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Trichothecene Mycotoxins Activate Inflammatory Response in Human Macrophages


Damp building-related illnesses have caused concern for years in many countries. Although the problem is extensive, the knowledge of the molecular immunology behind damp building-related illnesses is still quite limited. Trichothecene mycotoxins form one major group of toxins, which possibly contribute to the illnesses. Stachybotrys chartarum is a well-known, but also controversial damp building mold and many strains of this mold are capable of producing trichothecenes. In this report, we have examined the effect of S. chartarum and trichothecene mycotoxins on the proinflammatory cytokine response in human macrophages. As a result, satratoxin-positive S. chartarum activated inflammasome-associated caspase-1, which is needed for proteolytic processing of IL-1β and IL-18. Furthermore, purified trichothecene mycotoxins, roridin A, verrucarin A, and T-2 toxin activated caspase-1, and these mycotoxins also strongly enhanced LPS-dependent secretion of IL-1β and IL-18. The satratoxin-positive strain of S. chartarum and the trichothecenes also triggered the activation of caspase-3, which is an effector caspase of apoptosis. Satratoxin-negative S. chartarum was not able to activate either caspase-1 or caspase-3. In conclusion, our results indicate that human macrophages sense trichothecene mycotoxins as a danger signal, which activates caspase-1, and further enables the secretion of IL-1β and IL-18 from the LPS-primed cells. The Journal of Immunology, 2009, 182: 6418–6425.
caspase-1 (20). Once pathogen-associated molecular patterns and DAMP-induced innate immunity cascades through PRRs are initiated, high amounts of cytokines, which are essential for the activation of antimicrobial and inflammatory response via innate and adaptive immunity, are produced.

Water-damaged buildings harbor and provide substrates for several water mold species, which increases the risk of accumulation of harmful fungi. Stachybotrys chartarum is a notorious, but controversial damp building mold. This fungus is a potent toxin producer, and many of its toxins are thought to cause DBRI (1). One major group of fungal secondary metabolites produced by a number of fungi, including Fusarium sp., Stachybotrys sp. and Myrothecium sp., is trichothecene mycotoxins (21–23). The trichothecenes (type A–D trichothecenes) are roughly classified as nonmacrocyclic trichothecenes (types A–C) or macrocyclic trichothecenes (type D) (23). In general, trichothecenes are reported to cause many unfavorable effects on eukaryotic cells, including inhibition of RNA, DNA, and protein synthesis, as well programmed cell death, apoptosis (24). Type A, B, and D trichothecenes appear to be the most significant from the public health perspective. The T-2 toxin, which belongs to the type A group, is possibly the most studied nonmacrocyclic trichothecene. It is mainly produced by the Fusarium species, and is reportedly the most potent naturally occurring nonmacrocyclic trichothecene among diatoxocirpenol (another type A toxin) (24–27). The macrocyclic trichothecenes, including satratoxins, rodirdins, and verrucarins, which are produced by several mold species e.g., many strains of S. chartarum, reportedly mediate toxicity by interfering with ribosome function and activating stress responses (28, 29). These toxins may also have a DNA-damaging capacity (30, 31). We have previously shown in a mouse model of asthma that exposure to S. chartarum induces lung inflammation characterized by the infiltration of immune cells and the formation of granulomas and giant cells (32). However, the specific immunological mechanisms and pathways underlying S. chartarum-induced or trichothecine-induced apoptosis and inflammation have not yet been clearly defined. In our current study, we have focused on resolving the capability of trichothecenes, satratoxins, rodirdin A, verrucarin A, and T-2 toxins to activate inflammammasome-associated innate immunity reactions in human primary macrophages.

