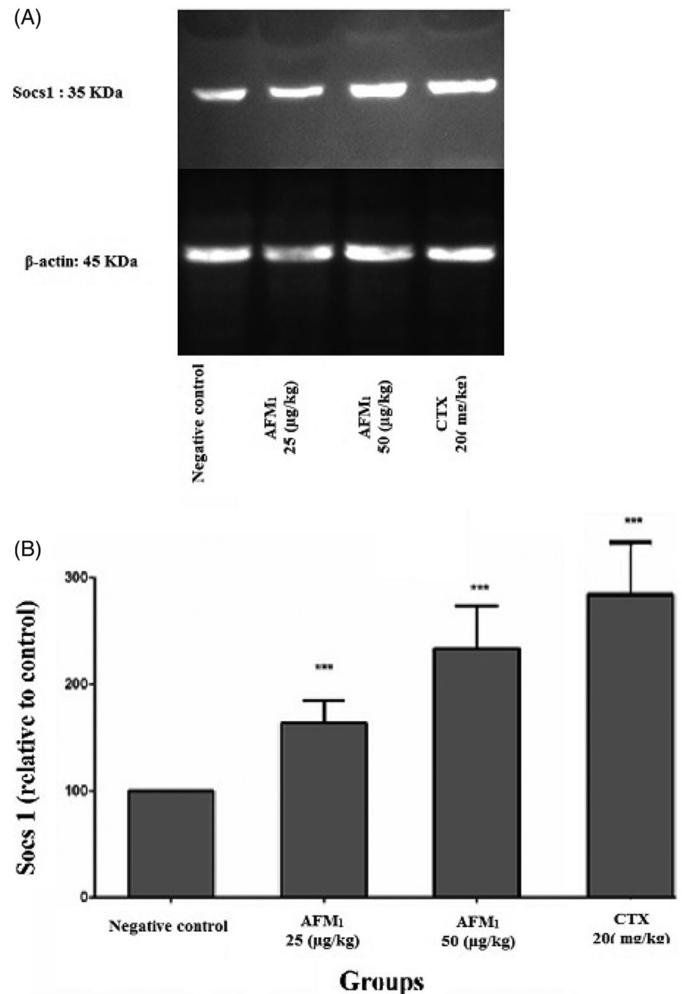

Figure 2. Effects of AFM_1 treatment on the concentration of Socs1 protein in purified T cells isolated from mouse spleen. (A) Western blot analyses were performed to determine the Socs1 protein levels. (B) The levels of Socs1 protein were determined using a densitometric analysis. Data are expressed mean ± SD ($n = 5$). The experiment was performed in triplicate. ***$p < 0.001$ versus negative control (NS). Intensity of the bands was normalized against β-actin. AFM_1: Aflatoxin M_1; CTX: Cyclophosphamide.

2.11. Western blot analysis

Western blot analysis was performed on protein extracts to investigate the expressions of Ship1, Socs1, and c-Maf in purified T cells isolated from mouse spleen. Amounts of various proteins, as well as β-actin, were measured by the immune blotting analysis. Briefly, equal amounts of protein extracts (50 μg) were electrophoresed on a SDS–PAGE gel. Then, proteins were transferred to a PVDF membrane. Blocking of the membrane was performed by use of 5% nonfat dry milk in Tris buffered saline tween (TBST). After blocking, membranes were incubated with suitable primary antibodies including, anti-c-Maf antibody (Abcam, # Ab76817), anti-Socs1 antibody (Abcam, # Ab137384), and anti-Ship-1 antibody (Biolegend, #

