Evaluation of immunomodulatory effects of zearalenone in mice

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ABSTRACT
Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by Fusarium species. The toxicity of ZEA has been evaluated for reproductive and developmental effects; however, there is little evidence about its acute toxicity or general immunotoxicity. In the present study, immune regulatory functions were investigated in mice that had been exposed to ZEA (5 or 20 mg/kg BW) daily for 14 days. Results showed that sub-populations of CD4⁺, CD8⁺ and CD11c⁺ cells in the spleen and CD4⁺, CD8⁺ and F4/80⁺ cells in the mesenteric lymph nodes (MLN) of ZEA (20 mg/kg)-exposed hosts were decreased compared to those in the control mice. However, CD19⁺ and CD11c⁺ cells were increased in the MLN of the ZEA mice and CD4⁺ CD25⁺ Foxp3⁺ cells were decreased in the spleen and MLN. There were differential changes in the immune cell populations of the small intestine of the ZEA mice as well, depending on small intestine location. In ex vivo experiments, ZEA treatments resulted in increased proliferative capacities of mitogen-induced splenocytes and MLN cells; such changes were paralleled by significant increases in interferon (IFN)-γ production. With regard to serum isotypes, IgM levels were decreased and IgE levels were increased in the 20 mg/kg ZEA-treated mice. Mucosal IgA levels were decreased in the duodenum and vagina of these hosts. Serum analyses also revealed that tumor necrosis factor (TNF)-α levels were decreased and interleukin (IL)-6 levels increased as a result of ZEA exposures. ZEA treatment also led to increased apoptosis in the spleen and Peyer’s patches; these changes were associated with changes in the ratios of Bax:Bcl-2. Following priming with different TLR ligands, ZEA exposure led to differentially modulated TLR signaling and variable production of pro- and anti-inflammatory cytokines in RAW 264.7 macrophage cells. Taken together, these results indicated that ZEA could alter the normal expression/function of different immune system components and this would likely lead to immunomodulation in situ.

Introduction

Zearalenone (ZEA, also known as F2 toxin) is a non-steroidal, estrogenic mycotoxin produced as a secondary metabolite by several species of Fusarium fungi (Kuiper-Goodman et al. 1987). The most important species of Fusarium genera are Fusarium graminearum and Fusarium culmorum (Ben Salah-Abbes et al. 2009). ZEA widely exists in many food products for human or animal consumption, including wheat, maize, oats, barley, sorghum and sesame seeds, as well as in hay and corn silage exposed to high moisture during storage (Scudamore and Pate 2000). ZEA has been found in cereals and cereal products at levels ranging from <1 mg/kg to >300 mg/kg, depending on climatic, harvest and storage conditions (Avantaggiato et al. 2003). Mean dietary ZEA intake has been estimated as 20 ng/kg/d in Canada, Denmark and Norway and 30 ng/kg/d in the USA (Zinedine et al. 2007).

Previous studies have suggested that consumption of ZEA-contaminated food presented a serious health hazard to humans, livestock and animal models (Conkov et al. 2001; Ouanes et al. 2003; Abbès et al. 2007; Ben Salah-Abbes et al. 2008a). ZEA causes severe reproductive toxicity in humans, laboratory animals and farm animals (Etienne and Dourmad 1994; Tomaszewski et al. 1998). As well as reproductive toxicity, histological alterations in reproductive organ was observed in the ZEA-treated mice, providing that ZEA treatment induced vacuoles, scarce array, and decreased intracellular connections in testes of mice (Yang et al. 2007). ZEA also has a significant genotoxic potential (Abbès et al. 2007) and can induce oxidative DNA damage (Fleck et al. 2012), chromosome aberrations and micronuclei (Ouanes et al. 2003). ZEA also causes DNA-adduct formation (Pfohl-Leszkowicz et al. 1995), DNA fragmentation, cell cycle arrest (Abid-Essefi et al. 2003), micronuclei formation and chromosomal aberrations (Ouanes et al. 2003, 2005). ZEA is also nephrotoxic and hepatotoxic (Ben Salah-Abbes et al. 2008b), and induces hepatocarcinoma development (Conková et al. 2001). ZEA also can cause changes in hematologic and immunologic endpoints (Marin et al. 1996; Murata et al. 2003; Ben Salah-Abbes et al. 2008a). ZEA has also been shown to be cytotoxic and pro-apoptotic (Kim et al. 2003) and enhance levels of lipid peroxidation (Conková et al. 2001) and oxidative damage in cells (El Golli et al. 2006; Hassen et al. 2007).
Because cytokines play a major role in inflammatory responses and act as critical modulators in humoral and cellular immune responses, any induced cytokine imbalance could aggravate inflammatory reactions and cause pathologies. Marin et al. (1996) reported that ZEA and α-zearalenol could stimulate cytokine production, including that of interleukin (IL)-2 and IL-5, by EL-4 thymoma cells. Ben Salah-Abbès et al. (2008a) reported that Balb/c mice treated with ZEA (40 mg/kg) for 2 weeks evidenced significant decreases in circulating levels of total white blood cells, immunoglobulin levels (IgG and IgM), B-cells, T-cell subtypes (CD3⁺, CD4⁺, CD8⁺), NK cells and pro-inflammatory cytokines. To date, mechanisms underlying many of these effects remain not fully understood.

Because of the clear risk to health, it is necessary to establish reasonable regulatory toxin levels based on immunotoxicology data to protect the public from adverse mycotoxin exposure. It is also necessary to clarify mechanisms underlying how these effects are induced. Accordingly, due to the limited information on potential immunotoxic effects of ZEA, this study examined such effects in mice and in RAW 264.7 murine macrophage cells.

