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Original article

The effect of *in vivo* exposure to zearalenone on cytokine secretion by Th1 and Th2 lymphocytes in porcine Peyer's patches after *in vitro* stimulation with LPS

K. Obremski

Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Oczapowskiego 13, 10-718 Olsztyn, Poland

Abstract

Most research studies investigating the estrogenic effects of zearalenone (ZEN) focus on the mycotoxin's effect on the reproductive system. Since estrogen receptors are present on various types of immunocompetent cells, ZEN can also modify diverse immune functions. This study analyzed immunocompetent cells isolated from Peyer's patches in the ileum of pigs administered ZEN in the estimated daily dose of $8 \mu\text{g kg}^{-1}$ BW (equivalent of $100 \mu\text{g kg}^{-1}$ feed per day⁻¹). The objective of the study was to determine whether long-term exposure to low ZEN doses below the NOEL threshold leads to changes in the percentages of lymphocyte subpopulations and cytokine secretion by Th1 (IL-2, IFN- γ) and Th2 (IL-4 and IL-10) lymphocytes in Peyer's patches of the ileum after *in vitro* stimulation with lipopolysaccharides (LPS). Immunocompetent cells isolated from Payer's patches on experimental days 0, 14, 28 and 42 were cultured *in vitro* and stimulated with LPS. The presence of IL-2, IFN- γ , IL-4 and IL-10 in culture media was determined by the ELISA method. The results of the study indicate that ZEN inhibits IL-2 and IFN- γ secretion and stimulates IL-4 and IL-10 production by Th1 and Th2 lymphocytes by shifting the Th1/Th2 balance towards the humoral immune response. The above can promote allergic responses, as demonstrated by the increase in the size of B₁ cell populations producing more autoantibodies. ZEN can also lower resistance to viruses and tumors by inhibiting the proliferation of NK cells and IFN- γ secretion.

Key words: zearalenone, porcine Peyer's patches, lymphocytes, cytokines, LPS

Introduction

The mucosa of the small intestine acts as a potential gateway for pathogenic invasions. In addition to its digestive and intrasecretory functions, the small intestine also serves as an immune barrier. The intestine

is constantly exposed to antigens, and it contains large numbers of lymphoid cells (B and T lymphocytes) and myeloid cells (macrophages, neutrophilic and acidophilic granulocytes, mast cells). The intestines have developed highly organized and effective responses to antigens as well as mechanisms of humoral

Correspondence to: K. Obremski, e-mail: kazimierz.obremski@uwm.edu.pl

and cellular immunity to mitigate the negative impacts of toxins and antigens. Gut-associated lymphoid tissue is found in three major regions in the form of Peyer's patches, lymphoid tissue in the lamina propria and endothelial lymphocytes. Peyer's patches, which are distributed most abundantly in the ileum, are part of gut-associated lymphoid tissue, and they recognize antigens through M (microfold) cells in the surrounding epithelium (Makala et al. 2002). Antigens penetrating Peyer's patches activate B and T lymphocytes. Stimulated Peyer's patch lymphocytes leave the gastrointestinal tract and are transported to mesenteric lymph nodes. Some lymphocytes are transported to the lamina propria, and B cells become Ig-A-producing lymphoblasts that play the key role in the mucosal immune system.

Animals and humans are constantly exposed to natural or synthetic estrogenic compounds. Natural estrogens, including several classes of phytoestrogens such as isoflavones, lignans, coumestans and resorcylic acid lactones, bind with both estrogen receptor subtypes and demonstrate agonistic or antagonistic activity (Nikov et al. 2000).

