Original Paper

Low-Level Aflatoxin B₁ Promotes Influenza Infection and Modulates a Switch in Macrophage Polarization from M1 to M2

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Key Words

Aflatoxin B₁ • Swine influenza virus • H1N1 • Mouse • Porcine alveolar macrophage • Polarization

Abstract

Background/Aims: Swine influenza virus (SIV) is a major pathogen of both animals and humans. Aflatoxin B₁ (AFB₁) is one of the most common mycotoxins in feed and food. However, the central contribution of AFB₁ to SIV infection remains unclear. **Methods:** Here, TCID₅₀ assays, fluorescence-based quantitative real-time PCR, western blotting, immunofluorescence staining, histopathological examination, flow cytometry and scanning electron microscopy were performed to investigate the involvement and underlying mechanism of AFB₁ in SIV infection *in vivo* and *in vitro* using mouse models and porcine alveolar macrophage (PAM) models, respectively. **Results:** The *in vivo* study showed that low levels of AFB₁ promoted SIV infection and increased its severity, as demonstrated by the increased mRNA expression of viral matrix protein (M); by the increased protein expression of nucleoprotein (NP), matrix protein 1 and ion channel protein; and by animal weight loss, lung index and lung histologic damage. In addition, the increased occurrence of SIV infection accompanied by increases in the level of IL-10 in sera and lungs, in the spleen index and in the number of CD206-positive mouse alveolar macrophages but decreases in the level of TNF-α in sera and lungs, in the thymus index and in the number of CD80-positive mouse alveolar macrophages was observed in SIV-infected mice after low-level AFB₁ exposure. The *in vitro* study showed that low concentrations of AFB₁ promoted SIV infection, as demonstrated by the increases in viral titers and viral M mRNA and NP expression levels in SIV-infected PAMs as well as by the number of cells positive for NP protein expression. Furthermore, AFB₁ promoted the polarization of SIV-infected PAMs to the M1 phenotype at 8 hpi and to the M2 phenotype at 24 hpi, as measured by the increases in IL-10 expression and in the number of CD206-positive PAMs as well as by the morphological changes observed by scanning electron microscopy. The administration of

the immune stimulant lipopolysaccharide (LPS) reversed the switch in PAM polarization from M2 to M1 and thereby counteracted the promotion of influenza virus infection induced by AFB₁. **Conclusion:** Our results are the first to confirm that low-level exposure to AFB₁ promotes SIV infection and modulates a switch in macrophage polarization from M1 to M2. The work reported here provides important data that point to a role for AFB₁ in SIV infection, and it opens a new field of study.

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Introduction

Swine influenza virus (SIV), a single-stranded negative-sense RNA virus, causes severe systemic manifestations, including fever, cough, headache, chills, diarrhea and vomiting; it results in significant economic losses in the animal husbandry industry and also causes instances of human disease, occasionally giving rise to human pandemics including the one caused by the H1N1/2009 virus [1]. An important reason that the virus spreads so rapidly and globally is that it has a complex genetic composition [2] and generates multiple recombinants. In addition, viral reassortment and cross-infection play a contributory role in the ineffectiveness of the vaccine. In particular, previous studies have provided evidence that the severity of viral infection is associated with some environmental, nutritional, and immune factors, such as mycotoxin contamination [3, 4], selenium deficiency [5, 6], and macrophage polarization [7]. These factors may partially explain the global differences in morbidity and mortality in animals and humans.

Aflatoxin B₁ (AFB₁), which is produced by *Aspergillus flavus*, is one of the most common mycotoxins in contaminated food and plant products, such as corn, wheat and peanuts, in tropical and subtropical areas with high temperatures and humidity [8, 9]. It is well known that AFB₁ is harmful to the liver and kidneys of mammals and that it is regarded as a representative orally ingested carcinogen [10]. However, accumulating evidence has indicated that AFB₁ is immunotoxic to mammals [11, 12]; these results showed that low-level AFB₁ (≤ 0.025 mg/kg) exposure significantly increased the expression of proinflammatory cytokines, such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), by T cells and NK cells in rats, although high-level AFB₁ (0.4 to 0.8 mg/kg) exposure significantly decreased both the lymphocyte response to mitogens and macrophage migration in pigs. Specifically, previous studies have proposed that mycotoxin exposure would eventually decrease resistance to infectious diseases [13], and aflatoxins have even been considered to feature prominently in the progression of some viral diseases, such as HIV [4]. Thus far, there are no studies investigating whether influenza virus infection in mammals exposed to AFB₁ is more severe than that in animals in a normal environment.