Materials and methods

Animals and ZEA administration

BALB/c mice (female, 7-week-old) were purchased from Samtako (Osan, South Korea). All mice were housed in a specific pathogen-free animal facility maintained under standard conditions (24 ± 2°C, 50 ± 5% relative humidity) with a 12-h light/dark cycle. All mice had ad libitum access to standard rodent chow and filtered water throughout the study. The Institutional Animal Care and Use Committee of Chonbuk National University approved all protocols used herein.

ZEA purchased from Sigma-Israel (Rehovot, Israel) was dissolved in corn oil (Sigma, St. Louis, MO) to prepare a stock solution. Stock solution was diluted –80°C body weight. Stock solution was diluted accordingly with corn oil to provide the needed final dose/mouse. Gavage volume was 8–10 μl/g body weight.

Preparation of immune cells

Following anesthesia using Zoletil (intramuscular injection; 0.2 ml/kg), mice were euthanized by cervical dislocation at 24 h after the final (Day 14) ZEA administration. Blood was then collected (by direct heart puncture) into 1.5-ml Eppendorf tubes and then incubated 30 min at room temperature (RT); after centrifugation (12000 rpm, 5 min), the generated serum was isolated and stored at –80°C until analyzed. At necropsy, each mouse had their spleen removed and weighed; both mediastinal lymph nodes (MLN) and Peyers’s patches (PP) were also isolated. A portion of each spleen and MLN from each mouse was isolated for use in histology (see below). Each isolated tissue was then mechanically disrupted by passage through a cell strainer fitted with a 70-μm nylon mesh to yield single cell suspensions. The cells from each tissue were then pelleted by centrifugation. Hypotonic RBC lysis buffer (2.42 g Tris/l and 7.56 g NH₄Cl/l water, pH 7.2) was added to the pellet to lyse red blood cells present. After sitting at RT for 10 min, the preparations were centrifuged. The resultant pellet in each was then re-suspended in complete RPMI 1640 medium (Gibco, Gaithersburg, MD), i.e. RPMI 1640 supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml and 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT). Cell density was adjusted to 10⁶ cells/ml. Viability of freshly isolated cells was consistently >90% as seen by a trypan blue exclusion test.

Lymphocyte isolation from small intestine intra-epithelium and lamina propria

Intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) of mice small intestines were prepared as described earlier with a minor modification (Davies and Parrott 1981). For IEL and LPL isolation, small intestines were harvested and washed by swirling in phosphate-buffered saline (PBS). The intestines were cut longitudinally and then into 0.5-cm pieces. Intestinal pieces were agitated in 25 ml extraction buffer (1 mM EDTA in PBS [pH 7.4]) for 30 min at 37°C. To remove aggregates, the slurry was passed through a loosely packed nylon wool column. To isolate LPL, the remaining tissues were digested with 20 ml of a solution of 1.5 mg/ml Type VIII collagenase for 15 min at 37°C (three times). The follow-through was layered atop a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Buckinghamshire, UK) and centrifuged at 600 g for 20 min. Cells at the interface of the 40/70% layer were collected and washed in staining buffer.

In vitro TLR (toll-like receptor) activity

The mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37°C in a humidified atmosphere with 5% CO₂. Macrophage cell numbers and viability were assessed by trypan blue dye exclusion using a hemocytometer. Prior to treatment, cells (10⁶/ml) were cultured in 24-well tissue culture plates for 24 h to achieve 80% confluency. For TLR ligand stimulation, cells were incubated with 10 μg zymosan/ml (TLR2), 25 μg poly I:C/ml (TLR3), 100 ng LPS/ml (TLR4), 100 ng flagellin/ml (TLR5) or 3 μg CpG ODN/ml (TLR9) for 12 h at 37°C. Zymosan, poly I:C, flagellin, CpG ODN (oligodeoxynucleotide) and LPS (lipopolysaccharide, from Escherichia coli O111:B4) were all obtained from Sigma. Medium was then replaced and the cells were incubated with 0, 1 or 5 μg ZEA/ml for 12 h at 37°C. The cells and supernatants were then carefully collected and stored at –80°C until analyzed via, respectively, RT-PCR and ELISA.

Flow cytometry

The immune cells obtained from spleens or MLN of each mouse were stained for the cell-surface molecules CD4, CD8, CD11c, CD19 and F4/80, or the intracellular molecule FoxP3 (all e-Biosciences, San Diego, CA) conjugated with FITC, PE, antigen-presenting cells (APC) or PerCP fluorochromes for flow cytometry. In a separate analysis, isolated lymphocytes from the small intestine intra-epithelium, lamina propria and PP were stained with fluorescent-conjugated CD4, CD8 and CD19 antibodies. In all cases, aliquots of the target immune cells (10⁶/sample) were first blocked with an unconjugated anti-CD3/16 monoclonal antibody (BD Biosciences, San Diego, CA) for 20 min at 4°C in FACS buffer (1% bovine serum albumin [BSA], 0.09% NaN₃, 1 mM EDTA in PBS). After centrifugation, the cells were re-suspended in buffer and stained with the respective fluoro-chrome-labeled antibodies – or with an isotype control antibody to show that the gates were placed in the optimal location – for an additional 30 min at 4°C in the dark. The cells were washed several times and then immediately analyzed in the
FACS Calibur system (BD Biosciences, San Diego, CA) and analyzed using FlowJo v0.8 software (TreeStar, Inc., Ashland, OR). A minimum of 10,000 events/sample was acquired.