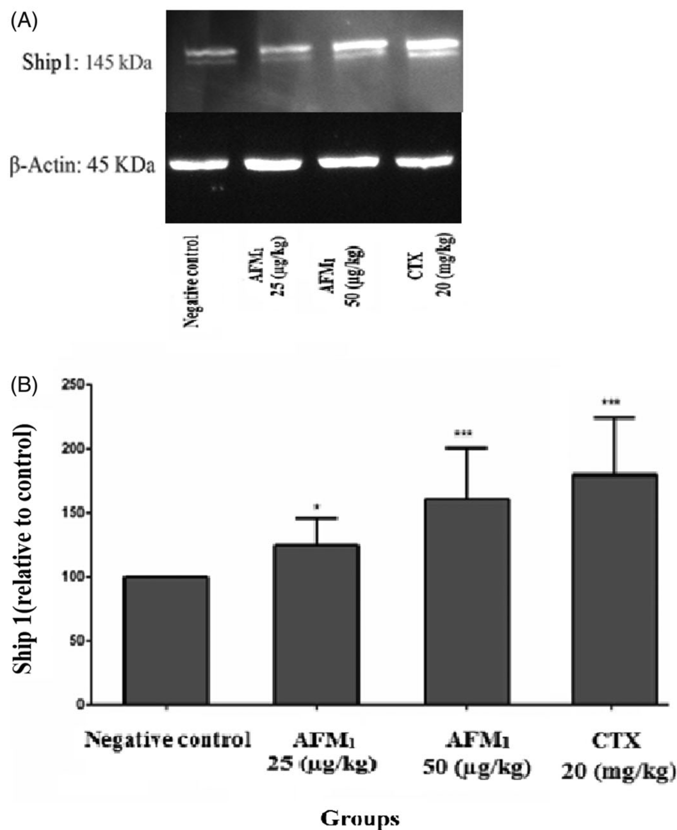

Figure 3. Effects of AFM_1 treatment on the concentration of Ship1 protein in purified T cells isolated from mouse spleen. (A) Western blot analyses were performed to determine the Ship1 protein levels. (B) The levels of Ship1 protein were determined using a densitometric analysis. Data are expressed mean ± SD ($n = 5$). The experiment was performed in triplicate. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus negative control (NS). Intensity of the bands was normalized against β-actin. AFM_1: Aflatoxin M_1; CTX: Cyclophosphamide.

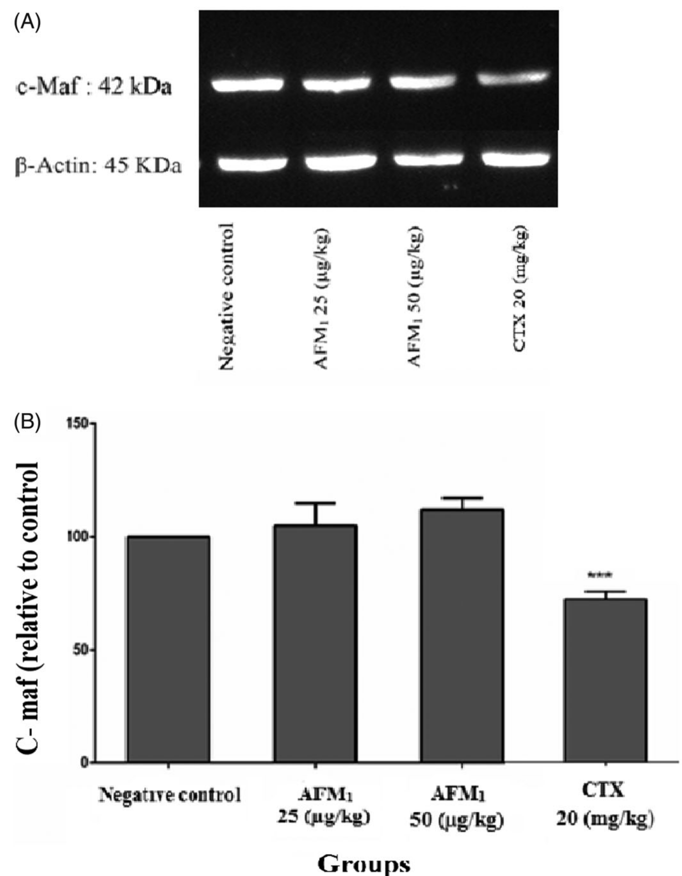

Figure 4. Effects of AFM_1 treatment on the concentration of c-MAF protein in purified T cells isolated from mouse spleen. (A) Western blot analyses were performed to determine the c-MAF protein levels. (B) The levels of c-MAF protein were determined using a densitometric analysis. Data are expressed mean ± SD ($n = 5$). The experiment was performed in triplicate. $^{***}p < 0.001$ versus negative control (NS). Intensity of the bands was normalized against β-actin. AFM_1: Aflatoxin M_1; CTX: Cyclophosphamide.

656601) and mouse monoclonal anti-serum against β-actin (Cell Signaling, # 4976) for 3 h. Following three washes with TBST buffer, the immunoassay was carried out by use of the appropriate anti-mouse IgG labeled with horseradish peroxidase (Cell Signaling, #7076). Finally, protein bands were visualized by chemiluminescence (Pierce ECL Western blotting substrate) and Alliance gel doc (Alliance 4.7 Gel doc, UK). Protein bands were semi-quantified using UV tec software (UK). All protein bands were normalized to β-actin (Razavi-Azarkhiavi *et al.* 2017). The experiment was performed in triplicate.