Materials and Methods

Differentiation of macrophages from human peripheral blood-derived monocytes

Monocytes from healthy blood donors (Finnish Red Cross Blood Transfusion Service) were isolated and purified as previously described (33, 34). In brief, we isolated PBMCs from normal human peripheral blood by low speed density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences). The cell layer containing lymphocytes, platelets, and monocytes was recovered from the interface, and further washed to remove platelets, excess plasma, and Ficoll-Paque Plus. After three sequential washes with PBS, 7.5–10 × 10⁶ mononuclear cells, which contained 0.75–1.5 × 10⁶ monocytes, were transferred to Multiwell six-well culture dishes (Falcon) in 1 ml of RPMI 1640 supplemented with 2 mM L-glutamate and antibiotics: 0.6 μg/ml penicillin and 60 μg/ml streptomycin (all from Life Technologies). The monocytes were allowed to attach to cell culture plates at 37°C and 5% CO2 for 1 h. The attached cells were washed three times with PBS and differentiated into macrophages by maintenance in Macrophage-SFM medium (Life Technologies) supplemented with 10 ng/ml GM-CSF (Biosource) and antibiotics. The following day, fresh medium was changed, and replaced every 2 days thereafter. After 7 days of culture, the resulting macrophages were used in experiments.

Microbe preparations

Stachybotrys chartarum strains 29 and 72 (NRRL 6084) have been characterized. They were grown on rice flour agar as previously described (35, 36). When grown on rice agar, S. chartarum strain 29 is not capable of satratoxin production (only minor amounts of stachybotryolactam and stachybotryolactone are produced, <1 ng per 10⁶ spores), whereas strain 72 produces satratoxin G and H, stachybotryolactone, and stachybotryolactam in the respective amounts of 4 ng, 10 ng, 8 μg, and 2 μg per 10⁶ spores. Previous analysis verified that strain 29 did not produce detectable amounts of either satratoxin G or H, and that in contrast, strain 72 produced 28 ng of satratoxin G and 6 ng of satratoxin H per 1 × 10⁶ spores (37). Strain 29 was used as the non-satratoxin producing strain (satratoxin negative S. chartarum), and strain 72 as the satratoxin-producing strain (satratoxin positive S. chartarum). The spores were suspended directly from the agar plates into PBS. S. chartarum suspensions were gamma-irradiated with 10 Kγ before use.

Cell stimulation

The functional concentrations of LPS and mycotoxins were pretreated, and we used the final amounts used in experiments for LPS (Escherichia coli 0111:B4) and rodirdin A, verrucarin A, and T-2 toxin (Sigma-Aldrich), with 100 μg/ml, 100 ng/ml. S. chartarum (s. 29) and satratoxin-negative S. chartarum (s. 29) were used at 3 × 10² or 1 × 10⁴ spores/ml. Macrophages were treated with LPS, rodirdin A, verrucarin A, T-2 toxin, or S. chartarum spores in Macrophage SFM medium. RPMI 1640 medium supplemented with 2 mM L-glutamate, 1 mM HEPES, and antibiotic was used when cell culture supernatants were prepared for concentration. After each stimulation period, the cells and cell culture supernatants were harvested, and samples prepared for the RT-PCR, Western blotting, and ELISA.

Quantitative real-time RT-PCR assay

We extracted total cellular RNA using TRizol Reagent (Life Technologies) according to the manufacturer’s instructions. Absorbance at A260 and A280 nm was measured to determine RNA concentration and purity in a spectrophotometer (GeneQuant Pro RNA/DNA calculator, Amersham Biosciences). Isolated RNA was temporarily stored at −70°C in nucleic-acid-free water. First, 0.5 μg of RNA was reverse transcribed into cDNA by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a 25-μl reaction mixture containing optimized reverse transcription buffer, random primers, deoxyNTP mixture, and MultiScribe reverse transcriptase. The conditions for cDNA synthesis were as follows: annealing at 25°C for 20 min and synthesis at 37°C for 120 min. The reverse transcription reaction was performed in an Applied Biosystems’ Thermal cycler version 2720. The quantitative Real Time-PCR and Taqman assay was performed in a 96-well optical reaction plate in ABI Prism 7500 Fast Sequence Detector (Applied Biosystems). The cDNA was amplified in 11 μl of 1× TaqMan Fast universal PCR master mix with Predesigned TaqMan assay primers and probes (IL-1β Hs0017497_m1, IL-18 Hs00155517_m1, HIF-1α Hs00985772_m1, IL-6 Hs00174178_m1, IL-8 Hs0017497_m1, IL-10 Hs00174078_m1, TGF-β Hs00985772_m1, and TNF Hs00174178_m1). The conditions for amplification were as follows: 40 cycles at 95°C for 15 s and 60°C for 60 s. RT-PCR was performed at least twice for each cDNA sample. No template control (NTC), in which molecular grade water was used instead of template, was included in each assay.