**Lymphoproliferation assay**

Immune cells from the spleen or MLN of each mouse were suspended in complete RPMI-1640, seeded into 96-well plates (at 10⁴/well), and then stimulated with Concanavalin A (5 μg ConA/ml) or LPS (10 μg/ml) for 48 h. After this period, 10 μl solution of cell counting kit-SK (CCK-SK; Dojindo Molecular Technologies, Inc., Rockville, MD) solution was added to each well and the plates were incubated further 4 h at 37°C. Absorbance in each well was then measured at 450 nm using an EMax plate reader (Molecular Devices, Sunnyvale, CA).

**Cytokine measures**

Serum levels of IL-6, IL-10, MCP-1 and tumor necrosis factor (TNF)-α were determined using a cytometric bead array (CBA) mouse cytokines flex set (BD Biosciences) according to the manufacturer’s instructions. In brief, a 1:1 mixture of diluted (here 1:4) serum and capture beads was incubated for 2 h at RT in the dark. The mixture was then incubated with PE-conjugated detection antibodies for 1 h at RT in the dark. Complexes were then evaluated by two-color flow cytometry using the FACS Calibur system. FCAP Array™ software (BD Biosciences) was used to analyze CBA results.

**Immunohistochemical study**

Frozen spleens and MLN in optimum cutting temperature (OCT) compound (Sakura Fine Technical Co., Tokyo, Japan) were used for immunohistochemical staining. Sections (5–μm thick) were cut, air-dried and fixed in cold solution (1:1 acetone-methanol) for 15 min at −20°C. A 3% peroxidase solution followed by 10% normal goat serum (Thermo Fisher Scientific, Waltham, IL) was used to block nonspecific binding. A Polink-2 HRP Plus Rat-NM DAB Detection kit (Golden Bridge International Inc., Mukilteo, WA) was used to immunostain. In brief, sections were incubated with manufacturer-recommended levels of anti-mouse CD4, CD8, F4/80, CD11c or CD19 antibody (e-Biosciences) in a moist chamber for 30 min. Sections were then incubated with PE-conjugated detection antibodies for 1 h at RT in the dark. Complexes were then evaluated using X-OMAT film (Kodak, Rochester, NY) according to manufacturer’s instructions. Relative intensities were measured using ImageJ software (NIH, Bethesda, MD).

**Semi-quantitative real-time PCR**

Total RNA was extracted from RAW 264.7 cells using Easy-Spin Total RNA extraction kits (iNitron Biotech, Seoul, Korea). Nucleic acid concentration and purity were measured with an ND-1000 system (NanoDrop Technologies, Wilmington, DE). Following incubation with RNase-free DNase I (Promega, Madison, WI), reverse transcription was performed using a PrimeScript RT kit (Takara, Shiga, Japan), following manufacturer’s instructions. The resulting cDNA was subjected to real-time PCR on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Green II as a double-strand DNA-specific binding dye to quantify resulting cDNA was subjected to real-time PCR on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Green II as a double-strand DNA-specific binding dye to quantify.

**Detection of cytokines by ELISA**

RAW 264 cell cytokine (IL-1β, TNFα, IL-10) production (i.e. by measures of their presence in culture supernatants after priming with various TLR ligands followed by ZEA treatment) was assessed using cytokine-specific ELISAs. Interferon (IFN)-γ from

**Western blotting**

Total cellular protein was extracted from cells isolated from spleen or PP by pelleting aliquots containing 10⁷ cells and then re-suspending the cells in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.01% NP-40 and 0.02% PMSF [phenylmethylsulfonyl fluoride]), 1 mM dithiothreitol and protease inhibitor cocktail for 15 min at 4°C. The samples were then centrifuged (13,000 rpm, 15 min, 4°C), and lysates were collected. The supernatant was assayed for protein concentration using a BCA Protein assay kit (Thermo Fisher, Rockford, IL) with BSA as a standard.

For Western blotting, 30 μg protein/sample was electrophoresed over a 15% poly-acrylamide gel and the resolved materials were then electrotransferred to a PVDF (polyvinylidene fluoride) membrane. The membrane was blocked for 1 h (at RT) with 5% nonfat dry milk in TBS-T (Tris-buffered saline +0.1% Tween-20), washed with TBS-T and then incubated overnight (at 4°C, with gentle rocking) with rabbit anti-bax (Cell Signaling Technology, Danvers, MA) and mouse anti-bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) (each at 1:1000 dilution). The membrane was then washed and incubated (90 min, RT, gentle rocking) with HRP-conjugated secondary anti-rabbit or mouse IgG antibody (Santa Cruz Biotechnology) in 2.5% nonfat dry milk/TBS-T. After three successive washes with TBS-T, the presence of the secondary antibody (and hence target proteins) was detected using a chemiluminescence assay kit and an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Boston, MA). Images were visualized using X-OMAT film (Kodak, Rochester, NY) according to manufacturer’s instructions. Relative intensities were measured using ImageJ software (NIH, Bethesda, MD).

**Table 1. Primer sequences used in quantitative RT-PCR.**

| Gene | Forward | Reverse |
|------|---------|---------|
| TNFα | GTCTACTCCGCCAGGTTCCTCCTGAAGG | GCAAACTGGCGTGACGGGTGTG' |
| IL1β | CTCGGACCCGACATCAACA | CCACCAGGAAAGACACAGGTGA |
| IL10 | GCTTGACCCACATGCCTAACGG | TTCGTAAGGCGCGACACCC |
| GAPDH | ACGGCAATTTCAACGGCACAG | GAAGTCCACGACATCTCGAC |

**TUNEL analysis of in situ apoptosis**

TUNEL (dUTP nick-end labeling) was performed to detect apoptotic immune cells in spleen and PP cryo-sections (prepared as above) using an ApopTag Peroxidase in situ apoptosis detection kit (Chemicon, Temecula, CA) following manufacturer’s instructions. Positive reactions were visualized with DAB substrate and nuclear counterstaining was done with methyl green dye. Sections were analyzed under light microscopy with analysis TS software (Olympus, Tokyo).