2.12. Statistical analysis

All data are presented as the mean ± standard deviation. Data normality was verified using the Kolmogorov–Smirnov test and homogeneity of variance was checked by Levene's test. If the data failed to pass the test, a logarithmic transformation of the data was performed and retested. Significant differences were assessed between each treatment and the control using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. A *p*-value <0.05 was considered to be statistically significant difference. All statistical tests were conducted using GraphPad PRISM (Version 6.00) software.

3. Results

3.1. Spleen weight

After 4 weeks, spleen weights of mice exposed to all doses of AFM_1 were significantly less than those of the control group (Table 1).

3.2. Delayed-type hypersensitivity response

Exposures to 25 or 50 μg AFM_1/kg resulted in a significant decrease in DTH response as compared to negative controls. CTX also suppressed the DTH response ($p < 0.001$) (Table 1).

3.3. Proliferative response to PHA

The proliferative response of splenocytes to PHA was decreased significantly in mice exposed to 25 or 50 μg AFM_1/kg and CTX as compared to negative control (Table 1). However, there was no statistically difference between animals injected with doses of 25 or 50 μg AFM_1/kg.

3.4. Productions of cytokines

The concentrations of IFN-γ was significantly decreased in mice exposed to AFM_1 while the concentration of IL-10

increased compared to negative control (Table 1). There was no significant difference in IL-4 in mice exposed to AFM_1 relative to the negative controls.

3.5. Spleen cell subtypes

Exposure to AFM_1 resulted in significantly ($p < 0.001$) fewer lymphocytes in spleens relative to controls (Table 2). Fewer CD^{3+}, CD^{4+}, and CD^{8+} cells were observed in mice exposed to either 25 or 50 μg AFM_1/kg. Proportions of both CD^{3+} and CD^{8+} T lymphocytes were less in mice exposed to 25 or 50 μg AFM_1/kg relative to the controls. AFM_1 at 50 μg/kg significantly reduced the percentage of CD^{4+} in Balb/c mice. However, the percentage of CD^{4+}exposed to 25 μg AFM_1/kg was not different from the controls (Table 2).

3.6. Quantitative Real-Time PCR

Expression of miR-155 in Balb/c mice exposed to AFM_1 was reduced dose dependently compared to the control group (Figure 1).

3.7. Western blot analysis

A significant upregulation of Ship1 and Socs1 was observed in mice injected with $AFM_{1;}$ however, the c-MAF was not significantly different from the unexposed control (Figures 2–4).

4. Discussion

The immune system which normally provides protection against infection can be adversely affected by exposure to the aflatoxins (Shirani *et al.* 2015). There is little information regarding the influence of AFM_1 on cell-mediated immunity (Mahmood *et al.* 2017). Luongo *et al.* (2014) showed that in the human lymphoblastoid Jurkat T-cell model, AFM_1 reduced proliferation in a dose-related manner but did not impact the production of IL-2 or IFN-γ.

In the current study, we evaluated the effects of AFM_1 on cell-mediated immune function by assessing miR-155 and its target proteins in T-cells. The doses were selected based on the predicted NOAEL which was estimated to be 2.5 μg AFM_1/kg. Doses 10- and 20-fold greater than the NOAEL were used to evaluate potency (Kuiper-Goodman 1990, Neishabouri *et al.* 2004).

Exposure to AFM_1 resulted in less mass of spleen which might be due to an overall reduction in lymphocyte subpopulations. Decrease in numbers of splenocytes (CD^{3+}, CD^{4+}, and CD^{8+} cells) might be associated with direct toxicity of AFM_1 (Kim *et al.* 2014).

Indices of proliferation in response to the mitogen PHA suggested that AFM_1 adversely affected normal T-cells function which was manifested as decreased DTH capacity (Yasuda *et al.* 1996, Song *et al.* 2013).