Real-time PCR data analysis

The real-time PCR data was developed by using Sequence detector system version 1.4 software (Applied Biosystems). We selected the cycle threshold value (Ct) of a sample according to the manufacturer’s guidelines. Relative units were calculated by a comparative Ct method. First, we subtracted the Ct of 18S rRNA sample from the corresponding target cytokine Ct to get the ΔCt. Next, we subtracted the average of Ct of 18S rRNA samples from the calibrator Ct value of 40, which was obtained from NTC, to get the calibrator ΔCt. The calibrator ΔCt was subtracted from the ΔCt of each experimental sample to get ΔΔCt. Finally, we calculated the amount of target normalized to an endogenous control, which was relative to the NTC calibrator, by the equation 2−ΔΔCt.

Western blot analysis

Western blot analyses were performed either by using whole cell extract or by using concentrated cell supernatants. First, cells were lysed in protein lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, and 25% ethylene glycol supplemented with complete mini protease inhibitor mixture (Roche Diagnostics) and homogenized with ultrasound sonicator.
S. chartarum and LPS synergistically activate caspase-1 and caspase-3-mediated inflammatory and apoptotic cascades in human macrophages

To analyze whether costimulation of macrophages by satratoxin-positive S. chartarum and LPS results in the secretion of mature IL-1β and IL-18, we stimulated the cells with S. chartarum spores and LPS for 12 h. IL-1β and IL-18 were determined by ELISA from the stimulated cell culture supernatants. Results showed that LPS and S. chartarum alone elicited very little secretion of IL-1β and IL-18. However, the enhancement in the release of the cytokines was seen after the costimulation: together S. chartarum and LPS caused increased secretion of IL-1β and IL-18 in human macrophages (Fig. 3, A and B).

IL-1β and IL-18 are synthesized as inactive 31 kDa (p31) and 24 kDa (p24) precursors, respectively, which remain in the cytoplasm. Their biological activity is dependent on caspase-1, which cleavages them into smaller subunits, to the conformation in which these cytokines can be secreted (15). To study the effect of satratoxin-positive S. chartarum and LPS on IL-1β and IL-18 protein processing, we performed Western blot analysis with anti-IL-1β and anti-IL-18 specific Abs. As shown in Fig. 3C, LPS induced synthesis of pro-IL-1β, which was not seen in the cells stimulated by satratoxin-positive S. chartarum. Pro-IL-1β was also absent in macrophages that were cotreated with LPS and S. chartarum spores. S. chartarum activated the formation of IL-18 p18, which is the caspase-1 cleavage product and the biologically active form of IL-18. In addition, we observed the caspase-3 processed cleavage products of IL-18, IL-18 p15/p16, in macrophages, which were activated by S. chartarum. In agreement with our previous results, LPS did not induce proteolytic processing of pro-IL-18. However,
LPS enhanced the *S. chartarum*-induced formation of both IL-18 p18 and IL-18 p15/p16 (Fig. 3C).

Caspase-1 is expressed as an inactive proenzyme (p45), which must be activated by proteolytic cleavage. The functional caspase-1 comprises of p10 and p20 subunits, which assemble into a heterotetramer (38). Like caspase-1, caspase-3 is a latent zymogen, which is processed upon activation into smaller polypeptide chains: p17 and p19, which in turn form the bioactive enzyme (10, 39, 40). To study the effects of LPS and *S. chartarum* on caspase-1 and caspase-3 activation, we performed Western blot analysis with anti-caspase-1 p20 and anti-caspase-3 p17/p19-specific Abs. As shown in Fig. 3C, satratoxin-positive *S. chartarum* activated the formation of caspase-1 p20 as well as caspase-3 p17/p19. Furthermore, *S. chartarum* and LPS together enhanced the formation of both caspase-1 p20 and caspase-3 p17/p19. LPS was unable to induce the formation of either caspase-1 p20 or caspase-3 p17/p19. Instead, it induced the caspase-1 p22 subunit (Fig. 3C). Thus, the satratoxin-positive *S. chartarum* activates both caspase-1 and caspase-3, a process which was enhanced by LPS costimulation.

