RESEARCH ARTICLE

Effects of Schiff base aromatic amino acid derivatives on antioxidant and immune system disturbances in a rat model of aflatoxin B1 induced experimental mycotoxicosis [version 1; referees: 1 approved with reservations]

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Abstract

Background: Aflatoxin B1 (AFB1) is the most hepatotoxic and hepatocarcinogenic of the aflatoxins and occurs as a contaminant in a variety of foods. The toxicity of AFB1 has been shown to be associated with a wide range of pathological events, such as enhanced apoptosis and oxidative events. Currently there is no treatment for mycotoxic exposure. The aim of this study was to evaluate the potential ability of picolinyl-L-phenylalaninate (PLP), picolinyl-L-tryptophanate (PLT), and nicotinyl-L-tryptophanate (NLT) Schiff base amino acid derivatives to act against damaging effects of AFB1 using a rat model of mycotoxicosis. For this purpose, a range of markers of immune and antioxidant systems in liver and blood plasma samples, as well as the apoptotic rate in neutrophils and monocytes was assessed.

Methods: Mongrel white pubescent rats (with 180-200g b/w) were used in all experiments. Concentration of the markers of immune and antioxidant systems was measured in plasma by ELISA, using commercially available kits according to manufacturers' instructions. The rate of apoptosis in neutrophils and monocytes was analyzed by flow cytometry. Results: AFB1 induced mycotoxicosis caused significant elevation of malonic dialdehyde contents (plasma and liver: p = 0.0001 compared with untreated rats), the levels of superoxide dismutase (p=0.005), total non-enzymatic water-soluble antioxidants (p = 0.0001), and terminal complement complex (p = 0.021). Moreover, the increased rates of early and late apoptosis in neutrophils and monocytes were observed as well. Treatment with PLP, PLT and NLT were shown to mitigate these effects, though to a different extent.

Conclusions: The results obtained in this study clearly demonstrated that chronic AFB1 exposure induced oxidative cell damage, immunosuppression and apoptosis of circulating immune cells. The oral administration of Schiff base cyclic amino acid derivatives was capable of minimizing the detrimental effects of mycotoxicosis by possessing multi-mechanistic effects that target AFB1-induced pathological events.
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Introduction

Aflatoxin B1 (AFB1) is a mycotoxin produced by Aspergillus flavus and related fungi that grow in staple foods, including cereals and nuts, such as corn, rice and peanuts, especially in areas with appropriate conditions of moisture and heat where these fungi are ubiquitous. AFB1 causes a serious threat for human and animal health and, in extreme cases, lead to death. The toxicity of AFB1 has been shown to be associated with a wide range of pathological events such as enhanced apoptosis, oxidative events and carcinogenesis. Moreover, AFB1 is the one of mycotoxins that were adopted for the use in bioterrorism.

AFB1 is metabolized into aflatoxin-8,9-epoxide, reactive oxygen species (ROS), which can react with proteins and DNA to form adducts and cause mutations in the p53 and other genes essential for cell malignant transformation. AFB1 has also been shown to be immunotoxic to animals and is suspected to be immunosuppressive in humans.

Currently there is no treatment for mycotoxin exposure, except of supporting therapy, such as diet and hydration. Development of the efficient measures neutralization of toxic effects of mycotoxins and prevention of associated pathological changes is an issue of great importance. The key aspect here is that the potential therapeutic should possess multi-mechanistic actions and simultaneously target a range of pathological processes caused by AFB1.

Our previous studies have demonstrated that Schiff base amino acid derivatives picolinyl-L-phenylalaninate (PLP), picolinyl-L-tryptophanate (PLT) and nicotinyl-L-tryptophanate (NLT) are capable of scavenging free-radicals, elevating the capacities of antioxidative and the immune system in radiation injury, and possessing anticytotoxic, antigenotoxic and antimutagenic properties. Based on these results, we suggest that the above-mentioned multifunctional compounds may have protective effects against mycotoxins. This suggestion is also supported by other results indicating that several Schiff base derivatives are capable of decreasing the concentrations of aflatoxin M1 in artificially contaminated raw milk, and have good neutralization activity against Aspergillus niger. In addition, L-tryptophan was shown to alleviate aflatoxin-induced chicken growth retardation and immunosuppression, while phenylalanine may prevent ochratoxin A-induced suppression of the immune response and inhibition of protein synthesis in spleen, kidney and liver.