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| Gene | Forward | Reverse |
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| TNFα | GTCTACTCCGCCAGGTTCCTCCTGAAGG | GCAAACTGGCGTGACGGGTGTG' |
| IL1β | CTCGGACCCGACATCAACA | CCACCAGGAAAGACACAGGTGA |
| IL10 | GCTTGACCCACATGCCTAACGG | TTCGTAAGGCGCGACACCC |
| GAPDH | ACGGCAATTTCAACGGCACAG | GAAGTCCACGACATCTCGAC |
cultures of ZEA-treated and ConA- or LPS-stimulated splenic and MLN cells was also evaluated. In general, ELISA plates (Greiner Bio-One, Kremsmünster, Austria) were coated with purified anti-mouse IL-1β, TNFα, IL-10 or IFNγ antibodies (e-Biosciences) in ELISA coating buffer (PBS, pH 7.4) and incubated overnight at 4 °C. The plates were then washed with 0.05% Tween 20/PBS and blocked with 10% FBS/PBS for 1 h at RT. After washing, serial twofold diluted samples and standards were added to dedicated wells (i.e. for a given cytokine) and the plates were incubated overnight at 4 °C. After gentle washing with PBS-T (PBS, 0.05% Tween-20), biotin-conjugated anti-mouse IL-1β, TNFα, IL-10 or IFNγ antibodies (1:250 dilution) were all added to the wells and the plates were then incubated further 1 h at RT. Avidin horseradish peroxidase (e-Biosciences) was then added to each well and incubated for 30 min at RT before kit-provided Avidin horseradish peroxidase (e-Biosciences) was then added to the plates. Reactions in the were stopped by the addition of 50 μl of 2 M H2SO4. Plates were then read at 450 nm in the EMax plate reader. The amount of cytokine/given well was then extrapolated from the standard curve generated in parallel for that cytokine using kit-provided IL-1β, TNFα, IL-10 or IFNγ standard.

Detection of humoral immune responses or complement C3a in serum by ELISA

Blood samples were collected by direct heart puncture from the mice treated with ZEA for 14 days. Sera were carefully collected from blood samples after centrifugation at 12 000 rpm for 5 min and stored at −80 °C until further use. The IgA, IgG, IgM, IgE and C3a concentrations were measured in serum following a standard ELISA method. In brief, ELISA plates (Greiner Bio-One, Germany) were coated with purified anti-mouse IgA, IgM, IgE (e-Biosciences), IgE (BD Biosciences), IgG-Fc (Bethyl Laboratories, Montgomery, TX) and anti-mouse C3a (BD Biosciences). They were then incubated at 4 °C overnight. Plates were washed with 0.05% Tween 20/PBS and blocked with 10% FBS/PBS for 1 h at RT. After washing, serial twofold diluted serum samples and standards were added to the wells, which were then incubated at 4 °C overnight. Plates were washed and incubated with biotin-conjugated anti-mouse IgA, IgM, IgE, C3a (BD Biosciences) or HRP-conjugated anti-mouse IgG-Fc (Bethyl) for 1 h at RT. Avidin-HRP (e-Biosciences) was added to plates for IgA, IgM, IgE and C3a. Finally, a TMB substrate reagent set (BD Biosciences) was used for color development, and the reaction was stopped with 2 M H2SO4. Plates were then read at 450 nm in the ELISA plate reader.

Statistical analysis

All data are expressed as mean ± SE. Differences between multiple groups were compared using a one-way analysis of variance (ANOVA). Individual comparisons were calculated using a Duncan’s multiple range test (DMRT). A p values < 0.05 was considered statistically significant. All data analyses were performed using SAS v.9.1 software (SAS Institute, Cary, NC).

Results

Modulation of immune cell populations in spleen and MLN after ZEA administration

In general, any clinical symptoms or changes in body and organs of interest (i.e. spleen, MLN) weights were not observed after ZEA administration. To observe any modulating effects of ZEA on immune cell populations in the mice, splenocytes and MLN cells from the ZEA-treated or control mice were isolated and then underwent staining and flow cytometry. Analyses showed that both the percentages and absolute numbers of CD4+ and CD11c+ cells in the spleen, and CD4+, CD8+ and F4/80+ cells in the MLN of the ZEA-treated (20 mg/kg) mice were significantly decreased (Figure 1(A)). In contrast, the percent-ages and absolute numbers of CD11c+ and CD19+ cells in the MLN significantly increased in the 20 mg/kg ZEA-treated mice. Populations of F4/80+ and CD19+ in the spleens were not altered by either ZEA exposures. In both spleens and MLN, CD4+CD25+Foxp3+ cells were significantly decreased in the ZEA-treated (20 mg/kg) mice. To further demonstrate changes in immune cell populations, immunohistochemistry was performed. As shown in Figure 1(B,C), similar results were observed with either flow cytometry, immunohistochemistry and/or image analysis. No significant differences of immune cell populations were mostly noted in the 5 mg/kg ZEA-treated mice. The results suggested to us that ZEA (at 20 mg/kg) modulated immune cell subpopulation patterns.

ZEA administration altered small intestine immune cell populations

To investigate effects of ZEA on immune cells in small intestine lymphocyte populations, IEL, LPL and PPL (Peyer’s patches lymphocytes) of the ZEA-treated or control mice were isolated, pooled and stained with CD4, CD8 and CD19 antibodies to identify various immune cells. As shown in Figure 2(A), the percentages of CD4+ cells decreased in the IEL, whereas those of CD8+ cells increased, due to either ZEA regimen. Similarly, the percentages of CD4+ cells in the LPL were decreased due to either ZEA regimen (Figure 2(B)). The percentages of CD19+ and CD8+ cells were not markedly altered by either ZEA dosage. As shown in Figure 2(C), the populations of CD8+ and CD19+ cells in the PPL slightly increased with ZEA administration. These results suggested to us that ZEA exposure could alter T- and B-cell populations in the small intestine.

