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Circulating Cytokine and Hematological Responses to Acute and Repeated Oral Tenuazonic Acid Exposure in Rats: An Exploratory Orthogonal Implicit Projection Analysis

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Abbreviations

AOH – alternariol

AME – alternariol monomethyl ether

EDTA – ethylenediaminetetraacetic acid

LDA – linear discriminant analysis

OIP – Orthogonal Implicit Projection

SCC – Symmetric Conditional Centering

TeA – tenuazonic acid

TEN – tentoxin

Abstract

Tenuazonic acid (TeA), a mycotoxin produced by *Alternaria* spp., is a common food contaminant whose potential immunomodulatory effects remain insufficiently characterized. This study investigated changes in circulating cytokine and routine hematological profiles following repeated oral TeA exposure in rats and comparatively reanalyzed a previously reported acute-exposure dataset to explore Orthogonal Implicit Projection (OIP) for multivariate interpretation of correlated biomarker responses. The repeated-dose experiment included 24 male Wistar rats (0, 0.36, and 1.55 mg/kg for 14 days; n = 8 per group), whereas the previously reported acute-exposure dataset comprised 30 rats (vehicle, purified TeA at 30 mg/kg, or an *Alternaria alternata* extract containing an equivalent TeA dose; n = 10 per group). Repeated TeA administration was associated with marked cytokine alterations, including increased IL-1β at the highest dose and intermediate-dose peaks of TNF-α, IL-2, IL-10, IL-13, and IL-17A, indicating monotonic and non-monotonic response patterns. Acute exposure to purified TeA and the *A. alternata* extract produced broadly similar cytokine profiles, with increased IFN-γ, IL-1β, IL-6, IL-17A, MCP-1, MIP-1α, and TNF-α, reduced IL-10, and lower IL-10/IL-17A ratios compared with controls. Routine hematological indices showed only modest changes. Exploratory OIP analysis complemented conventional statistical methods by facilitating interpretation of coordinated biomarker responses. Overall, repeated oral TeA exposure was associated with marked cytokine alterations, while comparative reanalysis of the acute-exposure dataset demonstrated a distinct cytokine response under acute exposure conditions. Further studies are needed to clarify the underlying biological mechanisms and their relevance to dietary exposure.

Keywords: tenuazonic acid; *Alternaria*; immunotoxicity; cytokines; orthogonal implicit projection; rats

Introduction

Alternaria species contaminate a wide range of agricultural commodities, including cereals, fruits, vegetables, oilseeds, animal feed, and processed food products. Their secondary metabolites include alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN), altertoxins, and tenuazonic acid (TeA). Although several of these compounds are classified as emerging mycotoxins, their frequent co-occurrence and incomplete toxicological characterization have raised increasing concerns regarding food safety (Aichinger et al. 2021; Louro et al. 2024; Nagda and Meena 2024; Zhang et al. 2025).

Recent monitoring studies have confirmed the presence of *Alternaria* toxins in a broad range of food and feed matrices. TeA, AOH, AME, and TEN have been detected in cereals, wheat grains, tomato products, foods purchased through online retail channels, and ruminant feeds (Ji et al. 2022a; Ji et al. 2023a, b; Mao et al. 2023; Daichi et al. 2025). In many surveys, TeA was among the most frequently detected and quantitatively predominant *Alternaria* toxins, although occurrence patterns varied according to commodity type, geographical region, and analytical methodology (Ji et al. 2022a; Ji et al. 2023a; Lu et al. 2025).

Dietary exposure assessments suggest that TeA may contribute substantially to overall exposure to *Alternaria* toxins, particularly among consumers with a high intake of cereal- and tomato-based products. However, considerable uncertainty remains because of limited occurrence data, variable analytical coverage, and incomplete toxicological information (EFSA 2011; EFSA et al. 2016; Ji et al. 2022a; Lu et al. 2025). Probabilistic risk assessments have further highlighted the importance of combined exposure to regulated and emerging mycotoxins, particularly in infants and young children (Ji et al. 2022b; Ji et al. 2023b). Reflecting these concerns, the European Commission has recommended monitoring TeA, AOH, and AME in selected foods while emphasizing the need for additional occurrence and toxicity data (EC 2022).

Among the major *Alternaria* toxins, TeA has long been regarded as one of the compounds with the greatest acute toxic potential. Early experimental studies demonstrated systemic toxicity in laboratory mammals and young chickens, including reduced body-weight gain, organ injury, and alterations in clinical and hematological parameters (Smith et al. 1968; Giambrone et al. 1978). More recent studies in mice have reported oxidative stress, impaired antioxidant defense, biochemical disturbances, histopathological lesions, and changes in differential leukocyte counts following TeA exposure (Kumari and Singh 2021, 2023).

TeA is a potent inhibitor of eukaryotic protein synthesis, and disruption of translational processes has long been considered one of its principal mechanisms of toxicity. Experimental evidence has additionally linked TeA exposure to oxidative stress, impaired antioxidant defense, cellular injury, and disturbances in metabolic and

mitochondrial function (Friedman et al. 1975; Kumari and Singh 2021; Louro et al. 2024; Nagda and Meena 2024; Qin et al. 2024). Nevertheless, the available mechanistic evidence has been obtained from heterogeneous experimental systems, including plants, cultured intestinal cells, and animal models, and many of the reported effects have not been independently confirmed in mammalian immune cells.

Despite substantial evidence of systemic toxicity, the immunotoxic effects of TeA remain poorly characterized. Most animal studies have focused on oxidative stress, biochemical alterations, organ pathology, or routine hematological indices rather than comprehensive analyses of circulating cytokine profiles (Kumari and Singh 2021, 2023; Louro et al. 2024). Recent *in vitro* studies indicate that TeA and other *Alternaria* mycotoxins can modulate inflammatory signaling in immune and intestinal epithelial cells; however, both the direction and magnitude of these effects appear to depend on the toxin, concentration, cell type, and inflammatory context (Partsch et al. 2026a, b).

Interpretation of fungal extract toxicity is further complicated by the simultaneous presence of multiple metabolites. AOH, AME, TEN, altertoxins, and other *Alternaria* metabolites exhibit cytotoxic, inflammatory, immunomodulatory, and receptor-mediated activities, and mixtures may produce biological responses that differ from those induced by individual compounds (Crudo et al. 2019; den Hollander et al. 2022; Gröstlinger et al. 2022; Saleh et al. 2024). Recent studies have additionally identified immunosuppressive properties of selected perylene quinone metabolites and demonstrated that different *Alternaria* toxins exert distinct effects in immune and intestinal cell models (Ismail et al. 2023; Partsch et al. 2026a).

AOH is of particular relevance for interpreting the fungal extract group because it has been shown to modulate inflammatory responses in stimulated intestinal epithelial cells and to induce toxic effects in experimental intestinal and hepatic models (Schmutz et al. 2019; Vilela et al. 2025). Although these observations support the biological plausibility that accompanying metabolites may contribute to the toxicity of fungal extracts, they do not demonstrate that AOH was responsible for the effects observed in the present study.

Cytokines are pleiotropic signaling molecules that regulate innate and adaptive immune responses through highly interconnected and context-dependent networks. Consequently, changes in circulating IL-1β, TNF-α, IL-6, IL-10, IL-12, IL-17A, IFN-γ, and chemokine concentrations may reflect altered inflammatory or regulatory signaling. However, plasma cytokine concentrations alone cannot identify the cellular source of these mediators or establish activation of specific immune pathways (Kany et al. 2019; Bou Zerdan et al. 2021).