This study is aimed to evaluate the ability of novel Schiff base cyclic amino acid derivatives to protect against oxidative stress and immunosuppression in an animal model of AFB1 mycotoxicosis.

Methods

Synthesis and characterization of PLT, NTL and PLP Schiff bases

The synthesis of PLT was performed as described previously by condensation of picolinaldehyde (2-pyridinecarboxaldehyde) and potassium salt of L-tryptophan in alcohol solution (ethanol, methanol) in a molar ratio 1:1 at the temperature range 5–25°C. A similar procedure was used for NLT synthesis, which is a condensation product of nicotinaldehyde (3-pyridinecarboxaldehyde) and L-tryptophan potassium salt, and PLP, which is a condensation product of picolinaldehyde (2-pyridinecarboxaldehyde) and L-phenylalanine potassium salt (Figure 1).

Animal models of AFB1-induced mycotoxicosis

Mongrel white pubescent male rats (180–200 g; Animal Facility of the Institute of Molecular Biology, National Academy of Sciences, Armenia) were used in all experiments. Twelve randomly selected animals were used in each of the following groups: 1) Controls – no treatment; 2) AFB1 mycotoxicosis – rats orally treated with AFB1 mycotoxin during 21 days at 25μg/kg per day dose level and left for 10 additional days without any treatment; 3) AFB1 mycotoxicosis + Schiff bases – rats orally treated with AFB1 mycotoxin during 21 days at 25μg/kg dose level and 10-day oral treatment with PLP, PLT or NLT at 10 mg/kg per day dose level; 4) Schiff-base only – rats received 10-day oral treatment with 10mg/kg PLP, PLT or NLT. Oral treatment with AFB1 and Schiff bases was delivered with water. At the end of the treatment period, animals were euthanized by decapitation.

During the treatment period animals were allowed free access to water and food and were kept (maximum 5 animals per cage) in pathogen free conditions at regular 12 hour day-night cycles. Animal care, handling and use in research were performed according to the international regulations adopted by the Ministry of Health of the Republic of Armenia. The described experimental protocols of animal studies were considered and approved.

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by the Ethical Committee acting at Institute of Molecular Biology. All efforts were made to ameliorate any suffering of the animals; animal decapitation was performed in dedicated room located distantly from animal care facility by trained personnel using sharpened guillotines regularly adjusted to ensure proper performance.

Plasma sampling
Fresh trunk blood samples were collected in EDTA containing tubes during decapitation. Fresh blood aliquots were immediately used for flow cytometry (see below). Plasma was separated with centrifugation (10 minutes at 3000g at 4°C) and stored at -30°C until further analyses.

Assessment of lipid peroxidation in plasma and liver
Malonic dialdehyde (MDA) content, as a marker of terminal phase of lipid peroxidation, was measured in blood plasma (0.1 mL)\(^3\) and liver homogenate. Homogenate was obtained by excising 100 mg of liver and pulverizing in solution containing 0.3 mL 40mM Tris–HCl buffer (pH = 7.4), 0.3 mL 12\(^*\)10\(^{-5}\) M Mohr salt and 0.3 mL of 0.8 mM of ascorbic acid\(^5\). The activity of lipid peroxidation in blood plasma and liver is based on the amount of MDA formation, which during interaction with 0.8 mL 0.12M thiobarbituric acid, gives a coloring reaction, determined at a wavelength 535 nm using UV-752 UV-VIS Spectrophotometer (Shanghai Phenix Optical Scientific Instrument Co. Ltd, China). Optical density was converted to concentration units using Excel 2007. The MDA concentration was calculated using an extinction coefficient and expressed as μMol MDA/g liver tissue or μMol MDA/mL plasma.

Assessment of water soluble antioxidant levels
The integral antioxidant activity (AOA) represents the sum of the antioxidant capacity of hydrophilic and lipophilic antioxidants of low-molecular non-enzymatic water-soluble antioxidants (ascorbic acid, glutathione and uric acid) of blood serum. AOA was analyzed by photochemiluminescence detection using a Photochem analyzer and ACW Kit (Analytik Jena AG, Jena, Germany), as per the manufacturer’s instructions. In this assay, generation of free radicals is partially eliminated by the reaction with the antioxidants present in the serum samples, and the remaining radicals are quantified by luminescence generation. An ascorbate calibration curve was used to evaluate AOA levels. The results were expressed as conventional units equal to mMol ascorbate with equivalent activity.