Lymphocytes from the ZEA-treated mice have altered proliferative capacities

To assess the potential impact of ZEA on lymphoproliferative responses, splenocytes and MLN cells from the ZEA-treated and control mice were isolated and then stimulated with ConA (T-cell mitogen) or LPS (B-cell mitogen) for 48 h. As shown in Figure 3(A), ZEA exposures led to splenocytes with increased proliferative capacity (relative to control cell activity) in response to ConA (ZEA 5 or 20 mg/kg) or LPS (ZEA 20 mg/kg). The effects were less obvious among the MLN cells; while a similar trend was noted in cell responses to LPS, with ConA the ZEA exposure led to cells yielding less IFNγ production by these cells was also differentially impacted by ZEA treatment and choice of mitogen (Figure 3(B)). With the splenocytes, responses to ConA or LPS were augmented by host treatments with either level of ZEA. Again, however, responses by the MLN cells were contradictory, i.e. with ConA, ZEA host exposure led to cells yielding less IFNγ than control cells.
ZEA administration modulated in situ antibody production

To examine the humoral immune response in all the ZEA-treated mice, IgA, IgG, IgM and IgE antibody levels in the serum were measured by sandwich ELISA. As shown in Figure 4, the level of IgM in serum of the ZEA-treated mice was significantly lower than that in the control mice. The levels of IgE were increased in the ZEA treatment group compared to that of the controls. IgG and IgA levels did not significantly change due to ZEA administration.
ZEA treatment reduces serum complement C3a levels

To investigate potential effects of ZEA exposure on host complement responses, complement C3a levels in serum of the ZEA and control mice were measured. As shown in Figure 5, the levels of C3a in the serum of all the ZEA-treated mice were significantly reduced compared to the control mice.

ZEA administration reduces mucosal IgA antibody production

To examine potential impact on mucosal immunity due to ZEA treatment, IgA antibody levels were measured in duodenal/vaginal washings obtained from the ZEA and control mice. Results indicated that IgA levels in both washings from the ZEA-treated mice (duodenum: 5 and 20 mg/kg, vagina: only 20 mg/kg) were significantly decreased compared to those of the control mice (Figure 6).

ZEA treatment induces TNFα and IL-6 production in situ

To observe any modulation of cytokine profiles in the ZEA-treated mice, isolated serum was analyzed for IL-6, IL-10, MCP-1 and TNFα. As shown in Figure 7, relative to levels in blood of the control mice, the level of IL-6 was significantly increased whereas that of TNFα was decreased in the 20 mg/kg ZEA-treated mice. The levels of the other cytokines were not significantly impacted by either ZEA treatment.

ZEA promotes apoptosis in immune cells and is related to changes in Bax/Bcl-2 ratio

The spleen, MLN and PP of the ZEA-treated mice were collected, cryo-sectioned, stained with TUNEL and positive apoptotic cells measured by image analysis. As shown in Figure 8(A), an increased number of apoptotic cells were observed in the spleens and PP of the 20 mg/kg ZEA-treated mice. Remarkable apoptosis was not observed in the MLN of the ZEA-treated mice. These data suggest that ZEA promoted apoptosis of spleen and PP immune cells in situ.

Densitometric analysis showed that the ratio of Bax:Bcl-2 in spleen and PP of the the 20 mg/kg ZEA-treated mice was significantly increased (Figure 8(B)), indicating there had been induction of apoptosis. ZEA treatment significantly decreased Bcl-2 levels ($p < 0.01$) with no change in Bax expression in splenocyte whole lysates, whereas Bax expression significantly increased with no change in Bcl-2 expression in lysates of PP cells. These results suggested to us that ZEA-induced apoptosis by altering Bax vs. Bcl-2 expression ratios in the spleen and PP.

ZEA modulates cytokine responses in macrophages post-stimulation with TLR ligands

To observe effects of ZEA on cytokine expression in murine macrophages pre-stimulated with different TLR ligands, RAW 264.7 macrophages were primed with TLR2 (zymosan), TLR3 (poly I:C), TLR4 (LPS), TLR5 (flagellin) or TLR9 (CpG ODN) ligands for 12 h and then treated with ZEA (at 1 or 5 μg/ml) for 12 h. Pro- (IL-1β and TNFα) and anti-inflammatory (IL-10) cytokine production was then measured from cell supernatants; gene expression was quantified by RT-PCR. After priming the RAW cells with different TLR ligands, zymosan-, LPS- and CpG ODN-induced production and mRNA expression of IL-1β were significantly enhanced by ZEA treatment. However, flagellin led to decreased IL-1β production and mRNA expression (Figure 9(A)). As shown in Figure 9(B), flagellin- and CpG ODN-induced IL-10 protein and gene expression by RAW cells were significantly inhibited by ZEA treatment. Conversely, zymosan-induced IL-10 protein and gene expression were up-regulated by ZEA. Figure 9(C) shows that both TNFα protein and gene expression were
Figure 2. Immune cell population in mouse small intestine. After 14 days of dosing with oil or ZEA (5 or 20 mg/kg), small intestine IEL (A), LPL (B) and PPL (C) of the control and ZEA-treated mice (n = 3/group) were isolated, pooled and extracellularly stained with different antibodies (CD19<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup>) to identify immune cells. Values are shown as percentage of total lymphocytes. ZEA, zearalenone; LPL, lamina propria lymphocytes; PPL, Peyer's patches lymphocytes; IEL, intra-epithelial lymphocytes.
significantly inhibited by ZEA when cells were primed with poly I:C, LPS, flagellin or CpG ODN. However, zymosan-induced TNFα protein and gene expression by the RAW cells were significantly enhanced by ZEA treatment.