Analysis of cytokine data presents several statistical challenges because immune mediators are biologically interdependent, frequently correlated, and may exhibit heterogeneous or non-monotonic responses. Evaluating each

cytokine independently may therefore overlook coordinated changes across the overall immune response while increasing the burden of multiple testing. Multivariate approaches can provide complementary information, although their stability and interpretation become increasingly challenging when the number of correlated outcomes is large relative to the available sample size (Bühlmann and van de Geer 2011).

The primary aim of this study was to characterize circulating cytokine and hematological responses following repeated oral exposure to TeA in rats. The previously reported acute-exposure dataset (Trushina et al. 2025), which included purified TeA and an *Alternaria alternata* extract containing an equivalent TeA dose together with AOH, AME, and TEN, was reanalyzed as a comparator for the repeated-dose experiment and for exploratory multivariate analysis using **Orthogonal Implicit Projection (OIP).** We hypothesized that repeated TeA exposure would be associated with coordinated changes in circulating cytokine profiles that would be more pronounced than alterations in routine hematological indices. Conventional statistical analyses were complemented by OIP, based on **Symmetric Conditional Centering (SCC),** as an exploratory framework for the multivariate interpretation of correlated biomarker responses. OIP was not intended to establish causal mechanisms, identify specific immune-cell populations, or validate the general statistical performance of the method.

Materials and Methods

Animals and Experimental Design

The study comprised a previously unpublished repeated-dose experiment and a comparative reanalysis of a previously reported acute-exposure experiment evaluating cytokine and hematological responses to TeA in male Wistar rats. The Ethics Committee of the Federal Research Centre of Nutrition, Biotechnology and Food Safety (Protocol No. 4, approved on 20 April 2017) approved the experimental protocol. Animal husbandry, housing, euthanasia, and all experimental procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 2011), the Russian National Standard GOST 33216-2014 governing the housing and care of laboratory rodents, and the principles of Replacement, Reduction, and Refinement (3Rs). The study was reported in accordance with the ARRIVE 2.0 guidelines (Percie du Sert et al. 2020).

A total of 54 male Wistar rats were used. Animals were housed under controlled environmental conditions with a 12 h light/12 h dark cycle and received a balanced semisynthetic diet and purified drinking water. Water was available *ad libitum* throughout the study. Animals were allocated to experimental groups on the basis of body weight.

Repeated-Dose Experiment

The repeated-dose experiment included 24 male Wistar rats with an initial mean body weight of approximately 106 g at study initiation. The animals were randomly assigned to three experimental groups (n = 8 per group):

- vehicle control receiving 10% aqueous ethanol;
- TeA group receiving 0.36 mg/kg body weight;
- TeA group receiving 1.55 mg/kg body weight.

The vehicle or TeA solution was administered once daily by intragastric gavage at a volume of 3 mL/kg body weight for 14 consecutive days. Twenty hours after the final administration, animals were euthanized by decapitation, and blood samples were collected for hematological and cytokine analyses.

Plasma cytokines and routine hematological parameters were measured in all animals.

Acute Experiment

The acute experiment included 30 male Wistar rats obtained at 4 weeks of age from the Stolbovaya breeding facility of the Scientific Center for Biomedical Technologies of the Federal Medical-Biological Agency. Following a 5-week acclimatization period, the animals had a mean body weight of approximately 238 g and were randomly assigned to three experimental groups (n = 10 per group):

- vehicle-control group receiving 10% aqueous ethanol;
- purified TeA group receiving 30 mg/kg body weight;
- *Alternaria alternata* extract group receiving an extract containing TeA at an equivalent dose of 30 mg/kg body weight.

All preparations were administered once by intragastric gavage at a volume of 3 mL/kg body weight. The ethanol concentration was identical in all administered formulations.

Food was withdrawn overnight before treatment, returned 3 h after administration, and withdrawn again before terminal blood collection. Twenty-four hours after administration, animals were euthanized by decapitation, and blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA).

The acute-exposure experiment and the corresponding cytokine and hematological data were previously reported by Trushina et al. (2025). In the present study, these data were reanalyzed and incorporated as a comparator dataset for the previously unpublished repeated-dose experiment and for exploratory multivariate analysis using OIP.

Tenuazonic Acid and *Alternaria alternata* Extract

Purified tenuazonic acid used in the acute experiment was purchased from Clearsynth (India; catalogue No. CS-BX-00058). Immediately before administration, TeA was dissolved in 10% aqueous ethanol.

The A. *alternata* extract was obtained from a toxin-producing fungal culture grown on tomato paste at 24 °C for two weeks. The administered extract contained:

- TeA: 30 mg/kg body weight;
- AOH: 0.276 mg/kg body weight;
- AME: 0.902 mg/kg body weight;
- TEN: 0.018 mg/kg body weight.

Other Alternaria metabolites, including altertoxins and altenuene, were not quantified in the extract.

The purified TeA and extract-treated groups therefore received equivalent doses of TeA but differed in formulation of the administered preparation. Consequently, comparisons between these groups reflected formulation-related differences rather than a dose-response relationship.

The acute dose of 30 mg/kg body weight was selected as a sublethal dose on the basis of historical acute-toxicity data for TeA and was intended for hazard characterization rather than simulation of typical dietary exposure (EFSA 2011; Smith et al. 1968). Because reported lethal-dose estimates vary according to species, sex, preparation, and route of administration, the dose should not be described as a universal fraction of the TeA LD_{50}.

Blood Sampling and Plasma Preparation

Blood samples were collected immediately after decapitation at terminal sacrifice. Samples intended for hematological analysis were collected into EDTA-containing tubes.

Whole-blood samples were analyzed for routine hematological parameters. Plasma was obtained by centrifugation and stored at -80 °C until cytokine analysis. All cytokine measurements were performed using plasma samples following a single freeze–thaw cycle in accordance with the manufacturer's protocol.

EDTA plasma was used for cytokine measurements. Sample matrix, anticoagulant type, processing time, and storage conditions can influence measured cytokine concentrations; therefore, the same collection and processing procedures were applied to all experimental groups (Thavasu et al. 1992; Biancotto et al. 2012; Hennø et al. 2017).

Cytokine Analysis

Plasma cytokine concentrations were determined using a Luminex 200 multiplex immunoassay system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The intra-assay coefficient of variation was below 10%, and cytokine concentrations were expressed as pg/mL.

The cytokine panel used in the repeated-dose experiment included granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, and tumor necrosis factor-α (TNF-α).

The cytokine panel used in the acute experiment included IFN-γ, IL-1β, IL-2, IL-5, IL-6, IL-10, IL-17A, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), and TNF-α.

For the acute experiment, the IL-10/IL-17A ratio was calculated for each animal from the corresponding plasma concentrations and used as an additional indicator of the balance between regulatory and pro-inflammatory cytokine responses.

The selected cytokines represented multiple components of the systemic immune response, including innate inflammatory mediators, chemokines, regulatory cytokines, and cytokines associated with T-cell-mediated immunity. However, circulating cytokine concentrations were not interpreted as direct measures of the abundance or functional activity of specific immune-cell populations.

Hematological Analysis

Routine hematological analyses were performed using a Sysmex XN-1000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan).

The evaluated erythrocyte variables included erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Leukocyte evaluation included total leukocyte count together with differential leukocyte analysis. Neutrophils, lymphocytes, monocytes, eosinophils, and basophils were evaluated as both relative values (%) and absolute counts ($\times 10^9$/L).

Platelet-related variables included platelet count, mean platelet volume (MPV), and plateletcrit (PCT).

Conventional Statistical Analysis

The individual animal was considered the experimental unit. The acute and repeated-dose experiments were analyzed separately because they differed in exposure duration, administered dose, treatment formulation, animal characteristics, and measured cytokine panels.