Assessment of plasma levels of markers of immune and antioxidant systems
Plasma levels of circulating immune complexes (CIC) (Rat Circulating Immune Complexes ELISA kit; BlueGene Biotech, Shanghai, China), terminal complement complex (C5b-C9) (Rat Terminal complement complex (C5b-9) ELISA kit; BlueGene Biotech), superoxide dismutase (SOD) (Rat Superoxide Dismutase Copper (SOD) ELISA kit; BlueGene Biotech), and catalase (CAT) (Rat Catalase ELISA kit; BlueGene Biotech) were measured using commercially available ELISA kits, according to manufacturer’s instructions using StatFax-2100 plate reader (Awareness Technology Inc, USA). The detection limit for CICs, C5b9, SOD, CAT was 0.1ng/mL, 0.1pg/mL, 0.1μg/mL, 0.1ng/mL, respectively.

Assessment of apoptotic rate in neutrophils and monocytes
100 μL of whole blood from each studied rat was used to quantify apoptotic rate and percentage of non-viable cells. Erythrocytes were discarded by lysis (ammonium chloride lysis buffer); white blood cells were washed in Annexin-binding buffer and stained with 5 μL of Annexin V–FITC conjugate for 20 minutes, followed by staining with 1 μg/mL of propidium iodide (PI). Apoptotic rate and cell viability were analyzed on a Partec CyFlow Space (Partec, Germany). 10 000 events were collected from each sample. The neutrophil and monocyte populations in peripheral blood were distinguished by forward scatter and side scatter. Gating and determination of early and late apoptotic rates were done by FlowJo vX0.7 software (Tree Star Inc, USA). The positively stained apoptotic cells were counted, and the apoptotic index was calculated as the percentage of apoptotic cells within the total number of cells. Cells that stained only for Annexin V were considered early apoptotic (Annexin V+/PI), and cells that dually stained for both Annexin V and PI were considered late apoptosis (Annexin V+/PI).

Statistical analysis
Data is presented as the mean ± SD, unless otherwise specified. Comparison of intergroup mean differences between the levels of studied markers in controls, AFB1-exposed, as well as treated groups, was performed using one-way analysis of variance (ANOVA). P values <0.05 were considered as significant. Statistical analysis of plasma markers was performed using GraphPad Prizm 5.0 software (GraphPad Software, Inc, USA).

Results and discussion
Effects of Schiff bases on mycotoxin induced oxidative stress
First we evaluated the effect of Schiff bases on the studied parameters in intact animals (Schiff-base only group). In blood plasma (Figure 2A) and liver (Figure 2B) of the intact animals treatment with PLP (blood: p = 0.0023, liver: p = 0.0001), PLT (blood: p= 0.0002, liver: p=0.0342), and NLT (blood: p = 0.0001, liver: p = 0.0001) caused a significant decrease in MDA levels. No changes in SOD and CAT levels were observed during treatment with Schiff bases (Figure 3A and B), while a significant increase in total soluble AOA of blood serum was observed (Figure 4).

Next we analyzed the changes of the above mentioned parameters in AFB1 and AFB1+Schiff bases groups of animals. We assessed the intensity of AFB1-induced lipid peroxidation and the effects of the Schiff bases. According to our results, AFB1 induced a significant increase of MDA both in plasma (59%, p = 0.0001) and liver (85%, p = 0.0001) in rats of mycotoxicosis group (Figure 2), which suggests about activation of lipid peroxidation processes. Treatment of mycotoxicosis with Schiff bases caused a significant decrease of MDA levels both in blood and liver (Figure 2).
Next, we tested the status of enzymatic free radical defense system during AFB1 mycotoxicosis and Schiff base treatment 10 mg/kg dose level. The levels of SOD were significantly increased (p=0.005) in the plasma of AFB1 treated rats, while no difference was observed for CAT levels (Figure 3A). Treatment with PLT and NLT decreased SOD levels to its control values, while treatment with PLP reduced its levels further (p=0.001) (Figure 3A).