**Trichothecene mycotoxins activate caspase-1 and caspase-3 in human macrophages**

Trichothecene mycotoxins such as satratoxins, roridins, and verrucarins are secondary metabolites of several strains of *S. chartarum* (41–44). Trichothecenes have been linked to many harmful health effects (1). To study the potential role of satratoxin in *S. chartarum*-induced caspase-1 and caspase-3 activation, macrophages were exposed to satratoxin-positive (s. 72) and satratoxin-negative (s. 29) *S. chartarum* strains and we analyzed caspase-1 and caspase-3 activation with Western blotting. In accordance with the results shown in Fig. 4A, satratoxin-positive *S. chartarum* activated the formation of caspase-1 p20 and caspase-3 p17/p19. Activation of caspase-1 and caspase-3 was seen 6 to 10 h after fungal exposure. In accordance with these results, processing of pro-IL-18 to smaller IL-18 p18 and IL-18 p15/16 pieces were seen, which further confirms activations of caspase-1 and caspase-3, respectively. In contrast, satratoxin-negative *S. chartarum* was not able to activate caspase-1 or caspase-3, nor the processing of IL-18 at any of the time-points studied (Fig. 4A). Our results suggest that the trichothecenes, satratoxin G and H, produced by *S. chartarum* strain 72, are the probable reason for the activation of caspase-1 and caspase-3 in human macrophages.

To further study the role of trichothecenes in the activation of caspase-1 and caspase-3, macrophages were stimulated with the purified trichothecenes, roridin A, verrucarin A, and T-2 toxin for...
18 h, after which caspase-1 activation was studied by Western blotting. Results presented in Fig. 4B show that caspase-1 was activated in response to all trichothecenes used. The trichothecenes also induced further degradation of caspase-1 p20 to a smaller fragment (the lowest arrow in caspase-1 picture in Fig. 4B). In addition, caspase-3 was clearly activated in response to toxins (Fig. 4B).

Roridin A dose-dependently activates secretion of IL-1β in LPS-primed cells

It is commonly known that LPS is able to activate the expression of pro-IL-1β, but it is unable to induce processing of pro-IL-1β to its biological active form. LPS-treated cells need second stimulus, such as ATP, for caspase-1 activation before pro-IL-1β is processed and mature IL-1β is secreted (45, 46). Therefore, we studied the capability of roridin A to activate secretion of IL-1β in LPS-primed cells. Macrophages were first stimulated with LPS for 6 h, and the cells were further incubated with roridin A concentration at 100, 10, or 1 ng/ml for 18 h. As ELISA results show in Fig. 5, the stimulation of LPS-primed cells with roridin A concentration at 100 and 10 ng/ml induced the highest secretion of IL-1β into the cell culture supernatants. Also 1 ng/ml of roridin A induced minor IL-1β secretion levels in human macrophages. Roridin A had a synergistic effect on IL-1β secretion also with lower LPS concentrations (10 ng/ml; data not shown). In further studies, the trichothecene mycotoxins and LPS were used at a concentration 100 ng/ml.

Costimulation with roridin A, verrucarin A, or T-2 toxin and LPS enhance secretion of IL-1β and IL-18

To determine the effect of the cooperation between the trichothecenes and LPS on cytokine production, macrophages were first stimulated with LPS for 6 h. After this, the cells were further stimulated with the trichothecene mycotoxins, roridin A, verrucarin A, and T-2 toxin, for 18 h. We then collected the cell culture supernatants, and the secretion of IL-1β and IL-18 was studied using ELISA. As a result, all the trichothecenes, together with LPS, elicited a high secretion of both IL-1β and IL-18 (Fig. 6). The synergistic effect of mycotoxins and LPS on IL-18 production was not
statistically significant due to high interindividual variation of IL-18 secretion levels. However, in each separate experiment, a synergistic effect of mycotoxins and LPS on IL-18 secretion was clearly seen (data not shown). Hence, IL-1β and IL-18 release, in LPS-primed cells, indicated that the trichothecenes activate the inflammasome-associated caspase-1 mediated inflammatory response in human macrophages (Fig. 4, 5, and 6).