The profile of cytokines in the supernatant fraction of splenocytes exhibited decreased IFN-γ but an unchanged IL-4. Furthermore, there was a statistically significant increased concentration of IL-10 which confirmed the inhibitory effects of AFM_1 on the propagation of the cell-mediated immune system toward Th_1 (Mittal and Roche 2015). These results indicated that the DTH response, which is known to be largely Th_1-mediated, was suppressed (Akahira-Azuma *et al.* 2004)

AFM_1 has been reported to be a potent producer of free oxygen radicals (ROS) (Marin and Taranu 2012, Zhang *et al.* 2015). It is possible that the generation of free oxygen radicals could inhibit immune cell function via membrane lipid peroxidation which in turn could lead to an impairment of lymphocyte reaction with antigen, lymphokines, or other cell subsets (Riahi *et al.* 2010). Therefore, the observed immunotoxicity resulting from exposure to AFM_1 could be from the formation of DNA adducts (AFM_1-N_7-guanine). Also, it might be due to a blockage of DNA which would suppress the cell proliferation required for an immune response (Ginsberg *et al.* 1989, Egner *et al.* 2003).

Vigorito *et al.* (2007) have reported that mice deficient in miR-155 exhibited attenuated germinal center reaction and cell-mediated immunity by regulating cytokine production. We found that miR-155 is significantly downregulated in Balb/c mice exposed to AFM_1. Recent studies have indicated that inhibition of miR-155 expression alleviates the IFN-γ-mediated activation of downstream inflammatory cytokines, suggesting that miR-155 is a positive feedback regulator in the anti-inflammatory response (Hsu *et al.* 2016). Alternatively, Banerjee *et al.* (2010) study showed that miR-155 suppression promotes Th_2 development. A deficiency of miR-155 decreased the response of CD^{8+} T-cells, whereas overexpression of miR-155 increased the response of CD^{8+} T-cells during inflammation (Gracias *et al.* 2013). Hence, a lack of miR-155 in cells producing IFN-γ resulted in functional impairment of T-cells and defective antigen-presenting cells (Testa *et al.* 2017).

In addition, our results demonstrated a reverse correlation between IL-10 and miR-155 in Balb/c mice injected with AFM_1. IL-10 is a cytokine with potent anti-inflammatory properties that can inhibit the synthesis of diverse inflammatory cytokines (IFN-γ, TNF-α, and IL-12) and activation of T lymphocytes (Cavalcanti *et al.* 2012). It has been reported that the immune response of mice deficient in miR-155 was directed toward a Th_2 pattern with greater production of IL-10 which can then mediate immunosuppressive effects against cell-mediated responses (Cyktor and Turner 2011).

Similar to miR-155, dysregulated expression of proteins can result in untoward outcomes (O'Connell *et al.* 2009). miR-155 has various roles in different cell types and physiological situations. Investigating targets such as c-MAF, Socs1, and Ship1, likely make significant contributions to understanding the function of miR-155 in a context-dependent manner (O'Connell *et al.* 2009, Testa *et al.* 2017).

Results of this study indicated that AFM_1 significantly downregulated miR-155 and enhanced expression of Ship1. Since in T cells, Ship1 is a functional target of miR-155 that modulates production of IFN-γ, it is suggested that miR-155 might play a role in AFM_1-induced Th_1 response suppression through targeting of this protein (Gracias *et al.* 2013).

Furthermore, the results of the Western blot analysis revealed that the expression of Socs 1 was upregulated in T

cells compared to control mice. There is evidence that miR-155 inhibits expression of Socs1 (Wu *et al.* 2012, Yao *et al.* 2012). A deficiency of miR155 in T cells resulted in greater expression of Socs1, accompanied by diminished activation of STAT5 factor in response to reduction of IL-2 which resulted in less proliferation of T cells (Lu *et al.* 2009, Tili *et al.* 2009, Seddiki *et al.* 2014).

In this study, expression of the c-MAF protein was did not alter in AFM_1 exposed animals at the end of the experiment compared to unexposed controls. c-MAF has been identified as a target of miR-155 which affects normal production of IL-4 during activation of T-cells (Kim *et al.* 1999). So, a lack of change in the concentration of IL-4 can be explained by the lack of effect of AFM_1 on c-MAF expression. However, additional studies will be required to elucidate mechanistic details (Su *et al.* 2013).

5. Conclusion

Exposure to AFM_1 resulted in reduced amounts of IFN-γ, whereas IL-10 was increased. Also, AFM_1 decreased the expression of miR-155. Significant upregulation of Ship1 and Socs1 was observed in isolated T cells from spleens of mice treated with AFM_1, but the c-MAF was not affected. These results suggest that miR-155 and targeted proteins might be involved in the immunotoxicity observed in mice exposed to AFM_1. However, the role of miR-155 in AFM_1 immunotoxicity needs to be confirmed in future studies to determine if over expression or inhibition of miR-155 is the basic for AFM_1 induced immunotoxicity.

Disclosure statement

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