Zearalenone (ZEN), a macrocyclic β -resorcylic acid lactone, is a non-steroidal estrogenic mycotoxin that is produced by polyketide biosynthesis by various *Fusarium* species, including *F. graminearum*, *F. culmorum* and *F. equiseti* (Richard 2007). Various degrees of ZEN contamination are observed in food and feed around the world. ZEN is a biologically active and relatively non-toxic compound, but its chemical structure resembles that of 17β -estradiol (E2) and supports binding with estrogen receptors (ERs). ZEN and its derivatives, α - and β -zearalenol (α - and β -ZEL), compete with E2 for specific ER binding sites. Numerous studies have demonstrated that by binding to ERs, ZEN and its derivatives trigger a series of estrogen-stimulated events (Mueller 2002, Tiemann et al. 2003). The binding efficacy of ZEN to ERs in target tissues is 1-10% lower in comparison with E2. α -ZEL demonstrates stronger affinity and β -ZEL – weaker affinity for ERs (Zatecka et al. 2014). ZEN is a full agonist of estrogen receptor α (ESR1) and a partial agonist of estrogen receptor β (ESR2) (Zinedine et al. 2007). For this reason, ZEN and its metabolites can significantly disrupt the hormonal balance and reproductive functions. ZEN induces hyperestrogenism, disrupts the physiology of the reproductive system (Obremski et al. 2003b), contributes to fertilization problems, inhibits embryo implantation and fetal development. ZEN disrupts the course of estrous cycle and contributes to weight loss, which was observed in pigs and rats (Zinedine et al. 2007). Female pigs are most susceptible to ZEN, and pre-pubertal gilts are more sensitive than adult indi-

viduals. The ovaries, uterus and vulva are most susceptible to ZEN's estrogenic effects (Obremski et al. 2003a). The LOEL (Lowest Observed Effect Level) ZEN dose for the above tissues in differently aged pigs ranges from 17 to 200 $\mu\text{g kg}^{-1}$ BW per day, whereas the NOEL (No Observed Effect Level) dose has been determined at 10 $\mu\text{g kg}^{-1}$ BW per day (EFSA 2011).

Most studies investigating the estrogenic effects of ZEN focus on its influence on the reproductive organs. It should be noted, however, that ERs are present on various types of immune cells, and the immune system is a potential target of estrogenic compounds (Ahmed 2000, Salem 2004). For this reason, ZEN can affect various immune functions (Marin et al. 2011). Luongo et al. (2008) demonstrated that ZEN delivers immunotoxic effects, but its role in inflammations has not been fully explained because ZEN can both suppress and induce the production of proinflammatory cytokines (Ben Salah-Abbes et al. 2008, Marin et al. 2010, 2011). ZEN also induces changes in immune parameters in humans (Berek et al. 2001) and mice (Abbès et al. 2013). In an *in vitro* experiment, ZEN inhibited the proliferation of mitogen-stimulated lymphocytes and stimulated the production of IL-2 and IL-5 (Murata et al. 2003). Lipopolysaccharides (LPS) play an important role in immune and inflammatory responses. Humans and animals are commonly exposed to LPS via the gastrointestinal system, translocation of the intestinal microflora in inflammatory bowel diseases or intestinal damage (Islam and Pestka 2006). According to our best knowledge, the effect of ZEN on the secretory function of Th1 and Th2 lymphocytes in ileal Peyer's patches after LPS stimulation has not been studied to date. According to research, ZEN could act as a mitogenic factor for T lymphocytes (Vecchiet et al. 2003).

The objective of the study was to determine whether long-term exposure to low ZEN doses below the NOEL threshold leads to changes in lymphocyte counts and cytokine secretion by Th1 (IL-2, IFN- γ) and Th2 (IL-4 and IL-10) lymphocytes in Peyer's patches of the ileum after *in vitro* stimulation with LPS.

Materials and Methods

Animals and experimental design

The study was performed on 35 Polish Large White female pigs (aged 2 months, body weight 15-18 kg) obtained from a commercial fattening farm in Baldy, Poland. The animals were housed and handled in accordance with the procedures laid down by the

local Ethics Committee No. 55/2008 (affiliated with the National Ethics Committee for Animal Experimentation of the Polish Ministry of Science and Higher Education). The healthy pigs were divided into two groups: the control group (group C, $n=20$; five animals from this group constituted "day 0" pigs for groups C and E) and the experimental group (Group E, $n=15$) comprising pigs administered ZEN toxin (Sigma Aldrich, USA, Cat No. Z2125) at $100 \mu\text{g kg}^{-1}$ feed day⁻¹ (European Commission, Commission Recommendation 2006).