Continuous data are presented as the first quartile (Q1), median, and third quartile (Q3). This summary was chosen because several variables showed asymmetric distributions and the experimental groups were of modest size.

For the repeated-dose experiment, treatment effects were evaluated using orthogonal polynomial contrasts to estimate linear and quadratic response components. The linear component described a consistent increase or decrease across the dose levels, whereas the quadratic component was used to identify non-monotonic response patterns, including intermediate-dose maxima or minima.

For the acute experiment, treatment groups were analyzed as categorical experimental conditions. Pairwise comparisons were performed between the vehicle-control group and each treatment group and between the purified TeA and A. *alternata* extract groups.

Effect magnitude was quantified using η^2, and 95% confidence intervals were calculated to characterize the precision of the estimates. Effect sizes and their confidence intervals were interpreted together with p-values rather than using statistical significance as the sole criterion of evidence (Kelley 2007; Lakens 2013).

Because the purified TeA and A. *alternata* extract groups received equivalent doses of TeA (30 mg/kg body weight), they were considered different treatment formulations rather than as successive dose levels. Consequently, comparisons between these groups were interpreted as formulation-related rather than dose-dependent effects.

Orthogonal Implicit Projection Analysis

Because cytokine and hematological variables are biologically interrelated and often exhibit correlated responses, conventional statistical analyses were complemented by an exploratory Orthogonal Implicit Projection (OIP) analysis based on the concept of Symmetric Conditional Centering (SCC). OIP was used as a complementary multivariate framework to summarize coordinated biomarker responses after removing variation associated with a predefined reference component. It was not intended to replace prespecified conventional statistical analyses, establish biological causality, or provide a general validation of the OIP methodology.

The SCC procedure is conceptually related to residualization and partial-regression approaches, in which the variation explained by predefined variables is removed before estimating treatment-associated effects. Let X denote the matrix of treatment predictors, Y the response vector or multivariate response matrix, and Z the predefined reference component used for conditional centering. The conditionally centered variables were defined as

$$X^* = X - \hat{E}(X|Z)$$

and

$$Y^* = Y - \hat{E}(Y|Z)$$

where $\hat{E}(X|Z)$ and $\hat{E}(Y|Z)$ denote the estimated conditional expectations of X and Y, respectively.

The treatment-associated coefficient matrix was subsequently estimated as

$$\hat{\theta} = (X^{*T}X^*)^+ X^{*T}Y^*$$

where $(\dots)^+$ denotes the Moore–Penrose generalized inverse and $\hat{\theta}$ represents the estimated association between the residualized treatment predictors and biomarker responses. The generalized inverse was used because $X^{*T}X^*$ may be singular or poorly conditioned.

The general OIP framework is not restricted to linear conditional expectation models and can be implemented using different estimators of conditional expectation, including penalized regression methods such as LASSO when appropriate (Tibshirani 1996; Hastie et al. 2009). In the present study, however, conditional expectations were estimated using simple linear regression applied to standardized variables.

Before OIP analysis, all variables were standardized as z-scores using the pooled mean and standard deviation. Because the variables were standardized, the regression intercept was zero and the regression slope was numerically equal to the Pearson correlation coefficient. Accordingly, the fitted conditional expectation was expressed as

$$\hat{E}(X|Z) = rZ$$

where r denotes the Pearson correlation coefficient. Residualized variables were therefore obtained as

$$X^* = X - rZ$$

with an analogous expression for Y.

For each experiment, the predefined reference component (Z) consisted of measurements from the third experimental group and was used solely for conditional centering during the exploratory OIP analysis. Thus, Z served as a statistical reference component rather than a biological covariate or adjustment variable. Residualization was therefore performed relative to this predefined reference component.

The repeated-dose and acute experiments were analyzed separately during the conventional statistical analyses. For exploratory multivariate OIP analysis, biomarkers measured within each experiment were first residualized using the corresponding reference component, after which the transformed datasets were combined as a stress-test application of the OIP framework for multivariate discrimination. Because the two experiments differed in exposure protocol, treatment formulation, and cytokine composition, only biomarkers common to both experiments were included in the integrated multivariate analysis.

Following residualization, the transformed variables were analyzed using linear discriminant analysis (LDA) and Hotelling's T^2 statistic to evaluate multivariate separation between the predefined experimental conditions.

Ninety-five percent confidence intervals were estimated using bootstrap resampling (1000 iterations). Bootstrap resampling was performed after the OIP transformation while preserving the original experimental-group structure. The complete computational workflow, specification of the reference component, and a step-by-step illustrative calculation are provided in the Supplementary Materials.

OIP was regarded as an exploratory, hypothesis-generating analytical framework. Its purpose was not to improve statistical significance or replace conventional hypothesis testing but to facilitate interpretation of correlated

multivariate biomarker responses after conditional centering. The present study demonstrates one practical implementation of the general OIP framework in experimental toxicology; it does not constitute formal mathematical validation of the method. Because the analysis was performed on relatively small experimental datasets without simulation studies or external validation, the statistical properties and comparative performance of OIP should be evaluated in future dedicated methodological investigations.

Integrated Multivariate Analysis

As a complementary multivariate analysis, cytokines measured in both experiments were used to compare the overall standardized cytokine response profiles of the acute and repeated-dose datasets. This analysis was intended to characterize differences in the overall biomarker patterns rather than to estimate the independent effect of exposure duration.

Overall differences between the two datasets were assessed using Hotelling's T^2 statistic and the corresponding multivariate F-test. Only cytokines measured in both experiments were included in the analysis. Individual coefficients were examined for exploratory purposes and were not interpreted as independent estimates of biological effects.

Because the acute and repeated-dose experiments differed with respect to exposure duration, administered dose, treatment formulation, animal characteristics, and cytokine panel composition, the observed multivariate separation was interpreted as reflecting overall differences between the experimental datasets rather than the isolated effect of any single experimental factor.

Sample Size and Interpretation of Statistical Evidence

The repeated-dose experiment included three groups of eight animals each, whereas the acute experiment comprised three groups of ten animals each. Plasma cytokines and routine hematological parameters were measured in all animals.

Because numerous correlated biological endpoints were evaluated in relatively small experimental groups, the results were interpreted by considering p-values, η^2 effect sizes, 95% confidence intervals, consistency across related biomarkers, and biological plausibility, rather than statistical significance alone.

Accordingly, the interpretation of individual cytokine changes should be regarded as exploratory. Findings associated with large estimated effects but wide confidence intervals were interpreted cautiously and should be confirmed in independent studies.

Results

Plasma Cytokine Responses after Repeated TeA Administration

Repeated administration of TeA was associated with marked alterations in plasma cytokine concentrations, although both the magnitude and direction of the responses differed among individual biomarkers (Table 1).

IL-1β exhibited a pronounced increase at 1.55 mg/kg, with the median concentration rising from 3.01 pg/mL in the vehicle-control group to 53.02 pg/mL. TNF-α reached its highest median concentration at 0.36 mg/kg, increasing from 5.04 pg/mL in controls to 130.00 pg/mL, and remained elevated at 1.55 mg/kg.

Intermediate-dose maxima were observed for IFN-γ, IL-2, IL-10, IL-13, and IL-17A. For example, the median IL-2 concentration increased from 5.62 pg/mL in the control group to 41.09 pg/mL at 0.36 mg/kg before decreasing to 12.86 pg/mL at 1.55 mg/kg. A similar pattern was observed for IL-10, with median concentrations of 1.23, 22.97, and 5.49 pg/mL in the vehicle-control, 0.36 mg/kg, and 1.55 mg/kg groups, respectively.