Finally, we observed fourfold elevation of total soluble AOA of blood serum non-enzymatic water-soluble antioxidants in AFB1 (p = 0.0001) mycotoxicosis compared to control. Treatment with PLT (p = 0.0001) and NLT (p = 0.0013) had a tendency to normalize AOA levels in AFB1-exposed animals, while PLP had no clear impact on their levels (Figure 4).

The increase of the malonic dialdehyde in blood plasma and liver homogenate suggests the intensification of lipid peroxidation in the organ and systemic levels. It is well known that AFB1 metabolizes into aflatoxin-8,9-epoxide, which aggressively interacts with DNA and forms adducts\(^{25}\). Moreover, in line with our results it has been shown that AFB1 induces lipid peroxidation in liver\(^{25}\). Meanwhile, we observed the increase of the levels of SOD and water-soluble non-enzymatic antioxidants, which can be a compensatory reaction to counterbalance oxidative stress. Schiff bases (PLT and NLT) were shown to normalize the levels of both SOD and AOA levels, as well as decrease the levels of MDA, which indicates their ability to interfere with the process of ROS generation in response to AFB1 exposure. Though the precise mechanism of their action is unknown, it was proposed that it might be related to the contents of active hydroxyl and amino groups of the Schiff bases\(^{26,27}\).

**Effects of Schiff bases on mycotoxin-induced changes of the immune system markers**

In order to evaluate the immune system changes caused by exposure to AFB1 and the effects of Schiff bases, the levels of terminal...
Figure 3. Effect of Schiff bases on SOD and CAT levels in AFB1 mycotoxicosis. Levels (μg/mL) of SOD (A) and CAT (B) in blood plasma of studied groups. CNTRL – intact animals (n = 10); PLP, PLT and NLT intact animals (for SOD: n = 10 in each group; for CAT: n = 10 in PLP and PLT, n = 9 in NLT groups) received 10-day oral treatment with corresponding Schiff bases at 10mg/kg dosage; AFB1 – rats (n = 5) treated with AFB1 for 21 days at 25μg/kg dosage; AFB1+PLP, AFB1+PLT, AFB1+NLT – AFB1 exposed rats (n = 5 in each group) treated with corresponding Schiff bases (mycotoxin and Schiff base dosages were similar in all treated groups). Data presented as mean±SD. *p<0.05 vs. CNTRL; #p<0.05 vs. AFB1. AFB1, aflatoxin B1; SOD, superoxide dismutase; CAT, catalase; PLP, picolinyl-L-phenylalaninate; PLT, picolinyl-L-tryptophanate; NLT, nicotinyl-L-tryptophanate.

Figure 4. Effect of Schiff bases on the total soluble AOA in AFB1 mycotoxicosis. Total soluble antioxidant activity (expressed as conventional units, c.u.) in blood plasma of studied groups. CNTRL – intact animals; PLP, PLT and NLT intact animals received 10-day oral treatment with corresponding Schiff bases at 10mg/kg dosage; AFB1 – rats treated with AFB1 for 21 days at 25μg/kg dosage; AFB1+PLP, AFB1+PLT, AFB1+NLT – AFB1 exposed rats treated with corresponding Schiff bases (mycotoxin and Schiff base dosages were similar in all treated groups). Number of animals in all groups was 6. Data presented as mean±SD. *p<0.05 vs. CNTRL; #p<0.05 vs. AFB1. AFB1, aflatoxin B1; AOA, antioxidant activity; PLP, picolinyl-L-phenylalaninate; PLT, picolinyl-L-tryptophanate; NLT, nicotinyl-L-tryptophanate.
complement component (C5b-C9, TCC), circulating immune complexes, as well as the rates of early and late apoptosis of neutrophils and monocytes were assessed.

The results showed a statistically significant increase (p = 0.021) of TCC levels (marker of complement activation) and reduced (p = 0.003) levels of CICs in the blood plasma of AFB1-exposed animals (Figure 5). The treatment with NLT and PLP further increased the levels of TCC (p = 0.001 and p = 0.003 compared to controls, respectively), in the meantime normalizing CIC levels to that of controls. By contrast, treatment with PLT decreased the levels of TCC to the control levels without affecting low CIC levels. Neither CIC nor TCC levels were affected by the treatment with Schiff bases alone (Figure 5).