Discussion

The mycotoxin ZEA, mainly produced by fungi in the genus *Fusarium*, contaminates many cereals/foodstuffs consumed by humans and animals (Zinedine et al. 2007). Many studies have noted significant adverse effects in exposed hosts or model systems. Several reports showed ZEA was genotoxic (Abbès et al. 2007), hepatop-nephrotoxic (Ben Salah-Abbès et al. 2008b) and immunotoxic (Ben Salah-Abbès et al. 2008a). Other findings have been that ZEA caused reproductive problems in animals (Ruhr et al. 1983), early-onset of puberty in children (Sáenz de Rodíguez et al. 1985), as well as hyperplasia, endometrial adenocarcinomas and breast cancer in women (Schoental 1983; Tomaszewski et al. 1998). At a cellular level, several studies have...
shown that ZEA was pro-apoptotic (Abid-Essefi et al. 2003), enhanced lipid peroxidation (Conková et al. 2001), and could induce micronuclei, chromosome aberrations, DNA fragmentation and cell cycle arrest (Ouanes et al. 2003, 2005).

Mycotoxin-induced immunosuppression arises from a vulnerability within proliferating and differentiating lymphoid cells that participate in immune-mediated activities and within the complex communications that exist among cellular and humoral immune system components. In the current study, ZEA exposures resulted in a decreased presence of CD4⁺, CD8⁺, CD11c⁺ and Foxp3⁺ regulatory T (Treg)-cells in the spleen as well as CD4⁺, CD8⁺, F4/80⁺ and Foxp3⁺ Treg cells in the MLN of mice. In contrast, the presence of CD19⁺ and CD11c⁺ cells increased in the MLN of ZEA-treated mice. Lymphocyte population in the small intestines in the ZEA-treated mice had decreased levels of CD4⁺ T-cells. To date, information from comparative studies of lymphocyte sub-populations in mouse lymphoid organs after host treatment with ZEA remain not widely available. Ben Salah-Abbès et al. (2008a) reported mice treated daily with ZEA (40 mg/kg, 2 weeks) had significant decreases in total white blood cells, specifically B-, T- and NK cells.

As CD4⁺ T-cells activate and direct other immune cells to promote activation and growth of cytotoxic CD8⁺ T-cells (which play a major role in protecting organisms against intracellular infections) (Harty et al. 2000), the present results showing ZEA-induced decreases of CD4⁺ and CD8⁺ T-cells in the MLN, spleen and lamina propria of small intestines suggested to us these hosts would likely be more susceptible to infections. Similarly, Treg-cells play indispensable roles in self-tolerance maintenance and control/regulation of immune responses (Sakaguchi 2004). In the current experiment, along with CD4⁺ and CD8⁺ T-cells, Treg cell levels were decreased due to ZEA treatments indicating that ZEA-induced changes in Treg cells did not impact on host CD4⁺ and CD8⁺ T-cells. Interestingly, even while there were decreases in levels of the various T-cell populations, mitogen-treated splenocytes and MLN cells obtained from ZEA-treated hosts had increases in proliferative capacity and production of IFNγ. This indicated that even though ZEA had an ability to reduce levels of T-/B-cells in a host, it concomitantly enhanced the activation capacity of remaining cells. The implications of this are unclear as these outcomes should give rise to diverging effects in the scheme of an actual immune response.

B-cells act as APC and produce antibodies against antigens; thus, as main mediators of humoral immunity, a deficiency in these cells would affect the host response to infection (Moise et al. 2010). In the present study, ZEA administration reduced IgM production in mice, though B-cell levels were increased in the small intestine MLN and PP. As such, these outcomes partially contradict the findings of Ben Salah-Abbès et al. (2008a) who noted significant decreases in circulating B-cells and serum IgG and IgM levels in mice treated daily with ZEA for 14 days. Because complement C3a causes B-cells to have a suppressive effect on the production of IgG, TNF and IL-6 (Fischer and Hugli 1997), levels of this factor were also analyzed in the ZEA-treated hosts. In conjunction with the other results here, the present findings of decreased levels of C3a in the serum of these hosts meant that ZEA exposure likely affected IgM (and thus indirectly, formation of select cytokines) production in mice.

IGA plays a critical role in mucosal defense from pathogen as well as mucosal homeostasis. In the present study, the levels of IgA were decreased in the mucosal layer of the host duodenum and vagina. These results indicated that hosts treated with ZEA might be more vulnerable to mucosal infection(s). Although this study did not test host resistance to various infectious agents, it was seen in another study (data not shown) that infant mice treated with ZEA were more susceptible to rotavirus infection, had higher rate of diarrhea, and expressed more NSP3 antigens in their small intestine and MLN.

An ability of ZEA to affect inflammatory responses has not been well-investigated. Mycotoxins either suppress or stimulate immune responses by modulating cytokine production (Wichmann et al. 2002; Johannessen et al. 2005, 2009). Marin et al. (2011) reported exposure of PMA-stimulated swine mononuclear cells to 5 or 10 μM ZEA led to reduced TNFα production. In the current study, after priming of RAW 264.7 cells with different TLR ligands, IL-1β, TNFα and IL-10 production were each modulated by a subsequent ZEA exposure. This study also examined the effect of ZEA on production of several key inflammatory cytokines in mice. The results show that ZEA treatment significantly decreased circulating levels of TNFα and increased IL-6 in the mice. It is difficult to discern if this is in line or in contrast with other studies in that information from reports assessing effects of ZEA on inflammatory cytokines is itself contradictory. Studies have indicated that ZEA either induces or suppresses (Marin et al. 2010; Salah-Abbès et al. 2010) pro-inflammatory cytokine synthesis. The in vitro data here from studies of TLR ligand-stimulation and then ZEA treatment suggested there was differential modulation of cytokine production due to ZEA and that this was depended on the given TLR ligand. When taken in the context of other studies of effects of ZEA on cytokines, this study could lead to the expectation that ZEA may cause an imbalance in immune cell secretion of inflammatory cytokines and that this may aggravate inflammatory reactions in certain pathological states (Johannessen et al. 2005). Clearly, more detailed mechanistic studies are needed to clarify how ZEA is acting to impact on TLR-related responses and on cytokine formation in general.