Macrophages, which have the greatest degree of plasticity among all cells, are found in all tissues of mammals and have roles in the maintenance of homeostasis and immunity [14]. In the healthy lung, at least two macrophage populations exist, namely, alveolar macrophages and interstitial macrophages [15]. Alveolar macrophages are classified as M1 and M2 macrophages according to their inflammatory state [16]. M1 macrophages produce proinflammatory cytokines, thus contributing to host defense against pathogens and tissue injury; M2 macrophages produce anti-inflammatory cytokines, thus promoting tissue repair [15, 16]. The phenomenon of the existence of the two contrasting M1/M2 phenotypes is referred to as 'macrophage polarization'. Macrophage polarization can occur at any point in an inflammatory process [17]; multiple phenotypic markers, cytokines and growth factors, such as nitric oxide synthase (iNOS), TNF- α and interleukin 10 (IL-10), interact to determine the final polarization state [18]. Previous studies indicated that AFB₁ was immunotoxic to porcine alveolar macrophages (PAMs) and that AFB₁ leads to time- and dose-dependent decreases in the viability and phagocytic activity of PAMs [19]; furthermore, AFB₁ decreases proinflammatory cytokine levels and increases anti-inflammatory cytokine levels in macrophages [20]. It is notable that macrophages are the first line of defense against viral infection, and it has been indicated that they are critical for the defense against influenza

virus infection [21-23]. Moreover, macrophages undergo specific differentiation programs, depending on the local environment [18]. Nevertheless, scant literature is available about the polarization states of AMs infected with influenza virus and exposed to AFB₁ or about the roles played by AFB₁-induced polarization in viral infection.

Thus, given the differences in morbidity and mortality following SIV infection, we hypothesize that AFB₁ promotes SIV infection.

Materials and Methods

Ethics statement

This research protocol was approved by the Ethics Committee for Animal Experimentation of Nanjing Agricultural University. All animal care and use procedures were conducted in strict accordance with the Animal Research Committee guidelines of the College of Veterinary Medicine at Nanjing Agricultural University, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Reagents

AFB₁ (Sigma-Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, 1mg/ml), packaged, and stored frozen at -20°C until used. Lipopolysaccharide (LPS), the human recombinant cytokines IFN-γ and interleukin-4 (IL-4) and TNF-α were purchased from R&D Systems (USA), and the stock solutions were prepared in phosphate-buffered saline (PBS), packaged, stored frozen at -80°C until used. The final working concentrations of above all reagents were diluted with PBS or serum-free medium (RPMI-1640, Gibco, USA).

Cell culture

PAM (3D4/21) and Madin-Darby canine kidney (MDCK, NBL-2) cell lines, free of any respiratory or systemic diseases, were purchased from the China Institute of Veterinary Drug Control (Beijing, China). PAMs were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal calf serum (FCS; Gibco, USA), 1% penicillin-streptomycin (Solarbio, China) and 1% nonessential amino acid (Gibco, USA) at 37°C in 5% CO₂. MDCK cells were grown in DMEM (Gibco, USA) medium containing 10% FCS and 1% penicillin-streptomycin at 37°C in 5% CO₂. During infection of virus, all cell lines were transferred to serum-free medium supplemented with 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, USA).

Viral titration and growth kinetics

Influenza virus strain A/swine/Guangxi/18/2011 (H1N1) was kindly provided by Dr. Weiye Chen, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Harbin, China). The virus was propagated and titrated in MDCK cells, and 50% tissue culture infectious doses (TCID₅₀) were determined. Briefly, MDCK cells were cultured in a 96-well plate (Corning, USA) for 24 h and were then infected with 10-fold serial dilutions of virus in serum-free medium supplemented with 1 μg/ml TPCK-treated trypsin. The cytopathic effect induced by the virus was observed and recorded at 48 h post-infection (hpi), and viral titers were then calculated by the Reed-Muench method. Next, MDCK cells were cultured in cell culture flasks (Corning, USA) and infected with virus at a multiplicity of infection (MOI) of 0.1; the virus was then passaged, harvested and stored at -80°C. Similarly, viral titration used for further assays was determined in PAMs by calculating the TCID₅₀. A biosafety level 3 facility was used for all experiments with the H1N1 virus.

Mouse model

Six to eight-week old male BALB/c mice were purchased from Yangzhou University (Yangzhou, China). All mice were housed at BioSupport AG (Zurich, Switzerland) under specific pathogen-free conditions in individually ventilated cages maintained at a temperature of 22 ± 2°C, a relative humidity of 40 ± 5%, and a 12-h light/dark cycle. Water and food were available *ad libitum* to all mice throughout the whole study. Body weight changes and illness indicators were monitored daily. A nonlethal dose of virus was selected for use

in the model experiments [24]. Briefly, mice were anesthetized by the inhalation of 2.5% isoflurane, and the anesthetized mice were then infected intranasally with H1N1 virus at a dose of 1000 TCID₅₀ or with PBS in a volume of 100 μ l (50 μ l/nostril) at d 1, d 7 and d 14, as described previously [25, 26].

Study design

All mice were acclimatized for one week before the start of experiments and were randomly divided into 6 groups. Four groups were challenged intranasally with 0.1 ml of H1N1 virus prior to treatment with AFB₁, and the other two groups were intranasally administered an equivalent volume of PBS. Of the 4 infected groups, three were injected intraperitoneally with 0.1 ml of AFB₁ daily for 15 days, and the fourth group was intraperitoneally administered an equivalent volume of PBS. The 3 AFB₁ levels used were 20, 40 and 80 μ g/kg b.w. The two uninfected groups were injected intraperitoneally with 0.1 ml of PBS and AFB₁ (80 μ g/kg).

BAL collection and macrophage classification

Bronchoalveolar lavage (BAL) was conducted to obtain alveolar macrophages. Briefly, each lung was flushed 10-15 times with 1 ml PBS, and cells were harvested from the BAL fluid by centrifugation. Cells from the BAL were resuspended and incubated with an Fc receptor blocker (BD Biosciences) for 15 min at 4°C to reduce nonspecific binding. Next, cells were incubated with specific F4/80-APC, CD11b-PerCP-Cy5.5, CD80-PE and CD206-FITC antibodies (eBioscience; BD Biosciences) for 30 min at 4°C. Subsequently, cells were washed twice with PBS and were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo for Mac 10.7 (Tree Star, Inc.).