**Trichothecenes, roridin A, verrucarin A, and T-2 toxin, active proteolytic processing and secretion of IL-1β and IL-18 in LPS-primed human macrophages**

To further characterize the proteolytic processing of pro-IL-1β and pro-IL-18 proteins, we performed Western blot analysis from cell lysates, and from concentrated cell culture supernatants. The samples were prepared from macrophages, which were left either unstimulated or stimulated with LPS and/or the purified trichothecene mycotoxins. Western blot samples performed from the cell lysates show that, unlike LPS, the mycotoxins were not able to activate the synthesis of pro-IL-1β protein (Fig. 7A). Instead, the costimulation of macrophages with the toxins and LPS caused both the production and the processing of pro-IL-1β, and this cotreatment also triggered the appearance of the biologically active 17-kDa form of IL-1β (Fig. 7A). In addition, results from the cell lysates showed that stimulation of macrophages with roridin A, verrucarin A and T-2 toxin activates the processing of pro-IL-18 to IL-18 p15/p16 isofoms, which are the caspase-3 cleavage products of IL-18 (Fig. 7A). The costimulation of macrophages with LPS and the mycotoxins also resulted in the appearance of the biologically active 18-kDa form of IL-18 in whole cell lysates (Fig. 7A). Further, the secretion of the biologically active form of IL-1β and IL-18, as well as the pro-forms of these cytokines, was clearly seen in concentrated cell culture supernatants, which were prepared from macrophages that were costimulated with LPS and the mycotoxins (Fig. 7B). These results are in line with the ELISA results shown in Fig. 6, and further confirm that costimulation of macrophages with LPS and trichothecenes is required for the efficient secretion of biologically active forms of IL-1β and IL-18.

**Discussion**

Multicellular organisms have evolved to live in the presence of microbes. During evolution, the host cells have developed many mechanisms to keep the interaction in balance. However, for some reason this harmony is easily broken, resulting in different clinical symptoms, which are mainly caused by the reaction of our own immune systems. *Stachybotrys chartarum* is a notorious but also controversial damp building mold. This toxic mold has often been associated to the DBRI (1). Mycotoxins, especially the trichothecene mycotoxins it produces, are probably a reason behind the symptoms (1, 47, 48). Trichothecenes have immunoregulatory and cytotoxic effects on immune cells (28, 36, 49–52). In addition, it has been concluded that these mycotoxins cause MAPK-mediated ribotoxic stress and apoptosis (53–55). We have previously shown in a mouse model of asthma that exposure to toxic *S. chartarum* induces lung inflammation characterized by the infiltration of immune cells, as well as the formation of granulomas and giant cells (32). However, the exact mechanism for *S. chartarum*-induced lung inflammation has not been determined. A potential explanation for the inflammation may found in the ability of the trichothecene mycotoxins to cause these reactions. In the present work, we have studied the effect of *S. chartarum* and purified trichothecene mycotoxins on proinflammatory cytokine response in human primary macrophages.

Activated macrophages are the primary source of proinflammatory cytokines, including IL-1β and IL-18. It is well characterized that TLR ligands, including LPS, induces transcription of IL-1β and expression of pro-IL-1β protein (15). A second stimulus is needed to engage inflammasome-associated caspase-1 activation, which leads to pro-IL-1β processing and secretion of mature IL-1β protein (56). Caspase-1 also processes pro-IL-18 for its biologically active form and enables the secretion of IL-18 (2). In our experiment, the satratoxin-positive *S. chartarum* activated gene expression of IL-1β and IL-18 (Fig. 1). Furthermore, satratoxin-positive *S. chartarum* and LPS synergistically activated IL-1β and IL-18 gene expression (Fig. 2). This enhancement was also seen in the secretion level of the cytokines promoted by costimulation, indicating activation of inflammasome-associated caspase-1 (Fig. 3). These results also suggest that *S. chartarum* spores include some substance(s), which is sensed as a danger signal by human macrophages.