The investigated material comprised sections of the ileum sampled from pigs on days 0, 14, 28 and 42 of the experiment. Five randomly selected pigs from each group were euthanized on each of the above experimental days. The excised segment of the ileum was opened, and Peyer's patches were identified. The mucosa and submucosa was scraped, minced and placed in 1.5 ml of ice-cold phosphate buffered saline (PBS, pH 7.4, 0.1 M). Minced tissue specimens were shaken for 1 min in PBS, the sediment was allowed to settle for 2 min, and the suspensions were removed. The 'extraction' was repeated with 1.5 ml of ice-cold PBS. The pooled suspensions were filtered through polyester wool in 2 ml disposable syringes. The number of lymphocytes was established in a hemocytometer.

Determination of the percentages of T lymphocyte subpopulations

The percentages of lymphocyte subpopulations obtained from Peyer's patches was determined with the use of mouse monoclonal antibodies against porcine CD4, CD5, CD8, CD21 antigens (CD4 VMRD, 74-12-4, IgG2b; CD5 VMRD, PG114A, IgG1, CD8 VMRD, 76-2-11, IgG2a; CD21 VMRD, BB6-11C9, IgG1) and corresponding secondary antibodies (Biotinylated rat anti-mouse IgG2b, DB Pharmingen 550333, streptavidin-PE, DB Pharmingen 554061, FITC rat anti-mouse IgG2a, DB Pharmingen 553390). Payer's patch leukocytes for cytometry were obtained in accordance with the procedure described by Kalezczyk et al. (2010).

The samples were analyzed in a flow cytometer (FACScalibur, Becton Dickinson, USA), and the results were analyzed in the Cell Quest™ program (Becton Dickinson). Lymphocytes were gated based on forward/side scatter cytograms, and lymphocyte subpopulations were identified based on the fluorescence intensity of dot-plot quadrant statistics.

Cells and cell cultures

Immunocompetent cells isolated from ileal Peyer's patches were suspended at the concentration of

3×10^6 cells ml^{-1} in the RPMI 1640 medium containing L-glutamine (R8758, Sigma Aldrich, USA), 10% fetal calf serum (FCS, 12133C, Sigma) and 10% penicillin-streptomycin (P4333, Sigma Aldrich, USA) in 48-well culture plates with a flat bottom. Cultures containing 1 ml of cell suspensions were established on the first day of the experiment. 50 ng ml^{-1} LPS (Lipopolysaccharides, rough strains) from *Salmonella enterica* serotype typhimurium SL1181 (Sigma Aldrich, USA) was added to stimulate PPMC cells. The resulting cell cultures were incubated for 7 days at 37°C in moist air with 5% CO_2 and the addition of LPS on day 6.