Sample collection and histopathological examination

Blood was collected and allowed to clot at room temperature (RT). Serum was separated by centrifugation and was stored at -80°C until analysis. Lung and spleen tissues were harvested from each mouse. Approximately 75% of the lung tissue was stored at -80°C for subsequent experiments, and the other 25% of the lung tissue, along with the whole spleen, was fixed in 4% formaldehyde for 48 h, embedded in paraffin, cut into 5- μ m-thick sections, and processed for hematoxylin-eosin (H&E) staining according to previously described standard protocols [27, 28] with some modifications.

Cytotoxicity and apoptosis assays by MTT and flow cytometry

PAMs (5×10^3 cells/well) were cultured in a 96-well plate for 24 h and were then exposed to various concentrations of AFB₁ and LPS for 24 or 48 h before being used in a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Subsequently, the absorbance was measured at 490 nm with a reference wavelength of 655 nm, and all experiments were performed in triplicate.

For the apoptosis assay, PAMs (1×10^5 cells/well) were cultured in 6-well plates for 24 h and were then exposed to various concentrations of AFB₁ for 48 h. Subsequently, PAMs were washed twice with PBS and were then stained with an annexin V-FITC and/or a PI staining buffer (Vazyme, China) according to the manufacturer's instructions. Finally, cells were shocked gently and were then analyzed with the above mentioned flow cytometer. The apoptosis rate was evaluated as the sum of the early and late apoptosis rates.

Fluorescent quantitative real-time PCR

Lung tissues and PAMs were collected for quantitative real-time PCR to determine the relative expression of viral matrix (M) protein [29], iNOS, TNF- α and IL-10 mRNA. Primers for target genes and reference genes were designed using Primer Premier software (Premier Biosoft International, USA) based on the known sequences, and forward and reverse primers were as follows, respectively:

M: 5' GGGAAGAACACCGATCTTGA 3' and 5' CTCCGTTCCATTAAAGAGCA 3'; 18 S: 5' TTGACGGAAGGGACACACAG 3' and 5' GCACCACCACCCACGGAATCG 3'; iNOS: 5' CCAAGCCCTCACCTACTTCC 3' and 5' CTCTGAGGCTGACACAAGG 3'; TNF- α : 5' CCACCAACGTTTCTCCTCACT 3' and 5' TTGATGGCAGAGAGGAGGTT 3'; IL-10: 5' CTGCCTCCACTTTCTCTTG 3' and 5' TCAAAGGGGCTCCCTAGTTT 3'; GAPDH: 5' CCACCCAGAAGACTGTGGAT 3' and 5' AAGCAGGGATGATGTTCTGG 3'. Mouse, TNF- α : 5' GACTCAGATCATCGTCTC 3' and 5' GGAGTAGATGAGGTACAG; IL-10: 5' CAGCCGGAAGACAATAACT 3' and 5' TCATTTCCGATAAGGCTTGG; GAPDH: 5' CGTCAAGCTCATTTCTCTGGT 3' and 5' TGGGATGGAACTGGAAGTC 3'.

Western blotting

PAMs and lung tissues were collected for western blotting to assess the relative expression levels of viral nucleoprotein (NP), matrix protein 1 (M1) and ion channel protein (M2). Briefly, total protein was extracted, and protein concentrations were then measured by a BCA kit (Beyotime, China). Proteins were denatured and were then subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA). Next, membranes were blocked for 2 h at RT with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST), followed by overnight incubation at 4°C with the specific primary antibodies (Abcam, UK) and by incubation with the appropriate secondary antibodies (horseradish peroxidase-labeled anti-mouse or anti-rabbit antibodies) (Cell Signaling Technology, USA) for 1 h at RT. Finally, the bound antibodies were visualized using an enhanced chemiluminescence kit (Beyotime, China).

Immunofluorescence staining

PAMs were seeded on glass coverslips and grown for 24 h to 80-90% confluence. PAMs were infected with H1N1 virus at an MOI of 0.1 in the presence or absence of AFB₁. After 24 h of infection, cells were fixed with 4% paraformaldehyde for 20 min at 4°C. To reduce nonspecific binding, PAMs were perforated with 0.2% Triton X-100 for 20 min and were then blocked with 1% BSA for 30 min at 37°C. Next, PAMs were incubated with an anti-NP monoclonal primary antibody (Abcam, UK) for 1 h at 37°C and were then incubated with an Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Beyotime, China) for 1 h at 37°C in the dark. After three washes with TBST, cell nuclei were counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (Beyotime, China) for 5 min. Images were acquired with a confocal laser scanning microscope (ZEISS LSM 710, Germany).

Measurements of TNF- α , IL-10 and nitric oxide (NO) with kits

PAM supernatants and sera from all mice were collected for the measurement of TNF- α , IL-10 and total NO. TNF- α and IL-10 were measured by a radioimmunoassay using ELISA kits (Jiancheng, China) according to the manufacturer's instructions. NO production was determined using a nitrate assay kit (Beyotime, China) according to the manufacturer's protocol. The absorbance was measured at 540 nm using a microplate reader, and the detection limit for NO concentration was 2-80 μ mol/l. All tests were performed in triplicate.