Many bacterial toxins are now known to be involved in the activation of innate immune response. However, because toxins are structurally a diverse group of molecules, they cannot be recognized directly by specific PRRs. In contrast, toxins may, for example, trigger changes in cellular ion composition, which the cells of the innate immunity system sense as a danger signal (20). Complexes formed by the NLR-family components, adapter molecules, and caspases are important in the sensing of different DAMP signals, including ATP release of the host cells (2). The recently described NLRP3-inflammasome complex is activated by a potassium (K⁺) efflux, triggered by pore-forming bacterial toxins (57). This inflammasome-induced caspase-1 activation enables the secretion of IL-1β and IL-18 (2). In our experiments, the satratoxin-positive *S. chartarum* induced the formation of the active caspase-1 p20 subunit, but the satratoxin negative strain of *S. chartarum* did not (Fig. 4A). LPS also failed to activate the formation of caspase-1 p20. Instead, the biologically inactive caspase-1 p22 was generated, which is not capable of processing pro-IL-1β or pro-IL-18 into the biologically active forms of the cytokines (Fig. 3C). Our result also shows that the purified trichothecenes, roridin
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A, verrucarin A, and T-2 toxin activate caspase-1 and they also seem to have a potency for further caspase-1 degradation (Fig. 4B). The role of degraded caspase-1 is still unclear. The costimulation with LPS and the trichothecene-induced enhancement in the secretion of IL-1β and IL-18, which further verifies the role of the trichothecenes as DAMPs, triggers the activation of inflammasome-associated caspase-1 (Figs. 5–7). Further studies are needed for the detailed characterization of the mechanisms for inflammasome activation triggered by trichothecenes.

In addition to inflammation, exposure of mouse alveolar cells to S. chartarum toxins has also been associated with cell death (53). Cell death may be due to necrosis or apoptosis. Most of the morphological changes associated with cell death are caused by a set of cysteine proteases, which are specifically activated in apoptotic cells (10). These death proteases are functionally and structurally homologous to each other, and belong to a large caspase protein family (12). Caspase-3 is a central executioner of the apoptotic pathway. It is expressed as a latent 32 kDa zymogen that cannot exert its enzymatic activities unless it is proteolytically processed (10). Our data shows that only satratoxin positive S. chartarum was able to induce the processing of caspase-3 and hence, its activation (Fig. 4A). This caspase-3 activation occurred at 6 h after stimulation and was associated with appearance of active form of caspase-1 (Fig. 4A). These results suggest that inflammatory and apoptotic responses are activated simultaneously in macrophages that have been stimulated with satratoxin positive strain of S. chartarum. In addition, roordin A, verrucarin A, and T-2 toxin were able to trigger the activation of caspase-3 (Fig. 4B). It has also been reported that apoptosis is involved in the deaths of S. chartarum toxin-induced mouse alveolar macrophages, manifested as DNA damage and an oxidative stress response (29, 58). In addition, data from mouse models indicates that the mycotoxins can cause lung injury that is associated with massive inflammation and cell death (36, 50). Furthermore, our results show that the activation of caspase-3 induced by satratoxin positive S. chartarum was further enhanced by the costimulation of LPS (Fig. 3C). Moreover, the capacity of LPS to potentiate the toxicity of trichothecenes in mouse models and cell cultures has been reported (59–61). We suggest that the activation of caspase-3, and the following apoptosis of human macrophages, is at least partly due to the trichothecene mycotoxins, and that caspase-3 mediated cell death is potentiated by coexposure with the trichothecenes and LPS.

In water-damaged buildings, toxic S. chartarum grows together with other molds and bacteria. Furthermore, many toxins, including different trichothecene mycotoxins, and bacterial endotoxins, such as LPS, are measured from material samples and air samples from water-damaged buildings (41, 44, 62–64). In addition, exposure of the pulmonary airways to LPS occurs relatively commonly with no necessary connection between the host and damp buildings (64–66). However, in buildings with dampness related problems, endotoxin levels up to 390 ng/m3 have been reported (67). Similarly, Brasel and coworkers (62) have shown that the concentration of trichothecenes can be 1.3 ng/m3 in water-damaged buildings. Therefore, our findings regarding the proinflammatory reactions, which are caused by costimulation with trichothecenes and LPS, should be taken into account when trying to explain the reasons for the symptoms seen in people suffering DBRI. This association certainly needs more investigation.

In conclusion, the data presented in our study shows that the trichothecenes, satratoxins, roordin A, verrucarin A, and T-2 toxin, are involved in the activation of the inflammatory and the apoptotic pathways in human primary macrophages. These mycotoxins, together with LPS, synergistically activate IL-1β and IL-18 mediated inflammatory response in the cells. The results suggest a novel role for the trichothecenes as a danger signal, which activates inflammasome-associated caspase-1 as a part of the mycotoxin-triggered innate immune response.

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Disclosures

The authors have no financial conflict of interest.

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