Lastly, cytotoxic effects of mycotoxins are thought to correlate with an ability to induce apoptosis (Gopee et al. 2003; Kamp et al. 2005). Many fungal toxins, including citrinin, ochra-toxin A, trichothecenes and fumonisin B₁, trigger cellular apoptosis. In the current study, ZEA caused apoptosis in the spleens and PP of the treated mice. This effect was likely related to the same pro-apoptotic effects of ZEA that had been observed earlier in testicular germ cells (Kim et al. 2003). ZEA has an ability to induce apoptosis in human leukemia cells via activation of mitochondrial Cyt-c release, reactive oxygen species production, caspase-3 and -8 activation and induction of endoplasmic reticulum stress.

Figure 7. Serum IL-6, TNFα, IL-10 and MCP-1 in mice. Serum isolated from mice (n = 8/group) treated with oil or ZEA for 14 days was analyzed for TNFα, IL-6, IL-10 and MCP-1 levels. Values shown are means ± SE. Different letters indicate significant differences (p < 0.05, DMRT). ZEA, zearalenone; DMRT: Duncan's multiple range test.
Additional investigations found ZEA also up-regulated Bax expression and down-regulated Bcl-xL expression in cells. In the present study, ZEA induced a prominent change in Bax:Bcl-2 ratios in splenocytes and PP. From this data, it might be concluded the ratio change of Bax:Bcl-2 activated caspases that led to the ZEA-induced apoptosis that was seen in splenocytes and PP cells. Further clarifying studies on this endpoint are also warranted.

(Banjerdpongchai et al. 2010). Additional investigations found ZEA also up-regulated Bax expression and down-regulated Bcl-xL expression in cells. In the present study, ZEA induced a prominent change in Bax:Bcl-2 ratios in splenocytes and PP.

Figure 8. Apoptosis in spleen, MLN and PP, and changes in Bax/Bcl-2 expression ratios. (A) Based on TUNEL, spleen and PP from oil or ZEA-treated mice (5 or 20 mg/kg, 14 days) (n = 3 mice/group) underwent apoptosis (dose-related manner). Apoptotic cells were stained positive with ApopTag kits. Bar =50 μm. For quantitative image analysis, DAB⁺ areas were quantified by image analysis. Values expressed as percentage DAB⁺ area/field. (B) Mice (n = 3/group) were treated with oil or (C) ZEA (5 or 20 mg/kg) for 14 days, and proteins were then extracted from isolated spleen and PP cells. Bax and Bcl-2 levels were analyzed by Western blot and densitometry. Values shown are means ± SE. Results shown are a representative band from three independent experiments. Different letters indicate significant differences (p < 0.05, DMRT). TUNEL: TdT-mediated dUTP nick-end labeling; MLN: mesenteric lymph nodes; PP: Peyer’s patches; ZEA, zearalenone; DMRT: Duncan’s multiple range test.
Conclusions

We herein demonstrated the immunomodulatory and/or immunotoxic effects of ZEA in mice showing differential broad-spectrum effects on a host immune system. As the exact mechanisms of ZEA toxicity remain incompletely understood, further studies are needed to understand the possible (molecular) mechanisms of how ZEA differentially regulates immune cells and likely influences inflammatory responses against pathogens.

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Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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References

Abbès S, Ouanes Z, Ben Salah-Abbes J, Abdel-Wahha M, Oueslati R, Bacha H. 2007. Preventive role of alumino-silicate clay against induction
of micronuclei and chromosome aberrations in bone marrow cells of Balb/c mice treated with zearalenone. Mutat Res. 631:85–92.

Abid-El-Etr S, Bauburger J, Hassel W, Ouanes Z, Mobio T, Anane R, Creppy E, Bacha H. 2003. DNA fragmentation, apoptosis and cell cycle arrest induced by zearalenone in cultured DOK, Vero and Caco-2 cells: Prevention by vitamin E. Toxicology. 192:237–248.

Avantaggiato G, Havenaar R, Visconti A. 2003. Assessing the zearalenone-binding activity of adsorbent materials during passage through a dynamic in vitro gastrointestinal model. Food Chem Toxicol. 41:1283–1290.

Banjerdpongchai R, Kongtawelert P, Khatramat O, Srisomsap C, Subhasitanont P, Svasti J. 2010. Mitochondrial and endoplasmic reticulum stress pathways cooperate in zearalenone-induced apoptosis of human leukaemic cells. J Hematol Oncol. 3:50.

Ben Salah-Abbès J, Abbès S, Houas Z, Abdel-Wahhab M, Oueslati R. 2008a. Zearalenone induces immunotoxicity in mice: Possible protective effects of Radish extract (Raphanus sativus). J Pharm Pharmacol. 60:761–770.

Ben Salah-Abbès J, Abbès S, Ouanes Z, Houas Z, Abdel-Wahhab M, Bacha H. 2007. Protein oxidation and anti-inflammatory cytokines produced by zearalenone in Balb/c mice. J Appl Toxicol. 28:6–14.