IL-12p70 concentrations were higher in both TeA-treated groups than in controls, whereas IL-4 and IL-6 concentrations were markedly lower at 1.55 mg/kg. In contrast, IL-5 reached its highest median concentration at the highest administered dose. GM-CSF exhibited considerable within-group variability and showed no consistent exposure-related pattern.

Analysis of the linear and quadratic dose components demonstrated heterogeneous response patterns across the three dose levels. The largest effect-size estimates were observed for IL-10, TNF-α, IL-1β, IFN-γ, and IL-4. Significant quadratic components were identified for TNF-α, IL-2, IL-10, IL-13, and IL-17A, indicating response patterns consistent with intermediate-dose maxima. Because only three dose levels were evaluated, these findings should not be interpreted as a definitive characterization of the underlying dose–response relationships.

Plasma Cytokine Responses after Acute Administration

As noted above, the acute-exposure dataset was previously reported by Trushina et al. (2025) and was reanalyzed here to enable comparison with the previously unpublished repeated-dose experiment and the exploratory multivariate analysis.

Twenty-four hours after administration, both purified TeA and the A. *alternata* extract were associated with changes in several plasma cytokine concentrations compared with the vehicle-control group (Table 2).

Median concentrations of IFN-γ, IL-1β, IL-6, IL-17A, MIP-1α, and TNF-α were higher in both exposed groups than in controls. MCP-1 reached its highest median concentration following administration of purified TeA. In contrast, IL-5 and IL-10 concentrations were lower in both exposed groups.

The median IL-10/IL-17A ratio decreased from 3.07 in the vehicle-control group to 0.81 in both exposed groups, reflecting reduced IL-10 concentrations together with increased IL-17A concentrations.

Purified TeA and the A. *alternata* extract produced broadly similar cytokine response profiles, although the magnitude of the response differed for several biomarkers between the two treatment formulations. Because both exposed groups received equivalent doses of TeA, these differences were interpreted as formulation-related rather than dose-dependent effects. The largest effect-size estimates were observed for IL-17A, the IL-10/IL-17A ratio, IL-5, IL-6, and IL-10. Detailed pairwise comparisons together with η^2 estimates and their corresponding 95% confidence intervals are presented in Supplementary Table S2.

Hematological Responses after Repeated TeA Administration

Compared with the cytokine findings, repeated TeA administration produced relatively modest changes in routine hematological parameters (Table 3). Most erythrocyte variables, including erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), showed only minor differences between treatment groups. Likewise, platelet count, mean platelet volume (MPV), and plateletcrit (PCT) exhibited no consistent exposure-related pattern.

Greater variability was observed among leukocyte parameters. Total leukocyte and absolute lymphocyte counts were lower in both TeA-treated groups than in the vehicle-control group, whereas the relative proportion of lymphocytes was higher. Absolute monocyte counts decreased from 0.48 × 10^9/L in controls to 0.23 × 10^9/L and 0.22 × 10^9/L following administration of 0.36 and 1.55 mg/kg TeA, respectively. Similar descriptive decreases were observed for absolute neutrophil and granulocyte counts.

Detailed statistical results, including effect-size estimates and corresponding 95% confidence intervals, are provided in Supplementary Table S3.

Hematological Responses after Acute Administration

Consistent with the previously reported acute-exposure dataset (Trushina et al. 2025), reanalysis of the hematological data showed only modest differences among the experimental groups (Table 4). Most erythrocyte and platelet variables showed largely overlapping distributions among the vehicle-control, purified TeA, and A. *alternata* extract groups.

Total leukocyte and absolute lymphocyte counts were lower in both exposed groups than in the vehicle-control group, whereas the relative proportion of lymphocytes remained broadly similar across groups. The purified TeA group exhibited a higher median relative monocyte percentage than the vehicle-control group, whereas the A. *alternata* extract group showed a lower median value. Differences in absolute monocyte counts were comparatively small, and neutrophil, eosinophil, and basophil parameters showed no consistent treatment-related pattern.

Detailed pairwise comparisons, η^2 effect-size estimates, and their corresponding 95% confidence intervals are presented in Supplementary Table S4.

Integrated Multivariate Analysis of Cytokine Responses

To complement the conventional univariate analyses, an exploratory multivariate analysis was performed to compare the overall cytokine response profiles of the acute and repeated-dose experiments. Rather than evaluating each cytokine individually, this approach considered the cytokine measurements as a correlated multivariate response.

The global Hotelling's T^2 test indicated an overall difference between the two datasets, whereas none of the individual cytokine coefficients reached the conventional threshold for statistical significance. Because the experiments differed substantially in administered dose, treatment formulation, exposure duration, animal characteristics, and cytokine panel composition, the observed multivariate separation was interpreted as reflecting an overall difference between the experimental datasets and should not be attributed to exposure duration, treatment formulation, any individual cytokine, or any other single experimental factor.

Discussion

The present study demonstrated marked alterations in circulating cytokine profiles following repeated oral exposure to TeA, whereas routine erythrocyte and platelet parameters were affected to a much lesser extent. Comparative reanalysis of the previously reported acute-exposure dataset showed a distinct cytokine response pattern following acute administration of purified TeA and an *A. alternata* extract. The observed responses were heterogeneous and included both monotonic and non-monotonic patterns. These findings extend the limited in vivo evidence on TeA, which has largely focused on general toxicity, oxidative stress, histopathological injury, and conventional hematological alterations rather than comprehensive systemic cytokine responses (Louro et al. 2024; Smith et al. 1968; Giambrone et al. 1978; Kumari and Singh 2021, 2023).

Cytokine responses following repeated exposure

Repeated TeA administration was associated with marked but heterogeneous alterations in circulating cytokine concentrations. The most pronounced increase was observed for IL-1β, whose median concentration increased from 3.01 pg/mL in controls to 53.02 pg/mL in the 1.55 mg/kg group. IL-1β is a key mediator of innate inflammatory responses and is released in response to cellular stress and tissue injury (Kany et al. 2019). However, because inflammasome components, caspase-1 activity, tissue cytokine expression, and immune-cell activation were not evaluated, the observed increase in circulating IL-1β cannot be interpreted as direct evidence of inflammasome activation.

TNF-α also exhibited a marked response, with the highest median concentration observed at 0.36 mg/kg and a lower value at 1.55 mg/kg. Similar intermediate-dose maxima were observed for IFN-γ, IL-2, IL-10, IL-13, and IL-17A. Collectively, these findings do not support a simple proportional relationship between administered dose and circulating cytokine concentrations.

Non-monotonic responses may result from several mechanisms, including opposing signaling pathways, compensatory regulation, differences in response kinetics, shifts in cytokine-producing cell populations, or increasing toxic burden at higher doses. TeA has been reported to inhibit eukaryotic protein synthesis, providing one possible explanation for attenuated cytokine production under conditions of greater toxic stress (Louro et al. 2024; Friedman et al. 1975). However, protein synthesis, cell viability, and intracellular signaling were not assessed in the present study. Consequently, the biological mechanisms underlying the descending portions of the observed dose–response patterns remain uncertain.

IL-12p70 differed from cytokines exhibiting intermediate-dose maxima by showing progressively higher concentrations across the analyzed dose groups. Because IL-12 promotes natural killer (NK)-cell and T-cell activation and stimulates IFN-γ production, the absence of a corresponding progressive increase in IFN-γ illustrates that circulating cytokine concentrations should not be interpreted as a simple linear signaling cascade (Kany et al. 2019). Differences in sampling time and regulatory feedback mechanisms may have contributed to the divergent response profiles.

IL-4 and IL-6 concentrations were lower at the highest repeated dose. These findings alone do not demonstrate suppression of Th2 responses or inhibition of a specific inflammatory pathway. Both cytokines are produced by multiple cell types, and their circulating concentrations reflect the combined effects of synthesis, secretion, tissue distribution, receptor binding, and clearance. Immunophenotyping together with functional immune assays would therefore be required to determine whether the observed changes resulted from altered immune-cell abundance, altered secretory activity, or secondary systemic toxic effects.