Determination of cytokine levels in culture supernatants

Cytokine levels were determined with the use of ELISA kits in accordance to the manufacturer's instructions (IFN- γ – Cat. No. 3130-1A-20, Mabtech, Sweden; IL-2 – Cat. No. CSC1243, Invitrogen, Poland; IL-4 – Cat. No. DY 654 R&D, USA; IL-10 – Cat. No. DY 693B, R&D, USA) with minor modification. ELISA microplates (96-well) were coated with specific antibodies in carbonate buffer (16 h, 4°C) and stabilized with 1% BSA (Sigma Aldrich, USA) in PBS (2 hours, 37°C). The supernatants of cell cultures were added, and microplates were incubated for 2 hours at 37°C . Biotinylated antibodies specific for porcine cytokines were added and incubated for 2 hours at 37°C . HRP-conjugated streptavidin solution was added, and the microplates were incubated for 20 minutes. In each stage, microplates were rinsed in PBS with 0.05% Tween 20 (Sigma Aldrich, USA). The specimens were stained with OPD (*o*-Phenylenediamine) (Sigma Aldrich, USA) and hydrogen peroxide (Avantor Performance Materials Poland S.A., Poland). The enzymatic reaction was stopped with 2 M HCl (Avantor Performance Materials Poland S.A., Poland). Absorbance was measured in a spectrophotometer plate reader (TECAN Infinite M200, Switzerland) at $\lambda = 492 \text{ nm}$. The resulting cytokine levels were expressed in pg ml^{-1} . The values of the intra- and inter-assay coefficients of variation (CV%) for IFN- γ , IL-2, IL-4 and IL-10 were respectively 3.86, 4.19, 4.14, 4.66, 4.48, 5.02, 2.84, 2.19.

Statistical analysis

The results were processed in Excel (Microsoft, USA) and GraphPad Prism 5 (GraphPad Software, USA) applications. Mean values and standard error of the mean (SEM) were determined for all the groups. Population distributions were evaluated by the