The spontaneous apoptotic rates (early and late) of whole blood neutrophils and monocytes from control rats were not different compared with those from the rats treated with NLT, PLT, and PLP (Figure 5). The only exception was significantly reduced late apoptosis of neutrophils in the blood of rats treated with PLT (p = 0.032) and PLP (p = 0.034). AFB1 aflatoxin had a profound effect on the apoptosis of neutrophils and monocytes (Figure 6 and Figure 7). Particularly, in mycotoxicosis induced rats significantly accelerated early apoptosis of neutrophils and monocytes (p = 0.045) and late apoptosis of monocytes (p = 0.0027) were observed. Meanwhile, the treatment of AFB1-exposed rats with NLT significantly decreased the apoptotic rate of neutrophils (p = 0.05 and p = 0.036, respectively) and monocytes (p = 0.011 and p = 0.0025, respectively). We also observed a

Figure 5. Effect of Schiff bases on the levels of CICs and complement terminal complex in AFB1 mycotoxicosis. Levels (pg/mL) of terminal complement complexes (A) and CICs (B) in blood plasma of studied groups. CNTRL – intact animals (n = 10); PLP (n = 9), PLT (n = 8) and NLT (n = 10) intact animals received 10-day oral treatment with corresponding Schiff bases at 10mg/kg dosage; AFB1 – rats treated with AFB1 for 21 days at 25μg/kg dosage (n = 8); AFB1+PLP (n = 6), AFB1+PLT (n = 6), AFB1+NLT (n = 9) – AFB1 exposed rats treated with corresponding Schiff bases (mycotoxin and Schiff base dosages were similar in all treated groups). Data presented as mean±SD. *p<0.05 vs. CNTRL; #p<0.05 vs. AFB1. AFB1, aflatoxin B1; CICs, circulating immune complexes; PLP, picolinyl-L-phenylalaninate; PLT, picolinyl-L-tryptophanate; NLT, nicotinyl-L-tryptophanate.
Figure 6. Effect of Schiff bases on the spontaneous and induced apoptotic rates of neutrophils in AFB1 mycotoxicosis. Spontaneous (A) and induced apoptosis (B) of neutrophils in studied groups. CNTRL – intact animals (n = 5); PLP, PLT and NLT intact animals received 10-day oral treatment with corresponding Schiff bases at 10mg/kg dosage (n = 9 in each group); AFB1 – rats treated with AFB1 for 21 days at 25μg/kg dosage (n = 9); AFB1+PLP, AFB1+PLT, AFB1+NLT – AFB1 exposed rats treated with corresponding Schiff bases (mycotoxin and Schiff base dosages were similar in all treated groups) (n = 9 in each group). Data presented as mean±SD. *p<0.05 vs. CNTRL; #p<0.05 vs. AFB1. AFB1, aflatoxin B1; PLP, picolinyl-L-phenylalaninate; PLT, picolinyl-L-tryptophanate; NLT, nicotinyl-L-tryptophanate.

significant effect of PLT on the late apoptosis of neutrophils (p = 0.035) and monocytes (p = 0.0075) in the rats with developed aflatoxicosis compared to untreated rats. A less prominent effect was shown for PLP. The only significant difference was found in the late apoptosis of monocytes (p = 0.016).

Mycotoxins may induce severe immunosuppression by down-regulation of T and B lymphocyte activity, inhibition of antibody production and synthesis of complement components and interferon as well as impairment of macrophage-effectors cell function. While the exact mechanisms of mycotoxins action on immune system are presently unknown, oxidative stress, DNA damage, inhibition of gene expression and protein synthesis can be involved in immunosuppressive action of mycotoxins.