Overall, repeated TeA exposure was associated with concurrent alterations in cytokines involved in innate inflammation, T-cell-mediated immunity, and immune regulation. The simultaneous increases in both pro-inflammatory and regulatory cytokines at 0.36 mg/kg are more consistent with a complex immunomodulatory response than with a uniform shift toward either inflammation or immunosuppression.

Acute exposure and comparison of purified TeA with the fungal extract

The acute-exposure findings discussed below derive from the dataset previously reported by Trushina et al. (2025) and are reconsidered here specifically in relation to the previously unpublished repeated-dose experiment and the exploratory multivariate analysis.

Acute exposure produced a cytokine profile distinct from that observed following repeated administration. Compared with vehicle-treated animals, both purified TeA and the *Alternaria alternata* extract were associated with higher median concentrations of IFN-γ, IL-1β, IL-6, IL-17A, MCP-1, MIP-1α, and TNF-α, together with lower concentrations of IL-5 and IL-10.

The marked reduction in the IL-10/IL-17A ratio reflected the combination of decreased IL-10 and increased IL-17A concentrations in both exposed groups. Although this pattern is consistent with a shift toward a more pro-inflammatory cytokine balance, it should not be interpreted as direct evidence of altered regulatory T-cell or Th17 activity because neither cell population was quantified, and both cytokines can originate from multiple cellular sources.

The increased concentrations of MCP-1 and MIP-1α are consistent with enhanced chemokine-mediated inflammatory signaling. However, because tissue infiltration and immune-cell migration were not assessed, these findings should be interpreted as evidence of altered systemic cytokine responses rather than direct evidence of leukocyte recruitment.

Both exposed groups received the same dose of TeA (30 mg/kg BW) but differed in treatment formulation. Consequently, differences between purified TeA and the A. *alternata* extract should be interpreted as formulation-related rather than dose-dependent effects. The extract additionally contained AOH, AME, and TEN, all of which have been reported to modulate inflammatory or cytotoxic responses in experimental systems (Crudo et al. 2019; den Hollander et al. 2022; Gröstlinger et al. 2022; Saleh et al. 2024; Schmutz et al. 2019; Vilela et al. 2025). Accordingly, the broadly similar cytokine profiles observed in both exposed groups suggest that TeA contributed substantially to the overall response but do not demonstrate that it was the sole biologically active component. Clarifying the individual and combined contributions of these mycotoxins will require dedicated mixture studies (Partsch et al. 2026a, b; Crudo et al. 2019; den Hollander et al. 2022; Gröstlinger et al. 2022). Moreover, the extract was characterized for TeA, AOH, AME, and TEN, but other potentially bioactive Alternaria metabolites were not quantified. Therefore, contributions from additional uncharacterized metabolites to the observed effects cannot be excluded.

Hematological findings

Routine hematological parameters were less responsive than circulating cytokines. Following repeated TeA administration, erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) remained within relatively narrow ranges across the experimental groups. Likewise, platelet count, mean platelet volume (MPV), and plateletcrit (PCT) showed no consistent dose-related pattern. Collectively, these findings provide no evidence of substantial disruption of erythrocyte or platelet homeostasis under the conditions of the present study.

Several leukocyte parameters showed descriptive differences between groups. Total leukocyte and absolute lymphocyte counts were lower in the exposed groups, whereas the relative proportion of lymphocytes was higher. This apparent discrepancy is expected because relative leukocyte percentages depend on changes in the total leukocyte count and in the distribution of the remaining leukocyte populations. Consequently, the observed increase in the relative proportion of lymphocytes should not be interpreted as absolute lymphocytosis.

Similarly, absolute monocyte counts were lower in both repeated-exposure groups than in controls. These findings do not support a dose-dependent increase in circulating monocytes. Any interpretation in terms of altered leukocyte redistribution, reduced production, enhanced tissue migration, or increased cell loss would remain speculative because bone marrow, lymphoid tissues, and tissue leukocyte populations were not examined.

Previous studies have reported neutropenia, lymphocytosis, monocytosis, eosinophilia, and basophilia following TeA exposure in mice; however, the reported findings varied according to dose, route of administration, exposure duration, and sampling time (Kumari and Singh 2021, 2023). Differences between those studies and the present findings may additionally reflect species-specific responses, toxin preparation, analytical methodology, and the distinction between absolute and relative leukocyte measurements.

Acute exposure likewise produced only limited changes in conventional hematological parameters. Most erythrocyte and platelet variables showed substantial overlap among the vehicle-control, purified TeA, and A. *alternata* extract groups. Accordingly, the hematological alterations appeared less pronounced than the accompanying cytokine responses. However, these findings do not demonstrate that cytokines are inherently more sensitive biomarkers of TeA toxicity. Rather, they indicate that, within the present experimental design and at the selected sampling time points, the measured cytokines exhibited greater between-group differences than routine hematological parameters.

Integrated multivariate analysis

The secondary multivariate analysis revealed an overall difference between the cytokine profiles of the acute and repeated-dose experiments. This finding indicates that the measured cytokines formed coordinated multivariate

response patterns but does not identify which experimental factor was primarily responsible for the observed separation.

Because the two experiments differed with respect to administered dose, treatment formulation, exposure duration, animal characteristics, and cytokine panel composition, the observed multivariate separation was interpreted as reflecting the combined influence of these design characteristics rather than the independent effect of any single factor.

Although the global Hotelling's T^2 statistic indicated significant separation between the datasets, none of the individual cytokine coefficients reached the conventional threshold for statistical significance. Accordingly, the findings support interpretation at the level of the overall cytokine response profile rather than identification of any single cytokine as the principal discriminator between the two experiments.

OIP provided a complementary framework for the multivariate interpretation of correlated cytokine responses following residualization with respect to the predefined reference component. Because the study included relatively small experimental groups and did not incorporate external or simulation-based validation, these findings should be regarded as exploratory and require confirmation in independent datasets.

Toxicological interpretation and study limitations

The previously unpublished repeated-dose experiment demonstrated substantial alterations in circulating cytokine profiles following TeA exposure. Comparative reanalysis of the previously reported acute-exposure dataset provided complementary evidence of altered cytokine profiles under a substantially different exposure scenario. Together, these analyses extend the available evidence on TeA toxicity, which has primarily focused on oxidative stress, histopathological injury, and conventional hematological endpoints. Nevertheless, circulating cytokine concentrations alone cannot establish impaired immune competence or identify the molecular mechanisms underlying TeA-associated immunotoxicity (Smith et al. 1968; Giambrone et al. 1978; Kumari and Singh 2021, 2023).

A major strength of the present study was the assessment of an extensive cytokine panel together with routine hematological parameters in the previously unpublished repeated-dose experiment, complemented by comparative reanalysis of the previously reported acute-exposure dataset. In addition, reanalysis of the previously reported comparison between purified TeA and a chemically characterized *A. alternata* extract provided complementary context for interpreting the repeated-dose findings.

Several limitations should be acknowledged. Only male rats were included, and cytokines were measured at a single terminal time point in each experiment, precluding assessment of the temporal dynamics of the immune response. An additional limitation is that the acute-exposure dataset was not newly generated for the present study but

had been reported previously (Trushina et al. 2025). Its inclusion here was restricted to comparative reanalysis alongside the previously unpublished repeated-dose dataset and to exploratory multivariate analysis. Furthermore, the study did not include immunophenotyping, functional immune assays, cytokine gene-expression analyses, lymphoid-organ histopathology, markers of NF-κB or inflammasome activation, oxidative-stress measurements, or toxicokinetic analyses. Consequently, mechanistic explanations involving inflammasome activation, altered immune-cell function, compensatory regulation, or translational inhibition remain speculative.