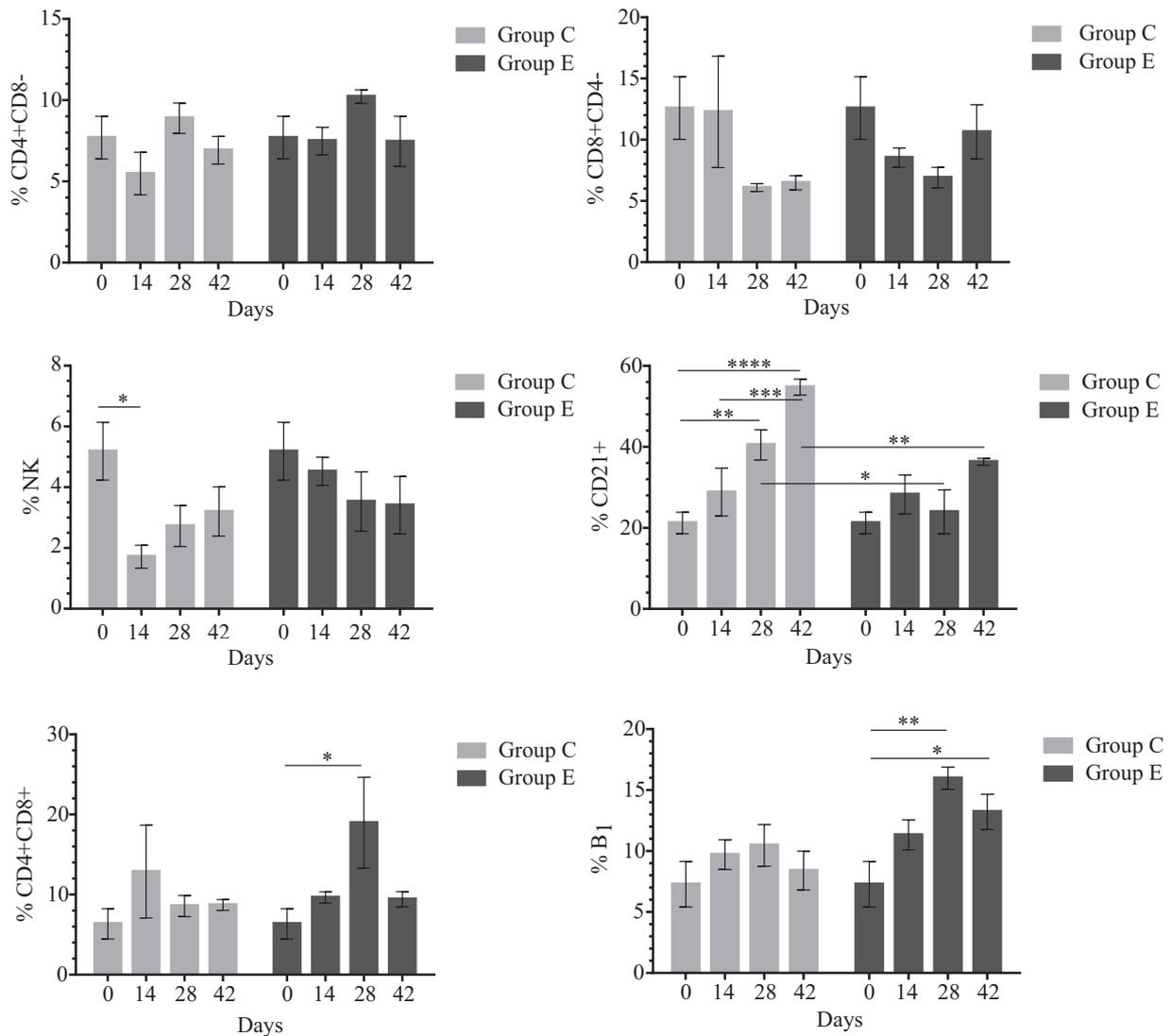


Fig. 1. Cytometric analysis of CD4+CD8-, CD4-CD8+, NK, CD21+, CD4+CD8+ and B₁ cells isolated from Payer's patches of the control (Group C) and zearalenone-administered (Group E) gilts and cultured *in vitro*. The graphs present mean values \pm SEM (n=5 each). *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001.

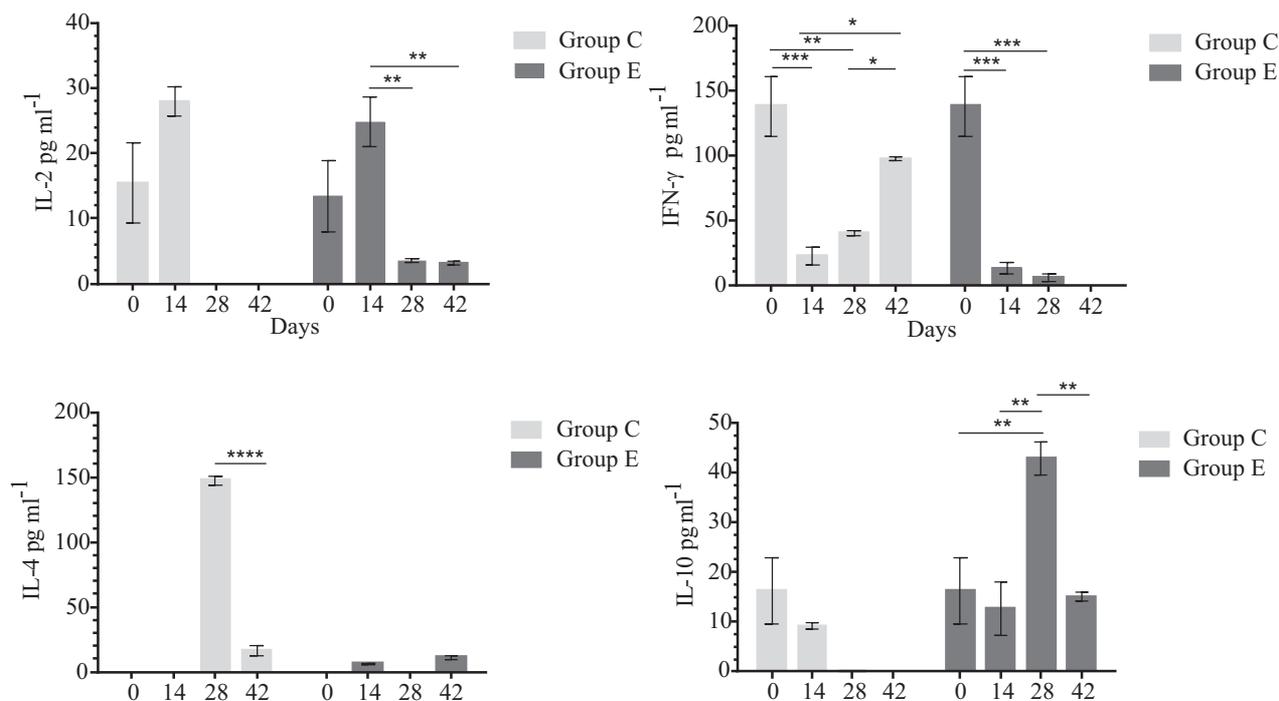
Kolmogorov-Smirnov test. The results were processed by the unpaired Student's t-test and two-way ANOVA. The results were regarded as statistically significant at p<0.05, p<0.01, p<0.001 and p<0.0001.