Observation of PAM polarization by scanning electron microscopy

To observe the effects of AFB₁ on the polarization of SIV-infected PAMs, PAMs were seeded on coverslips and infected with SIV for 24 h. Subsequently, groups of cells were further exposed to polarizing cytokines and AFB₁ for an additional 24 h, as follows: Group 1 (M1 control): LPS (100 ng/ml) plus IFN- γ (25 ng/ml); Group 2 (M2 control): IL-4 (25 ng/ml) [30-32]; Group 3: AFB₁ (0 μ g/ml); Group 4: AFB₁ (0.02 μ g/ml); and Group 5: AFB₁ (0.04 μ g/ml). At the end of the incubation period, PAMs were washed three times with PBS and were then fixed in 2.5% glutaraldehyde for 4 h at 4°C. Next, cells were processed according to the instructions for scanning electron microscopy established by the Electron Microscope Room of Nanjing Agricultural University. Images were acquired with a scanning electron microscope (HITACHI S-3000N, Japan).

Identification of PAM polarization by flow cytometry

PAMs (1×10^5 cells/well) were cultured in 6-well plates for 24 h and infected with SIV for 24 h. Subsequently, cells were further exposed to polarizing cytokines and AFB₁ for an additional 24 h, as follows: LPS (1 μ g/ml), IL-4 (100 ng/ml), AFB₁ (0 μ g/ml), AFB₁ (0.02 μ g/ml) and AFB₁ (0.04 μ g/ml). Subsequently, PAMs were washed twice with PBS and were then incubated with an Fc receptor blocker for 15 min at 4°C. Next, cells were incubated with specific CD80-PE or CD206-FITC antibodies (R&D Systems) for 30 min at 4°C. Finally, stained cells were washed twice with PBS and were analyzed using a flow cytometer.

Statistical analysis

Statistical analysis was conducted using Prism 6 (GraphPad Software, La Jolla, CA). Data are presented as the means $\pm$ SEM. To evaluate statistical significance, unpaired two-tailed Student's t-test was performed for two-group comparisons, and ordinary one-way (nonparametric) ANOVA with Tukey's post hoc test or two-way ANOVA with Dunnett's post hoc test was performed for multigroup comparisons. A value of $P < 0.05$ was considered significant. A * indicates $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ns, not significant.

Results

Low levels of AFB₁ confer a poorer outcome in response to SIV infection in mice

To assess the impact of AFB₁ on viral infection *in vivo*, we examined the outcome of SIV-infected mice intraperitoneally injected with various levels of AFB₁. As expected, SIV-infected mice exhibited enhanced weight loss, lung index, lung injury and inflammatory cell infiltration after low-level AFB₁ exposure (Fig. 1). However, none of the above changes were

Fig. 1. Low levels of AFB₁ confer a poorer outcome in response to SIV infection in mice. Anesthetized mice were intranasally infected with H1N1 virus at a level of 1000 TCID₅₀ or with PBS, injected intraperitoneally with various concentrations of AFB₁ daily, and monitored for body weight changes until the end of the experiment. (A) Comparison of weight change expressed as percentage of baseline weight. (B) The lung index was calculated by measuring the lung weight. Data are presented as means $\pm$ SEM of eight mice in each group. A * indicates $P < 0.05$; **, $P < 0.01$. (C) Representative images from mice in the six groups, as indicated. (D) Pathological changes in the lungs. The mouse lungs were removed, sectioned and stained with H&E for histological examination. Representative images from mice in each group were obtained at 100 $\times$ magnification. Inflammatory cell infiltration, inflammatory cells in the bronchial lumen and areas of hemorrhage are denoted with the black, yellow and blue arrows, respectively.

Fig. 2. Low levels of AFB₁ promote SIV infection in mice. Anesthetized mice were infected intranasally with H1N1 virus at a level of 1000 TCID₅₀, injected intraperitoneally with various concentrations of AFB₁ daily, and sacrificed. The lung tissues were harvested as described in Materials and Methods to assess viral infection, as measured by the mRNA levels of (A) viral M protein, (B, C) nucleoprotein (NP), matrix protein 1 (M1) and ion channel protein (M2). Data are presented as means ± SEM of three independent experiments. A * indicates P < 0.05; ***, P < 0.001.

seen in mice from the high-level AFB₁ group. In addition, a level of 80 µg/kg AFB₁ had no effect on these parameters in mice from the blank group. Our data suggest that compared with high levels of AFB₁, low levels of AFB₁ confer a poorer outcome in response to SIV infection and that 80 µg/kg AFB₁ is safe to mice.

Low levels of AFB₁ promote SIV infection in mice

Lung tissues were harvested to assess viral infection by the mRNA level of viral M (Fig. 2A) and the protein levels of nucleoprotein (NP), matrix protein 1 (M1) and ion-channel protein (M2) (Fig. 2B and 2C). As expected, AFB₁ at levels of 20 and 40 µg/kg markedly increased the mRNA expression level of viral M and the protein expression levels of M1, M2 and NP in the lungs of SIV-infected mice. However, we also detected no difference in viral protein and gene expression levels in the lungs of SIV-infected mice exposed to levels of AFB₁ between 0 and 80 µg/kg. In summary, these data suggest that low levels of AFB₁ promote SIV infection in mice.