The acute TeA dose was substantially higher than expected dietary exposure, and bolus intragastric administration does not fully reproduce typical human exposure scenarios (EFSA 2011; EFSA et al. 2016; Lu et al. 2025). In addition, plasma and tissue concentrations of TeA were not measured, limiting extrapolation from administered dose to internal exposure and, consequently, to potential human health risk.

Finally, the relatively small experimental groups, the large number of correlated endpoints, and multiple statistical comparisons increase uncertainty. Accordingly, numerical trends that lacked statistical support should be interpreted as exploratory observations rather than established biological effects. OIP should likewise be regarded as an exploratory analytical framework in the present study rather than as a formally validated methodology. Its statistical properties, robustness, and comparative performance require evaluation in dedicated methodological studies using simulation experiments and independent datasets.

Overall, the previously unpublished repeated-dose experiment demonstrated marked and heterogeneous alterations in circulating cytokine profiles following oral TeA exposure, including a pronounced increase in IL-1β at the highest analyzed dose and intermediate-dose maxima of TNF-α and several T-cell-associated cytokines. Comparative reanalysis of the previously reported acute-exposure dataset showed broadly similar cytokine response profiles following administration of purified TeA and an *A. alternata* extract, including reduced IL-10/IL-17A ratios compared with vehicle-treated animals. Routine erythrocyte and platelet parameters showed comparatively limited variation, whereas leukocyte changes were modest and did not exhibit a consistent dose-related pattern. Under the conditions of the present study, circulating cytokine profiles therefore showed more pronounced between-group differences than routine hematological parameters.

Exploratory multivariate analysis provided a complementary framework for interpreting coordinated biomarker responses but did not establish the underlying biological mechanisms or validate the statistical methodology. Future toxicological studies should evaluate repeated oral exposure across a wider range of lower doses and incorporate toxicokinetic measurements, serial cytokine sampling, immunophenotyping, functional immune assays, and analyses of inflammatory signaling pathways. Studies comparing purified TeA with individual Alternaria

mycotoxins and defined toxin mixtures will also be required to determine whether accompanying metabolites modify TeA-associated immunotoxic responses (Partsch et al. 2026a, b; Crudo et al. 2019; den Hollander et al. 2022; Gröstlinger et al. 2022). In parallel, dedicated methodological studies using simulations, independent datasets, and comparisons with established multivariate approaches are needed to evaluate the statistical properties and broader applicability of OIP.

Statements and Declarations

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Competing Interests

The authors declare no competing interests.

Ethics Approval

The animal study protocol was approved by the Ethics Committee of the Federal Research Centre of Nutrition, Biotechnology and Food Safety (Protocol No. 4, approved on 20 April 2017).

Data Availability

The data supporting the findings of this study are available within the article and its Supplementary Materials. Additional data are available from the corresponding author upon reasonable request.

Authors' Contributions

All authors contributed to the conception and design of the study, data acquisition, data analysis, interpretation of the results, and manuscript preparation. All authors have read and approved the published version of the manuscript.

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References

Aichinger G, Del Favero G, Warth B, Marko D (2021) Alternaria toxins—Still emerging? Compr Rev Food Sci Food Saf 20:4390–4406. https://doi.org/10.1111/1541-4337.12803

Biancotto A, Feng X, Langweiler M, Young NS, McCoy JP (2012) Effect of anticoagulants on multiplexed measurement of cytokine/chemokines in healthy subjects. Cytokine 60:438–446. https://doi.org/10.1016/j.cyto.2012.05.019

Bou Zerdan M, Moussa S, Atoui A, Assi HI (2021) Mechanisms of immunotoxicity: stressors and evaluators. Int J Mol Sci 22:8242. https://doi.org/10.3390/ijms22158242

Bühlmann P, van de Geer S (2011) Statistics for high-dimensional data: methods, theory and applications. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-20192-9

Crudo F, Varga E, Aichinger G, Galaverna G, Marko D, Dall'Asta C, Dellafiora L (2019) Co-occurrence and combinatory effects of Alternaria mycotoxins and other xenobiotics of food origin: current scenario and future perspectives. Toxins 11:640. https://doi.org/10.3390/toxins11110640

Daichi MB, Masiello M, Haidukowski M, De Girolamo A, Moretti A, Bencheikh A, Rouag N, Somma S (2025) Assessing Alternaria species and related mycotoxin contamination in wheat in Algeria: a food safety risk. Toxins 17:309. https://doi.org/10.3390/toxins17060309

den Hollander D, Holvoet C, Demeyere K, De Zutter N, Audenaert K, Meyer E, Croubels S (2022) Cytotoxic effects of alternariol, alternariol monomethyl-ether, and tenuazonic acid and their relevant combined mixtures on human enterocytes and hepatocytes. Front Microbiol 13:849243. https://doi.org/10.3389/fmicb.2022.849243

EC – European Commission (2022) Commission Recommendation (EU) 2022/553 of 5 April 2022 on monitoring the presence of Alternaria toxins in food. Off J Eur Union L 107:90–92

EFSA Panel on Contaminants in the Food Chain (CONTAM) (2011) Scientific opinion on the risks for animal and public health related to the presence of Alternaria toxins in feed and food. EFSA J 9:2407. https://doi.org/10.2903/j.efsa.2011.2407

European Food Safety Authority, Arcella D, Eskola M, Gómez Ruiz JA (2016) Dietary exposure assessment to Alternaria toxins in the European population. EFSA J 14:4654. https://doi.org/10.2903/j.efsa.2016.4654

Friedman JH, Hastie T, Tibshirani R (2010) Regularization paths for generalized linear models via coordinate descent. J Stat Softw 33:1–22. https://doi.org/10.18637/jss.v033.i01

Friedman MA, Aggarwal V, Lester GE (1975) Inhibition of epidermal DNA synthesis by cycloheximide and other inhibitors of protein synthesis. Res Commun Chem Pathol Pharmacol 11:311–318

Giambrone JJ, Davis ND, Diener UL (1978) Effect of tenuazonic acid on young chickens. Poult Sci 57:1554–1558. https://doi.org/10.3382/ps.0571554

Gröstlinger J, Spindler V, Pahlke G, Rychlik M, Del Favero G, Marko D (2022) Alternaria alternata mycotoxins activate the aryl hydrocarbon receptor and Nrf2-ARE pathway to alter the structure and immune response of colon epithelial cells. Chem Res Toxicol 35:731–749. https://doi.org/10.1021/acs.chemrestox.1c00364