In this study, we observed massive induction of apoptosis of neutrophils and monocytes induced by AFB1, which is in line with a previous report. It is known that apoptotic cells activate complement. Subsequently, complement binding by apoptotic cells in normal human plasma occurs mainly to late apoptotic, secondary necrotic cells, and the dominant mechanism involves the classical pathway of complement activation by antibodies. Depletion of antibodies abolishes most complement fixation by apoptotic cells and causes delayed clearance of them. Furthermore, we for the first time, reported the decrease of circulating immune complexes and the increase of circulating terminal complement component in AFB1-treated mice, which is in line with these findings. In this regard, Schiff bases (PLP and NLT) were shown to have modulating effect on immunity, by decreasing apoptosis rate and restoring the
Figure 7. Effect of Schiff bases on the spontaneous and induced apoptotic rates of monocytes in AFB1 mycotoxicosis. Spontaneous (A) and induced apoptosis (B) of monocytes in studied groups. CNTRL – intact animals (n = 5); PLP, PLT and NLT intact animals received 10-day oral treatment with corresponding Schiff bases at 10mg/kg dosage (n = 9 in each group); AFB1 – rats treated with AFB1 for 21 days at 25μg/kg dosage (n = 9); AFB1+PLP, AFB1+PLT, AFB1+NLT – AFB1 exposed rats treated with corresponding Schiff bases (mycotoxin and Schiff base dosages were similar in all treated groups) (n = 9 in each group). Data presented as mean±SD. *p<0.05 vs. CNTRL; #p<0.05 vs. AFB1. AFB1, aflatoxin B1; PLP, picolinyl-L-phenylalaninate; PLT, picolinyl-L-tryptophanate; NLT, nicotinyl-L-tryptophanate.

Conclusions
The results obtained in this study clearly demonstrated that AFB1 administration induced oxidative cell damage, immunosuppression and apoptosis of circulating immune cells. The oral administration of Schiff base cyclic amino acid derivatives is capable of minimizing the detrimental effects of mycotoxicosis by possessing multi-mechanistic effects that target AFB1-induced pathological events.

Data availability
Dataset 1. Raw data for raw values for all the commercial ELISA kits (SOD, CAT, TCC and CIC), the values from the MDA, AOA.
http://dx.doi.org/10.5256/f1000research.11756.d169180

Dataset 1 contains tables with raw data for all measured assays except flow cytometry. CNTRL – intact animals; PLP, PLT and NLT intact animals received 10-day oral treatment with corresponding Schiff bases at 10mg/kg dosage; AFB1 – rats treated with AFB1 for 21 days at 25μg/kg dosage; AFB1+PLP, AFB1+PLT, AFB1+NLT – AFB1 exposed rats treated with corresponding Schiff bases (mycotoxin and Schiff base dosages were similar in all treated groups). Measurement units provided in corresponding table legend.
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Open Peer Review

Current Referee Status: ?

Version 1

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- The authors showed here more interesting effects of Schiff base aromatic amino acid derivatives on antioxidant and immune system disturbances caused by AFB1 and Schiff base aromatic amino acid derivatives as potential good candidates. I appreciate the authors’ focus on this interesting topic, strategy and detoxification, but regardless of its potentials there are some questions and uncertainties needed to be clarified in the text, methods and interpretation. Also the way of addressing at its current format with some unnecessary words, speculations and incomplete methods. We also like to see the interesting point of the immunological mechanistic effects of this work in the rat model.

- The way of writing should be switched towards the following direction and ...

- The acute toxicity is not an issue for the world, the chronic and invisible exposure is an issue. The authors should reformat their points and direction and in the introduction the authors should more precisely explain the issues related to chronic aspects/exposure/toxicity of AFB1, and some immunosuppressing effects of this toxin in human and animals’ immune cells and molecules. Also apply this direction for your interpretation of the works. Some useful papers it might be useful for this work to mention are referenced below this report.

- In the methods the authors should improve their methodology of work; Flow cytometry needs to be improved and data related to gating, and selecting the specific cells for the assays should be shown, and readers might want to see and learn. How you gated the neutrophils and monocytes and the process of flow cytometry needed for improving its quality. How you chose the cells in the gates?

- Also in the methods it is necessary how you prepared and dissolved AFB1 and mixed them with your Schiff base aromatic amino acid derivatives?

- And better explain the administration schedule for your rats? They are unclear; please make them clearer.

- The detailed methods for antioxidant assessments and luminometry should be addressed.
The paper needs some revision and improvement and it is the authors’ duty to improve their nice works.

For the caption of each figs please add the main message of each figure?

English and typing errors through the text should be improved and cleaned…

Terminology issues should be improved. For example, what do you mean for the “apoptotic rat? I do not understand. AFB₁ (1 should be subscripted through the text). Sometimes you should use rat rather than animals.

Good luck

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

*Competing Interests:* No competing interests were disclosed.

*Referee Expertise:* Immunology and single-immune cell technologies
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.