Low levels of AFB₁ promote inflammatory responses and immune organ damage in SIV-infected mice

From Fig. 3A and 3B, it can be seen that TNF-α markedly increased but IL-10 significantly decreased in sera of SIV-infected mice exposed to low levels of AFB₁ compared with the TNF-α and IL-10 levels in mice not exposed to AFB₁. To determine the kinetics of AFB₁-induced TNF-α and IL-10 expression, additional time-course measurements of the serum TNF-α and IL-10 levels in SIV-infected mice with or without AFB₁ exposure were performed. As shown in Fig. 3C and 3D, the TNF-α levels increased markedly at 12 and 15 dpi, and then returned to basal levels at 18 and 21 dpi, in mice exposed to 20 or 40 µg/kg AFB₁; in contrast, the IL-10 levels decreased significantly at 15 dpi, but then increased at 18 and 21 dpi, in SIV-infected mice exposed to 40 µg/kg AFB₁. As shown in Fig. 3E, the thymus index was significantly decreased in SIV-infected mice exposed to AFB₁ at levels of 20 or 40 µg/kg compared with that in mice not exposed to AFB₁. As shown in Fig. 3F, the spleen index was significantly increased in SIV-infected mice exposed to 40 µg/kg AFB₁ compared with that of those not exposed to AFB₁, which suggests that AFB₁ may cause swelling of the spleen; this result is consistent with those shown in Fig. 3G. Taken together, these data suggest that low levels of AFB₁ promote inflammatory responses and immune organ damage in SIV-infected mice.

Fig. 3. Low levels of AFB₁ promote inflammatory responses and immune organ damage in SIV-infected mice. Anesthetized mice were infected intranasally with H1N1 virus at a level of 1000 TCID₅₀, injected intraperitoneally with various concentrations of AFB₁ daily. Sera were collected every 3 days through 9 dpi. The levels of (A) TNF- α and (B) IL-10 in sera at 15 dpi; and dynamic (C) TNF- α and (D) IL-10 levels in sera at 9, 12, 15, 18 and 21 dpi. Data are presented as the means $\pm$ SEM of three independent experiments. (E, F) The thymus and spleen weights were measured at 15 dpi. to calculate the organ indexes. Data are presented as means $\pm$ SEM of eight mice in each group. A * indicates $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ns, not significant. (G) Pathological changes in the spleen. Spleens were removed, sectioned and stained with H&E for histological examination. Representative images from mice in each group were obtained at 100 $\times$ magnification. White pulp destruction and edema in the spleen are denoted with the yellow arrows and circles, respectively.

Low levels of AFB₁ induce a switch in alveolar macrophage polarization from M1 to M2 in SIV-infected mice

To further investigate the effects of AFB₁ on the inflammatory response phase, the phenotypic switch in macrophage polarization in the lungs of SIV-infected mice was analyzed by flow cytometry (Fig. 4A and 4B). The results indicated that levels of 20 and 40 $\mu\text{g/kg}$ AFB₁ significantly decreased the high proportions of M1 macrophages induced by SIV infection (Fig. 4C) but noticeably increased the proportion of M2 macrophages in the total population of macrophages in the lungs (Fig. 4D). In addition, we detected no significant difference in the percentages of either M1 or M2 macrophages in SIV-infected mice between the group of mice not exposed to AFB₁ and the group of mice exposed to 80 $\mu\text{g/kg}$ AFB₁. Next, a gene expression analysis of M1 and M2 markers, including TNF- α and IL-10, in lung tissue was performed to further confirm the data from the flow cytometry analysis. The results showed

Fig. 4. Low levels of AFB₁ induce alveolar macrophage polarization to M2 in the SIV-infected mice. (A, B) Analysis of bronchoalveolar lavage (BAL) fluid for M1 and M2 macrophages. Cells in the BAL fluid were stained with antibodies against F4/80, CD11b, CD80, and CD206 and were analyzed using flow cytometry. Gating was performed on F4/80⁺ and CD11b⁺ cells, and cells expressing CD80 (M1 marker) or CD206 (M2 marker) were detected. (C, D) The proportions of M1 and M2 macrophages in the total population of CD80⁺ and CD206⁺ cells were calculated. (E, F) Relative TNF- α and IL-10 mRNA levels in the lungs were measured by quantitative real-time PCR. Data are presented as means $\pm$ SEM of eight mice in each group. A * indicates P<0.05; **, P<0.01; ***, P<0.001; and ns, not significant.

that levels of 20 and 40 μ g/kg AFB₁ significantly decreased the level of SIV-induced TNF- α mRNA (Fig. 4E) but markedly increased the level of IL-10 mRNA in SIV-infected lungs (Fig. 4F). Taken together, these data indicate that low levels of AFB₁ induce a switch in alveolar macrophage polarization from M1 to M2 in SIV-infected mice.

Effects of various concentrations of AFB₁ and LPS on PAMs

To assess whether any of the effects of AFB₁ and LPS on SIV infection result from an induction of toxicity, the effects of various concentrations of LPS or AFB₁ on cell viability were determined by MTT and flow cytometric assays. As shown in Fig. 5, the viability of PAMs was not affected by either LPS or AFB₁ at concentrations between 0.01 and 0.04 μ g/ml for (A) 24 or (B, C) 48 h. Thus, concentrations of 0.01, 0.02 and 0.04 μ g/ml AFB₁ were used in our subsequent experiments.