610 Hastie T, Tibshirani R, Friedman J (2009) The elements of statistical learning: data mining, inference, and
611 prediction. 2nd edn. Springer, New York. https://doi.org/10.1007/978-0-387-84858-7
612 Hennø LT, Storjord E, Christiansen D, Bergseth G, Ludviksen JK, Fure H, Barene S, Nielsen EW, Mollnes
613 TE, Brekke OL (2017) Effect of the anticoagulant, storage time and temperature of blood samples on the
614 concentrations of 27 multiplex assayed cytokines: consequences for defining reference values in healthy humans.
615 Cytokine 97:86–95. https://doi.org/10.1016/j.cyto.2017.05.014
616 Ismail AM, Elshewy ES, El-Ganainy SM, Magistà D, Hamouda AF, Alhudaib KA, Ebrahim W, Almaghasla
617 MI (2023) Mycotoxins from tomato pathogenic Alternaria alternata and their combined cytotoxic effects on human
618 cell lines and male albino rats. J Fungi 9:282. https://doi.org/10.3390/jof9030282
619 Ji X, Xiao Y, Jin C, Wang W, Lyu W, Tang B, Yang H (2022a) Alternaria mycotoxins in food commodities
620 marketed through e-commerce stores in China: occurrence and risk assessment. Food Control 140:109125.
621 https://doi.org/10.1016/j.foodcont.2022.109125
622 Ji X, Xiao Y, Lyu W, Li M, Wang W, Tang B, Wang X, Yang H (2022b) Probabilistic risk assessment of
623 combined exposure to deoxynivalenol and emerging Alternaria toxins in cereal-based food products for infants and
624 young children in China. Toxins 14:509. https://doi.org/10.3390/toxins14080509
625 Ji X, Deng T, Xiao Y, Jin C, Lyu W, Wu Z, Wang W, Wang X, He Q, Yang H (2023a) Emerging Alternaria
626 and Fusarium mycotoxins in tomatoes and derived tomato products from the China market: occurrence, methods of
627 determination, and risk evaluation. Food Control 145:109464. https://doi.org/10.1016/j.foodcont.2022.109464
628 Ji X, Jin C, Xiao Y, Deng M, Wang W, Lyu W, Chen J, Li R, Li Y, Yang H (2023b) Natural occurrence of
629 regulated and emerging mycotoxins in wheat grains and assessment of the risks from dietary mycotoxins exposure in
630 China. Toxins 15:389. https://doi.org/10.3390/toxins15060389
631 Kany S, Vollrath JT, Relja B (2019) Cytokines in inflammatory disease. Int J Mol Sci 20:6008.
632 https://doi.org/10.3390/ijms20236008
633 Kelley K (2007) Confidence intervals for standardized effect sizes: theory, application, and implementation.
634 J Stat Softw 20:1–24. https://doi.org/10.18637/jss.v020.i08
635 Kumari A, Singh K (2021) Evaluation of prophylactic efficacy of cinnamaldehyde in murine model against
636 Paradendryphiella arenariae mycotoxin tenuazonic acid-induced oxidative stress and organ toxicity. Sci Rep
637 11:19420. https://doi.org/10.1038/s41598-021-98319-8

Kumari A, Singh K (2023) Preventive role of cinnamaldehyde against tenuazonic acid- and Freund's adjuvant-induced histopathological and biochemical alterations in the mouse model. Front Microbiol 14:1159881. https://doi.org/10.3389/fmicb.2023.1159881

Lakens D (2013) Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. Front Psychol 4:863. https://doi.org/10.3389/fpsyg.2013.00863

Louro H, Vettorazzi A, López de Cerain A, Spyropoulou A, Solhaug A, Straumfors A, Behr AC, Mertens B, Žegura B, Fæste CK, et al (2024) Hazard characterization of Alternaria toxins to identify data gaps and improve risk assessment for human health. Arch Toxicol 98:425–469. https://doi.org/10.1007/s00204-023-03636-8

Lu Z, Zhang R, Wu P, Zhao D, Chen J, Pan X, Wang J, Zhang H, Qi X, Ye S, et al (2025) Prevalence and exposure assessment of Alternaria toxins in Zhejiang Province, China. Foods 14:3298. https://doi.org/10.3390/foods14193298

Mao X, Chen W, Wu H, Shao Y, Zhu Y, Guo Q, Li Y, Xia L (2023) Alternaria mycotoxins analysis and exposure investigation in ruminant feeds. Toxins 15:495. https://doi.org/10.3390/toxins15080495

Nagda A, Meena M (2024) Alternaria mycotoxins in food and feed: occurrence, biosynthesis, toxicity, analytical methods, control and detoxification strategies. Food Control 158:110211. https://doi.org/10.1016/j.foodcont.2023.110211

National Research Council (2011) Guide for the care and use of laboratory animals, 8th edn. National Academies Press, Washington, DC

Partsch V, Crudo F, Marko D (2026a) Opposing immunomodulatory effects of the Alternaria mycotoxin tenuazonic acid in immune and intestinal epithelial cells. bioRxiv. https://doi.org/10.64898/2026.06.24.734282

Partsch V, Selimagić A, Crudo F, Marko D (2026b) Immunomodulatory potential of Alternaria mycotoxins on immune and intestinal cells: a comparative systematic in vitro study. Arch Toxicol 100:3519–3534. https://doi.org/10.1007/s00204-026-04346-7

Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, et al (2020) The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol 18:e3000410. https://doi.org/10.1371/journal.pbio.3000410

Qin Y, Zhou H, Yang Y, Guo T, Zhou Y, Zhang Y, Ma L (2024) Metabolome and its mechanism profiling in the synergistic toxic effects induced by co-exposure of tenuazonic acid and patulin in Caco-2 cells. Toxins 16:319. https://doi.org/10.3390/toxins16070319

667 Saleh I, Zeidan R, Abu-Dieyeh M (2024) The characteristics, occurrence, and toxicological effects of
668 alternariol: a mycotoxin. Arch Toxicol 98:1659–1683. https://doi.org/10.1007/s00204-024-03743-0
669 Schmutz C, Cenk E, Marko D (2019) The Alternaria mycotoxin alternariol triggers the immune response of
670 IL-1β-stimulated, differentiated Caco-2 cells. Mol Nutr Food Res 63:1900341.
671 https://doi.org/10.1002/mnfr.201900341
672 Smith ER, Fredrickson TN, Hadidian Z (1968) Toxic effects of the sodium and the N,N′-
673 dibenzylethylenediamine salts of tenuazonic acid (NSC-525816 and NSC-82260). Cancer Chemother Rep 52:579–
674 585
675 Thavasu PW, Longhurst S, Joel SP, Slevin ML, Balkwill FR (1992) Measuring cytokine levels in blood:
676 importance of anticoagulants, processing, and storage conditions. J Immunol Methods 153:115–124.
677 https://doi.org/10.1016/0022-1759(92)90313-I
678 Tibshirani R (1996) Regression shrinkage and selection via the lasso. J R Stat Soc Series B Stat Methodol
679 58:267–288. https://doi.org/10.1111/j.2517-6161.1996.tb02080.x
680 Trushina EN, Riger NA, Mustafina OK, Timonin AN, Aksenov IV, Tutelyan VA (2025) Alternaria toxins as
681 risk factors for cellular immunity disorders and cytokine profile imbalance (based on an in vivo experimental model).
682 Health Risk Analysis 3:104–113. https://doi.org/10.21668/health.risk/2025.3.11
683 Vilela RS, Pina-Martins F, Ventura C (2025) From food to humans: the toxicological effects of Alternaria
684 mycotoxins in the liver and colon. J Xenobiot 15:205. https://doi.org/10.3390/jox15060205
685 Zhang Y, Liu C, van der Fels-Klerx HJ (2025) Occurrence, toxicity, dietary exposure, and management of
686 Alternaria mycotoxins in food and feed: a systematic literature review. Compr Rev Food Sci Food Saf 24:e70085.
687 https://doi.org/10.1111/1541-4337.70085

688 Table 1. Plasma cytokine concentrations after 14 days of repeated oral administration of tenuazonic acid (n =
689 8 per group).