Results

The percentages of lymphocyte subpopulations in porcine Payer's patches determined *in vitro* after *in vivo* administration of ZEN

The results are presented in Fig. 1. In cells isolated from ileal Peyer's patches before the establishment of *in vitro* cultures, the percentages of selected cell subpopulations – T helper lymphocytes (CD4+CD8-), cytotoxic T lymphocytes

(CD4-CD8+), NK cells, B lymphocytes (CD21+), double-positive T lymphocytes (memory cells, CD4+CD8+) and B₁ lymphocytes (CD5+CD8-) – were determined by flow cytometry. No significant differences in CD4+CD8-, CD4-CD8+ and B₁ cells were determined between groups C and E or within groups on successive days of the experiment. B₁ cell populations in group E were characterized by a higher growth rate until day 28. A significant increase (p<0.01, p<0.05) in the percentage of B₁ cells were observed on days 28 and 42 in the group E in comparison with day 0. Similar changes were reported in the percentage of T memory cell (CD4+CD8+) populations in both groups, but a significant increase was observed in group E on day 28 relative to day 0 (p<0.05). Notable changes were observed in the percentages of NK cell populations. In group C, NK



of the control (Group C) and zearalenone-administered (Group E) gilts (3×10^6 cells ml⁻¹), stimulated with LPS on day 6. The graphs present mean values \pm SEM (n=5 each). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

populations continued to increase, but a significant difference was noted between days 0 and 14 ($p<0.05$). A reverse trend was reported in group E where the percentages of NK cell populations continued to decrease throughout the experiment. In group C, CD21+ B cells were characterized by significant linear growth throughout the experiment (Fig. 1). In group E, the percentage of CD21+ B cell populations were smaller in comparison with group C, and significantly lower values were noted on days 28 ($p<0.05$) and 42 ($p<0.001$).

Cytokine concentrations in culture supernatants of Payer's patch lymphocytes after LPS stimulation

Cytokine concentrations in culture media are presented in Fig. 2. No significant differences in culture supernatants IL-2 levels were observed between pigs whose feed was supplemented with ZEN (group E) and control group animals (group C). A significant decrease ($p<0.01$) in culture supernatants IL-2 levels were observed on days 14 and 28 in the experimental group in comparison with day 14. A significant decrease ($p<0.001$, $p<0.01$) in concentrations of IFN- γ were observed on days 14 and 28 in the group C in comparison with day 0, moreover a significant in

crease ($p<0.01$) in culture supernatants IFN- γ levels was observed on days 28 and 42 in comparison with day 14. A significant decrease ($p<0.001$) in culture supernatants IFN- γ levels were observed on days 14 and 28 in the experimental group in comparison with day 14. No significant differences in culture supernatants IL-4 levels were observed between pigs whose feed was supplemented with ZEN but the values showed an increasing trend. A significant decrease ($p<0.01$) in concentrations of IL-4 was observed on day 42 in the group C in comparison with day 28. A significant increase ($p<0.01$) in concentrations of IL-10 was observed on day 28 in the experimental group in comparison with days 0 and 14 and then a significant decrease ($p<0.01$) in day 42 on comparison with day 28.