Ben Salah-Abbès J, Abbès S, Abdel-Wahhab M, Oueslati R. 2009. Raphanus sativus extract protects against zearalenone induced reproductive toxicity, oxidative stress and mutagenic alterations in male Balb/c mice. Toxicon. 53:525–533.

Cetin Y, Bullerman L. 2005. Cytoxicity of Fusarium mycotoxins to mammalian cell cultures as determined by the MTT bioassay. Food Chem Toxicol. 43:755–764.

Conkova E, Laciakova A, Pástorová B, Seidel H, Kováč G. 2001. The effect of zearalenone on some enzymatic parameters in rabbits. Toxicol Lett. 121:145–149.

Corrier D. 1991. Mycotoxicosis: mechanisms of immunosuppression. Vet Immunol Immunopathol. 30:7–37.

Davies MD, Parrott DM. 1981. Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. Gut. 22:481–488.

El Golli E, Hassen W, Bouzilmi A, Bouaziz C, Ladjimi M, Bacha H. 2006. Induction of Hsp 70 in Vero cells in response to mycotoxins cytoprotection by sub-lethal heat shock and by vitamin E. Toxicol Lett. 166:122–130.

Etienne M, Dourmad J. 1994. Effects of zearalenone on sheep in vivo: A review. Livestock Prod Sci. 48:99–113.

Farber E. 1994. Programmed cell death: Necrosis versus apoptosis. Mod Pathol. 7:605–609.

Fischer W, Hugli T. 1997. Regulation of B cell functions by sub-lethal heat shock and by vitamin E. Toxicol Lett. 166:122–130.

Flepk SC, Hildebrandt AA, Müller E, Pfeiffer E, Metzler M. 2012. Genotoxicity of ochratoxin A and its derivatives on the innate immune response of swine. Toxicon. 63:564–573.

Fusarium mycotoxins to mammalian cell cultures as determined by the MTT bioassay. Food Chem Toxicol. 43:755–764.

Fukusaki S, Oueslati R. 2009. Zearalenone induces immunotoxicity in mice: Possible protective effects of Radish extract (Raphanus sativus). J Pharm Pharmacol. 60:761–770.

Ben Salah-Abbès J, Abbès S, Ouanes Z, Houas Z, Abdel-Wahhab M, Bacha H. 2007. Protein oxidation and anti-inflammatory cytokines produced by zearalenone in Balb/c mice. J Appl Toxicol. 28:6–14.

Ben Salah-Abbès J, Abbès S, Abdel-Wahhab M, Oueslati R. 2009. Raphanus sativus extract protects against zearalenone induced reproductive toxicity, oxidative stress and mutagenic alterations in male Balb/c mice. Toxicon. 53:525–533.

Cetin Y, Bullerman L. 2005. Cytoxicity of Fusarium mycotoxins to mammalian cell cultures as determined by the MTT bioassay. Food Chem Toxicol. 43:755–764.

Conkova E, Laciakova A, Pástorová B, Seidel H, Kováč G. 2001. The effect of zearalenone on some enzymatic parameters in rabbits. Toxicol Lett. 121:145–149.

Corrier D. 1991. Mycotoxicosis: mechanisms of immunosuppression. Vet Immunol Immunopathol. 30:7–37.

Davies MD, Parrott DM. 1981. Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. Gut. 22:481–488.

El Golli E, Hassen W, Bouzilmi A, Bouaziz C, Ladjimi M, Bacha H. 2006. Induction of Hsp 70 in Vero cells in response to mycotoxins cytoprotection by sub-lethal heat shock and by vitamin E. Toxicol Lett. 166:122–130.

Etienne M, Dourmad J. 1994. Effects of zearalenone on sheep in vivo: A review. Livestock Prod Sci. 48:99–113.

Farber E. 1994. Programmed cell death: Necrosis versus apoptosis. Mod Pathol. 7:605–609.

Fischer W, Hugli T. 1997. Regulation of B cell functions by C3a and C5a(desArg); suppression of TNFα, IL-6, and the polyclonal immune response. J Immunol. 159:4279–4286.

Fleck SC, Hildebrandt AA, Müller E, Pfeiffer E, Metzler M. 2012. Genotoxicity and immunotoxicity of Fusarium mycotoxins to mammalian cell cultures. Myco toxicology. 28:267–273.

Girgis G, Sharif S, Barta J, Boermans H, Smith T. 2008. Immunomodulatory effects of feed-borne Fusarium mycotoxins in chickens infected with coccidian. Exp Biol Med. 233:1411–1420.

Gopee N, He Q, Sharma R. 2003. Fumonisin B1-induced apoptosis is associated with delayed inhibition of protein kinase C, NF-κB, and TNFα in LLC-PK1 cells. J Biol Chem. 288:14621–14629.

Harty J, Tvinnereim A, White D. 2000. CD8+ T-cell effector mechanisms in resistance to infection . Annu Rev Immunol. 18:275–308.

Hassen W, Ayed-Boussema I, Oscoz A, Lopez A, Bacha H. 2007. The role of oxidative stress in zearalenone-mediated toxicity in Hep G2 cells: oxidative DNA damage, glutathione depletion and stress proteins induction. Toxicology. 232:294–302.

Johannesson L, Lüdvik M, Lydersen S, Nilsen A. 2009. Combined cell wall polysaccharide, mycotoxin and bacterial lipopolysaccharide exposure and inflammatory cytokine responses. Apmis. 117:507–517.

Johannesson L, Nilsen A, Lüdvik M. 2005. The mycotoxins citrinin and gliotoxin differentially affect production of the pro-inflammatory cytokines TNFα and IL-6, and the anti-inflammatory cytokine IL-10. Clin Exp Allergy. 35:782–789.