Low concentrations of AFB₁ promote SIV infection in PAMs

To investigate the potential role of AFB₁ in SIV infection, viral titers, viral M mRNA and NP protein expression levels in SIV-infected PAMs were measured by TCID₅₀ assay, real-time PCR, and western blotting and immunofluorescence staining, respectively. PAMs were infected with SIV (MOI: 0.1) and were then treated with various concentrations of AFB₁ for 48 h because the viral titers peaked at 48 hpi and then plateaued [33]. As shown in Fig. 6A, viral titer was significantly increased in PAMs treated with 0.04 μ g/ml AFB₁ compared with

Fig. 5. Effects of various concentrations of AFB₁ and LPS on PAMs. PAMs were exposed to various concentrations of AFB₁ or LPS for 24 or 48 h and were then subjected to MTT and flow cytometric assays for the detection of (A, B) cell proliferation and (C) apoptosis. Cells not treated with AFB₁ or LPS were used as the control group (A, B). The total apoptosis rate was equal to the sum of the early apoptosis rate (Q2, red ellipse) and the late apoptosis rate (Q3, pink ellipse). Data are presented as means ± SEM of three independent experiments. Significance compared with control, *P < 0.05; **P < 0.01; and ***P < 0.001.

that in cells not treated with AFB₁. Correspondingly, the viral M mRNA expression level was also markedly increased in cells treated with 0.02 and 0.04 µg/ml AFB₁ (Fig. 6B). To further verify the results, the NP expression level in SIV-infected PAMs was assessed. As shown in Fig. 6C and 6D, AFB₁ at the concentrations of 0.02 and 0.04 µg/ml significantly increased viral NP expression in PAMs compared with that in cells not treated with AFB₁. Consistent with the above results, immunofluorescence staining demonstrated that compared with no treatment, AFB₁ treatment markedly increased the percentage (data not shown) of cells staining positive for the viral NP protein (Fig. 6E) and that no NP expression was observed in PAMs from the mock group. Taken together, our results suggest that exposure to low concentrations of AFB₁ promotes influenza virus infection in PAMs.

AFB₁ induces a switch in SIV-infected PAM polarization from M1 to M2

SIV-infected PAMs were exposed to various concentrations of AFB₁, and total RNA was extracted at 4, 8, and 24 hpi. The real-time PCR results showed that TNF-α and iNOS mRNA expression levels in PAMs exposed to 0.04 µg/ml AFB₁ were significantly elevated at 8 hpi and then returned to basal levels at 24 hpi; IL-10 mRNA expression was sharply elevated at 24 hpi

Fig. 6. Low concentrations of AFB₁ promote SIV infection in PAMs. PAMs were seeded in 96-well plates, infected with H1N1 virus (MOI: 0.1), and then treated with various concentrations of AFB₁. (A) Viral titers. Infectious viral particles were quantified at 48 hpi by a TCID₅₀ assay. (B) Levels of viral M protein mRNA and (C, D) nucleoprotein (NP) were analyzed by real-time PCR and western blotting. Significance compared with control, *P < 0.05; **P < 0.01; and ***P < 0.001. (E) Immunohistochemistry of H1N1 influenza virus NP expression. PAMs were infected with H1N1 virus (MOI: 0.1) or mock-infected (medium) and were immediately treated with various concentrations of AFB₁. The infected cells were stained for NP expression (green, as indicated by red arrows) at 24 hpi, and nuclei (blue) were counterstained with DAPI.

in cells exposed to AFB₁ at concentrations of 0.02 and 0.04 µg/ml (Fig. 7A). The ELISA and the total NO assay showed results for macrophage phenotype verification similar to those of the gene expression analysis. Particularly, IL-10 production was markedly increased at 24 hpi in cells exposed to AFB₁ at concentrations of 0.02 and 0.04 µg/ml (Fig. 7B). Moreover, we also demonstrated the morphological change in polarized macrophages by using scanning electron microscopy analysis. As shown in Fig. 7C, the surface of M1 macrophages (M1 control) was rough, and the cilia were shorter than those of M2 macrophages; however, M2 macrophages (M2 control) were smooth, and the cilia were longer. With increasing AFB₁ concentrations, the macrophages clearly transformed to the M2 phenotype. To further confirm whether AFB₁ exposure induced macrophage polarization to M2, we used flow cytometry to identify the percentages of M1 and M2 macrophages (Fig. 7D). The polarization of macrophages to M1 or M2 was determined by the expression of CD80 or CD206, respectively. Our results showed that the percentages of CD206-positive cells in the AFB₁-treated groups were much higher than those in the group with SIV infection only. However, there was no significant difference in the percentages of CD80-positive cells among the AFB₁ and SIV groups. Taken together, our results demonstrated the dynamic polarization of macrophages induced by AFB₁ and suggested that AFB₁ induced the switch in PAM polarization from M1 to M2.