Discussion

Peyer's patches in the ileum facilitate the generation of the immune response (Brandtzaeg 2011). The immune response requires macrophages, antigen-presenting cells (APC) and T and B cells (Burkey et al. 2009). When a supportive cytokine environment is not created in Peyer's patches due to various factors, the disrupted Th1/Th2 balance can have serious health implications because each cell population plays distinctive roles in the body. Th1 lymphocytes are in-

involved in the cellular immune response and host defense against viruses, protozoa and tumors, and they inhibit allergic reactions. Th2 lymphocytes play a key role in the humoral immune response, and they promote allergies by shifting the Th1/Th2 balance in favor of the Th2 response.

LPS, the major inflammatory component of the outer membrane of Gram-negative bacterial cells, induce a distinctive pattern of cytokine release. This type of stimulation induces immune responses mediated not only by B lymphocytes, macrophages, mast cells or dendritic cells, but also by T lymphocytes and NK cells (Ulmer et al. 2000) to stimulate the secretion of IL-2 and IFN- γ . LPS can stimulate T lymphocytes and NK cells indirectly by activating dendritic cells (DC) or macrophage TLR4 receptors (Toll-like receptor 4) or directly by activating TLR4 receptors on T lymphocytes and NK cells (Mian et al. 2010). The discussed mechanism of LPS activity can play a major role in many inflammatory diseases and hypersensitivity reactions classified by Coombs. The initiation of an immune response to LPS in the presence of estrogenic compounds can be associated with immune disorders, and it can lead to chronic inflammations and autoimmune disorders.

The applied method of isolating immunocompetent cells from ileal Peyer's patches supported the acquisition of a wide range of Th lymphocytes (CD4+CD8-), cytotoxic T lymphocytes (CD4-CD8+), B lymphocytes (B₁, CD21+), memory cells (CD4+CD8+), NK cells and macrophages (adherent cells, data not published) for *in vitro* cultures and the control of the rate of changes in cell populations under physiological conditions (group C) and under exposure to ZEN (group E) throughout the experiment (Obremski et al. 2014).

The results of the study indicate that exposure to low doses of orally administered ZEN contributed to changes in the size of the analyzed lymphocyte populations, which were successively cultured *in vitro* and stimulated with LPS. ZEN exerted a particularly notable influence on NK and B₁ cell populations. The observed decrease in NK cell counts validates the theory that estrogenic substances inhibit the proliferation of NK cells. An increase was reported in the populations of B₁ cell populations, which, according to Yurino et al. (2004), are susceptible to environmental estrogens (in this case, ZEN) and produce more autoantibodies due to an age-related increase in mRNA expression of ER- α (Erlandsson et al. 2003). Numerous studies have demonstrated that selected mycotoxins affect the Th1/Th2 balance. Li et al. (2005) observed that doxynivalenol administered to mice activated Th2 populations and inhibited the Th1 immune response. Subclinical doses of patulin can al-

ter the Th1/Th2 balance in favor of the Th2 immune response and promote allergic reactions and ulcerative colitis (Luft et al. 2008). Marin et al. (2010, 2013) reported on the immunomodulatory effects of ZEN. There is an absence of published data on the effects of mycotoxin exposure, in particular ZEN and its metabolites, on the Th1/Th2 cytokine profile in ileal Peyer's patches. ZEN and other mycotoxins influence various immune and intestinal functions, but the potential correlations between mycotoxin exposure and chronic inflammatory diseases in humans, such as celiac disease, Crohn's disease or ulcerative colitis, have not been explored to date (Maresca and Fantini 2010).