Dose (mg/kg)	0			0.36			1.55		
	Q1	Me	Q3	Q1	Me	Q3	Q1	Me	Q3
GM-CSF	1.17	1.57	3.66	1.73	4.93	11.94	1.29	1.84	10.70
IFN-γ	1.13	6.47	8.09	13.75	18.36	19.39	1.94	2.43	6.47
IL-1β	1.73	3.01	8.16	2.47	4.45	8.90	45.03	53.02	67.76
IL-2	3.62	5.62	9.35	25.98	41.09	49.37	4.41	12.86	20.61
IL-4	6.34	13.07	17.85	9.22	12.94	21.71	0.43	0.52	0.60
IL-5	0.60	0.64	0.70	0.55	0.79	1.16	0.83	1.46	2.06
IL-6	4.96	8.94	13.12	7.05	7.68	8.94	0.38	1.20	2.86
IL-10	1.13	1.23	1.34	14.08	22.97	28.66	4.99	5.49	7.31
IL-12p70	1.12	2.69	6.16	5.73	16.45	25.16	7.93	10.17	20.15
IL-13	2.09	2.57	7.81	20.88	23.31	31.80	3.01	10.15	25.85
IL-17A	0.77	1.78	2.20	4.36	9.34	18.93	0.52	0.73	0.95
TNF-α	2.68	5.04	8.99	108.03	130.00	144.56	51.53	71.78	85.51

690

691 Table 2. Plasma cytokine concentrations 24 h after acute administration of purified tenuazonic acid or an
692 *Alternaria alternata* extract (n = 10 per group).

Parameter	Vehicle control			Purified TeA (30 mg/kg)			A. *alternata* extract (TeA-equivalent, 30 mg/kg)		
	Q1	Me	Q3	Q1	Me	Q3	Q1	Me	Q3
IFN-γ	0.46	0.59	0.94	0.85	0.98	1.37	0.96	1.09	1.26
IL-1β	0.22	0.36	0.53	0.44	0.57	0.74	0.53	0.63	0.71
IL-2	3.17	4.62	5.24	4.86	6.32	6.94	4.88	5.04	5.59
IL-5	0.94	1.03	1.20	0.38	0.41	0.46	0.38	0.42	0.44
IL-6	1.12	1.25	1.35	1.93	2.14	2.32	1.81	2.02	2.27
IL-10	1.03	1.28	1.47	0.52	0.76	0.96	0.64	0.71	0.83
IL-17A	0.37	0.42	0.48	0.95	1.01	1.07	0.76	0.91	1.02
MCP-1	1.92	1.97	1.99	2.29	2.32	2.36	2.03	2.15	2.42
MIP-1α	0.31	0.49	0.62	0.53	0.71	0.84	0.48	0.66	0.79
TNF-α	0.31	0.45	0.51	0.65	0.84	0.92	0.57	0.74	0.81
IL-10/17A	2.61	3.07	3.65	0.53	0.81	0.89	0.66	0.81	1.06

693

694 Table 3. Hematological parameters after repeated oral tenuazonic acid administration (Q1, median, Q3).

Dose (mg/kg)	0			0.36			1.55		
	Q1	Me	Q3	Q1	Me	Q3	Q1	Me	Q3
Erythrocytes, $\times 10^{12}$/L	6.10	6.29	6.42	6.29	6.48	6.77	6.31	6.51	6.65
Hemoglobin, g/L	117	123	128	120	131	132	121	127	130
Hematocrit, %	33.48	34.70	35.63	34.60	36.30	36.63	34.13	35.45	36.25
MCV, fL	53.50	55.21	56.25	54.00	55.50	56.25	52.25	55.00	55.25
MCH, pg	18.85	19.42	20.05	18.55	20.05	20.55	18.08	19.75	20.03
MCHC, g/L	349	353	357	350	357	363	354	359	361
Leukocytes, $\times 10^9$/L	8.53	10.74	11.78	7.70	8.30	8.73	5.65	7.25	9.13
Neutrophils, %	9.08	10.81	14.95	8.28	10.00	11.43	8.40	9.05	9.68
Eosinophils, %	0.80	0.91	1.00	0.78	1.00	1.48	0.28	0.50	0.83
Basophils, %	0.48	0.60	0.80	0.48	0.85	1.13	0.40	0.65	1.10
Lymphocytes, %	78.1	81.6	85.1	82.2	85.1	87.3	84.8	86.7	87.6
Monocytes, %	3.48	4.41	6.40	2.50	2.85	3.15	2.38	3.10	4.13
Platelets, $\times 10^9$/L	538	643	754	440	557	677	461	537	775
MPV, fL	6.48	6.64	6.70	6.40	6.70	6.73	6.40	6.45	6.58
Thrombocrit, %	0.36	0.43	0.49	0.28	0.38	0.45	0.31	0.35	0.50
Neutrophils, $\times 10^9$/L	0.81	1.18	1.65	0.67	0.79	0.99	0.49	0.62	0.80
Eosinophils, $\times 10^9$/L	0.07	0.10	0.12	0.05	0.11	0.15	0.02	0.03	0.07
Basophils, $\times 10^9$/L	0.06	0.06	0.07	0.05	0.07	0.09	0.04	0.04	0.06
Lymphocytes, $\times 10^9$/L	7.33	8.54	9.45	6.69	6.96	7.42	4.87	6.27	7.99
Monocytes, $\times 10^9$/L	0.36	0.48	0.57	0.15	0.23	0.26	0.19	0.22	0.25
Granulocytes, $\times 10^9$/L	0.99	1.34	1.83	0.76	0.98	1.20	0.63	0.69	0.90

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696 Table 4. Hematological parameters after acute exposure to purified TeA or *Alternaria alternata* extract (Q1,
697 median, Q3).

Parameter	Vehicle control			Purified TeA (30 mg/kg)			A. *alternata* extract (TeA-equivalent, 30 mg/kg)		
	Q1	Me	Q3	Q1	Me	Q3	Q1	Me	Q3
Erythrocytes, $\times 10^{12}$/L	7.19	7.41	7.58	7.14	7.52	7.84	7.45	7.62	7.71
Hemoglobin, g/L	137	140	149	135	143	145	141	147	152
Hematocrit, %	37.15	38.85	40.75	37.58	39.12	40.83	39.95	40.65	42.38
MCV, fL	52.00	53.00	53.75	51.25	52.17	53.75	52.25	54.00	54.00
MCH, pg	18.30	18.80	19.70	18.23	18.72	19.00	19.10	19.25	19.38
MCHC, g/L	352	360	367	352	360	365	355	361	367
Leukocytes, $\times 10^9$/L	7.93	9.35	10.83	6.23	7.30	8.27	6.50	8.00	9.85
Neutrophils, %	6.10	7.35	8.28	5.55	6.94	9.53	6.90	7.85	8.48
Eosinophils, %	0.55	1.00	1.93	0.40	0.70	1.35	0.65	0.80	1.53
Basophils, %	0.53	1.10	1.45	0.63	0.99	1.38	0.63	0.70	1.05
Lymphocytes, %	86.2	88.4	89.4	83.7	88.5	91.5	85.7	88.4	89.5
Monocytes, %	1.93	2.25	2.63	2.25	2.82	3.35	1.33	1.95	2.58
Platelets, $\times 10^9$/L	417	557	627	398	500	531	465	480	553
MPV, fL	6.33	6.40	6.50	6.33	6.53	6.80	6.30	6.40	6.73
Thrombocrit, %	0.26	0.36	0.41	0.28	0.32	0.35	0.29	0.30	0.35
Neutrophils, $\times 10^9$/L	0.56	0.59	0.68	0.44	0.57	0.65	0.56	0.69	0.75
Eosinophils, $\times 10^9$/L	0.06	0.08	0.16	0.03	0.06	0.08	0.05	0.10	0.14
Basophils, $\times 10^9$/L	0.06	0.09	0.10	0.05	0.07	0.08	0.05	0.09	0.16
Lymphocytes, $\times 10^9$/L	6.84	8.25	9.64	5.19	6.32	7.41	5.76	7.12	8.71
Monocytes, $\times 10^9$/L	0.13	0.21	0.28	0.19	0.22	0.27	0.10	0.17	0.25

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