AFB₁ promotes SIV infection through inducing a switch in PAM polarization from M1 to M2

To investigate the relationship between the increase in viral infection and the macrophage polarization to M2, LPS was added to PAMs to reverse the switch in polarization from M2 to M1. In one trial, after 24 h of incubation with LPS, PAMs were infected with SIV and were then exposed to AFB₁; in the other trial, LPS was added to PAMs exposed to various concentrations of AFB₁ at 24 h post-SIV infection. As shown in Fig. 8A, TNF-α and IL-10 mRNA expression levels were not affected, suggesting that LPS treatment before viral infection could not reverse the PAM polarization to M1. Likewise, LPS treatment prior to viral infection could not attenuate the AFB₁-induced promotion of viral infection, as demonstrated by the indistinctive decrease in viral M mRNA expression (Fig. 8B). In contrast, LPS treatment post-viral infection reversed the PAM polarization to M1, as demonstrated by the significantly decreased IL-10 mRNA (~25-fold) but increased TNF-α mRNA (~10-fold) expression

Fig. 7. AFB₁ induces the switch in SIV-infected PAM polarization from M1 to M2. PAMs were infected with H1N1 virus (MOI: 0.1) and treated with or without AFB₁. Then, (A, B), levels of the M1 and M2 macrophage polarization markers (TNF- α and IL-10) were determined as described in Materials and Methods. (C) Macrophage surface morphology. Red arrows denote the surface of macrophages, and green arrows denote macrophage cilia. (D) SIV-infected PAMs were treated with 0, 0.02 or 0.04 μ g/ml AFB₁ for 24 h. LPS (1 μ g/ml) and IL-4 (100 ng/ml) were used simultaneously for 24 h to stimulate macrophage polarization to M1 and M2, respectively. Cells positive for CD80 (M1 marker) or CD206 (M2 marker) expression were analyzed by flow cytometry. Data are presented as means $\pm$ SEM of three independent experiments. Significance compared with control, *P < 0.05; **P < 0.01; ***P < 0.001; and ns: not significant.

in PAMs compared with those levels in PAMs not treated with LPS (Fig. 8C). Importantly, compared with no treatment, LPS treatment post-viral infection significantly reduced viral M mRNA expression and thoroughly counteracted the promotion of viral infection induced by AFB₁ at the concentration of 0.02 μ g/ml (Fig. 8D). Taken together, our results suggest that AFB₁ promotes SIV infection via a switch in PAM polarization from M1 to M2.

Fig. 8. AFB₁ promotes SIV infection through inducing a switch in PAM polarization from M1 to M2. LPS was added to PAMs exposed to various concentrations of AFB₁ at 24 h (A, B) before or (C, D) after SIV infection (MOI: 0.1) to investigate the relationship between the increase in viral infection and the PAM polarization. Cells were collected to measure the mRNA expression of polarization markers and viral M protein by using real-time PCR. Data are presented as means ± SEM of three independent experiments. Significance compared with control, **P < 0.01; ***P < 0.001; and ns: not significant. Different lowercases indicated significant difference (P < 0.05).

Discussion

AFB₁ is considered the most toxic aflatoxin, and its LD₅₀ is just 0.36, 1, and 500 mg/kg for ducklings, rats, and sheep, respectively [34, 35]. Swine are one of the most mycotoxin-sensitive species, and a previous study has shown that the maximum tolerance level of AFB₁ for pigs was approximately 0.385 mg/kg of feed [36]. In contrast, mice are highly resistant to AFB₁ (TD₅₀ > 5400 mg/kg b.w.) [37]. In humans, exposure to AFB₁ at concentrations of 30 to 50 µg/kg b.w., 50 to 100 µg/kg b.w. and 100 to 1000 µg/kg b.w. was considered to lead to mild, moderate and severe intoxication, respectively, according to the World Health Organization. According to the guidelines established by the US Food and Drug Administration and the National Food Safety Standard (GB2761-2017, China), maximum dietary AFB₁ concentrations of 20 and 300 µg/kg are allowed for humans and animals, respectively. However, we did not know whether low-level AFB₁ may cause or exacerbate other secondary diseases. Therefore, concentrations of 20, 40 and 80 µg/kg b.w. were used in this study. Our findings confirmed that 80 µg/kg AFB₁ has no effects on the body weight and lung function of mice, which is consistent with a previous report [37]. Likewise, in this study *in vitro*, the safe concentrations of AFB₁ (0.01 to 0.04 µg/ml) were screened for use in subsequent experiments by cytotoxicity and apoptosis assays.

Since the initial report in 1979 that AFB₁ decreased interferon production related to influenza virus infection [38], scant literature has been available about the effects of AFB₁ on viral load or infection. Our study demonstrates for the first time that low levels of AFB₁ promote SIV infection *in vivo* and *in vitro*. The results of the *in vivo* study showed that the body weight of SIV-infected mice exposed to 40 µg/kg AFB₁ decreased, but the lung index

increased. These findings are consistent with the outcomes of viral infection [39-41], suggesting that AFB₁ exacerbated viral infection. In addition, levels of 20 and 40 µg/kg AFB₁ also increased lung injury and inflammatory cell infiltration in SIV-infected mice. All these results suggested that low levels of AFB₁ promoted SIV infection in mice. Previous studies have reported that PAMs were likely to be the first targets of an invading pathogen [23], and influenza virus was detectable in a PAM cell line [42]. Thus, it was essential for PAM to be one of the cell types in our experimental studies. We also observed increased viral infection in the PAM model of SIV infection, supporting the conclusion of the *in vivo* experiment.