ZEN is an estrogenic mycotoxin, and its influence can be compared to that of E2. Under physiological conditions, E2 stimulates the secretion of cytokines IL-4, IL-10 and IFN- γ in the ovulatory phase (Staub 2007). ZEN shows weaker affinity for ERs than E2, but it can bind with ERs in immunocompetent cells to modulate their immune response. An *in vitro* analysis of LPS-stimulated cells sampled from pigs administered ZEN revealed higher concentrations of IL-4 and lower concentrations of IL-2 and IFN- γ in comparison with control cultures where an increase in IFN- γ levels was observed on day 42. ZEN's influence resembles the role played by cytokines in allergies and type I hypersensitivity reactions according to the Coombs classification system. Exposure to IL-4 stimulates the production of IgE antibodies, which is a common environmental response to antigens. In this study, immunocompetent cells isolated from ileal Peyer's patches and stimulated with LPS produced a 'more pro-allergic' response under the influence of ZEN and shifted the immune balance towards humoral immunity. Both IL-4 and IL-10 produced by Th2 lymphocytes inhibit the activity of Th1 lymphocytes (Mosser and Zhang 2008). In this experiment, IFN- γ and IL-2 concentrations decreased after stimulation with LPS. IFN- γ is secreted by Th1 cells, whereas IL-2 is crucial for the development of all Th lymphocytes (Malek 2003). IL-2 is also essential for the development of immunological memory, which was represented by double-positive cells (CD4+CD8+) in this experiment, and the regulation of Th FoxP3CD25, Th3 and Tr1 lymphocytes. In the present study, IL-2 was detected in the culture media of cells stimulated with LPS in group E, whereas IL-2 concentrations in group C were below the limit of detection. ZEN's stimulating influence on IL-4 and IL-10 secretion seems to point to the dominant role of Th2 lymphocytes in cell cultures because both cytokines are specific for the Th2 cell population. The presence of IL-4 and IL-10 was also found in control culture media, but they were accompanied by IFN- γ , in particu-

lar on day 42. The immune responses of LPS-stimulated immunocompetent cells isolated from Peyer's patches of pigs exposed to ZEN suggest that the discussed mycotoxin promotes allergic reactions (Gajęcki et al. 2006).

Interestingly, IFN- γ was co-secreted with the antagonistic IL-4 on day 42 in group C, but the above was not observed in cell culture supernatants in group E. According to Morris et al. (2006), activated NKT cells can secrete both IL-4 and IFN- γ , and selected populations of CD8+ Tc lymphocytes produce IFN- γ (Tc1) and IL-4 (Tc2) (Romagnani 1996, Seder et al. 1996). The above suggests that a cellular immune response was developed in control cultures where immunocompetent cells had not been exposed to ZEN *in vivo*. Stronger humoral immune responses or weaker cellular immune responses were observed in the cell culture supernatants of lymphocytes from animals treated with ZEN. IL-4 and IL-10 concentrations increased and IFN- γ levels decreased in cultures of LPS-stimulated Peyer's patch cells sampled from pigs that had been exposed to ZEN-contaminated feed (day 42). Unlike in control cultures, where IL-4 could have been secreted by Tc lymphocytes (due to the simultaneous production of IFN- γ), in experimental cultures analyzed on day 42, IL-4 was probably produced by Th2 lymphocytes. The above observation indicates that IFN- γ did not inhibit the development of the analyzed lymphocyte populations. Our observations are validated by the results of other studies, which reported changes in the type of Th cells involved in the immune response under exposure to an estrogenic substance (Karpuzoglu-Sahin et al. 2001).

The results of this study indicate that long-term exposure to ZEN at doses below the NOEL threshold ($10 \mu\text{g kg}^{-1} \text{ BW day}^{-1}$), which had no estrogenic effects on pre-pubertal pigs, can alter the sensitivity of T and B cells to LPS stimulation. ZEN inhibits IL-2 and IFN- γ production, and stimulates IL-4 and IL-10 secretion by Th1 and Th2 cells, thus shifting the Th1/Th2 balance towards the humoral immune response. The above can probably contribute to allergic reactions, as demonstrated by the increase in the size of B₁ cell populations producing more autoantibodies. ZEN can also decrease resistance to viral infections and tumors by inhibiting the proliferation of NK cells and IFN- γ secretion.

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