Cytokine secretion is closely related to the host immune status after viral infection [43]. Here, increased SIV infection accompanied by increases in TNF-α but decreases in IL-10 in sera were observed at 15 dpi. after low-level AFB₁ exposure. A previous study reported that proinflammatory cytokines not only induce host resistance to influenza virus but also instruct adaptive immune responses to reduce viral load; however, the effects of proinflammatory cytokines are antagonized by anti-inflammatory cytokines such as IL-10 [44]. In addition, a delicate balance between pro- and anti-inflammatory cytokine production is essential for the recovery from and defense against viral infection [45]. Accordingly, our data suggested that the inflammatory response was aggravated to defend against SIV infection. Furthermore, the thymus index was significantly decreased in SIV-infected mice exposed to AFB₁ at levels of 20 and 40 µg/kg compared to that of mice not exposed to AFB₁, which suggests that low-level AFB₁ exposure may cause thymus atrophy, consistent with the results of a previous report [46]. Moreover, the spleen index was significantly increased in SIV-infected mice exposed to 40 µg/kg AFB₁ compared with that of mice not exposed to AFB₁, which suggests that AFB₁ may cause swelling of the spleen, consistent with the results of the HE staining. In general, M1 macrophages produce proinflammatory cytokines, thus contributing to host defense against pathogens and inducing tissue injury through persistent inflammation; M2 macrophages produce anti-inflammatory cytokines, thus inhibiting inflammatory responses and promoting tissue repair [47]. Therefore, we concluded that AFB₁ might accelerate the inflammatory response phase, thereby promoting a switch to the protective M2 phenotype. Our findings also demonstrated that compared with no exposure to AFB₁, 80 µg/kg AFB₁ caused decreases in the levels of both TNF-α and IL-10 in sera of mice, which is consistent with the results of these previous studies [19, 48] but inconsistent with the findings of Long et al., who reported that AFB₁ exposure increased the TNF-α mRNA expression in mouse spleens [46].

To further confirm the inflammatory status in SIV-infected mice exposed to AFB₁, models of SIV-infected PAMs and SIV-infected mice were established and exposed to AFB₁. Our data showed that AFB₁ induced a switch in macrophage polarization from M1 to M2 *in vivo* and *in vitro*. Our findings also showed that the promotion of influenza virus infection requires the phenotypic transformation of PAMs from M1 to M2 and is dependent on the polarization status induced by AFB₁, which confirms that AFB₁ promotes SIV infection via the switch in PAM polarization from M1 to M2. Interestingly, there was an inconsistency in the cytokine levels of TNF-α and IL-10 between the *in vivo* and *in vitro* conditions in this study. TNF-α and IL-10 were the markers of M1 and M2, respectively, and their change trends in sera were not consistent with the alveolar macrophage polarization results from the flow cytometric assays *in vitro* and *in vivo*. We try to explain the reason for the inconsistency as follows. First, the inflammatory response is a consecutive process; macrophages first display the M1 phenotype and upon return to homeostasis, they convert to the M2 phenotype [18]. Our *in vitro* results indicated that TNF-α was significantly elevated at 8 hpi and then returned to basal levels at 24 hpi, whereas IL-10 was sharply elevated at 24 hpi; these results confirmed macrophage polarization to M1 at 8 hpi and to M2 at 24 hpi. In addition, increasing numbers of studies have reported that M1/M2 polarization depends on the amount of pathogen and the duration of exposure [15-17]. Therefore, TNF-α and IL-10 could present inconsistent trends at different time points. To confirm our hypothesis, dynamic measurements of the serum TNF-α and IL-10 levels in SIV-infected mice with or without AFB₁ exposure were performed at 9, 12, 15, 18 and 21 dpi. The *in vivo* kinetics of serum TNF-α and IL-10 showed

that the TNF- α level began to increase at 12 and 15 dpi. and then returned to the basal level at 18 and 21 dpi. in mice exposed to AFB₁; in contrast, the IL-10 content significantly decreased at 15 dpi. but then increased at 18 and 21 dpi. in SIV-infected mice exposed to AFB₁. These data suggest that AFB₁ first promotes proinflammatory cytokines and then induces anti-inflammatory cytokines, thereby inducing a switch in macrophage polarization from M1 to M2. *In vivo*, alveolar macrophages are the first line of defense against pathogens, and systemic reactions often occur later than the local reaction in alveolar macrophages. Therefore, the sera could still contain cytokines related to the M1 phenotype when the alveolar macrophages just began to convert from M1 to M2. Second, macrophages are not the only cells that produce TNF- α and IL-10, so the concentrations of TNF- α and IL-10 in the sera could be inconsistent with those in alveolar macrophages at the same time. Finally, the bodies of animals are often sufficiently complex and robust to display a strong buffering ability, and the IL-10 level in sera may not quickly increase relative to the speed of its increase in local macrophages.

Conclusion

Our study indicated that low-level AFB₁ exposure promotes SIV infection and increases its severity and that the switch in PAM polarization from M1 to M2 may be one of the mechanisms of action underlying this phenomenon. The work reported here provides important data that point to a role for AFB₁ in SIV infection, and it opens a new field of study. It is likely that toxic contamination will increase the risk to a range of viral infections beyond influenza.

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Disclosure Statement

The authors declare no conflict of interests.

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